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Pseudomonads and bacilli as important spoilage organisms in the dairy industry — a taxonomic study —

An Coorevits

promotors

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Dissertation submitted in fulfillment of the requirements for the degree of
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En nu . . . , op naar de volgende uitdagingen, met enorm veel goesting, samen lukt ons dat wel!

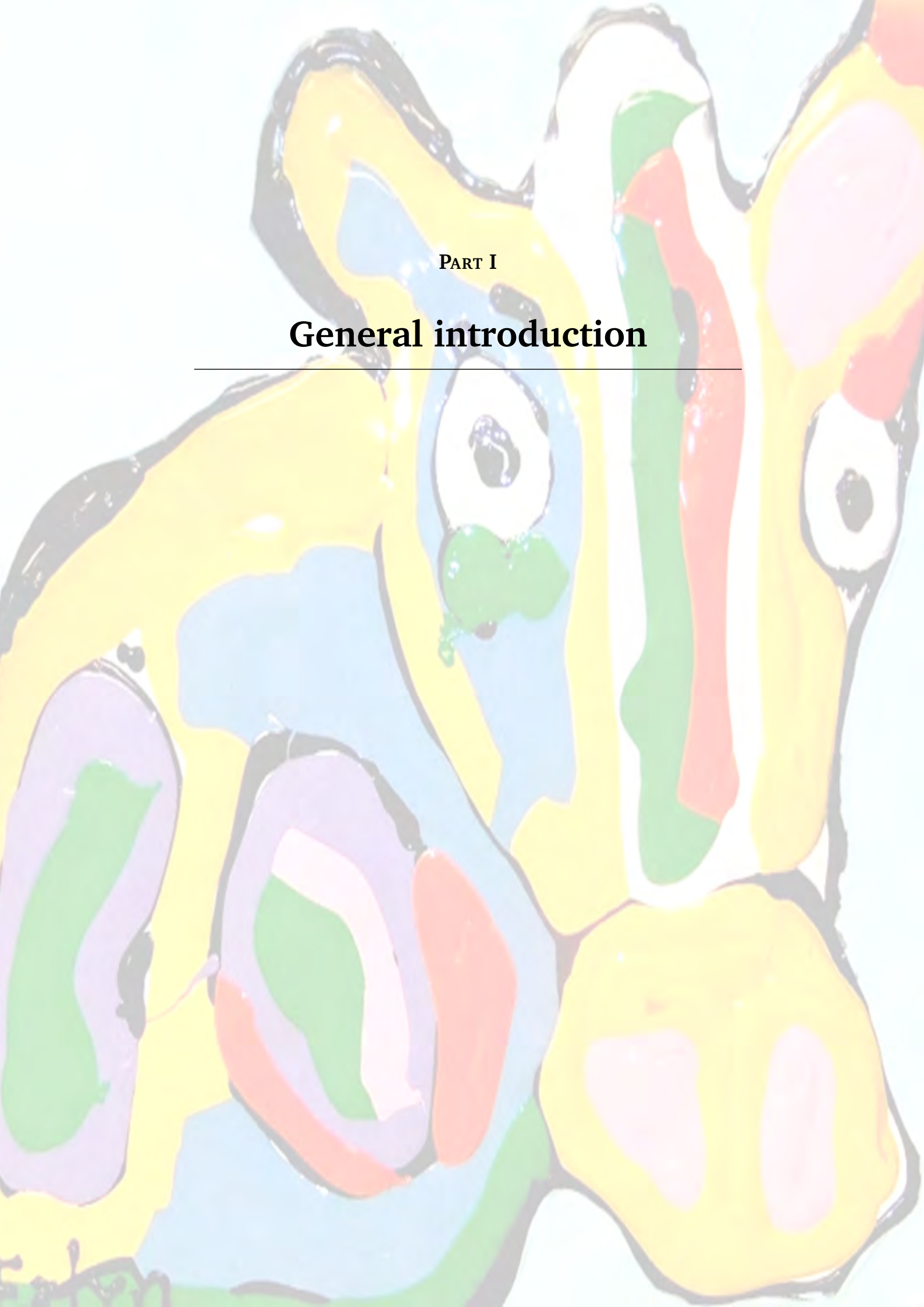
List of abbreviations

AAI	Average Amino Identity
AL	aminolipid
aLRT	approximate Likelihood Ratio Test
ANI	Average Nucleotide Identity
APGL	aminophosphoglycolipid
APL	aminophospholipid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
B	*Bacillus* Collection at Glasgow Caledonian University
BHI	Brain Heart Infusion
BFA	*Bacillus fumarioli* agar
BLAST	Basic Local Alignment Search Tool
bp	basepairs
BRC	Biological Resource Center
CECT	Coleccion Espanola de Cultivos Tipo
CI	confidence interval
CIP	Cleaning In Place
CIP	Collection de l'Institut Pasteur
CFC	Cetrimide Fucidine Cefaloridine
cfu	colony forming unit
CTAB	hexadecyl-trimethyl-ammonium bromide
DAP	diaminopimelic acid
DDH	DNA-DNA hybridization
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DPG	diphosphatidylglycerol
DSM	Deutsche Sammlung von Microorganismen
EC	European Commission
EDTA	ethylenediaminetetraacetic acid
EG	Europese Gemeenschap

EMBL	European Molecular Biology Laboratory
ESL	Extended Shelf Life
EU	European Union
FAME	Fatty Acid Methyl Ester
FASTA	Fast All
FATAL	Fast Automatic Tracking And Loading
FID	Flame Ionization Detector
GC	Gas Chromatograph(y)
GES	guanidiumthyocyanate EDTA sarkosyl
GL	glycolipid
GLM	generalized linear model
GMP	guanosine monophosphate
GTR	General Time Reversible
HACCP	Hazard Analysis and Critical Control Point
HPLC	High Pressure Liquid Chromatography
HRS	highly heat-resistant spore
HTST	High Temperature Short Time
ID	identification
IG	intrageneric
IKM	Integrale Kwaliteitszorg Melk
ILVO	Instituut voor Landbouw en Visserij Onderzoek
IPTG	isopropyl-β-D-thiogalactopyranoside
LMG	Laboratorium voor Microbiologie, Gent
LTLT	Low Temperature Long Time
LTP	Living Tree Project
LUDA	Leeds University Department of Agriculture
MCC	Melk Controle Centrum
MIS	Microbial Identification System
MQ	MilliQ
ML	Maximum Likelihood
MLSA	Multi Locus Sequence Analysis
MP	Maximum Parsimony
MPCA	Milk Plate Count Agar
MSC	monophyletic subgeneric cluster
NA	Nutrient Agar
NCA	National Canners Association
NJ	Neighbor Joining
OD	Optical Density
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
OTU	Operational Taxonomic Unit
PAL	phosphoaminolipid
PBS	Phosphate Buffered Saline

PC	*Pseudomonas* count
PCA	Plate Count Agar
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGL	phosphoglycolipid
PL	phospholipid
PS	phosphatidylserine
R	Research
Rep	repetitive element
RIVM	RijksInstituut voor Volksgezondheid en Milieu
RMO	Rijdende Melk Ophaling
RNA	ribonucleic acid
rpm	rounds per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
s.l.	sensu lato
SNA	Supplemented Nutrient Agar
S_G	similarity coefficient of Gower
S_P	Pairwise similarity values
s.s.	sensu stricto
SSC	saline sodium citrate
SSM	PCA supplemented with Skim Milk Medium
T_A	Annealing Temperature
TA	Tributyrin Agar
TAE	Tris base-Acetic acid-EDTA
TAPC	Total Aerobic Plate Count
TASC	Total Aerobic Spore Count
TE	Tris base-EDTA
T_H	Hybridisation Temperature
TLC	Thin Layer Chromatography
TSA	Tryptone Soy Agar
TSBA	Trypticase Soy Broth Agar
UHT	Ultra High Temperature
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
VKM	All-Russian Collection of Microorganisms
WGS	whole genome sequencing
WHO	World Health Organization
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Contents

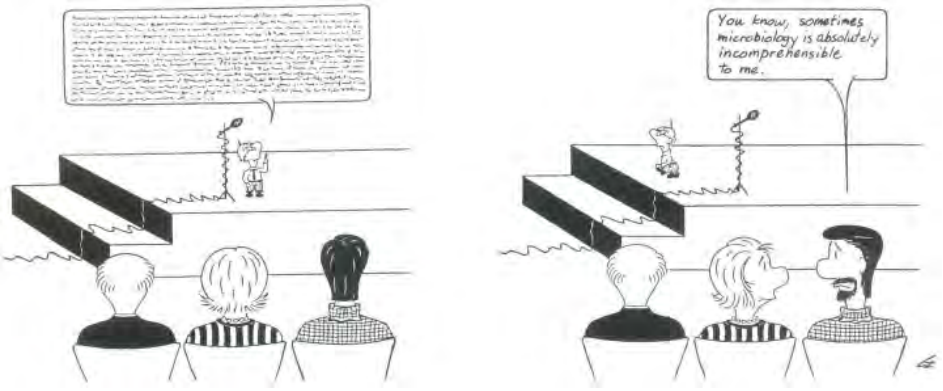


PART I

General introduction

CHAPTER 1

Outline



This thesis is the result of a joint cooperation between University College Ghent, Ghent University, Glasgow Caledonian University and the Institute for Agricultural and Fisheries Research. The research topic was quite diverse, and often went astray, but main focus was on the identification of pseudomonads and bacilli recovered from milk, as indicated in the background and objectives (**Part I**).

The literature overview (**Part II**) presents a general introduction to the spoilage issue in the dairy industry (**Chapter 2**) and to bacterial taxonomy (**Chapter 3**). The experimental set-up and results (**Part III**) of two diversity studies, one on pseudomonads and one on bacilli are presented in **Chapter 5** and **6**; both studies were performed in collaboration with the Institute for Agricultural and Fisheries Research. These chapters are preceded by a short overview of techniques frequently applied by the author throughout this study (**Chapter 4**). Taxonomic work on bacilli was performed in collaboration with Glasgow Caledonian University, one study describing the novel species *Bacillus thermolactis* (**Chapter 6**) and one resolving the taxonomic mix-up within the genus *Geobacillus* (**Chapter 7**). Additionally, **Chapter 5** shortly discusses the applicability of a Multi Locus Sequence Analysis scheme for identification purposes in *Pseudomonas* taxonomy.

A conclusion of the work performed, as well as future perspectives within both fields (milk spoilage and taxonomy of pseudomonads and bacilli), are given in **Chapter 8**. A summary of this work can be found in **Part I**.

1.1 Background and objectives

Raw cow's milk is a product of high nutritional value but this automatically implies it is a medium highly suitable for growth of spoilage organisms that negatively affect milk quality and safety through production of proteolytic and lipolytic enzymes and toxins. Two major groups of spoilage bacteria are recognized, namely pseudomonads and bacilli.

Identification of members of these groups is hampered by their confusing taxonomic situation. Both taxa historically grew as dumping grounds for aerobic Gram-positive spore-forming rods in the case of bacilli, and aerobic Gram-negative rods in the case of pseudomonads, resulting in two very large, heterogeneous groups. As a consequence, members of these groups were often poorly identified based on identification tools with insufficient resolution, and usually only two major species were recognized in the issue of milk spoilage, namely *Bacillus cereus* and *Pseudomonas fluorescens*. Nonetheless, several studies on the identity of bacterial milk flora indicated diversity was much bigger than originally thought, and the dairy product spoilage issue is clearly not a story of *Bacillus cereus* and *Pseudomonas fluorescens* alone.

The main objectives of this study were two-fold. The first goal was to accurately map the diversity of bacterial milk flora through a polyphasic identification approach of milk isolates, with focus on bacilli and pseudomonads. Additionally, the spoilage potentials of these isolates were assessed. The second goal was an attempt to resolve the complex taxonomic situation at least for subgroups within the bacilli (the genus *Geobacillus*) and pseudomonads (the *Pseudomonas fluorescens* group).

1.2 Summary

Raw cow's milk is a product of high nutritional value but this automatically implies it is prone to spoilage organisms that negatively affect milk quality and safety through production of proteolytic and lipolytic enzymes, and toxins. Two major groups of spoilage organisms in milk are recognized, namely the pseudomonads and bacilli. However, identification of members of both groups is hampered by their confusing taxonomic situation.

Main objectives of this study were *i*) to accurately map the diversity of bacterial milk flora through a polyphasic identification approach of milk isolates, with focus on bacilli and pseudomonads, and *ii*) an attempt to resolve the complex taxonomic situation at least for subgroups within both taxa (the genus *Geobacillus* and the *Pseudomonas fluorescens* group).

The first diversity study **(Chapter 5)** focused on the psychrotrophic aerobic bacterial fraction, that benefits from the prolonged cold storage of raw milk prior to processing. The dominant *Pseudomonas* microbiota was identified as members of the *Pseudomonas fluorescens* group, *Pseudomonas lundensis* and *Pseudomonas fragi*. It was shown these species have an important proteolytic and lipolytic spoilage potential, and *P. fragi* and *P. lundensis* were predominantly isolated at the end of the dairy chain which indicates these organisms may be controlled by adequate cooling and rapid processing at the dairy plant. Suboptimal cooling conditions greatly favored growth of these pseudomonads, and of some as yet undescribed strains with considerable spoilage potential. Strains assigned to the *Pseudomonas fluorescens* group could often not be identified onto the species level for two reasons. Firstly, the complex taxonomic situation of this group hampers reliable species identification. Secondly, several strains probably represent as yet undescribed

species, the so-called 'likes' throughout the study. Additionally, the applicability of a recently proposed MLSA scheme to identify *Pseudomonas* strains was validated, but preliminar results indicate there are some discrepancies between different taxonomic markers used in this scheme, and identification onto the species level remains problematic. Further research is required to resolve this complex situation.

In a second study **(Chapter 6)** focus was on diversity of the aerobic endospore-formers. Isolates were subjected to fatty acid methyl ester (FAME) analysis, and representatives of FAME-groups were further identified on the basis of 16S rRNA gene sequencing and rep-fingerprinting. The predominant species were *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus circulans* and members of the *Bacillus cereus* group, as confirmed by other studies focusing on the diversity of aerobic spore-formers in milk. However, this study was the first to demonstrate the presence of representatives of the genus *Oceanobacillus*, *Bacillus clausii*, *Lysinibacillus massiliensis*, *Paenibacillus odorifer*, *Solibacillus silvestris*, *Bacillus simplex* and *Paenibacillus lautus*. This indicates the importance of a polyphasic identification approach. Furthermore, a considerable fraction of spore-forming isolates (7%) probably represent as yet undescribed species, of which some show proteolytic and lipolytic actitivity, indicating their spoilage potential. One group of these isolates was further investigated and described as the novel species *Bacillus thermolactis*. It was also shown that differences in feeding and housing strategy at the farm (e.g. conventional versus organic dairy farming) did influence the bacterial quality of raw milk in terms of aerobic spore content and bacterial diversity and implies the need for further investigation to exactly point out the contamination sources. Study on the impact of these isolates on milk quality and safety indicated members of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Lysinibacillus fusiformis*, *Bacillus amyloliquefaciens* and *Paenibacillus polylmyxa* showed strong to intermediate proteolytic activity and members of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens* showed lipolytic activity. The ability to reduce nitrate to nitrite was shown for *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Lysinibacillus sphaericus* and *Bacillus clausii*, implicating these species might cause structural defects during cheese production. Even more, for *Paenibacillus polymyxa*, this effect is fortified due to gas production during fermentation.

Within the large group of bacilli, several strains belonging to the genus *Geobacillus* were thoroughly investigated to assess a reliable taxonomy of this taxon **Chapter 7**. This resulted in the merger of species *Geobacillus kaustophilus, Geobacillus lituanicus, Geobacillus vulcani* to *Geobacillus thermoleovorans*, together with "*Geobacillus thermoleovorans* subsp. *stromboliensis*", "*Bacillus caldovelox*", "*Bacillus caldotenax*" and "*Bacillus caldolyticus*", and the description of *Geobacillus thermoleovorans* was emended accordingly. *Geobacillus gargensis* appeared to be a later heterotypic synonym of *Geobacillus thermocatenulatus*, and description of the latter species has been emended. Descriptions of the genus itself and for *Geobacillus stearothermophilus, Geobacillus jurassicus, Geobacillus toebii, Geobacillus thermodenitrificans* and *Geobacillus thermoglucosidans* (formerly *thermoglucosidasius*) have been emended. *Bacillus thermantarcticus* was transferred to the genus *Geobacillus* as *Geobacillus thermantarcticus*. *Geobacillus debilis* should be accommodated in the genus *Caldibacillus* (gen. nov.), and species *Geobacillus caldiproteolyticus* and *Geobacillus tepidamans* should be transferred to the genus *Anoxybacillus*.

This thesis clearly shows the need for reliable identification methods in diversity studies. Diversity of aerobic spore-formers seemed to be much bigger than first thought, and the impact of *Pseudomonas fluorescens* as main spoilers seems to be overestimated, as *Pseudomonas fragi*, *Pseudomonas lundensis* and several as yet undescribed species also contribute to milk spoilage. However, it also shows taxonomy within both groups is far from satisfying and a lot of further

work is needed to resolve the taxonomic complex situation for both genera *Bacillus* and *Pseudomonas*, which would probably result in more reliable identification tools.

1.3 Samenvatting

Rauwe koemelk is een product met een hoge voedingswaarde maar dit heeft automatisch tot gevolg dat het een geschikte habitat vormt voor tal van micro-organismen, waaronder bederfbacteriën. Deze bedervers kunnen de melkkwaliteit en -veiligheid negatief beïnvloeden door onder andere de productie van extracellulaire proteasen en lipasen die structurele defecten veroorzaken van de melk, en door productie van toxines die verantwoordelijk kunnen zijn voor voedselvergiftiging en/of -infectie. Twee grote groepen van melkbedervers worden onderscheiden, namelijk de pseudomonaden en de bacilli. Identificatie van leden van deze groepen wordt echter bemoeilijkt door hun complexe taxonomische situatie.

De belangrijkste doelen van deze thesis waren enerzijds om de diversiteit van deze melkbedervers in kaart te brengen, met nadruk op psychrotrofe enzymproducerende bedervers, de pseudomonaden, en de sporenvormende bedervers, de bacilli. Het aanwenden van een polyfasische aanpak voor identificatie van de isolaten zorgt voor een nauwkeurig beeld van de complexe bacteriële flora in melk. Anderszijds werd gepoogd de complexe taxonomische situatie binnen subgroepen van beide taxa (gedeeltelijk) op te helderen. Daartoe werd een vooropgesteld MLSA-schema voor de *Pseudomonas fluorescens* groep gevalideerd, en werden stammen van het genus *Geobacillus* uitgebreid bestudeerd om zo tot nieuwe voorstellen te komen.

De eerste diversiteitsstudie **(hoofdstuk 5)** was gericht naar koudebestendige *Pseudomonas* stammen, die profiteren van de verlengde koele bewaarperioden die nu vaak in de zuivelindustrie worden toegepast. De meest voorkomende pseudomonaden waren *Pseudomonas fragi*, *Pseudomonas lundensis* en leden van de *Pseudomonas fluorescens* groep. Er werd tevens aangetoond dat deze species aanzienlijk bederfpotentieel vertonen door de productie van lipasen en proteasen, en voor *P. fragi* en *P. lundensis* werd tevens aangetoond dat deze species voornamelijk uitgroeien op het eind van de zuivelketen bij suboptimale bewaaromstandigheden van de rauwe melk. Dit wijst er op dat uitgroei van deze bacteriën zou kunnen beperkt worden door de rauwe melk snel te verwerken, en door strikte gekoelde bewaaromstandigheden aan te houden. Bij suboptimale bewaring kunnen niet alleen de meest voorkomende pseudomonaden goed uitgroeien, maar werd ook een grotere diversiteit waargenomen, o.a. van tot nu toe ongekende stammen met een niet te verwaarlozen bederfpotentieel. Naast de diversiteitsstudie werd ook een recentelijk voorgestelde identificatietool in de vorm van een MLSA-schema gevalideerd. Resultaten waren echter niet bevredigend met belangrijke verschillen tussen de gebruikte taxonomische merkergenen, waardoor identificatie binnen de *Pseudomonas fluorescens* groep problematisch blijft. Verder onderzoek is vereist om deze complexe situatie op te helderen.

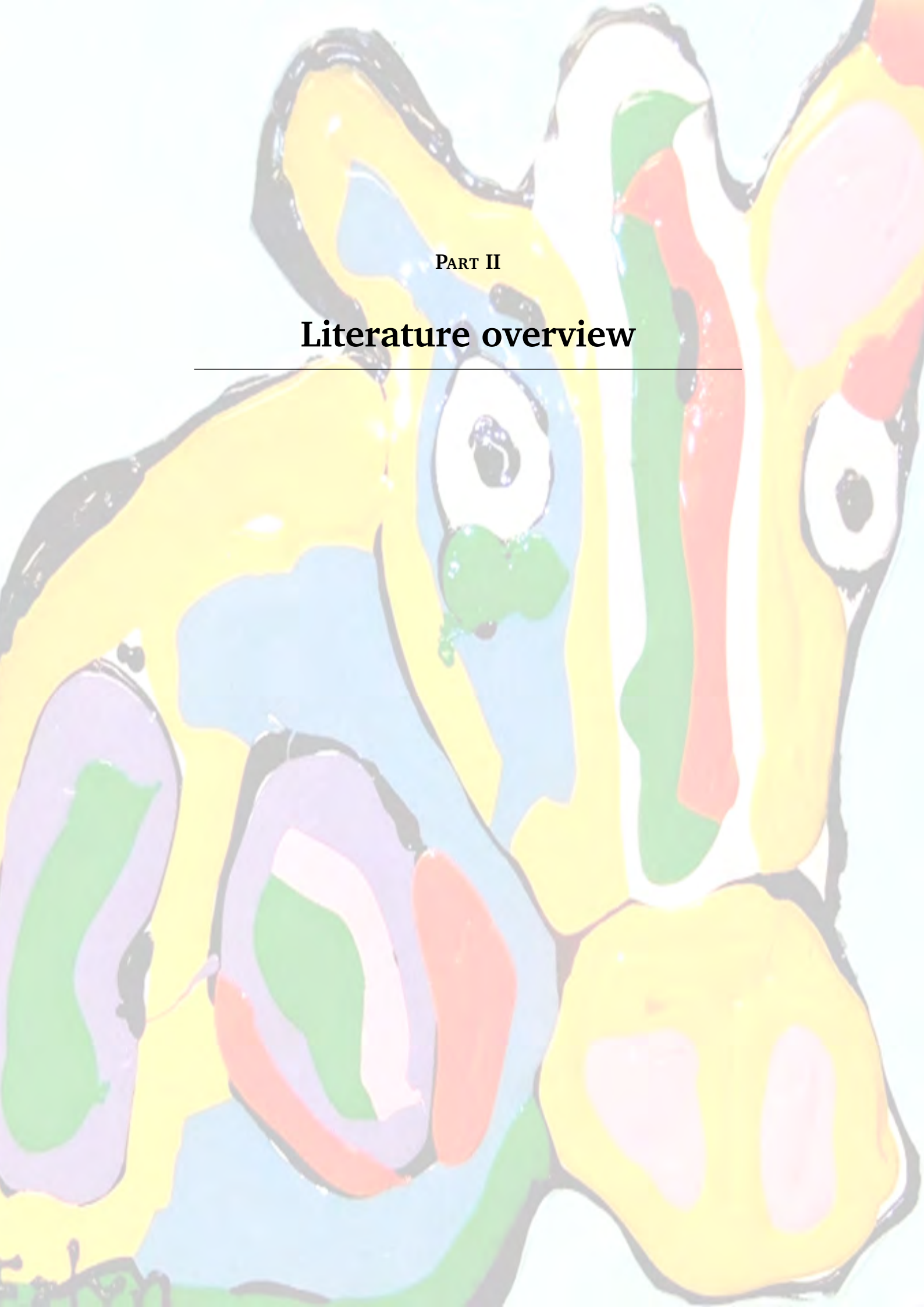
Een tweede studie (**hoofdstuk 6**) was toegespitst op de aërobe endosporen-vormers, de bacilli. Isolaten werden eerst gegroepeerd op basis van analyse van de vetzuursamenstelling van de celmembraan, en vervolgens werden vertegenwoordigers van die verschillende groepen verder geïdentificeerd aan de hand van 16S rRNA gen sequenering, en DNA-typering. De meest voorkomende species waren *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus circulans* en leden van de *Bacillus cereus* groep, en deze bevindingen werden bevestigd door eerder uitgevoerde studies omtrent microbiële diversiteit van melk. Nieuw echter was de aanwezigheid van *Bacillus clausii*, *Paenibacillus odorifer*, *Lysinibacillus massiliensis*, *Solibacillus silvestris*, *Bacillus*

simplex, *Paenibacillus lautus* en leden van het genus *Oceanobacillus*. Deze bevindingen benadrukken het belang van een verregaande, nauwkeurige identificatie van de isolaten (via polyfasische aanpak), om een betrouwbaar beeld te verkrijgen van de aanwezige microbiële populatie. Daarenboven is aangetoond dat een belangrijke fractie van de isolaten (7%) waarschijnlijk behoren tot nieuwe species, en dat deze ook bederfpotentieel vertonen door hydrolase activiteit. Eén groep isolaten werd verder geanalyseerd, en beschreven als *Bacillus thermolactis*. Door de microbiële samenstelling van melk van twee types bedrijfsvoering, nl. biologische en conventionele, met elkaar te vergelijken, kon vastgesteld worden dat verschillen in voeding en behuizing naar alle waarschijnlijkheid een invloed hebben op de bacteriële diversiteit in melk. Het bederfpotentieel van de isolaten werd tevens in kaart gebracht, en hieruit bleek dat *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Lysinibacillus fusiformis*, *Bacillus amyloliquefaciens* en *Paenibacillus polymyxa* aanzienlijke protease-activiteit vertoonden, terwijl *Bacillus subtilis*, *Bacillus pumilus* en *Bacillus amyloliquefaciens* lipase-activiteit vertoonden. Nitraatreductie (tot nitriet) werd aangetoond voor *Paenibacillus polym*yxa, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Lysinibacillus sphaericus* en *Bacillus clausii*, wat betekent dat deze species verantwoordelijk kunnen zijn voor structurele defecten in de kaas. Aangezien *Paenibacillus polymyxa* daarenboven ook gas produceert tijdens de fermentatie kan dit effect zelfs nog versterkt worden.

Binnen de grote groep bacilli, werden verscheidene stammen die behoren tot het genus *Geobacillus* verder geanalyseerd om de fylogenetische verwantschappen binnen vertegenwoordigers van dit genus nauwkeurig te bepalen **(hoofdstuk 7)**. Dit onderzoek resulteerde in de samensmelting van de species *Geobacillus kaustophilus, Geobacillus lituanicus, Geobacillus vulcani* met het species *Geobacillus thermoleovorans*, alsook de tot nu niet erkende species "*Geobacillus thermoleovorans* subsp. *stromboliensis*", *Bacillus caldovelox*", "*Bacillus caldotenax*", "*Bacillus caldolyticus*" werden aan dit species *Geobacillus thermoleovorans* gekoppeld. De beschrijving van dit species werd dan ook gecorrigeerd. *Geobacillus gargensis* bleek een heterotypisch synonym te zijn van *Geobacillus thermocatenulatus* en de beschrijving van dit laatste species werd dan ook aangepast. Verder werd de beschrijving van het volledige genus *Geobacillus*, alsook van *Geobacillus stearothermophilus, Geobacillus jurassicus, Geobacillus toebii, Geobacillus thermodenitrificans* en *Geobacillus thermoglucosidans* (vroeger *thermoglucosidasius*) verbeterd. *Bacillus thermantarcticus* werd overgeplaatst naar het genus *Geobacillus* onder de naam *Geobacillus thermantarcticus*. *Geobacillus debilis werd* ondergebracht in het nieuwe genus *Caldibacillus* als *Caldibacillus debilis* en de species *Geobacillus caldiproteolyticus* en *Geobacillus tepidamans* werden ondergebracht in het genus *Anoxybacillus*.

Het werk in deze thesis toont duidelijk aan dat er nood is aan betrouwbare methodes om de belangrijkste bedervers in melk goed te kunnen identificeren. Ontwikkeling van dergelijke methodes is echter onlosmakelijk verbonden met de classificering, en dus de taxonomie van de taxa waartoe deze bedervers behoren. Het gebruik van een polyfasische identificatie-aanpak toonde duidelijk aan dat de diversiteit van aërobe sporenvormers in melk veel groter is dan algemeen aangenomen. Bovendien bleek ook dat het aandeel van *Pseudomonas fluorescens* in melk veel kleiner is dan algemeen aangenomen, en dat eerder *Pseudomonas fragi* en *Pseudomonas lundensis* belangrijke spelers zijn in melkbederf. Het werk dat werd uitgevoerd om de taxonomisch complexe situatie van de *Pseudomonas fluorescens* groep en het genus *Geobacillus* op te helderen, heeft voor het genus *Geobacillus* geleid tot een grondige herziening van het genus, met als resultaat onder andere het samensmelten van verscheidene species. Binnen de *Pseudomonas fluorescens* groep echter, is duidelijk gebleken dat het vooropgestelde MLSA-schema, dat momenteel kan beschouwd worden als de beste identificatiemethode die er is voor leden van dit genus, niet voldoet om de leden tot op speciesniveau van elkaar te onderscheiden. Er is dus nood aan nieuwe

taxonomische merkers, die de fylogenetische relaties binnen leden van deze groep beter kunnen voorstellen, en de zoektocht naar dergelijke merkers zal aanzienlijk vergemakkelijkt worden met de uitbreiding van het aantal volledige genomen binnen deze groep.



PART II

Literature overview

Chapter 2 has been redrafted from

1. **Coorevits A.**, De Jonghe V., Vandroemme J., Van Landschoot A., Heyndrickx M. & De Vos P. (2010). How can the type of dairy farming influence the bacterial flora in milk? In 'Organic Farming and Peanut Crops', pp. 123-136. Eds. D.C. Grossman, T.L. Barrios. Nova Science Publishers, Inc., New York. ISBN: 978-1-60876-187-6.

2. De Jonghe V., **Coorevits A.**, Marchand S., Van Landschoot A., De Block J., De Vos P. & Heyndrickx M. (2011a). Microbial contamination and spoilage of consumer milk – facts and fiction. In 'Raw milk: production, consumption and health effects'. Ed. Momani, J. & Natsheh, A. Nova Science Publishers, Inc., New York, in press. ISBN: 978-1-61470-751-6.

CHAPTER 2

Microbial spoilage of milk and derived products

"Melk is goed voor elk". This catchphrase was used by the Dutch and Belgian government in the sixties to promote the consumption of milk and derived products. Milk is produced by mammals and provides the necessary nutrients for youngsters before they are able to digest other types of food. Thus, it should not be surprising that the nutritional value of milk is high, and that consumption of milk and derived products is stimulated and regarded as positive for human health.

2.1 Milk composition

Raw cow's milk consists of 3.5% proteins, 3.7% fat and 4.8% carbohydrate, as well as 0.7% minerals and vitamins (Webb *et al.*, 1974; Shearer *et al.,* 1992; Bylund, 1995). These percentages represent average values as milk composition can vary according to species, breed, feed, stage of lactation and stage of milk removal (Verdier-Metz *et al.*, 2009; Hurley, 2010). In comparison, the composition of goat, sheep, horse and human milk is presented in Table 2.1.

Although cows are not the only milk-producing animals, focus in this thesis will be on cow's milk. The contribution of goat and sheep milk to the daily dairy intake of man should not be denied as it is of major importance in Mediterranean countries and large parts of Africa and Asia (Bylund, 1995; Rohner-Tielen, 2008), but consumption of these types of milk in Belgium is rather low (less than 5% (Rohner-Thielen, 2008)). Fat is the most variable component, while carbohydrate

	cow	goat	sheep	horse	human
proteins	3.5	3.1	5.5	2.7	1.1
fat	3.7	3.5	5.3	1.6	4.5
carbohydrate	4.8	4.6	4.6	6.1	6.8
minerals and vitamins	0.7	0.8	0.9	0.5	0.2

Table 2.1: Composition of milk for various species; values are represented in percentages (Jensen, 1995).

is the least variable. At first sight, milk appears to be a homogeneous liquid, however it is a complex mixture of a wide range of compounds. It is actually an emulsion of fat globules and a suspension of casein (protein) micelles, all of which are suspended in an aqueous phase that contains dissolved carbohydrate, whey protein and some minerals.

2.1.1 Milk proteins

Milk contains hundreds of types of proteins, and three major classes can be differentiated, namely casein, whey and fat globule membrane proteins. The concentration of proteins in milk is represented in Table 2.2.

	conc. in milk (g/kg)	% of total protein (w/w)
casein		
α_{s1}-casein	10.0	30.6
α_{s2}-casein	2.6	8.0
β-casein	10.1	30.8
κ-casein	3.3	10.1
whey protein		
α-lactalbumin	1.2	3.7
β-lactoglobulin	3.2	9.8
blood serum albumin	0.4	1.2
immunoglobulins	0.7	2.1
others	0.8	2.4
fat globule membrane proteins	0.4	1.2

Table 2.2: Concentration of proteins in milk (Bylund, 1995).

Casein proteins are present in milk as colloidal structures, called micelles, due to polymerization of hundreds up to thousands of casein molecules, held together by hydrophobic interactions and calcium ions. The exact structure of casein micelles is still not completely unraveled and several models have been proposed (Walstra, 1979; Holt, 1992; Horne, 1998; Lucey, 2002) but they all consider micelles as colloidal particles formed by casein aggregates (mainly α- and β-casein) wrapped up in soluble κ-casein molecules. In Figure 2.1 a casein micelle is represented as a complex of sub-micelles (A), each having a diameter of 10 to 15 nm. A medium-sized micelle can consist of up to 500 sub-micelles. These micelle structures are very stable due to calcium phosphate (C) and hydrophobic interactions (E) between the sub-micelles. The outside situated κ-casein molecules (D) contain carbohydrate groups (B), protruding from the outer side of the micelle, giving it a 'hairy' look.

Whey contains everything that is soluble from milk when it coagulates (so no caseins and fat),

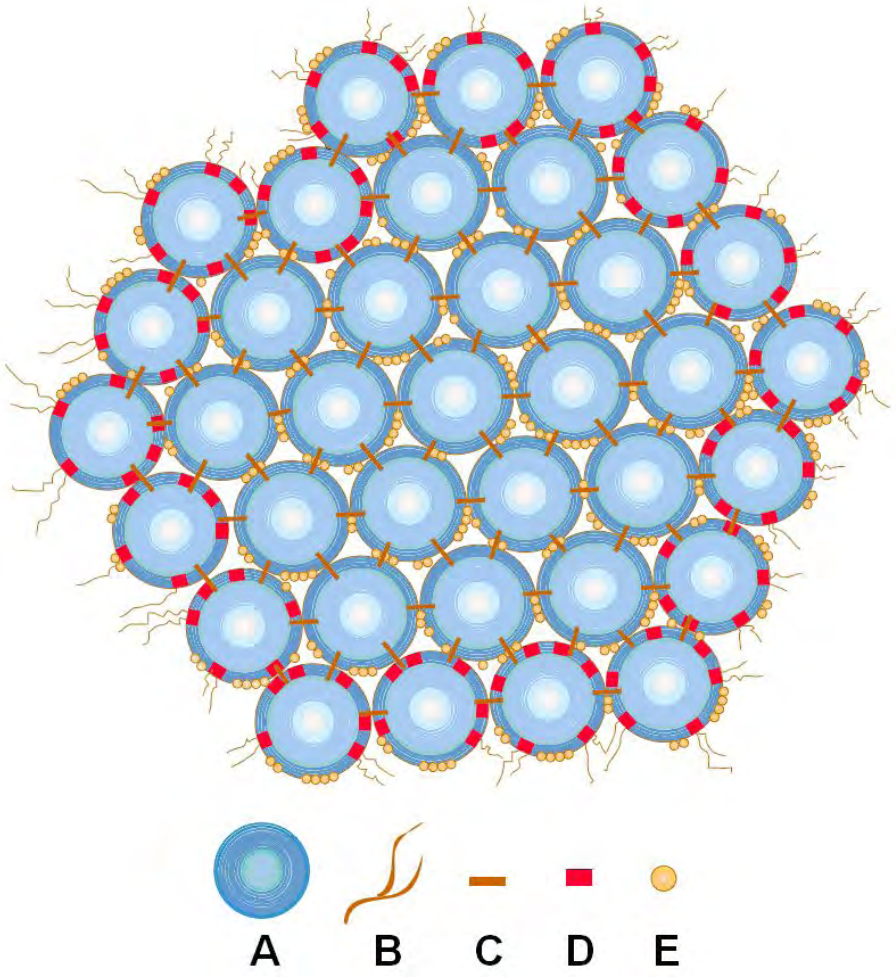


Figure 2.1: Micelle structure consisting of sub-micelles (A) held together through calcium phosphate (C), and hydrophobic interactions (E). On the outside, carbohydrate groups (B) protrude from the κ-casein molecules (D) (Bylund, 1995; Food-info.net, 2011).

and is also called milk serum. Proteins found in that fraction are mainly lactalbumines and lactoglobulines and are called whey proteins. β-lactoglobulin is the major whey protein in bovine milk and plays a role in the typical 'cooked' flavor of heat-treated milk due to release of sulphurous compounds. Indeed, if milk is heated over 60°C, the β-lactoglobulines start to denature and sulphur bridges are formed between the protein molecules, resulting in the gradual release of these sulphurous compounds (Bylund, 1995). α-Lactalbumin was found to be a subunit of lactose synthetase (Brodbeck *et al.*, 1967).

The fat globule membrane proteins form a protective layer around fat globules in milk and thus play a major role in stabilization of the milk emulsion (Bylund, 1995). Proteins in milk have high nutritional value, and particularly whey proteins are considered as the 'biological optimum' due to an almost ideal amino acid composition (Bylund, 1995; Ha & Zemel, 2003).

2.1.2 Milk fat

Milk is an example of an oil-in-water emulsion with fat occurring as small globules dispersed in the milk serum. Some 15 billion globules are found per mL milk, with a diameter ranging from 0.1 to 20 μm, the average size being 3 – 4 μm. Fat globules consist of mainly triglycerides, di- and monoglycerides, fatty acids, sterols, carotenoids (accounting for the yellowish color of milk fat) and vitamins, surrounded by a very thin membrane, the milk fat globule membrane. Raw cow's milk consists mainly of long chain fatty acids, namely oleate ($C_{18:1}$), palmitate (C_{16}) and sterate (C_{18}). The globule membrane consists of phospholipids, lipoproteins, glycosphingolipids, proteins, nucleic acids, enzymes, trace elements and bound water. As these compounds are constantly exchanged with the milk serum, the composition of the membrane is not constant. Fat globules are not only the largest particles in milk, but also the lightest, meaning they tend to rise to the surface when milk is left to stand for a while, a process called creaming (Bylund, 1995).

2.1.3 Milk carbohydrate

The most important carbohydrate and energy source of milk, which is only found in milk, is lactose. In addition, very low concentrations of monosaccharides (glucose and galactose), oligosaccharides and protein-bound carbohydrates (*e.g.* in κ-casein) can be present (Banks *et al.*, 1981). Lactose is a disaccharide, soluble in milk serum, and consists of a glucose and a galactose monomer, linked β-1-4 (Bylund, 1995). Some people, mainly adults, are unable to digest lactose due to lack of the enzyme lactase (= β-galactosidase), an ailment called lactose-intolerance. Normally lactose is cleaved by lactase into its building blocks, glucose and galactose, and these monomers are absorbed through the small intestine into the bloodstream. However, persons lacking lactase accumulate lactose molecules in the colon, where it is digested by enteric bacteria that switch to lactose fermentation. This is coupled to gas production, which might cause symptoms such as nausea, cramps, acid reflux and flatulence. The inability to produce lactase is actually the normal condition for adult mammalian species. During the first period of their life, young mammals are fed with mother's milk, and need lactase to digest the lactose that's in the milk. After the weaning period, when switching to other types of food, production of lactase usually drops, as there is no need to digest lactose anymore. However, some human populations continue to produce lactase after the weaning period, and appear to be adapted to dairy consumption. This has occurred independently in both northern Europe and east Africa in populations with a historically pastoral lifestyle (Heyman, 2006; Coles, 2007).

2.1.4 Milk minerals and vitamins

Milk is a source of vitamins A, B, C and D. Vitamin A and D are fat soluble, the others are soluble in water. One liter of milk fulfills at least 20% of the adult daily vitamin requirements (Bylund, 1995). Milk minerals are found in solution in milk serum or in casein compounds. The total concentration is less than 1%. The most important minerals are calcium, sodium, potassium and magnesium.

2.2 Milk consumption

The composition of milk clearly shows its high nutritional value, and consumption of milk and derived products is consequently considered beneficial for human health. In the European Union, almost 133 million tonnes of milk were collected in 2008, of which 2.85 million tonnes were in Belgium. In 2004, the Belgian dairy industry accounted for 11.3% of the Belgian food industry, thus making it one of the most important sectors, with a yearly turnover of 3.4 billion euro (IKM, 2005). The last 20 years the consumption of dairy products has steadily grown. Although the consumption of drinking milk and buttermilk has decreased from 76.3 and 4 liter per year per person in 1980 to 55.8 and 1 liter per year per person in 2004, the consumption of yoghurt and cream has increased two- to threefold in that same period. In the European Union, approximately only 10% of the collected milk was consumed as drinking milk. Cheese and butter production accounted for 33.2% and 24.9% of the total milk load. Other derivatives of milk were milk powder (4.6%), cream for direct consumption (11.5%) and other fresh or manufactured products (Eurostat, 2011). For Belgium, the consumption of dairy products per person on the family scale in 2004 is represented in Figure 2.2. Drinking milk (including buttermilk and flavored milk drinks) accounted for 65% of all dairy consumption, yoghurt for 11.6%, cream and butter for approximately 2.5% and cheese for 18.2%.

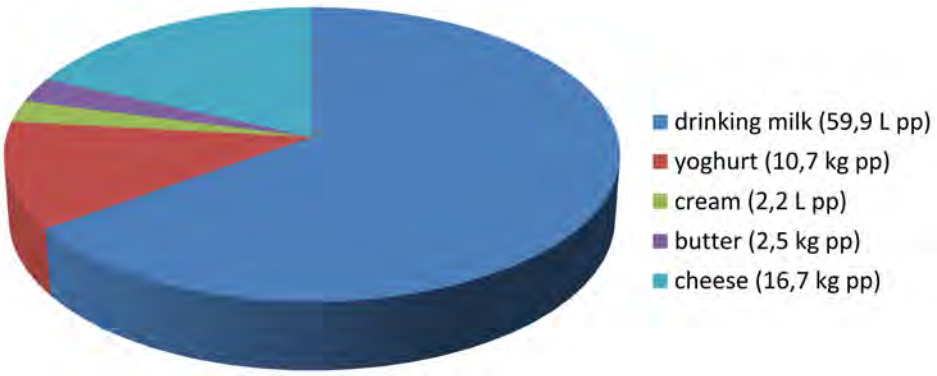


Figure 2.2: Consumption of dairy products in Belgium on family scale in 2004 (IKM, 2005).

2.3 Milk production

The history of milk production starts more than 6000 years ago, when man managed to tame ruminants, mostly cows, for their milk. It evolved from one cow per family and immediate intake

of the raw milk to a complex large-scale industrial process, with herds of thousands of animals on dairy farms, long storage of raw milk on that farm and in the dairy factory and intake up to several months after milking. In 2004 there were 15817 dairy farms in Belgium, but it is expected this number will continue to decrease as it did the last 25 years (66% since 1984). However, milk production remained stable. This clearly demonstrates the evolution of milk production from many small dairy farms to few large dairy farms (IKM, 2005). The production chain of milk will be discussed based on the processing of cow's milk in Belgium.

2.3.1 From grass to glass

The primary stage of the production process is milking itself at the dairy farm. The usual practice nowadays is to perform this automatically with a milking machine. Freshly harvested milk, with a temperature equal to the body temperature of the cow, namely 37°C, is immediately chilled to 4°C on the dairy farm to prevent bacterial outgrowth. Approximately 97% of all collected milk on the farm is meant for the dairy industry. The remaining 3% is processed and sold on the farm itself (IKM, 2005).

The collected milk is transported from dairy farm to dairy plant every two or three days, in specialized trucks, called RMO's (Rijdende Melk Ophaling). At the plant, fresh milk is mostly immediately processed. If this is not possible (due to cleaning procedures, change of production line or heavy milk supply causing overload of the plant) the incoming raw milk is stored in vertical cooled tanks with gentle agitation to prevent cream separation by gravity. A common procedure is then to heat the milk to a temperature just below pasteurization temperature to temporarily inhibit bacterial growth, a process called thermization. Dependant on the final destination of the milk, different production lines can be followed as presented in a simplified scheme in Figure 2.3. A first step in processing milk is the separation step where the fat phase, which has a lower density than the aqueous phase, is separated by centrifugal force. Furthermore, impurities present in raw milk such as hay and soil particles will be separated from the milk due to their higher density. After separation, the two resulting flows, cream (fat) and skim milk (low fat milk), are subjected to pasteurization, or for the milk, a more severe heating step, such as Ultra High Temperature (UHT)-treatment and sterilization, may be applied. These heating steps are introduced to kill and/or reduce bacterial contaminants and thus ensure milk quality and safety. UHT-treatment and sterilization may be preceded by a pasteurization step, or may be performed directly on the raw skim milk.

Pasteurized cream is then further processed to its derivatives, and also used to standardize the pasteurized skim milk. Standardization of skim milk involves adjustment of the fat content by re-adding cream to obtain a standardized fat content of the milk (products). This results in three types of milk commonly sold, namely whole milk (fat content of at least 3.5%), reduced-fat milk (fat content between 1.5 and 1.8%) and skimmed milk (fat content less than 0.3%). After standardization, the milk is subjected to homogenization, a mechanical process to disrupt the fat globules into much smaller ones, thus preventing creaming of milk. This heat-treated, homogenized, standardized milk is further processed into pasteurized UHT-treated or sterilized milk products.

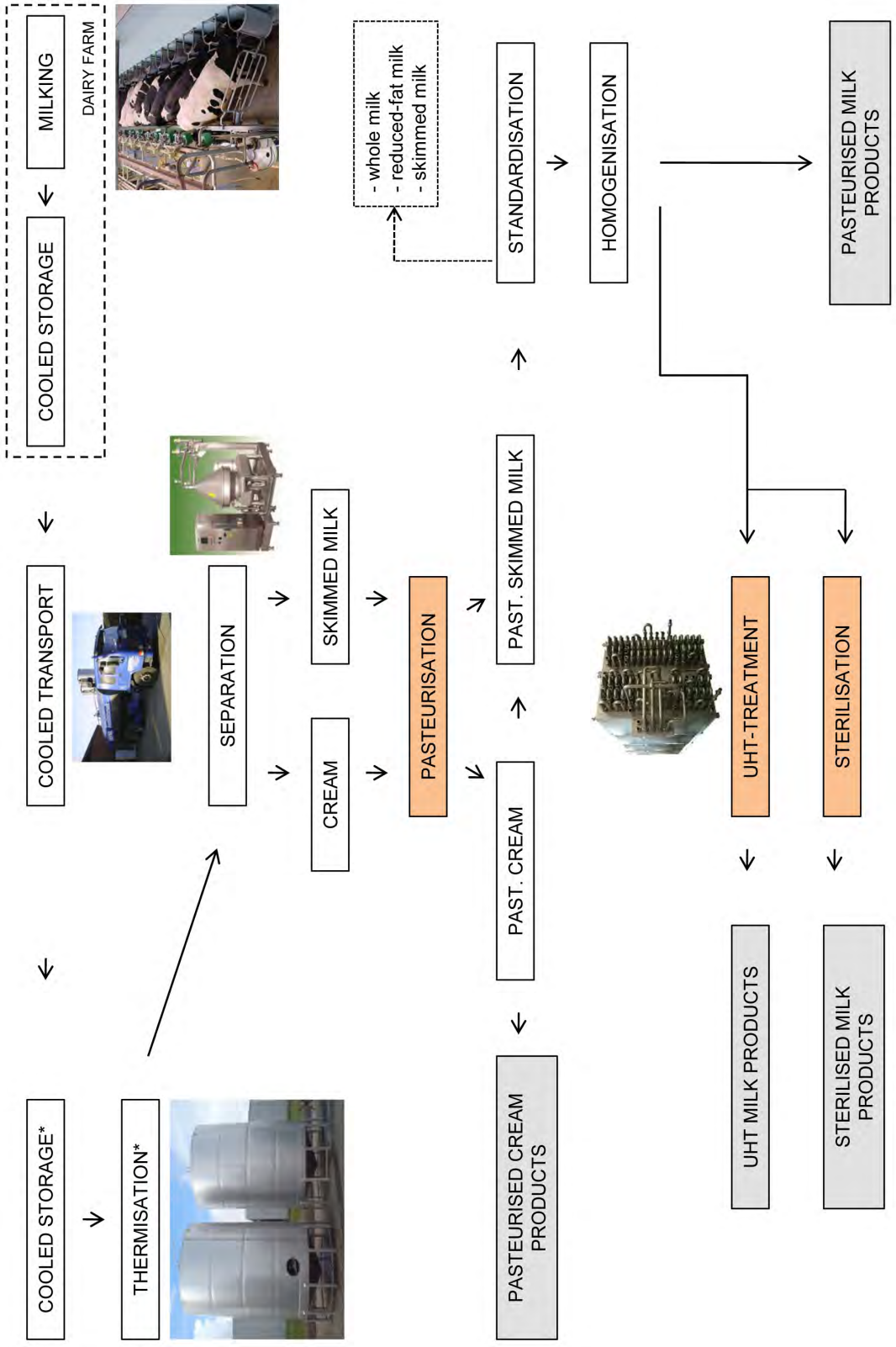


Figure 2.3: Simplified scheme of milk production and processing.

2.3.2 Milk products

Cream is produced by separating the fat fraction from raw milk, and has a fat content of around 40%. It is usually referred to as 'whipping' cream, as it can be whipped into a thick froth. Lower fat content cream (10 – 18% fat) also exists and is obtained by re-adding skimmed milk to the cream after separation. Other milk derivatives (butter, yoghurt and cheese) are dependant of the addition of a bacterial culture that gives the product its specific characteristics (acidity, flavor, aroma and consistency) during fermentation. Starter cultures are added to the milk after heat treatment and cooling to the appropriate inoculation temperature, which is around 20 – 30°C for mesophilic bacteria (*Lactococcus* and *Leuconostoc*), and around 42 – 45°C for thermophilic bacteria (*Streptococcus* and *Lactobacillus*). After an incubation period of 3 to 20 hours, during which bacteria mainly ferment lactose into lactic acid, the milk products are cooled and stored (Bylund, 1995). For yoghurt production, the milk is usually evaporated to obtain a higher dry matter content which results in a firmer yoghurt, and sweeteners or sugars, fruits and stabilizers may be added. Butter is made by subjecting pasteurized cream to a specific cooling procedure causing the fat to solidify. Bacteria are added to ferment lactose to lactic acid and to obtain the desired flavor and aroma for the butter. This ripening process is followed by the churning process, where cream is violently agitated to break down the fat globules, causing the fat to coalesce into butter grains. The remaining liquid, butter milk, is drained and butter is further worked to a continuous fat phase with a finely dispersed water phase. For cheese making, bacteria and rennet are added to the milk to coagulate the milk proteins, and render curd. The curd is cut into small pieces, and heated to separate the liquid, called whey, from the curd grains. The finished curd is then placed in cheese moulds, and further compressed, after which it is placed in a brine bath to promote taste, shelf life, firmness and rind formation. Finally, during ripening procedure (which takes at least 4 weeks), aromas are formed giving the cheese its typical flavor (Bylund, 1995). Milk powder is made by gently removing water from milk in two steps. A first step involves boiling of the milk under reduced pressure at low temperature, the evaporation step; a second step involves spraying the boiled milk in a fine mist into hot air to further evaporate moisture, and obtain a powder.

2.4 Milk and microbiology

Freshly drawn raw milk from the udder is considered a sterile fluid, meaning free of micro-organisms. However, contact with the environment implicates immediate contamination of milk through air, water, soil, faeces, grass, straw, feed and milking equipment (Waes, 1976; Fleet, 1990; te Giffel *et al*., 1995; Slaghuis *et al*., 1997; Vaerewijck *et al.*, 2001; Ellis *et al.*, 2007). Bacteria frequently recovered from milk belong to the genera *Pseudomonas, Arthrobacter, Alcaligenes, Achromobacter, Aeromonas, Brucella, Enterobacter, Serratia, Campylobacter, Chromobacterium, Flavobacterium, Bacillus, Clostridium, Corynebacterium, Streptococcus, Lactobacillus, Lactococcus, Leuconostoc* and *Microbacterium* (Bylund, 1995; Cempirkova, 2002; Chen *et al*., 2003a; Raats *et al*., 2011; Vacheyrou *et al*., 2011). Yeasts are also encountered, although less frequently, and belong to the genera *Candida*, *Pichia*, *Rhodotorula*, *Kluyveromyces* and *Saccharomyces* (Fleet, 1990).

Some of these micro-organisms are responsible for severe milk-borne diseases of humans, and thus negatively affect milk safety (Brown, 2000). For instance, *Brucella melitensis*, mostly found in goat's raw milk, can be responsible for brucellosis, a disease inducing fever, sweating, weakness,

anaemia, headaches, depression and muscular pain, and can persist from a few weeks up to months (Hamdy & Amin, 2002). *Campylobacter jejuni* is not very abundant in raw milk, but even in low concentrations, it is the most common bacteria to cause food-borne enteritis (Lovett *et al.*, 1983; Peterson, 2003; Heuvelink *et al.*, 2009). *Coxiella burnetii* might be responsible for the so-called Q-fever, a disease showing similar symptoms to influenza that can last up to one year. It is transferred from cattle to humans via the consumption of raw milk, or via the air (RIVM, 2011). Listeriosis, caused by *Listeria monocytogenes*, is an infectious disease characterized by fever and headache. Infections are rare, but particularly problematic for pregnant woman, as it might cause early delivery, infection or even death of the fetus. *Salmonella typhimurium* has been reported as the cause of a disease associated with high fever and diarrhea due to the consumption of recontaminated milk after pasteurization (Olsen *et al.*, 2004). *Mycobacterium tuberculosis* causes tuberculosis (an infectious long disease, often lethal) in cows and can be passed to humans through consumption of raw milk, where it exhibits similar symptoms. It was one of the most common contaminants of raw milk before the introduction of pasteurization of milk (products). Finally, *Staphylococcus aureus*, a frequent cause of intramammary infections of dairy cows, produces toxins that cause vomiting in humans (Akineden *et al.*, 2001). *Bacillus cereus* is also known for its toxin production causing diarrhea or vomiting, but the effects of bacilli on milk safety will be dealt with in detail in §2.4.1.2.

All the above mentioned diseases are mostly associated with consumption of raw milk. The frequency of these diseases decreased dramatically thanks to the establishment of heating procedures in the dairy industry to kill off these contaminants. Nonetheless, outbreaks still do occur and reports by the World Health Organization (WHO) commenting such outbreaks are published on a regular base (WHO, 2011).

Some micro-organisms do not cause infections in humans but can have a detrimental effect on milk and derived products as they can cause structural defects, as well as off-flavors and undesirable smell of milk products, thus negatively affecting milk quality, and making milk (products) unsuited for consumption. Main focus in this study will be on those micro-organisms that affect milk quality, the so-called milk spoilers.

2.4.1 Overview of milk spoilers

Yeasts are single cell fungi, whose fermentation capacities are widely appreciated in alcoholic beverages and bread production. However, yeast spoilage, although less frequently reported, can have detrimental effects on milk product quality. Hazardous impact on milk itself is low, because yeast growth is often inhibited by growth of psychrotrophic bacteria during cold storage of the milk. Nevertheless, there are strong indications that yeasts might survive pasteurization, and thus play a role further on in the production chain. Yeast contamination might also occur from production equipment surfaces, as often reported in yoghurt contamination. Spoiled milk products, such as yoghurt, cream and cheese are rendered yeasty in odour and flavor, and sometimes even structural defects are observed due to gas production during fermentation (Fleet, 1990).

Coliform bacteria (e.g. *Enterobacter*) are facultative anaerobes, mainly found in the intestines of warm-blooded animals. They ferment lactose to lactic acid and other organic acids, carbon dioxide and hydrogen and they break down milk protein causing off-flavor and smell. These bacteria are especially problematic in cheese making due to gas production which changes the texture of the cheese, a phenomenon known as 'blowing' (Figure 2.4).

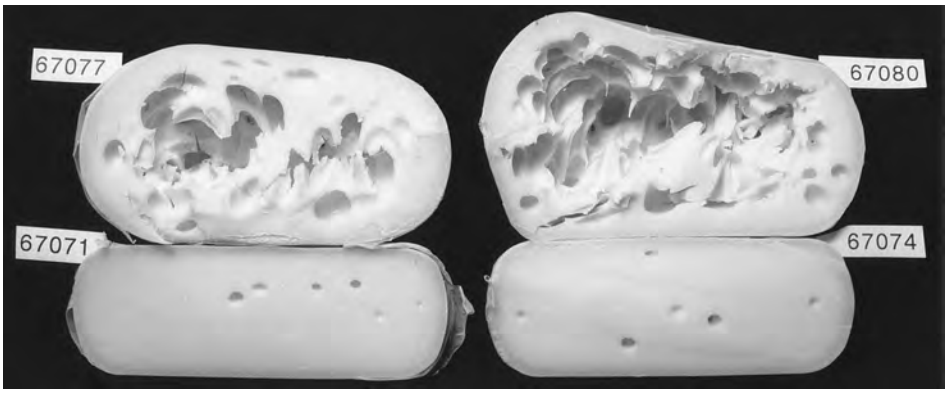


Figure 2.4: Blowing in cheese (top cheeses), an unwanted textural change due to gas production during fermentative growth of bacteria (Klijn *et al.*, 1995).

Butyric acid bacteria (e.g. *Clostridium*) are anaerobic spore-forming soil organisms that thrive extremely well in cheese. They produce large quantities of carbon dioxide, hydrogen and butyric acid during fermentation causing a ragged textured cheese with a rancid, sweetish taste, a defect also called 'blowing'. Clostridia are able to form endospores, cell forms that are extremely resistant to heat, implying these bacteria can't be destroyed by common heat treatment procedures. Alternatives are the addition of potassium nitrate or sodium chloride, both of which have an inhibitory effect on the growth of butyric acid bacteria. However, as potassium nitrate has a carcinogenous effect, its use is banned in many countries, and the concentration of added sodium chloride should not be too high because it can also inhibit the growth of lactic acid bacteria that are beneficial during cheese production.

Putrefaction bacteria produce protein-splitting enzymes (= proteases) and fat-degrading enzymes (= lipases). Sometimes a rennet-like enzyme is produced that causes coagulation of the milk without souring it, a phenomenon known as 'sweet curdling'. Some pseudomonads and bacilli belong to these putrefaction bacteria and are the main study object of this thesis. Their effects on milk quality will be discussed in detail below.

2.4.1.1 Pseudomonads in the dairy industry

The dairy production industry is a typical example of a cold-chain food industry, where a constant temperature between 2 to 8°C is maintained in order to suppress bacterial outgrowth. In Figure 2.5 the impact of temperature on bacterial outgrowth in milk is presented, and it clearly shows that milk not properly cooled allows rapid growth of bacteria.

However, this cold chain does offer a selective advantage for bacteria that thrive well in these cold temperatures, the so-called psychrotrophic bacteria. Psychrotrophic bacteria are defined as bacteria that are able to grow at 7°C or less, regardless of their optimal growth temperature (Suhren, 1989). Furthermore, due to prolonged storage times of raw milk at the farm before processing (which is nowadays common practice in modern dairy farming), this effect became even stronger. Approximately 65 - 70% of these psychrotolerant isolates from raw milk are assigned to the genus *Pseudomonas* (Garcia *et al.*, 1989). However, recent studies, also applying culture-independent techniques to map psychrotolerant bacterial diversity in raw milk, indicate this number to be an overestimation, and attribute 45 - 63% to the genus *Pseudomonas* (Raats

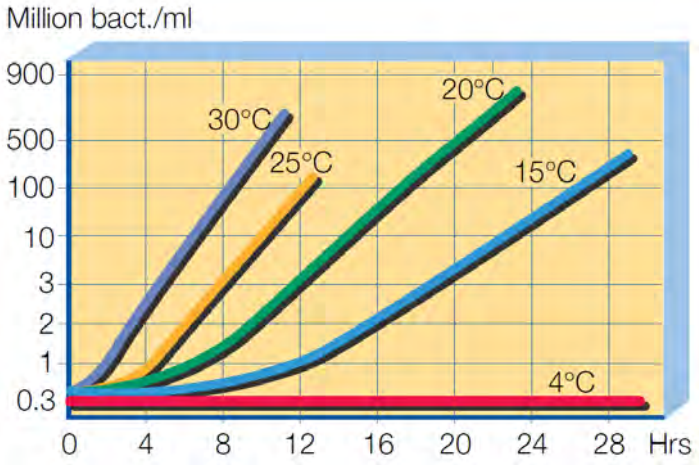


Figure 2.5: Influence of temperature on bacterial outgrowth in milk (Bylund, 1995).

et al., 2011), which is of course still a considerably high number. Pseudomonads can thus be considered as the predominant psychrotolerant micro-organism in raw milk (Adams *et al.,* 1975; Sørhaug & Stepaniak, 1997). Other psychrotolerant species found in raw milk were *Aeromonas*, *Acinetobacter*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium*, *Serratia*, *Streptococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Clostridium*, *Corynebacterium*, *Micrococcus* and *Microbacterium* (Cousin, 1982; Champagne *et al.*, 1994; Lafarge *et al.*, 2004; Raats *et al.*, 2011; Vacheyrou *et al.*, 2011).

These psychrotolerant bacteria have soil, water, animal and plant material as their natural habitat (Cousin, 1982). Plant materials that are commonly used for animal feed (*e.g.,* grass, hay) may contain over 10^8 psychrotolerant bacteria per gram (Thomas, 1966) and the bedding materials on which cows are housed in the winter show a count of 10^9 psychrotolerant bacteria per gram on average (Cousins & Bramley, 1981). The milking equipment, storage tanks and milk tankers are generally considered the major contamination sources for psychrotolerant bacteria (Cousin, 1982). The equipment is mainly made from stainless steel, glass, plastics and rubber. The use of untreated water supplies for the final rinse of the milking equipment may contribute to contamination of raw milk with psychrotolerant microorganisms, dominated by *Pseudomonas*, *Achromobacter*, *Alcaligenes* and *Flavobacterium* (Thomas, 1966). Because psychrotolerant bacteria isolated from water are proven to be vigorous producers of extracellular enzymes and grow rapidly in refrigerated raw milk, contaminated water can be considered an important source of milk spoilage bacteria regardless of the possible low initial contamination level (Cousin, 1982). A likely reservoir from which contamination of these water supplies originate, is the soil (Thomas, 1966). Even though proper cleaning of the milking equipment effectively reduces contamination from these sources, the rubber materials used to connect different pipelines are quite susceptible to deterioration caused by a combination of high cleaning temperatures and strongly oxidizing products in the disinfectants (used to kill off a considerable fraction of spores). The resultant microscopic cracks and cuts form an ideal attachment place for the formation of biofilms (Morse *et al.,* 1968). These multispecies structures (harboring among others *Bacillus* and *Pseudomonas* species) often possess greater combined stability to mechanical treatments and resilience to chemical sanitizers than do the component individual species (Simões *et al.,* 2009).

The explanation for the predominance of pseudomonads in cold stored raw milk is two-fold. First, their short generation times at 0 - 7°C allow rapid growth, thus overwhelming other bacteria

present (Chandler & McMeekin, 1985). Secondly, *Pseudomonas* species are able to colonize the processing line by adhering strongly to the surface of milk processing equipment. This may enable them to persist unless removed by proper cleaning and sanitizing procedures (Cousin, 1982; Bishop & White, 1986).

Even though *Pseudomonas* species are easily inactivated by various heat treatments, an important fraction of the spoilage enzymes produced during growth, remains active because of their resistance to high temperatures. *Pseudomonas* species are known to produce heat stable spoilage enzymes that retain significant activity even after UHT processing and production of milk powders (Chen *et al.*, 2003b). These enzymes can then cause spoilage and structural defects in pasteurized and UHT-treated milk (products). It needs to be noted that proteolytic psychrotolerant bacteria can also produce heat-labile proteases (Richardson & Te Whaiti, 1978; Griffiths *et al.*, 1981), but since these enzymes are readily inactivated by heat treatment applied, they are of no particular importance to the dairy industry. Although optimal synthesis of heat-stable enzymes mostly occurs at 20 - 30°C, considerable synthesis occurs even at lower temperature, for example, production of extracellular protease by *Pseudomonas fluorescens* at 5°C was 55% of that produced at 20°C (McKellar, 1982). Furthermore, the enzymes remain active at temperatures well under their optimum temperature, for instance even at 2°C for *P. fluorescens*. Various types of lipases exist that are produced by members of the genus *Pseudomonas*, as listed by Jaeger and Eggert (2002), but the majority of *Pseudomonas* species produce only one type of protease that is thought to be responsible for milk spoilage, the alkaline metalloprotease AprX (Liao & McCallus, 1998; Kumeta *et al.*, 1999; Chabeaud *et al.*, 2001; Marchand *et al.*, 2009b).

P. fluorescens has traditionally been accepted as the most important spoilage organism (Jayarao & Wang, 1999; Dogan & Boor, 2003). Nowadays, the importance of *P. fluorescens* is under debate as it seems to be overestimated in the past due to an incorrect identification (Marchand *et al.*, 2009a). Marchand and co-workers (2009a) identified *P. lundensis* and *P. fragi* as the most important proteolytic spoilers in raw milk based on a thorough identification of the strains, using a polyphasic approach. Studies discussed later on in this thesis acknowledge the predominant presence and spoilage capacity of *Pseudomonas* species closely related to *P. fluorescens* and *P. gessardii*, but clearly distinct from the *P. fluorescens* type strain.

2.4.1.2 Bacilli in the dairy industry

Members of the genus *Bacillus* and closely related genera, *Bacillus sensu lato* (s.l.), further referred to as aerobic endospore-formers, show a wide diversity in metabolism and habitat, but there is one common trait that makes them particularly annoying for the dairy industry, namely their capacity to form endospores. These special cell forms are extremely resistant to heat, dehydration, UV-radiation and chemicals (Brown, 2000; Setlow, 2006). They are formed when environmental conditions are harsh (mainly nutrient depletion) and can stay dormant for up to several years (Nicholson *et al.,* 2000). When environmental conditions again turn favorable, the endospore germinates and becomes a vegetative cell, capable of growth and reproduction (Setlow, 2006). A schematic overview of endospore formation is given in Figure 2.6.

Aerobic endospore-forming bacteria are the cause of many problems in the dairy industry concerning food safety and product quality. These problems arise due to the production of spoilage enzymes, toxins or through interference with cheese preparation due to gas formation or nitrate reduction. Aerobic endospore-formers contaminate raw milk through both the vegetative form

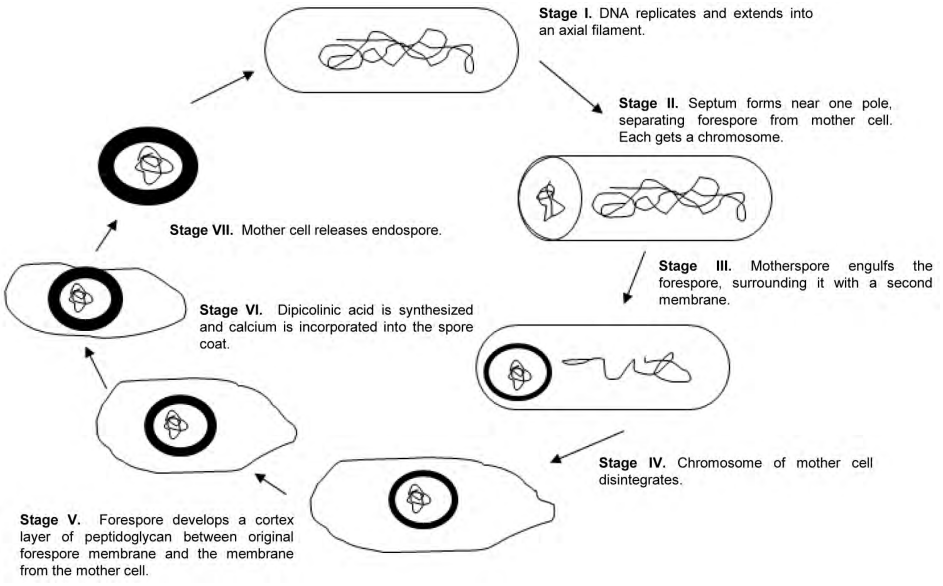


Figure 2.6: Schematic overview of endospore formation (redrafted from Slonczewski & Foster, 2011).

and the heat resistant endospore structure, and it is specifically this endospore configuration that poses problems. A lot of studies have been performed on the contamination sources of these aerobic endospore-formers (most research focussed on *Bacillus cereus*), but there is no consensus as to their origin in raw milk (Van Heddeghem & Vlaemynck, 1992).

Very diverse contamination sources are indicated: soil, bedding, feed, hay, dust, air, faeces, dirty teats or teat cups and milking equipment (De Jonghe *et al.*, 2011a). Once these endospores have contaminated the milk, they cannot be destroyed by conventional heating processes - which are applied to destroy bacteria and thus assure safety of the product - such as pasteurization (Andersson *et al.*, 1995b). Some endospores are even known to survive Ultra High Temperature (UHT)-treatment (Pettersson *et al.*, 1996; Scheldeman *et al.*, 2005; Scheldeman *et al.*, 2006). After the sterilization step, endospores can germinate into vegetative cells, a process which is even activated by heat, and subsequently grow in the milk or derived products, possibly causing problems mainly due to enzyme or toxin production. Furthermore, their growth is not restricted anymore by other micro-organisms because most of them have been killed off during the heating step (Andersson *et al.*, 1995b).

Spoilage enzymes such as proteases, lipases and lecithinases are responsible for off-flavors and structural defects in pasteurized milk (Meer *et al.*, 1991). The most important spoilage organism in the dairy industry is undoubtedly *B. cereus*, causing 'bitty cream' (floating clumps of fat) due to lecithinase activity and 'sweet curdling' (curdling of the milk without acidification) due to protease activity (Heyndrickx & Scheldeman, 2002). The latter also causes bitter and rotten off-flavors, whereas fruity and rancid off-flavors are caused by lipolytic activity (Meer *et al.*, 1991). Furthermore, these enzymes have also been found in *Bacillus* species other than *B. cereus*, and appear to have heat resistant characteristics (Chen *et al.*, 2003a).

Another important trait of *B. cereus,* and some other bacilli too, is their ability to grow at the storage temperature of milk (4 - 7°C), which mainly determines the shelf life of pasteurized milk and derived milk products (Meer *et al.*, 1991; te Giffel *et al.*, 1995). Contrary to these

psychrotolerant bacteria, some thermophilic species that thrive well at temperatures above 40°C can form biofilms, initiated by the attachment of their spores to dairy plant equipment. These biofilms are difficult to remove, and can recontaminate every fresh load of milk that passes the production line (Andersson *et al.*, 1995b).

Spoilage caused by aerobic endospore-formers is mostly limited to pasteurized milk. Spoilage of UHT and sterilized milk only occurs occasionally and can mostly be attributed to recontamination with proteolytic *Bacillus* species or Gram-negative spoilage organisms during the filling step (Westhoff & Dougherty, 1981; Schröder, 1984; Foschino *et al.*, 1990). Despite this fact, there are problems with aerobic spore-formers in UHT-milk, not necessarily related to spoilage but more to a non-achievement of sterility standards. EG-regulation 92/46 requires that the number of colonies counted from incubated (30°C during 15 days) unopened UHT-cartons, does not exceed 10 colony forming units (cfu) per 0.1 mL. This requirement could not be met in Italy, Austria (1985) and Germany (1990) and later-on in other European (France, Benelux, Spain) and non-European (Mexico, USA) countries (Hammer *et al.,* 1995; Scheldeman *et al.,* 2006). Hammer and co-workers (1995) linked this problem to highly heat resistant spores (HRS) of a mesophilic micro-organism described later as *Bacillus sporothermodurans* (Pettersson *et al.*, 1996). Since this organism does not have any pathogenic or toxic activity (Hammer *et al.*, 1995; Hammer & Walte, 1996), and also only rarely causes minor spoilage defects in the form of a slight pink discoloration (Lembke, 1995; Klijn *et al.*, 1997), this is not so much an issue from a hygienic point of view, but all the more from a technological perspective, being a mere sterility issue.

Thermotolerant bacilli, and more specifically those belonging to the genus *Geobacillus*, are also specifically related to spoilage of milk powders. The high temperatures applied during milk powder production selectively create an optimal environment for these endospore-formers because members of this genus are highly thermophilic with optimal growth temperatures between 40 and 65°C. Initial contamination of the milk powder production unit probably occurs via pasteurized milk that contains low numbers of thermophilic cells and endospores. Both cell types can attach to the equipment surfaces where conditions are favorable to promote growth and start bio-film formation. Every fresh load of pasteurized milk gets contaminated by sloughing parts of the bio-film or shedding of individual cells. Again, this is not so much a problem for safety of the milk because these geobacilli are not pathogenic but their presence is unwanted because they are an indicator of plant hygiene during processing. Furthermore, the endospore forms are able to survive in the final product, and might germinate when the milk powder is reconstituted. Subsequent growth might eventually result in enzyme production and thus spoilage (Rueckert *et al.*, 2005; Rueckert *et al.*, 2006; Scott *et al.*, 2007). Other species involved in this type of spoilage are *Anoxybacillus*, *Bacillus licheniformis* and *Bacillus pumilus* (Rueckert *et al.*, 2004).

Another issue regarding aerobic endospore-formers in milk is the possible production of toxins affecting milk safety. *Bacillus cereus* is a well known food pathogen that can cause two types of food poisoning syndromes: an emetic and a diarrhoeal type. The former is caused by a small ring-formed heat-stable dodecadepsipeptide named cereulide. The diarrhoeal syndrome is caused by vegetative cells that are thought to produce a variety of heat-labile enterotoxins in the small intestine (Granum, 2002). Three pore-forming cytotoxins have been associated with diarrhoeal disease: haemolysin BL (Hbl) and nonhaemolytic enterotoxin (Nhe), which are both two homologous three-component toxins, and cytotoxin K (CytK), a single component cytotoxin. Two different forms for the latter have been described, the highly cytotoxic CytK-1 and the moderate cytotoxic CytK-2 variant, encoded by *cytK-1* and *cytK-2* genes (Fagerlund *et al.*, 2004). The CytK-1 variant has thus far only been detected in a limited number of *B. cereus* strains, that have been

proposed to form a novel bacterial species, for which the name "*Bacillus cytotoxis*" or "*Bacillus cytotoxicus*" is suggested (Lapidus *et al*., 2008).

Although *Bacillus* species other than *B. cereus* have been incriminated as food poisoning agents, the link between toxin production and foodborne illness has not been fully established. Increasing evidence for the production of both heat-stable and heat-labile toxins is becoming apparent through cellular assays that confirm both production and functionality of the toxins. In these assays, strains identified as *Brevibacillus brevis*, *Bacillus circulans*, *Bacillus lentus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens* are shown to produce heat-labile enterotoxins (Beattie & Williams, 1999; Lindsay *et al*., 2000; Phelps & McKillip, 2002). Strains identified as *B. licheniformis*, *B. pumilus*, *Bacillus mojavensis*, *B. subtilis*, *Bacillus simplex*, *Bacillus firmus, Bacillus megaterium*, *B. brevis*, *B. circulans* and *B. lentus* can produce novel heat-stable toxins, resembling the physico-chemical characteristics of cereulide (Beattie & Williams, 1999; Salkinoja-Salonen *et al.*, 1999; Mikkola *et al*., 2000; Suominen *et al*., 2001; From *et al*., 2005; Taylor *et al.*, 2005; From *et al*., 2007a; From *et al*., 2007b). Some of these toxins, heat-labile as well as heat-stabile, have been characterised as surfactin isoforms, namely lichenysin (described in *B. licheniformis* (Salkinoja-Salonen *et al.*, 1999; Mikkola *et al*., 2000)) and pumilacidin (described in *B. pumilus* (Suominen *et al*., 2001)). The surfactin superfamily is a family of structurally diverse, low molecular weight cyclic lactonic lipopeptides, that is well-described in strains of the *B. subtilis* group.

A third concern regarding aerobic spore-forming bacteria is interference with cheese production through nitrate reduction, whether or not in combination with gas production during fermentation. Nitrate is often added to raw milk during the cheese preparation process to prevent growth and germination of anaerobic spore-forming species belonging to the genus *Clostridium*. Specifically *Clostridium tyrobutyicum* can grow fermentatively with gas production, thereby causing 'late blowing' defects in Gouda type cheeses (Klijn *et al*., 1995). The reduction of nitrate to nitrite or nitrogenous gasses, is a process carried out by many *Bacillus* species (Ternström *et al.*, 1993; Verbaendert *et al.,* 2011), thus nullifying the growth inhibitory effect of supplemented nitrate. Recently, it became apparent that also gas producing aerobic spore-formers, namely *Paenibacillus polymyxa* and *Paenibacillus macerans*, can be directly implicated in this type of spoilage in Argentinian Cremoso and Mozarella cheeses (Quiberoni *et al*., 2008).

2.4.2 Enzymatic activity in milk

The different constituents of milk are a desired target for spoilage enzymes. These enzymes can either have an indigenous nature, or they can enter the milk through microbial contamination. Both groups of enzymes may differ in their specificity towards the different milk constituents (Nielsen, 2002).

2.4.2.1 Proteolysis of milk and derived products

Different enzymes can be responsible for proteolytic decay: indigenous proteolytic enzymes (= plasmins) and proteases of microbial origin. The effect of the two protease types in UHT milk is quite distinct: bacterial proteases lead to the formation of a curd or a gel with custard-like consistency throughout the whole milk sample (Hardham, 1998), while the native milk protease plasmin causes a creamy layer on the surface of the milk which eventually thickens to form a curd-like layer (Harwalker, 1982). Gels caused by bacterial proteases have a tighter protein network

with thicker strands and contain more intact casein micelles and micelle aggregates than plasmin-initiated gels (Fox, 1981; Harwalker, 1982); plasmin and bacterial proteases also show different affinities for the individual caseins. Plasmin in milk mainly occurs as the inactive precursor plasminogen (Prado *et al.*, 2006), and can be activated by plasminogen activators, among which of bacterial origin (Kohlmann *et al.*, 1991). Plasmin is quite heat-stable, and is known to survive pasteurization processes (Metwalli *et al.*, 1998) and even UHT-treatment (Alichandis *et al.*, 1986). Nevertheless, it is less heat-resistant than *Pseudomonas* proteases that retain 73% of their activity when conditions are applied that completely destroy plasmin (Marchand *et al.*, 2008).

For *Bacillus* enzymes a similar heat resistance pattern is suspected as for *Pseudomonas* enzymes, based on thermal stability tests in buffer systems (Chen *et al.*, 2003a). Proteases produced by bacteria are usually extracellular endopeptidases that can be classified as metallo- and serine proteins (Cousin, 1989; Chen *et al.*, 2003a). The degradation of milk proteins, mainly caseins, through proteolysis may have beneficial effects and even be essential to obtain desirable qualities in dairy products, as is the case for flavor development and texture changes during cheese ripening. However, uncontrolled or unwanted proteolysis can adversely affect food quality: proteases are known to cause off-flavors because of the formation of 'bitter peptides' (Ney, 1979).

It is generally agreed that whey proteins are not degraded by bacterial proteases in raw milk. There are some reports of minor whey degradation, but never to the extent as for caseins and it usually takes longer to occur. Their secondary and tertiary structure and globular nature probably makes it difficult for microbial proteases to degrade them (Cousin, 1989).

Even though bacterial proteases can have substantial activity at low temperatures and at the pH of milk (pH 6.7), they do not often cause noticeable off-flavors in pasteurized milk. This may be explained by the short storage period that does not allow more advanced proteolysis which is required to obtain these small peptides (Mottar, 1989). The shelf life of UHT-treated milk on the other hand, seems to be mainly limited by the action of heat resistant proteases during storage (Mottar *et al.*, 1979): at first, a bitter flavor may occur (McKellar *et al.,* 1984), and finally the deterioration can lead to gelation (Law *et al.*, 1977) caused by formation of a three-dimensional matrix of aggregated β-lactoglobulin-κ-casein-complexes (Datta & Deeth, 2001).

Proteolysis does not only influence the flavor of UHT-milk, it also has an impact in the dairy plant itself by fouling of the UHT-equipment. Fouling is the formation of deposits mainly composed of proteins and minerals on the walls of the heat exchangers. This is problematic because fouling reduces heat transfer efficiency which might result in insufficient kill-off of bacteria. Other side effects are product losses and increased cleaning costs. Additionally, these fouling crusts might present an ideal habitat for bacterial biofilm formation and thus contamination of heat-treated milk (de Jong, 1997; Bansal & Dong Chen, 2006). Furthermore, proteolysis might be responsible for sedimentation of coagulated milk proteins on the bottom of the UHT-equipment (Kelly & Foley, 1997), which in turn can cause severe obstruction of the production process through sediment burning-on on the heat surfaces implicating severe cleaning procedures or sometimes even a temporary shut-down of the process (Bylund, 1995).

Proteases from bacterial origin may have multiple effects on cheese production. Loss of cheese yield by breakdown of casein is usually associated with increased storage time of the milk and a high psychrotolerant count (Mottar, 1989; Yan *et al.*, 1983). Cheese quality can be affected during storage by the action of bacterial proteases, resulting in an increased growth of starter cultures due to greater accessibility of nitrogen sources; however, this effect is rather minor since these enzymes are usually removed in the whey during cheese production - unlike bacterial lipases that

are concentrated along with the fat in the curd (Fox, 1981). Texture problems have also been associated with proteolysis, but only with milk with a high bacterial count before pasteurization (Law, 1979).

Problems with the quality of fermented milk products due to proteolytic activity have rarely been reported, probably due to their high acidity and storage temperature below 10°C (Law, 1979).

2.4.2.2 Lipolysis of milk and derived products

Lipolytic enzymes is a term used to describe different classes of enzymes, including esterases (or carboxylases), true lipases (or triacylglycerol acylhydrolases) and phospholipases. Lipases are enzymes that catalyse the hydrolysis of carboxyl ester bonds present in triglycerides, the major lipid component of milk. The products of this so-called lipolysis are free, non-esterified fatty acids, and partial glycerids and in some cases even glycerol.

In milk, lipases can not readily access the fat because it is encapsulated by the milk fat globule membrane. Lipolysis can be categorized into two types: spontaneous and induced. Spontaneous lipolysis acts on the free fat molecules present in fresh raw milk, but the biochemical basis of this process is poorly understood (Deeth & Fitz-Gerald, 1983). Induced lipolysis is initiated by disruption of the milk fat globule membrane, so that the enzyme can easily access the fat fraction of milk. This can happen either mechanically, due to agitation, pumping, stirring and freezing / thawing of milk, or by enzymatic means, such as by phospholipases (Bylund, 1995).

As for proteases, two types of lipases can be differentiated, namely indigenous and bacterial lipases (Chen *et al.,* 2003b; Olivecrona *et al*., 2003). The importance of indigenous lipase in milk spoilage is limited because these enzymes are heat-labile and are thus destroyed during pasteurization or more severe heat treatments (Farkye *et al*., 1995). In contrast, bacterial lipases are heat-stable, and in reality appear not to be hindered by the milk fat globule membrane. The mode of access and mechanism of this activity are not yet known (Deeth & Fitz-Gerald, 1994), but a possible explanation is the action of accompanying enzymes such as phospholipases (Mabbit, 1981). The latter enzymes are produced by many types of bacteria including members of the genera *Pseudomonas, Bacillus* and *Clostridium* (Cousin, 1989), and are able to withstand various heat treatments (even UHT-treatment) of milk (Deeth & Fitz-Gerald, 1983; Griffiths, 1983; Koka & Weimer, 2001).

A specific type of phospholipases, lecithinase, causes bitty cream and is produced by members of *Bacillus*, *Clostridium*, *Listeria* and *Yersinia* (Billing & Cuthbert, 1958; Nakamura *et al*., 1969; Toora *et al*., 1989; Kathariou & Pine, 1991). This defect typically appears as small cream flakes floating on top of the milk (Barkworth, 1958). Bacterial lipases are mainly produced by members of *Bacillus, Pseudomonas* and *Burkholderia*, and have a catalytic centre similar to that of the serine proteases.

Although lipolysis is important for the development of cheese flavor, excessive lipolysis can cause rancid off-flavors in cheeses with a long shelf-life, possibly already after a period of ripening of 2 to 3 months (Cousin, 1982). Also, lipolysis is linked to some technical consequences in cheese production, as the released free fatty acids are known to inhibit starter bacteria such as *Streptococcus lactis* and *Streptococcus cremoris*, thus retarding acidification (Deeth & Fitz-Gerald, 1983). Free fatty acids have strong flavors, which are mostly considered undesirable (Scanlan *et al*., 1965). Several terms have been used to describe these lipolytic and oxidized flavor defects, such as rancid, bitter, goaty, soapy, unclean and butyric (Shipe *et al*., 1978).

2.4.3 Control mechanisms of the dairy industry

It can be concluded that milk is prone to bacterial spoilage due to its composition. This traditionally urged the dairy industry to apply good manufacturing practices at all different production levels. Four factors are important in the pursuit for a better microbiological quality of raw milk throughout the dairy chain: *i*) the amount of bacteria that are present in the milk, *ii*) the sort of bacteria, *iii*) storage temperature and *iv*) storage time. Hygiene in all aspects of milk handling, strict maintenance of refrigeration at 4°C or lower, minimization of the storage period of raw milk, combined with a suitable method to remove or kill as many micro-organisms as possible and followed by an effective Hazard Analysis and Critical Control Point (HACCP) system, are therefore important parameters of primary concern in the dairy industry.

2.4.3.1 Farm level

Bacterial contamination of raw milk can originate from various sources and is inevitable. Though, presence of bacteria can be kept to a minimum by handling good hygienic practices, and this starts at the dairy farm. General guidelines have been approved by the European Union and have been recorded in EC legislation 178_2002. The risk of contamination can be reduced by keeping the animals clean, especially the udder, as well as their environment (ventilated cowhouses, sufficient space for grazing and lying, well maintained passage ways and fields). Animals should be in a healthy condition. Milking equipment should be clean, and a cleaning in place (CIP) procedure should be performed after every use. Persons handling the milking equipment should maintain a high personal hygiene. Milk storage tanks at the farm should have a temperature at or below 4°C, and freshly harvested milk (app. 37°C) poured into the milk tank should reach that low temperature within 2 hours.

Quality of raw milk in Belgium is subject to firm control by 'MCC-Vlaanderen' in Flanders and 'Comité du Lait' in the Walloon provinces. These agencies sample each milk delivery and check six parameters on a regular base, namely germ count, somatic cell count, presence of antibiotics, purity, freezing point and determination of fat and protein content. Milk that does not fulfill the postulated requirements (Table 2.3) is withdrawn from production (MCC Vlaanderen, 2010). Although the importance of endospore-formers and psychrotrophic bacteria is clearly demonstrated above, there are no requirements for spore count, and psychrotrophic germ cell count.

parameter	**requirement**
germ number	= 100000 cells per mL
somatic cell count	= 400000 cells per mL
presence of antibiotics	none
purity	< 0.5 mg/L
freezing point	= -0.53°C

Table 2.3: Requirements for high quality milk in Belgium (MCC-Vlaanderen, 2010).

The yearly report of MCC Vlaanderen indicates the mean germ number fluctuates around 12000 cells per mL for the last three years (Figure 2.7), with a total average in 2010 of 11260 germ cells per mL raw milk. This is far below the limit of 100000 cells per mL and dairy farms in Flanders thus clearly deliver milk of high microbial quality.

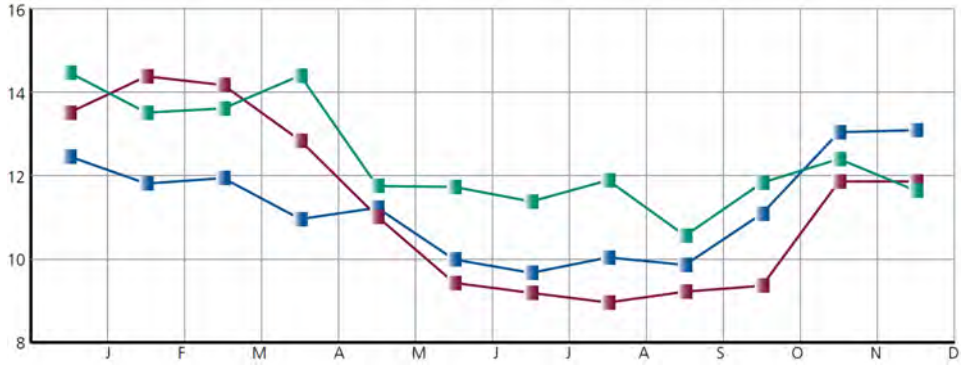


Figure 2.7: Mean germ number (Y-axis × 10000 cells per mL) for 5657 dairy farms in Flanders represented for each month (X-axis) in 2010 (blue), 2009 (red) and 2008 (green) (MCC Vlaanderen, 2010).

Mean somatic cell count for 2010 was 227545 somatic cells per mL raw milk. Only 0.05% of all analyses (749332) scored positive for presence of antibiotics; only 0.57% of all analyses (732684) scored a deviating freezing point value and 0.03% of all analyses (67793) failed for the purity test (MCC-Vlaanderen, 2010). It can thus be concluded that milk produced in the region of Flanders is high quality milk, largely fulfilling the European legislations.

A critical point, however, in modern dairy farming is the longer cold storage of raw milk at the dairy farm in storage tanks. In earlier days, milk was delivered daily or even twice a day to the dairy factory, but as dairies are fewer and larger, transport takes longer, and delivery to the dairy is usually carried out once every two days. This implies the storage time of raw milk at the farm is longer (72 hours at the most), which has its implications for the outgrowth of certain bacteria that thrive at those cold storage temperatures. Especially *Pseudomonas* species seem to benefit from this evolution.

2.4.3.2 Transport level

At least once every 72 hours the farm storage tank is emptied and transported to the dairy plant by specialized trucks (RMOs). RMO drivers are qualified to take milk samples that will be analyzed by the MCC-Vlaanderen or by the Comité du Lait, on each farm, and they also perform a quick organoleptic quality test by checking odour and appearance of the raw milk. Tanks of RMO-trucks are insulated to ensure raw milk keeps the same temperature as in the storage tank at the dairy farm. However, these truck tanks do not have a cooling system installed and it is thus of utmost importance to limit the time the raw milk is transported from the dairy farm to the dairy plant. Therefore, all farms are within a span of maximum 100 km from the dairy plant. After the last round at the end of the day, the RMO is cleaned (CIP) with a caustic soda solution, and rinse water is checked for microbial growth via an ATP-measurement on a regular base. The latter relies on the emission of light when the enzyme luciferase comes into contact with ATP, the cellular energy molecule. The amount of light measured is directly proportional to the amount of ATP, and thus to biochemical activity of bacteria, in other words to bacterial growth (Hansen *et al.*, 2008). Once every month, the RMOs are cleaned with nitric acid, to get rid of mineral sediments.

2.4.3.3 Dairy plant level

Every fresh load of raw milk (maximum volume of one RMO is 20000 L) is subjected to three quick tests, an antibiotics test, a pH and a temperature measurement. Milk is accepted if no antibiotics are present, if pH is between 6.65 and 6.80 and if temperature is below 10°C. If content of the RMO tank deviates for one of these three parameters, raw milk is destroyed. This test system of RMOs is a first barrier at the dairy plant, and is performed in three minutes. Because milk that arrives at the dairy plant is 2 or 3 days old, time is of utmost importance, and it is impossible to implement additional long-term tests before accepting milk from a RMO. Milk that arrives needs to be processed immediately, and normally time span between the arrival of fresh raw milk at the plant, and release of final products is about 12 hours. The pH test gives an idea about microbial quality of the milk because microbial growth will most likely result in a lowering of pH. For instance, growth of the pathogen *B. cereus* in sufficient amounts causes a shift of pH to 6.1 – 6.2. A first real 'defense mechanism' at the dairy plant is of course heat-treatment, of which the main purpose is to obtain a prolonged shelf-life for the end product by reducing the activity of micro-organisms. In general, milk (products) is heated to achieve this aim, however, severe heating mostly causes a loss of taste and/or nutritional value. A balance needs thus to be found between sterility and quality of the end product. The following heat treatments are commonly used in dairy industry (Table 2.4) (Bylund, 1995; Smit, 2000).

A valuable complement to these heat treatments might be centrifugation to separate micro-organisms from the milk with a so-called Bactofuge®. Micro-organisms, especially spores, have a higher density than milk, making the Bactofuge® an efficient tool to get rid of bacterial spores (efficiency of 95% for bacterial spores), thus extending the shelf-life of pasteurized products with three days. Inclusion of the Bactofuge® in the production process however is expensive, and the waste stream might be as high as 5%, a loss which often out-competes the benefit obtained from the extended shelf-life of milk products. Bactofugation is more effective for spores than for vegetative cells due to the higher density of spores (Smit, 2000). Bactofugation is mostly integrated in cheese production lines, where milk of very high microbial quality is required (Schutz *et al.*, 1990).

After pasteurization and standardization (see Figure 2.3), when milk is stored in cooled storage tanks before further processing, a second control barrier is built in. A sample of pasteurized milk from these tanks is mixed with methylene blue and incubated at 37°C. Methylene blue is a dye showing a blue color in oxidized form, and colorless in the reduced form. It is added in the oxidized form to the milk, and serves as an electron acceptor for bacteria, being reduced into the colorless form. The higher the number of bacteria present, the shorter the time required to reduce the dye. Quick discoloration of milk mixed with methylene blue indicates high bacterial activity. If a discoloration occurs within 2 hours, this milk should be immediately processed, preferably to the UHT or sterilization production lines, and if immediate processing is not possible, milk should be subjected to a second pasteurization step. If discoloration occurs before 8 hours, this again indicates milk should be processed as quick as possible.

For the UHT production line, all actions, from UHT-treatment until packing, are performed in a sterile environment thus ensuring a sterile product. The packing material (often milk cartons) is made at the dairy plant and sterilized before use with hydrogen peroxide. The application of a sterile circuit is a second 'defense mechanism' to prevent bacterial outgrowth at the dairy plant.

A third 'defense mechanism' is cleaning all equipment (CIP) on a regular base. Caustic soda and

process	temperature	time	remark
thermisation	63 - 65°C (57°C)[1]	15 s (15 s)[1]	Ideally, milk is heat-treated immediately upon arrival in the dairy. However, in practice, milk is stored in tanks for several hours. To prevent deterioration, milk is preheated to a temperature below pasteurization temperature to temporarily inhibit bacterial growth.
LTLT pasteurization of milk	63°C	30 min	Low Temperature Long Time pasteurization. This is the original type of heat treatment, with the milk being heated in an open batch process, but this method has almost completely been replaced by continuous processes.
HTST pasteurization of milk	72 - 75°C (72°C)[1]	15 - 20 s (15 s)[1]	High Temperature Short Time pasteurization. The applied temperature and time of heating is dependant of the quality of the raw milk, the type of product and the required keeping properties. The shelf life of pasteurized milk is dependant on the quality of the raw milk, and of good manufacturing practices at the dairy farm. In general, pasteurized milk should have a shelf life of 8 – 10 days at 5 – 7°C in unopened packages.
HTST pasteurization of cream	> 80°C	1 - 5 s	
ultra pasteurization	125 - 138°C	2 - 4 s	This process results in the so-called ESL-products, Extended Shelf Life products. The principle is to reduce the chance for re-infection of the pasteurized product which is mainly achieved by a more severe heating step, but even more importantly by rigourous hygienic measures during packaging, and a distribution and storage temperature never exceeding 7°C. Ultra pasteurization extends the shelf life a further 30 to 40 days.
UHT (flow sterilization)	135 - 140°C (135°C)[1]	a few seconds	Ultra High Temperature Treatment. In contrast to LTLT pasteurization this process is carried out in a closed continuous system preventing airborne micro-organisms from recontaminating the product. After heating and cooling, the product is aseptically filled, again preventing recontamination. UHT products can be stored at room temperature and shelf life is up to several months.
sterilization in container	115 - 120°C (F_0-value = 3)[1]	20 - 30 min	Traditional intense 2-step heating process, whereby the milk is pre-sterilized at 130°C for 3 – 4 s, then bottled 'hot' at 80°C and sterilized in its packaging at 115°C for 20 min. Alternatively, the milk is sterilized at 115°C for 20 min. and then aseptically filled in bottles. As for UHT treatment, products can be stored at room temperature for several months.

Table 2.4: Main categories of heat treatment in the dairy industry.
[1]Minimal EU requirements as defined by 92/46/EG; 852/2004

peracetic acid solutions are mostly applied for CIP. Peracetic acid is specifically applied for destroying endospore-forming bacteria as these often survive a caustic soda treatment. Equipment that is temporarily not in use is often filled with peracetic acid, to prevent endospore-formers outgrowth. Sterile UHT-equipment is cleaned by heating at 160°C for 20 to 30 minutes.

As a third barrier, all final products are subjected to a final quality control by plating milk samples on growth media for several days. Because of the short shelf life of pasteurized products, these should leave the plant as quick as possible to reach the consumer in a reasonable time span, and results of this last test are available after 8 hours, when products already left the plant. However, there is still enough time to recall a batch of products if necessary. For UHT products, the final control test takes 5 days, and products leave the plant only when these tests are favorable. If a sample turns out positive for growth, the whole batch is intensively sampled, to track the problem, and to define which products can still be released and which are not suited for consumption. Milk that has been rejected this way, re-enters the production line, and is thus subjected to an extra heating step. Sterilized products are not tested because sterilization takes place after packing, thus minimizing the risk for post-contamination.

Despite all these 'defense mechanisms' and barriers, which are summarized in Table 2.5, some bacteria do find a way to contaminate milk, and unfortunately some areas and some procedures at the plant are specifically available for these bacteria.

Barrier			
1	delivery of milk from RMO to the plant	→	acceptable or not
2	methylene blue testing	→	immediate processing or not
3	final quality control	→	release or recall or not

Defense mechanism	
1	heat-treatment
2	aseptic conditions
3	cleaning in place (CIP)

Table 2.5: Overview of barriers (passive measurements) and 'defense mechanisms' (active measurements) taken by the dairy industry to prevent bacterial outgrowth.

A first problem area is the buffer tank where raw milk from the RMOs is stored when immediate processing is not possible. These buffer tanks could pose a first contamination issue at the dairy plant because different loads of milks are mixed, and if one load is problematic, this could be passed on to the other loads. This contamination problem concerns pseudomonads as well as bacilli.

A second problem mostly occurs at dairy plants that produce a lot of different products, and thus have various production lines, some of which serve multiple purposes. This means during transport in the plant, milk has to pass pipes and taps where various types of milk pass. Various types of milk in this particular case is milk that underwent different heating procedures. For example, sometimes fresh milk needs to be transported with the same pipes mostly used for pasteurized milk. Or the UHT-equipment has to be used for the production of buttermilk, a production process carried out at lower temperatures than UHT. Although intensive cleaning steps are performed between different types of milk, this might be a source for recontamination problems of pasteurized and UHT-treated products. Main culprits in this recontamination issue are bacilli.

Surprisingly, a third problem occurs with sterilized products packed in glass bottles. These bottles are meticulously screened for the presence of cracks by a robot (the bottle-inspector). However, cracks smaller than 0.8 mm are not detected. Furthermore, the crown caps used nowadays do not always ensure complete sealing of the bottle. Both factors contribute to post-contamination of sterilized products, despite the sterilization procedure of the final product taking place in the glass bottle itself.

A fourth contamination issue at the dairy plant is that rejected UHT milk batches are recycled. This might carry a persistent contamination issue with it.

It can be concluded that psychrotrophic *Pseudomonas* species mainly pose a problem during prolonged storage at the dairy farm, where they can produce extracellular proteolytic and lipolytic enzymes that mainly hamper prolonged storage of UHT-products and, to a lesser extent, the milk production process, while endospore-formers are mainly a problem in pasteurized and UHT-treated/sterilized products because they survive pasteurization. Furthermore recontamination of heat-treated milk at the dairy plant can often be attributed to aerobic endospore-formers.

CHAPTER 3

Introduction to microbial taxonomy

Even the taxonomist has moments of glory.

The astonishing complexity and diversity of life urged biologists to sort organisms on the basis of mutual similarities in non-overlapping groups, called taxa (from the Greek *taxis*, meaning order). Taxonomy is the science that deals with the organization of organisms in an orderly manner and relies on three inter-dependent elements, namely *i*) classification, the orderly arrangement of organisms into taxonomic groups, *ii*) nomenclature, the assignment of names to taxonomic units defined and *iii*) identification, the allocation of an unknown to one of the defined taxonomic groups (Moore *et al*., 2010; Tindall *et al*., 2010).

Bacterial classification systems were first proposed in the late 19th century and were based on morphological characteristics mainly (e.g. the classification proposed by Migula was based on cell morphology and motility (Breed *et al.*, 1944)). In 1920, the Committee on Characterization and Classification of Bacterial Types published a system of classification not only based on morphology, but included physiology, growth characteristics and colorimetric reactions as well, hereby following the classification systems proposed by Kruse and Jensen (Winslow *et al.*, 1920).

These early classification systems were replaced by classification systems based on 'natural' relationships between organisms, the so-called phylogenetic classification system. Phylogeny (or evolutionary relationship) between organisms is contained in their genome sequences and the application of techniques that reveal similarity between genome sequences (whether direct from the nucleic acid molecule, called genotypic techniques, or indirect from expressed features, called phenotypic techniques) is nowadays common practice in bacterial taxonomy. Current classification is mainly based on the 16S rRNA gene, a highly conserved macromolecule that contains phy-

logenetic information in its sequence, and the classification system derived from this molecule is presented in the 'Bergey's Manual of Systematic Bacteriology'. However, classification systems are subject to continuous changes due to new insights in bacterial phylogeny and molecular techniques, and the classification system that is currently accepted, and regarded as 'modern' might be outdated within a few years.

Historically, taxonomy was regarded as a subjective area of study with little relevance to other scientific research fields, however, it evolved into a supportive phylogenetic framework which is embedded in every biological science (Cohan, 2002). The relevance of taxonomy is threefold. First, it provides the scientific community with a unique name for each organism, enhancing communication between different research groups. Secondly, it allows prediction of the functions and structures of similar organisms, and thirdly, it helps to uncover and understand potential evolutionary connections and their ecological and economical impact.

3.1 Classification of micro-organisms

The biological diversity of 'life' can be subdivided in three domains, namely Archaea, Bacteria and Eukarya. The domain of Bacteria is further subdivided in phyla, orders, families, genera and species. Two different phyla are addressed in this thesis, namely the *Proteobacteria* (harboring the genus *Pseudomonas*) and the *Firmicutes* (harboring the genera *Bacillus* and *Geobacillus*). A comprehensive overview of the current taxonomic outline can be found on the website of the Bergey's Manual Trust (http://www.bergeys.org).

The core unit in microbial taxonomy is a 'species', which could be defined as 'a group of isolates sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions' (Rosello-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). In practice, a species is defined as 'a group of strains characterized by a certain degree of phenotypic consistency, by a significant degree (50 – 70%) of whole genome DNA relatedness and over 97% of 16S ribosomal RNA gene sequence identity' (Coenye *et al.*, 2005). This species definition is dated 10 years ago, but it still is a pragmatic and universally applicable definition, widely accepted by prokaryotic taxonomists (Konstantinidis & Tiedje, 2004; Moore *et al.*, 2010). However, the prokaryotic species definition as it is now, is criticized for many reasons as listed by Konstantinidis and co-workers (2006): it is *i*) difficult to implement because of technical limitations in identifying diagnostic traits and in performing the DNA-DNA reassociation experiments, *ii*) not adequately predictive of phenotype, *iii*) too broad, and not encompassed by any of the eukaryotic species definitions, and *iv*) not consistently applied, for instance, some (mostly pathogenic) strains show more than 70% DNA relatedness but are classified in separate species.

Maybe one of the most important criticisms on this definition is that it is based upon empirically defined values (for DNA-DNA reassociation and 16S rRNA gene sequence identity) that confirmed the previously demarcated phenotypic-based species, but not on a general theory-based concept of what a species should be (Gevers *et al.*, 2005). Currently, focus in microbial taxonomy is on whole-genome sequencing (WGS), which might contribute to unravel evolutionary relationships between microbial organisms and to find a satisfying species accompanied by a workable species definition (Konstantinidis & Tiedje, 2004; Coenye *et al.*, 2005; Gevers *et al.*, 2005; Konstantinidis & Tiedje, 2005; Henz *et al.*, 2005; Doolittle & Papke, 2006; Staley, 2006; Konstantinidis & Tiedje, 2007).

Nonetheless, higher taxonomic ranks (genus, family, order) lack a clear definition and their descriptions are mainly based on distinctness and robustness of observed 16S rRNA gene sequence based clusters, in the best case supported by discriminatory phenotypic traits. It should not come as a surprise that the subjective manner of working with these higher taxonomic ranks does hamper a clear, logical bacterial classification system. Indeed, a study performed by Konstantinidis and Tiedje (2006) on 175 available whole genome sequences clearly indicated extensive overlap in genome relatedness between adjacent higher taxonomic ranks.

Classification of unknowns implicates their characterization to obtain a picture as complete as possible of their traits. An overview of techniques currently applied in characterization of bacteria is given below, and their applicability in microbial taxonomy is discussed. This overview is far from complete, but deals with some of the experiments performed and taxonomical challenges encountered during the course of this work.

3.2 Characterization of micro-organisms

The current consensus in microbial taxonomy is to use a polyphasic approach for characterizing and thus classifying bacteria. A range of genotypic and phenotypic techniques are applied to characterize an organism in the most comprehensive way as possible (Vandamme *et al.*, 1996; Moore *et al.*, 2010).

Phenotypic techniques are based on the phenotype of an organism, i.e. the expressed features of an organism. Classically, phenotyping of bacteria includes the analysis of morphology, physiology and biochemical features.

> **Morphology** of bacteria includes cellular (Gram-stain, shape, size, flagella, endospore, motility) and colonial (shape, size, colour, opacity) characteristics, whereas **physiological and biochemical analyses** involve growth characteristics (pH-, temperature and salt concentration growth range, atmospheric condition), utilization of carbon sources and activity of enzymes (Vandamme *et al.*, 1996). Often these tests are performed using miniaturized highly standardized commercially available test kits, such as API (bioMérieux) and Biolog (Inc.). Routine identification labs worldwide use these miniaturized tests for quickly identifying pathogens or food spoilers. The profiles of the unknowns are compared with profiles in reference databases comprising a range of economically and/or clinically relevant strains, and identification is obtained through matching profiles. However, dependency on a reference database is an important drawback of these miniaturized test kits, as these databases are often not complete, and/or not updated on a regular base, so unreliable identifications are no exception.

Other phenotypic tests that study differences in cell compounds / cell structure are often grouped as **chemotaxonomic techniques**. Examples are analysis of polar lipids using thin layer chromatography (TLC), of quinones and peptidoglycans. These techniques are very useful in differentiating higher taxonomic levels, but mostly fail at the genus level and lower. Two other chemotaxonomic techniques, analysis of whole cell proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and analysis of cellular fatty acid methyl esters through gas chromatography (FAME), often can differentiate onto the genus level, sometimes even species level, depending on the type of organisms under study.

In general, phenotypic techniques are very useful in characterizing an organism, and chemotaxonomic methods might help in drawing a picture of high-level taxonomy. But phylogeny mostly can't be determined based on phenotype alone. Therefore, genotypic methods, such as rRNA homology studies and 16S rRNA gene sequencing are mostly applied. It might sometimes feel like phenotypic techniques are regarded as outdated and surpassed by 'modern' genotyping, partly because the resolution of genotypic methods by far seems to exceed the resolution of any phenotypic technique used by now (Peix *et al.*, 2009). However, one should keep in mind that for routinely applied identification purposes in industrial or clinical laboratories, quick and easy-to-use tests will always be preferred. Ideally, genotypic analysis should result in the development of reliable, quick and accurate (phenotype-based) identification systems for daily use.

Genotypic techniques are directed towards RNA or DNA molecules, and currently dominate bacterial taxonomy for reasons stated above.

The backbone of the current classification system is **16S rRNA gene sequencing**. The 16S rDNA encodes a 16S rRNA molecule, which is part of the small ribosomal subunit that plays an essential role in translation of RNA to proteins. It is considered a good phylogenetic marker cause it *i*) is universally present, *ii*) is functionally stable, *iii*) contains highly conserved regions guaranteeing the identification of gene homology, and *iv*) contains a sufficient amount of variable sites, providing the informative portion (Yarza *et al.*, 2010). Impressive efforts have been made by several researchers to curate publicly available 16S rRNA gene sequences for all prokaryotic type strains described thus far (The Living Tree Project, LTP, (http://www.arb-silva.de/projects/living-tree/)), and to provide a robust, reliable alignment of these gene sequences based on secondary structure of the gene (Ludwig *et al*, 2004).

Importance of 16S rRNA gene sequencing in current taxonomy is reflected in its incorporation in the species definition, stating that strains exhibiting 97% sequence similarity or less represent separate species, and strains exhibiting more than 97% may or may not represent the same species, which should be evaluated by DNA-DNA hybridization (DDH) experiments. Stackebrandt and Ebers (2006) proposed to shift this 97% value to the new value 98.7 - 99%, but this has not yet been generally accepted by prokaryotic taxonomists. However, there are some constraints when using 16S rRNA gene sequencing. For instance, exceptions to this 97% rule have already been shown, with strains belonging to the same species showing more than 3% sequence diversity (e.g. in the genus *Helicobacter*; Harrington & On, 1999). Furthermore, the resolution of 16S rRNA gene sequences is often insufficient to elucidate affiliations between closely related organisms (Tayeb *et al.*, 2005; Ash *et al.*, 1991).

Apart from that, the use of a single characteristic to reconstruct entire bacterial phylogeny is subject to some devastating criticism for several reasons as listed by Gest (Gest, 1999; Gest, 2003). First, he denounces the lack of proof for the 16S rRNA molecule to represent cellular phylogeny, and strengthens his opinion by the words of Gupta (2000) doubting the correctness of rRNA gene phylogeny due to contradiction with other conserved molecules based phylogenies. Secondly, he emphasizes the underestimation and ignorance of horizontal gene transfer, the moving force in prokaryotic evolution (Doolittle, 1999), when reconstructing 16S rRNA gene phylogenetic trees. A third concern refers to Gupta (2000) that bacterial phyla defined by 16S rRNA genes are not stable, very arbitrary and ill-defined. Furthermore, 16S phylogeny does not correlate with some complex phenotypic character-

istics (e.g. the membrane cell structure), showing polyphyletic branching where one can assume such a complex feature would only have developed once (Gupta, 2000).

At the very least, one could conclude with the words of Palleroni (2003) saying that "rRNA phylogeny is the phylogeny of rRNA genes but not necessarily that of their hosts (Postgate, 1995), and therefore should be used with caution in the characterization of taxonomic units". Nonetheless, whole genome sequence data determined thus far support the 16S rRNA gene based phylogeny as backbone of bacterial taxonomy. Additionally, it might not be feasible to define other genetic markers due to shortage of genes that are common to all prokaryotes, or due to difficulties in universal primer design.

The second technique is considered the standard for species delineation and is also incorporated in the species definition, namely **DNA-DNA hybridization**, reflecting sequence identity of two whole genomes rather than a single small piece of DNA. Nonetheless, DDH is also subject to criticism for *i*) being time-consuming and labour-intensive, *ii*) being carried out properly in only a few specialized laboratories, *iii*) different methods yielding different results and most importantly, *iv*) being inapplicable for non-cultivable prokaryotes (environmental studies) and *v*) its comparative nature disabling the implementation of incremental database (Gevers *et al.*, 2005; Konstantinidis *et al.*, 2006; Goris *et al.*, 2007).

This urged researchers to seek for alternatives, of which **Average Nucleotide Identity (ANI)** and **Average Amino Identity (AAI)** for now are considered the best. Both parameters rely on the pairwise comparison of whole genome sequences to determine a set of orthologues genes conserved among both genomes. The sequence similarity (nucleotides or amino acids) for each individual pair of orthologues is calculated, and the average value for all similarity values thus obtained is determined (Rossello-Mora, 2005). ANI- and AAI-values have shown to correlate extremely well with experimentally determined DDH-values, and the comparison between these techniques resulted in a threshold value of 95% ANI and 95-96% AAI for species delineation, comparable to the 70% DNA-relatedness value (Rossello-Mora, 2005; Konstantinidis *et al.*, 2006; Moore *et al.*, 2010).

As stated earlier, 16S rRNA gene sequencing suffers from lack of resolution for closely related strains, and alternatives were found in the **analysis of housekeeping genes**, so called because these genes encode conserved enzymes that perform essential metabolic tasks in the cell. Usage of a single gene sequence is debatable due to stochastic genetic variation, horizontal gene transfer and recombination (Gevers *et al.*, 2005), so often a combination of 4 to 8 house-keeping genes is preferred, and the combined analysis of multiple genes buffers the above mentioned effects. Such approach is known as **Multi Locus Sequence Analysis (MLSA)** and MLSA-schemes often provide higher resolution than 16S rRNA gene sequencing allowing differentiation at the species, sometimes even strain level (Moore *et al.*, 2010). Drawbacks of MLSA are that *i*) due to the enormous diversity observed for all prokaryotes a universal MLSA-scheme, comparable to the universal 16S rRNA gene, is utopic; and *ii*) their development requires a large set of reliably identified strains which is not always available.

Whole-genome sequencing (WGS) is more and more being integrated in microbial taxonomy as new sequencing techniques develop quickly and allow continual cheaper and faster sequence determination. Currently, 1560 bacterial genomes are completely sequenced, and another 6178 are ongoing (GOLD database; http://www.genomesonline.org; Bernal *et al.*, 2001). Unfortunately, these genomes only cover a small part of the total bacterial diver-

sity currently known, due to preference for sequencing economically and clinically relevant strains first. But, as more and more genomes will be sequenced, taxonomists hope that this will reveal a better view on evolutionary relationships between prokaryotes and will result in a workable, satisfying species definition. Up to now, it seems species are characterized by a set of genes shared among all strains, the so-called core-genome, and a set of genes characteristic for that specific strain, the so-called accessory genome (Sim *et al.*, 2008; Kung *et al.*, 2010).

Other genotypic techniques include determination of the ratio of guanine and cytosine nucleosides within the total genomic DNA, the **%G + C content** (Marmur & Doty, 1962). This parameter is useful to reveal higher taxonomic levels (Wayne *et al.,* 1987), for instance it is commonly accepted that %G + C content divergence should not exceed 10% within a single genus (Bull *et al.*, 1992). Nonetheless, it is not possible to assign strains to a certain taxon, based on %G + C content alone. **DNA fingerprinting methods** are often used to determine intraspecies variability. An example is rep-PCR genomic fingerprinting that utilizes primers targeting repetitive elements in the genome, thus generating a mixture of random DNA fragments, which are separated and visualized through agarose gel electrophoresis (Ludwig, 2008). An overview of techniques discussed here and their resolution is represented in Figure 3.1. A more detailed description of the methodology of some of these techniques is represented in Chapter 4.

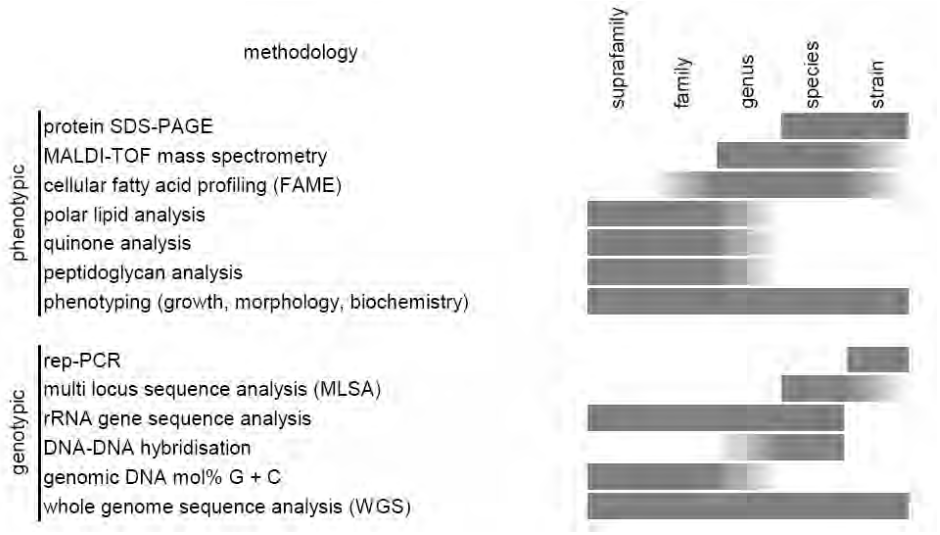


Figure 3.1: Methodologies for the characterization of prokaryotes and the approximate respective taxonomic levels of resolution (modified from Vandamme *et al.*, 1996).

3.3 Bacterial taxonomy in practice

Bacterial taxonomy is mostly practiced as a tool to reliably identify isolates and assign these unknowns to their corresponding taxon. However, there is no consensus approach or general guideline on how to start such an identification process (Vandamme *et al.*, 1996). As indicated above, numerous techniques are available and it largely depends on the identity of the isolates,

and the desired identification level to choose the appropriate identification methodology. Ideally, a minimum of foreknowledge is desired before starting the identification approach, although this is not always feasible.

When starting a diversity study, a common procedure is to dereplicate the large amount of isolates into groups of isolates showing high similarities for a well-choosen characteristic, either on the phenotypic or genotypic level. As already mentioned, the choice of technique depends on the expected identity of the isolates. For instance, it is known that BOX-fingerprinting, a type of rep-fingerprinting, does not generate good banding patterns for bacilli, showing too few bands to allow grouping of isolates. On the other hand, BOX fingerprinting does result in grouping of isolates onto the subspecies level for members of the genus *Pseudomonas*. Contrary, FAME-analysis does result in grouping of bacilli, mostly onto the species level, unlike for pseudomonads, where it can only be used to confidently allocate the isolates to the genus *Pseudomonas*, but no differentiation onto the species level can be obtained. Additional factors that might influence the choice of dereplication tool are the cost and fastness of the technique and the desired resolution. Another point of consideration is the information that can be deduced from the technique applied. For instance, the advantage of FAME compared to rep-fingerprinting is that it does not only allow to group the isolates, but also gives an idea about the identity of the isolates based on comparison of obtained fatty acid patterns with a reference database, while for rep-fingerprinting no information about the identity is obtained, unless coupled to an in-house database.

After dereplication, representatives are chosen from each group of isolates, and further analyzed. Mostly, 16S rRNA gene sequencing is the preferred method as it immediately provides information about the identity of the representative. Based on the outcome of 16S rRNA gene sequence analysis, one can decide whether the identification still needs to be refined, for instance by sequencing some well-chosen housekeeping genes (MLSA), or whether it needs to be confirmed through DNA-DNA hybridization experiments, or whether it is sufficiently reliable for confidently allocating the isolate (representative) to a certain species. Multiple MLSA schemes have already been developed for various taxonomic units, and a common approach in bacterial identification nowadays is first determination of the 16S rRNA gene sequence to allocate the isolate onto the genus level, and then refinement onto species level by using the MLSA scheme prescribed for that specific genus (or family).

Specifically for both diversity studies dealt with in this work, two somewhat different approaches were used as outlined in Figure 3.2.

For the pseudomonads (Chapter 5), it was chosen to dereplicate the large amount of isolates based on BOX-PCR. Major reasons for this choice were *i*) the fact that BOX-PCR was known to differentiate *Pseudomonas* species onto the subspecies level, *ii*) which could help in linking the spoilage potential to the observed species heterogeneity. Furthermore, *iii*) due to the specific isolation procedure, it was known that most isolates very likely would indeed be pseudomonads and *iv*) this way, obtained profiles could be compared with an in-house database constituting profiles from previous studies on pseudomonads, some of which were reliably identified with DDH experiments. Most groups however could not be linked to an in-house profile and representative isolates per group were further analysed with FAME, to confirm or repudiate their allocation to the genus *Pseudomonas*. 16S rRNA gene sequencing was performed on the confirmed pseudomonads. However, as resolution of the 16S rRNA gene sequence is poor within this genus, isolates were further identified based on *rpoB, rpoD* and *gyrB* gene sequencing (Tayeb *et al.,* 2005; Mulet *et al.*, 2010). For some representatives, a confirmation was obtained through DDH experiments. The

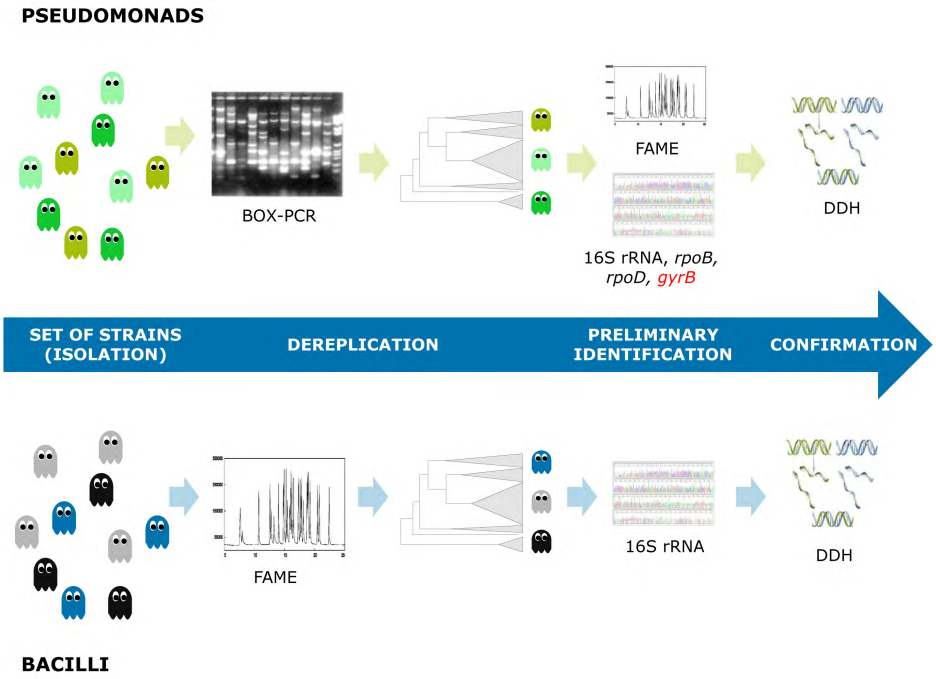


Figure 3.2: Outline of the identification approaches used for pseudomonads (Chapter 5) and bacilli (Chapter 6) in this study.

identification approach described here resulted in differentiation of the isolates onto the strain level.

For the bacilli (Chapter 6), it was chosen to dereplicate the large amount of isolates based on FAME patterns, because it was known that FAME analysis could be used to group major part of the bacilli onto the species level. Furthermore, it is a fast and cheap method, allowing relatively quick processing of all isolates. The preliminar identification based on FAME was further confirmed by 16S rRNA gene sequencing, and additionally, DDH experiments were performed for some isolates. The identification approach described resulted in differentiation of the isolates onto species level.

The identification approaches presented for the above studies illustrate the appropriate choice of identification tools largely depends on the desired outcome (what taxonomic level is achieved?), the speed and cost of the technique, the high-throughput capacity, the labor-requirements and facility requirements. Identification is mostly a stepwise process with allocation of the isolate to a certain major taxon (i.e. family or order level), and then further refinement onto the genus, species or strain level.

3.4 Taxonomy of the genus *Pseudomonas*

The genus *Pseudomonas* was proposed in 1894 by Migula, and belongs to the family *Pseudomonadaceae*, order *Pseudomonadales*, class γ-*proteobacteria* in the *Proteobacteria* division. Members of this genus historically confine to the following description as given by Stanier and co-workers (1966): “unicellular rods, with the long axis straight or curved, but not helical. Motile by means of one or more polar flagella. Gram-negative. Do not form spores, stalks, or sheaths. The energy-

yielding metabolism is respiratory, never fermentative or photosynthetic. All use molecular oxygen as terminal oxidant; and some can use denitrification as an alternative, anaerobic respiratory mechanism. All are chemo-organotrophs; some are facultatively chemolithotrophs that use H_2 as an energy source." This rather vague and broad definition resulted in the genus being a dumping ground for all Gram-negative, aerobic, polarly flagellated rods. Fortunately, substantial efforts have been made to resolve the taxonomic position of members of this genus, starting in the 1920's, and still ongoing, as will be discussed in the 'history of *Pseudomonas* taxonomy' (3.4.1).

Members of the genus *Pseudomonas* are of considerable scientific and practical importance and are ubiquitously found in water and soil related environments. They play a crucial role in the process of mineralization of organic matter in nature, which is reflected by their metabolic diversity. An unusually wide range of organic compounds, mainly low molecular weight molecules, can be used as carbon and energy sources (Palleroni, 1975). Some species are of economical importance, exhibiting either beneficial or detrimental effects in the fields of agriculture, biotechnology and food industry. For instance, *Pseudomonas syringae* is responsible for a variety of bacterial spot, speck and blight diseases in a wide range of important crops such as tomato and soy (Sarkar & Guttman, 2004). On the other hand, some *Pseudomonas* species (e.g. *Pseudomonas putida*) are considered plant growth-promoting rhizobacteria, stimulating plant growth and/or reducing incidence of plant disease by competitively colonizing plant roots (Haas & Defago, 2005). *Pseudomonas aeruginosa* strains have recently been adopted in the development of a biosensor detecting cephalosporins (Kumar *et al*., 2008), and several species (e.g. *Pseudomonas putida* and *Pseudomonas fluorescens*) have been reported as valuable helpers in soil and water bioremediation (Farhadian *et al.,* 2008; Jeyalakshmi & Kanmani, 2008). *Pseudomonas aeruginosa* is subject of many studies, as it might cause severe infections, associated with high mortality rates, in immune-compromised patients, and is also frequent the cause of infections in hospitalized patients, in this capacity often referred to as the hospital bacteria (Curran *et al*., 2004; Gershman *et al.*, 2008). The role of pseudomonads in the dairy industry as food spoilers has already extensively been discussed in §2.4.1.1.

3.4.1 History of *Pseudomonas* taxonomy

The genus *Pseudomonas* has always been an intriguing study object for taxonomists since it historically developed as a kind of dumping ground for aerobic, motile Gram-negative rods, and thus lacked a profound classification of its members. It was first described by Migula in 1894 (Migula, 1900) as a genus harboring bacteria characterized by 'cells with polar organs of motility; formation of spores occurs in some species but is rare'. Despite this very limited, rather useless description, which afterwards even turned out to be wrong as *Pseudomonas* strains do not form spores, it became accepted and *Pseudomonas aeruginosa* was designated the type species (Palleroni, 2010). Due to the vague definition above, many species were assigned to this genus without elaborate description, complicating the classification and identification of its members. A first milestone was achieved by den Dooren de Jong in 1926 who recognized the immense diversity in nutritional characteristics of pseudomonads and recommended to use this versatility in *Pseudomonas* taxonomy. Unfortunately, his valuable findings only reached a small number of taxonomists, and were for long neglected by the large community of scientists (Palleroni, 2010). A second attempt was made by Stanier, Palleroni and Doudoroff in 1966 who, through an extensive screening of 267 strains for biochemical, physiological and nutritional characters, subdivided the genus in three subgeneric groups, namely the 'fluorescent group', with species *P. aeruginosa*, *P. flu-*

orescens and *P. putida*, the 'acidovorans group' with species *P. acidovorans* and *P. testosteroni* and the 'alcaligenes group' with species *P. alcaligenes* and *P. pseudoalcaligenes*. Species *P. multivorans*, *P. stutzeri* and *P. maltophilia* remained ungrouped. A fourth group, the 'pseudomallei group', was composed of species *P. mallei* and *P. pseudomallei*, however data incorporated by Stanier and co-workers (1966) for this group were obtained by Redfearn and co-workers (1966). Likewise, data for the stand-alone species *P. lemoignei* were taken from Delafield and co-workers (1965). Stanier, Palleroni and Doudoroff (1966) provided the scientific community with a diagnostic key to distinguish different *Pseudomonas* species based on phenotype, and some of these are summarized in Table 3.1.

	P. aeruginosa	***P. fluorescens***	*P. putida*	*P. multivorans*	***P. pseudomallei***	*P. mallei*	***P. acidovorans***	*P. testosteroni*	***P. alcaligenes***	*P. pseudoalcaligenes*	*P. stutzeri*	*P. maltophilia*	*P. lemoignei*
n° of flagella	1	>1	>1	>1	>1	0	>1	>1	1	1	1	>1	1
poly-β-OH-butyrate reserve	-	-	-	+	+	+	+	+	-	v	-	-	+
fluorescent pigment	+	+	+	-	-	-	-	-	-	-	-	-	-
phenazine pigment	+	v	-	v	-	-	-	-	-	-	-	-	-
denitrification	+	v	-	-	+	v	-	-	-	-	+	-	-
growth at 4°C	-	+	v	-	-	-	-	-	-	-	-	-	-
growth at 41°C	+	-	-	v	+	+	-	-	+	+	v	-	-
gelatin hydrolase	+	+	-	+	+	+	-	-	+	v	+	+	-
starch hydrolase	-	-	-	-	+	v	-	-	-	-	+	-	-
oxidase reaction	+	+	+	+	+	+	+	+	+	+	+	-	+

Table 3.1: Characters of diagnostic value for differentiation of pseudomonad species (Stanier *et al.*, 1966). Groups as defined by Stanier *et al.*, (1966) are indicated in grey, and the name bearer of each group is indicated in bold.

That same study also pointed out on the one hand the heterogeneity within the fluorescent species *P. putida* and *P. fluorescens* (both can be differentiated based on gelatin liquefaction) resulting in 2 biotypes for *P. putida* and 7 biotypes for *P. fluorescens*, and on the other hand the homogeneity of the type species *P. aeruginosa*.

The accumulation of poly-β-hydroxybutyrate was considered a valuable taxonomic marker to define subgeneric groups within *Pseudomonas* as it is completely absent from the 'fluorescent group', *P. stutzeri* and *P. maltophilia*. It is present in all other groups, although variable for *P. pseudoalcaligenes*. The unique presence of a fluorescent pigment, pyocyanin, in the 'fluorescent group' is considered another valuable marker.

In 1972, Palleroni and coworkers studied a considerable fraction of the strain set used by Stanier and co-workers (1966) to determine deoxyribonucleic acid homologies between pseudomonads. They defined a '*Pseudomonas fluorescens* complex' characterized by the absence of poly-β-hydroxybutyrate accumulation and comprised of the 'fluorescent group', the 'alcaligenes group' and the 'stutzeri group'. The complex harbored fluorescent and non fluorescent strains that were clearly distinct from all other pseudomonad groups defined at that time, namely the 'pseudomallei group' and the 'acidovorans group' as well as other ungrouped species, such as *P. maltophilia*

and *P. delafieldii*. DNA hybridization data correlated quite well with phenotypic data gathered by Stanier and co-workers (1966). A remarkable exception was *P. putida,* with biotype B showing more DNA relatedness with *P. fluorescens* than with *P. putida* biotype A, again demonstrating the heterogeneity within this species. Various biotypes of *P. fluorescens* were also studied more in detail, and biotype A, B and F were found to be very heterogeneous. Biotype C might represent a novel species, while biotype D (*P. chlororaphis*) and biotype E (*P. aureofaciens*) were shown to be very similar to each other, which was later confirmed by Peix and co-workers (2007). With this study Palleroni and co-workers (1972) made a start in introducing molecular techniques in *Pseudomonas* taxonomy.

Their work was quickly followed by another paper of the same group (Palleroni *et al.*, 1973), describing a second milestone in *Pseudomonas* taxonomy with the subdivision of the genus into 5 distinct groups, some of which are as distantly related to each other as they are to *Escherichia coli*, again based on nucleic acid homologies of the rRNA genes. Their experiments on several *Pseudomonas* strains revealed five RNA homology groups. Group I corresponded with the '*P. fluorescens* complex' defined in their previous work, group II corresponded with the 'pseudomallei group', group III comprised two DNA homology groups, the 'acidovorans group' and the 'facilis - delafieldii group', group IV represented the 'diminuta group', while group V contained *P. maltophilia* and members of the genus *Xanthomonas* that were also taken into account in their study. Authors stated the five groups found are so distantly related that they, at least, deserved a separate genus status. However, unfortunately, no distinctive phenotypic characteristics could be found to unequivocally distinguish species from one rRNA homology group from those belonging to other rRNA groups.

Ten years later, the work of Palleroni and co-workers (1973), was extended by De Vos and De Ley (1983), who did not only study pseudomonads, but also included other aerobic heterotrophic Gram-negative bacteria (a total of 236 strains), to establish a general framework of Gram-negative taxa, and to point out the positions of the earlier proposed five *Pseudomonas* rRNA homology groups within this framework (Figure 3.3). They found 5 rRNA superfamilies with the first superfamily harboring all genera from the *Enterobacteriaceae, Vibrionaceae* and *Plesiomonas*. The second superfamily consisted of *Azotobacter, Azomonas, Pseudomonas* rRNA homology group I (*Pseudomonas fluorescens* complex)*, Alteromonas, Xanthomonas* and *Frateuria*. Members of the second *Pseudomonas* rRNA homology group (the 'pseudomallei group') could be found in the third rRNA superfamily, together with *Alcaligenes*, *Bordetella*, *Derxia*, *Janthinobacterium* and *Chromobacterium*. The fourth rRNA superfamily harbored *Agrobacterium, Rhizobium, Bradyrhizobium, Acetobacter, Gluconobacter, Zymomonas, Beijerinckia, Rhodo-pseudomonas* and *Paracoccus*, as well as the earlier proposed *Pseudomonas* rRNA homology group IV (the 'diminuta group'). The fifth rRNA superfamily consisted of authentic flavobacteria and *Cytophaga*.

Considering only the pseudomonads, the same five groups as described by Palleroni and co-workers (1973) could be differentiated and their position in the general framework has already been discussed partially above, but for the sake of clarity, it is schematically represented in Figure 3.4 (from the third to the fourth column). Surprisingly, the position of various *Pseudomonas* species was dispersed over the α-, β- and γ-subclasses of the division *Proteobacteria*.

Based on these results, De Vos and De Ley (1983) proposed an emended genus description of *Pseudomonas* (*P. aeruginosa* as the type species is mandatory), and this genus should correspond to the earlier proposed *P. fluorescens* complex or rRNA homology group I, an idea already suggested by Palleroni and co-workers (1973). The combined findings of both research groups (Palleroni

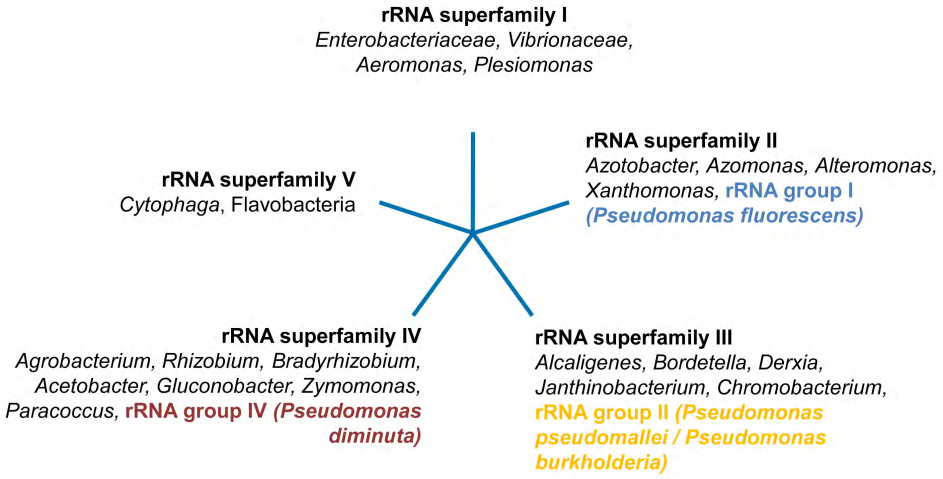


Figure 3.3: Schematic representation of five rRNA superfamilies within the Gram-negative aerobes.

et al., 1973; De Vos & De Ley, 1983) resulted in a considerable reshuffling of pseudomonad taxonomy, with the establishment of new genera, and the transfer of several species to already described genera.

In brief, members of rRNA homology group II (the 'pseudomallei group') were transferred to the new genus *Burkholderia* with type species *B. cepacia* (Yabuuchi *et al.*, 1992; Urakami *et al.*, 1994; Gillis *et al.*, 1995) and *Ralstonia* with type species *Ralstonia pickettii* (Yabuuchi *et al.*, 1995). Members of rRNA homology group III (the 'acidovorans group') have been reclassified in the *Comamonadaceae* (Willems *et al.,* 1991), harboring the genera *Comamonas* (De Vos *et al.*, 1985), *Acidovorax* (Willems *et al.,* 1990) and *Hydrogenophaga* (Willems *et al.*, 1989), while the pseudomonads belonging to rRNA homology group IV (the 'diminuta group') were transferred to the genus *Brevundimonas* (Segers *et al.*, 1994). The rRNA homology group V, *P. maltophilia,* was first transferred to the genus *Xanthomonas* (Swings *et al*., 1983), and later on has been assigned to the genus *Stenotrophomonas* (Palleroni & Bradbury, 1993). The history of pseudomonad taxonomy from the paper of Stanier and co-workers (1966) up to the allocation of its members to different genera (late 1980s – mid 1990s) is schematically represented in Figure 3.4. Numerous efforts in the field resulted in a reduction of the *Pseudomonas* genus from more than 250 species (whether well described or not) to about 30 'authentic' *Pseudomonas* species (Kersters *et al.*, 1996). Further attention in this overview of the history of *Pseudomonas* taxonomy will be drawn only on the real pseudomonads, namely *Pseudomonas sensu stricto* (s.s.), members of rRNA homology group I.

Moore and coworkers studied these pseudomonads *s.s.* more in detail based on 16S rRNA gene sequences and observed two intrageneric (IG) clusters (Moore *et al.*, 1996) that were well supported by FAME and phospholipid profiling (Vancanneyt *et al.*, 1996a). The first intrageneric cluster was called the '*P. aeruginosa* IG cluster', and the second '*P. fluorescens* IG cluster'. Species included in both clusters are represented in Table 3.2. Within these IG clusters, several distinct lineages were observed, namely the *P. aeruginosa* lineage, the *P. resinovorans* lineage, the *P. mendocina* lineage and the *P. flavescens* lineage for the *P. aeruginosa* IG cluster and the *P. syringae* lineage, *P. fluorescens* lineage, *P. putida* lineage and *P. agarici lineage* for the *P. fluorescens* IG cluster (Table 3.2). Palleroni already stated for the 5 rRNA homology groups that 'no distinctive

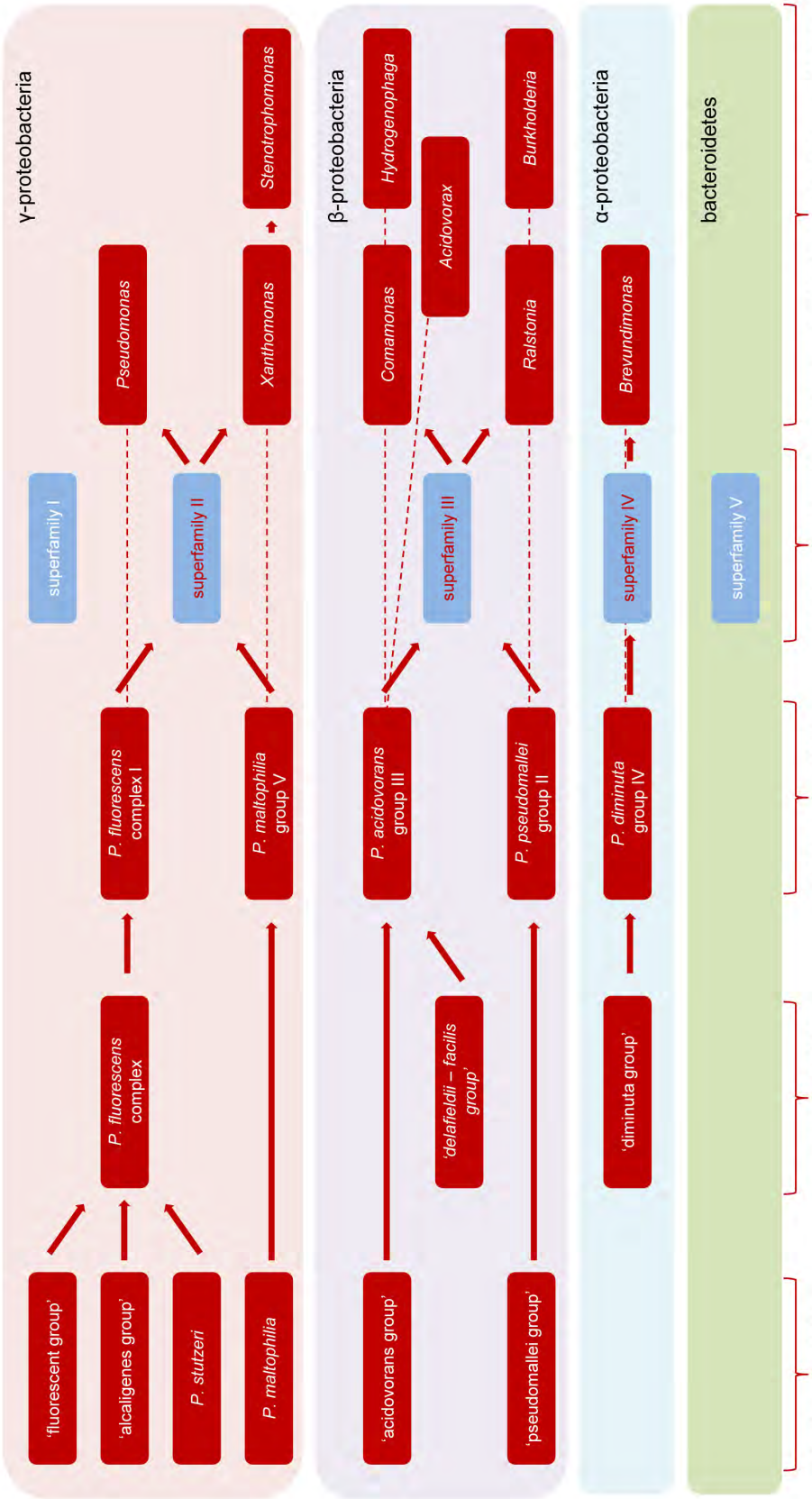


Figure 3.4: Schematic overview of the history of pseudomonad taxonomy.

phenotypic characteristics could be found to unequivocally distinguish all species from one rRNA homology group from all species harbored in other rRNA groups', and the same is unfortunately true for the intrageneric clusters and lineages: there is no obvious, consistent, metabolic trait that differentiates the species of one phylogenetic lineage from the others.

***P. aeruginosa* IG cluster**	
P. aeruginosa	*P. aeruginosa*
P. alcaligenes	
P. citronellosis	
P. stutzeri	
P. balearica	*P. resinovorans*
P. resinovorans	
P. mendocina	*P. mendocina*
P. oleovorans	
P. pseudoalcaligenes	
P. flavescens	*P. flavescens*

***P. fluorescens* IG cluster**	
P. fluorescens	*P. fluorescens*
P. marginalis	
P. tolaasii	
P. aureofaciens	
P. chlororaphis	
P. viridiflava	
P. syringae	*P. syringae*
P. coronafaciens	
P. ficuserectae	
P. amygdali	
P. cichorii	*P. cichorii*
P. putida	*P. putida*
P. asplenii	
P. agarici	*P. agarici*

Table 3.2: Intrageneric structure of *Pseudomonas* s.s. based on 16S rRNA gene sequences.

Moore *et al.* (1996) realized this division of the genus was not rigid but subject to changes as new organisms would be found and identified as *Pseudomonas* species. However, they provided the basic framework for subdividing the genus *sensu stricto* into several subgroups that is still used nowadays. A first reshuffling was presented by Anzai and co-workers (2000) who, again based on 16S rRNA gene sequences, split the *P. chlororaphis* lineage from the *P. fluorescens* lineage, and the *P. stutzeri* lineage from the *P. aeruginosa* lineage. The *P. resinovorans*, *P. mendocina* and *P. flavescens* lineages were merged to the *P. aeruginosa* lineage.

However, it became soon apparent, with further expansion of the genus, that resolution of the 16S rRNA gene was not sufficient to differentiate species within a lineage, and alternatives were sought. Various house-keeping genes (Table 3.3) were successfully applied to differentiate *Pseudomonas* species with a higher resolution. For example, biotypes A and B of the heterogeneous species *P. putida* could be easily differentiated from one another by *gyrB* and *rpoD* sequencing (Yamamoto & Harayama, 1998a). The same authors expanded their study to the whole genus, and observed two IG clusters, each bearing different sublineages, as firstly observed by Moore and co-workers (1996) based on 16S rRNA gene sequences. There were some striking differences between 16S rRNA and *gyrB* gene sequence phylogeny, nonetheless, authors stressed the applicability of *gyrB* sequencing in determining the phylogenetic relationships between organisms, as it correlated well with DDH values, even better than did 16S rRNA gene sequencing (Yamamoto *et al.*, 1999, 2000). It must be noted though, these findings were based on the analysis of the genus *Acinetobacter,* and one might wonder if these observations are automatically applicable for other taxa.

Other genes introduced for analyzing *Pseudomonas* phylogeny were *atpD*, *carA*, and *recA* (Hilario *et al.*, 2004) and *rpoB* (Tayeb *et al.*, 2005), all resulting in a phylogeny more or less resembling the 16S rRNA gene phylogeny. Probably, the recent study of Mulet and co-workers (2010), in-

gene	protein	function	study
rpoB	RNA polymerase β subunit	synthesis of RNA	Tayeb *et al.*, 2005 Mulet *et al.*, 2010
gyrB	DNA gyrase β subunit	introduction of neg. supercoils into bact. chromosomes during replication	Yamamoto & Harayama, 1995 Yamamoto *et al.*, 2000 Mulet *et al.*, 2010
atpD	ATP synthase F1 subunit	synthesis of adenosine trophosphate (ATP)	Hilario *et al.*, 2004
carA	carbamoyl phosphate synthase subunit A	provides glutamine amidotransferase activity for removal of the ammonia group from glutamine in the synthesis of pyrimidines and purines	Hilario *et al.*, 2004
recA	recombinase A	multifunctional protein involved in the SOS mechanism of DNA repair	Hilario *et al.*, 2004
rpoD	σ70-factor	confers to promotor-specific transcription initiation on RNA polymerase	Yamamoto & Harayama, 1995 Yamamoto *et al.*, 2000 Mulet *et al.*, 2010
oprF	OprF protein	non-specific outer membrane porin, permits passive diffusion of polar nutrients	Bodilis *et al.*, 2003

Table 3.3: Overview of genes other than 16S rRNA used for resolving *Pseudomonas* taxonomy, the encoded protein, its function and the study/studies the gene has been used in are given.

troducing a MLSA scheme based on four genes - 16S rRNA, *rpoB*, *rpoD* and *gyrB* - can now be considered the reference work for modern *Pseudomonas* taxonomy. Based on this work, the current state-of-the-art is the subdivision of the genus *Pseudomonas* into two intrageneric groups, IG *P. aeruginosa* and IG *P. fluorescens*. The first IG group consists of three main lineages, represented by the species *P. aeruginosa*, *P. stutzeri* and *P. oleovorans*. The second IG is divided in six main lineages, represented by *P. fluorescens*, *P. syringae*, *P. lutea*, *P. putida*, *P. anguilliseptica* and *P. straminea*. The *P. fluorescens* lineage itself approved to be very heterogeneous and nine subgroups can be observed, represented by the species *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata*, *P. chlororaphis* and *P. asplenii*. A schematic dendrogram of this subdivision is presented in Figure 3.5 (adopted from Mulet *et al.*, 2010).

The re-organization of the entire genus and its species is represented on the species level in Table 3.4, listing up the up-to-date genus and species names for all validly published *Pseudomonas* species.

Table 3.4: Current classification of the pseudomonads (redrafted from Kersters *et al.* (1996). Group names assigned to species are based on the subdivision of Mulet *et al.* (2010), as represented in Figure 3.5. The current classification is only given when different from the previous name.

previous name	current classification	references	group name
P. abietaniphila		Mohn *et al.*, 1999	*P. lutea* group
P. acidovorans	*Delftia acidovorans*	den Dooren de Jong, 1926 Tamaoka *et al.*, 1987 Wen *et al.*, 1999	
P. aeruginosa		Migula, 1900	*P. aeruginosa* group
P. agarici		Young, 1970	
P. alcaligenes		Monias, 1928	*P. aeruginosa* group
P. alcaliphila		Yumoto *et al.*, 2001b	*P. oleovorans* group
P. aminovorans	*Aminobacter aminovorans*	den Dooren de Jong, 1926 Urakami *et al.*,1992	
P. amygdali		Psallidas & Panagopoulos, 1975	*P. syringae* group
P. andropogonis	*Burkholderia andropogonis*	Smith, 1911 Gillis *et al.*, 1995	
P. anguilliseptica		Wakabayashi & Egusa, 1972	*P. anguilliseptica* group
P. antarctica		Reddy *et al.*, 2004	*P. fluorescens* subgroup
P. antimicrobica	*Burkholderia gladioli*	Attafuah & Bradbury, 1990 Yabuuchi *et al.*, 1992 Coenye *et al.*, 2000	
P. argentinensis		Peix *et al.*, 2005	*P. straminea* group
P. arsenicoxydans		Campos *et al.*, 2010	
P. asplenii		Savulescu, 1947	*P. asplenii* subgroup
P. aurantiaca	*P. chlororaphis subsp. aurantiaca*	Nakhimovskaya, 1948 Peix *et al.*, 2007	*P. chlororaphis* subgroup
P. aureofaciens	*P. chlororaphis subsp. aureofaciens*	Kluyver, 1956 Peix *et al.*, 2007	*P. chlororaphis* subgroup
P. avellanae		Janse *et al.*, 1996	*P. syringae* group
P. avenae	*Acidovorax avenae* subsp. *avenae*	Manns, 1909 Willems *et al.*, 1992	
P. avenae subsp. *citrulli*	*Acidovorax citrulli*	Hu *et al.*, 1991 Willems *et al.*, 1992 Schaad *et al.*, 2008	
P. avenae subsp. *konjaci*	*Acidovorax konjaci*	Hu *et al.*, 1991 Willems *et al.*, 1992	
P. azotifigens		Hatayama *et al.*, 2005	*P. stutzeri* group
P. azotoformans		Iizuka & Komagata, 1963	*P. fluorescens* subgroup
P. balearica		Bennasar *et al.*, 1996	*P. stutzeri* group
P. beijerinckii	*Chromohalobacter beijerinckii*	Hof, 1935 Peçonek *et al.*, 2006	
P. beteli		Savulescu, 1947	
P. borbori		Vanparys *et al.*, 2006	*P. anguilliseptica* group
P. boreopolis		Gray & Thornton, 1928	
P. brassicacearum subsp. *brassicacearum*		Achouak *et al.*, 2000	*P. corrugata* subgroup
P. brassicacearum subsp. *neoaurantiaca*		Ivanova *et al.*, 2009	*P. corrugata* subgroup
P. brenneri		Baïda *et al.*, 2001	*P. gessardii* subgroup
P. caeni		Xiao *et al.*, 2009	
P. cannabina		Gardan *et al.*, 1999	*P. syringae* group
P. carboxydohydrogena		Meyer *et al.*, 1980	
P. caricapapayae		Robbs, 1956	*P. syringae* group
P. caryophylli	*Burkholderia caryophylli*	Starr & Burkholder, 1942 Yabuuchi *et al.*, 1992	
P. cattleyae	*Acidovorax cattleya*	Savulescu, 1947 Willems *et al.*, 1992 Schaad *et al.*, 2008	
P. cedrina subsp. *cedrina*		Dabboussi *et al.*, 1999b	*P. fluorescens* subgroup

continued on next page ...

previous name	current classification	references	group name
P. cedrina subsp. *fulgida*		Behrendt *et al.*, 2009	*P. fluorescens* subgroup
P. cepacia	*Burkholderia cepacia*	Palleroni & Holmes, 1981	
		Yabuuchi *et al.*, 1992	
P. chloritidismutans	*P. stutzeri*	Wolterink *et al.*, 2002	
		Cladera *et al.*, 2006	
P. chlororaphis subsp. *chlororaphis*		Bergey *et al.*, 1930	*P. chlororaphis* subgroup
P. chlororaphis subsp. *piscium*		Burr *et al.*, 2010	*P. chlororaphis* subgroup
P. cichorii		Stapp, 1928	*P. syringae* group
P. cissicola		Burkholder, 1948	
P. citronellolis		Seubert, 1960	*P. aeruginosa* group
		Lang *et al.*, 2007	
P. cocovenenans	*Burkholderia gladioli*	Van Damme *et al.*, 1960	
		Gillis *et al.*, 1995	
		Yabuuchi *et al.*, 1992	
P. congelans		Behrendt *et al.*, 2003	*P. syringae* group
P. corrugata		Scarlett *et al.*, 1978	*P. corrugata* subgroup
		Sutra *et al.*, 1997	
P. costantinii		Munsch *et al.*, 2002	*P. fluorescens* subgroup
P. cremoricolorata		Uchino *et al.*, 2001	*P. putida* group
P. cuatrocienegasensis		Escalante *et al.* ,2009	
P. delafieldii	*Acidovorax delafieldii*	Davis, 1970	
		Willems *et al.*, 1990	
P. delhiensis		Prakash *et al.*, 2007	
P. diminuta	*Brevundimonas diminuta*	Leifson & Hugh, 1954	
		Segers *et al.*, 1994	
P. doudoroffii	*Oceanimonas doudoroffii*	Baumann *et al.*, 1972	
		Brown *et al.*, 2001	
P. duriflava		Liu *et al.*, 2008	
P. echinoides	*Sphingomonas echinoides*	Heumann, 1962	
		Denner *et al.*, 1999	
P. elongata	*Microbulbifer elongatus*	Humm, 1946	
		Yoon *et al.*, 2003a	
P. extremaustralis		López *et al.*, 2009	
P. extremorientalis		Ivanova *et al.*, 2002	*P. fluorescens* subgroup
P. facilis	*Acidovorax facili*	Davis, 1969	
		Willems *et al.*, 1990	
P. ficuserectae		Goto, 1983	*P. syringae* group
P. flava	*Hydrogenophaga flava*	Davis, 1969	
		Willems *et al.*, 1989	
P. flavescens		Hildebrand *et al.*, 1994	*P. straminea* group
P. flectens		Johnson, 1956	
P. fluorescens		Migula, 1895	*P. fluorescens* subgroup
P. fragi		Gruber, 1905	*P. fragi* subgroup
P. frederiksbergensis		Andersen *et al.*, 2000	*P. mandelii* subgroup
P. fulva		Iizuka & Komagata, 1963	*P. putida* group
P. fuscovaginae		Miyajima *et al.*, 1983	*P. asplenii* subgroup
P. gelidicola		Kadota, 1951	
P. geniculata		Chester, 1901	
P. gessardii		Verhille *et al.*, 1999a	*P. gessardii* subgroup
P. gladioli	*Burkholderia gladioli*	Severini, 1913	
		Yabuuchi *et al.*, 1992	
P. glathei	*Burkholderia glathei*	Zolg & Ottow, 1975	
		Vandamme *et al.*, 1997	
P. glumae	*Burkholderia glumae*	Kurita & Tabei, 1967	
		Urakami *et al.*, 1994	
P. graminis		Behrendt *et al.*, 1999	*P. lutea* group
P. grimontii		Baïda *et al.*, 2002	*P. fluorescens* subgroup
P. guineae		Bozal *et al.*, 2007	*P. anguilliseptica* group
P. halophila		Fendrich, 1988	
P. hibiscicola		Moniz, 1963	
P. huttiensis	*Herbaspirillum huttiense*	Leifson, 1962a	
		Ding & Yokota, 2004	
P. indica		Pandey *et al.*, 2002	*P. aeruginosa* group
P. indigofera	*Vogesella indigofera*	Migula, 1900	
		Grimes *et al.*, 1997	
P. iners	*Marinobacterium georgiense*	Iizuka & Komagata, 1964	
		González *et al.*, 1997	
		Satomi *et al.*, 2002	
P. japonica		Pungrasmi *et al.*, 2008	
P. jessenii		Verhille *et al.*, 1999b	*P. jessenii* subgroup
P. jinjuensis		Kwon *et al.*, 2003	*P. aeruginosa* group
P. kilonensis		Sikorski *et al.*, 2001	*P. corrugata* subgroup
P. knackmussii		Stolz *et al.*, 2007	*P. aeruginosa* group
P. koreensis		Kwon *et al.*, 2003	*P. koreensis* subgroup
P. lanceolata	*Curvibacter lanceolatus*	Leifson, 1962a	
		Ding & Yokota, 2004	
P. lemoignei	*Paucimonas lemoignei*	Delafield *et al.*, 1965	
		Jendrossek, 2001	
P. libanensis		Dabboussi *et al.*, 1999a	*P. fluorescens* subgroup
P. lini		Delorme *et al.*, 2002	*P. mandelii* subgroup
P. lundensis		Molin *et al.*, 1986	*P. fragi* subgroup
P. lurida		Behrendt *et al.*, 2007	
P. lutea		Peix *et al.*, 2004	*P. lutea* group
P. luteola		Kodama *et al.*, 1985	
P. mallei	*Burkholderia mallei*	Redfearn *et al.*, 1966	
		Yabuuchi *et al.*, 1992	
P. maltophilia	*Stenotrophomonas maltophilia*	Hugh, 1981	
		Swings *et al.*, 1983	

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previous name	current classification	references	group name
		Palleroni & Bradbury, 1993	
P. mandelii		Verhille *et al.*, 1999b	*P. mandelii* subgroup
P. marginalis		Stevens, 1925	*P. fluorescens* subgroup
P. marina	*Cobetia marina*	Baumann *et al.*, 1972	
		Baumann *et al.*, 1983a	
		Dobson & Franzmann, 1996	
		Arahal *et al.*, 2002	
P. marincola		Romanenko *et al.*, 2008	*P. anguilliseptica* group
P. mediterranea		Catara *et al.*, 2002	*P. corrugata* subgroup
P. meliae		Ogimi, 1977	*P. syringae* group
P. mendocina		Palleroni *et al.*, 1970	*P. oleovorans* group
P. mephitica	*Janthinobacterium lividum*	Claydon & Hammer, 1939	
		De Ley *et al.*, 1978	
P. meridiana		Reddy *et al.*, 2004	*P. gessardii* subgroup
P. mesophilica	*Methylobacterium mesophilicum*	Austin & Goodfellow, 1979	
		Green & Bousfield, 1983	
P. migulae		Verhille *et al.*, 1999a	*P. mandelii* subgroup
P. mixta	*Telluria mixta*	Bowman *et al.*, 1988	
		Bowman *et al.*, 1993	
P. mohnii		Cámara *et al.*, 2007	*P. jessenii* subgroup
P. monteilii		Elomari *et al.*, 1997	*P. putida* group
P. moorei		Cámara *et al.*, 2007	*P. jessenii* subgroup
P. moraviensis		Tvrzová *et al.*, 2006	*P. koreensis* subgroup
P. mosselii		Dabboussi *et al.*, 2002	*P. putida* group
P. mucidolens		Levine & Anderson, 1932	*P. gessardii* subgroup
P. multiresinivorans	*P. nitroreducens*	Mohn *et al.*, 1999	
		Lang *et al.*, 2007	
P. nautica	*Marinobacter hydrocarbonoclasticus*	Baumann *et al.*, 1972	
		Spröer *et al.*, 1998	
P. nitroreducens		Iizuka & Komagata, 1964	*P. aeruginosa* group
P. oleovorans subsp. lubricantis		Saha *et al.*, 2010	*P. oleovorans* group
P. oleovorans subsp. oleovorans		Lee & Chandler, 1941	*P. oleovorans* group
P. orientalis		Daboussi *et al.*, 1999b	*P. fluorescens* subgroup
P. oryzihabitans		Kodama *et al.*, 1985	*P. oryzihabitans* group
P. otitidis		Clark *et al.*, 2006	*P. aeruginosa* group
P. pachastrellae		Romanenko *et al.*, 2005a	
P. palleroniana		Gardan *et al.*, 2002	*P. fluorescens* subgroup
P. palleronii	*Hydrogenophaga palleronii*	Davis, 1970	
		Willems *et al.*, 1989	
P. panacis		Park *et al.*, 2005	*P. fluorescens* subgroup
P. panipatensis		Gupta *et al.*, 2008	*P. aeruginosa* group
P. parafulva		Uchino *et al.*, 2001	*P. putida* group
P. paucimobilis	*Sphingomonas paucimobilis*	Holmes *et al.*,1977	
		Yabuuchi *et al.*, 1990	
P. pelagia		Hwang *et al.*, 2009	
P. peli		Vanparys *et al.*, 2006	*P. anguilliseptica* group
P. perfectomarina	*P. stutzeri*	Baumann *et al.*, 1983b	
		Döhler *et al.*, 1987	
P. pertucinogena		Kawai & Yabuuchi, 1975	
P. phenazinium	*Burkholderia phenazinium*	Bell & Turner, 1973	
		Viallard *et al.*, 1998	
P. pickettii	*Ralstonia pickettii*	Ralston *et al.*, 1973	
		Yabuuchi *et al.*, 1992	
		Yabuuchi *et al.*, 1995	
P. pictorum		Gray & Thornton, 1928	
P. plantarii	*Burkholderia plantarii*	Azegami *et al.*, 1987	
		Urakami *et al.*, 1994	
P. plecoglossicida		Nishimori *et al.*, 2000	*P. putida* group
P. poae		Behrendt *et al.*, 2003	*P. fluorescens* subgroup
P. pohangensis		Weon *et al.*, 2006	
P. proteolytica		Reddy *et al.*, 2004	*P. gessardii* subgroup
P. pseudoalcaligenes	*P. oleovorans*	Stanier, 1966	*P. oleovorans* group
		Saha *et al.*, 2010	
P. pseudoflava	*Hydrogenophaga pseudoflava*	Auling *et al.*, 1978	
		Willems *et al.*, 1989	
P. pseudomallei	*Burkholderia pseudomallei*	Haynes, 1957	
		Yabuuchi *et al.*, 1992	
P. psychrophila		Yumoto *et al.*, 2001a	*P. fragi* subgroup
P. psychrotolerans		Hauser *et al.*, 2004	*P. oryzihabitans* group
P. putida		Migula, 1895	*P. putida* group
P. pyrrocinia	*Burkholderia pyrrocinia*	Imanaka *et al.*, 1965	
		Vandamme *et al.*, 1997	
P. radiora	*Methylobacterium radiotolerans*	Ito & Iizuka, 1971	
		Green & Bousfield, 1983	
P. reinekei		Cámara *et al.*, 2007	*P. jessenii* subgroup
P. resinovorans		Delaporte *et al.*, 1961	*P. aeruginosa* group
P. rhizosphaerae		Peix *et al.*, 2003	
P. rhodesiae		Coroler *et al.*, 1996	*P. fluorescens* subgroup
P. rhodos	*Methylobacterium rhodinum*	Heumann, 1962	
		Green & Bousfield, 1983	
P. rubrilineans	*Acidovorax avenae*	Stapp, 1928	
		Willems *et al.*, 1992	
P. rubrisubalbicans	*Herbaspirillum rubrisubalbicans*	Krasil'nikov, 1949	
		Baldani *et al.*, 1996	
P. sabulinigri		Kim *et al.*, 2009	
P. saccharophila	*Pelomonas saccharophila*	Doudoroff, 1940	
		Xie & Yokota, 2005	
P. salomonii		Gardan *et al.*, 2002	*P. fluorescens* subgroup

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previous name	current classification	references	group name
P. savastanoi		Gardan *et al.*, 1992	*P. syringae* group
P. segetis		Park *et al.*, 2006	
P. simiae		Vela *et al.*, 2006	*P. fluorescens* subgroup
P. solanacearum	*Ralstonia solanacearum*	Smith, 1911	
		Yabuuchi *et al.*, 1992	
		Yabuuchi *et al.*, 1995	
P. spinosa	*Malikia spinosa*	Leifson, 1962b	
		Spring *et al.*, 2005	
P. stanieri	*Marinobacterium stanieri*	Baumann *et al.*, 1983b	
		Satomi *et al.*, 2002	
P. straminea		Iizuka & Komagata, 1963	*P. straminea* group
P. stutzeri		Sijderius, 1946	*P. stutzeri* group
P. synxantha		Holland, 1920	*P. fluorescens* subgroup
P. syringae		Van Hall, 1902	*P. syringae* group
P. syzygii	*Ralstonia syzygii*	Roberts *et al.*, 1990	
		Vaneechoutte *et al.*, 2004	
P. taeanensis		Lee *et al.*, 2010	
P. taeniospiralis	*Hydrogenophaga taeniospiralis*	Lalucat *et al.*, 1982	
		Willems *et al.*, 1989	
P. taetrolens		Haynes, 1957	*P. fragi* subgroup
P. taiwanensis		Wang *et al.*, 2010	
P. testosteroni	*Comamonas testosteroni*	Marcus & Talalay, 1956	
		Tamaoka *et al.*, 1987	
P. thermotolerans		Manaia & Moore, 2002	*P. aeruginosa* group
P. thivervalensis		Achouak *et al.*, 2000	*P. corrugata* subgroup
P. tolaasii		Paine, 1919	*P. fluorescens* subgroup
P. tremae		Gardan *et al.*, 1999	*P. syringae* group
P. trivialis		Behrendt *et al.*, 2003	*P. fluorescens* subgroup
P. tuomuerensis		Xin *et al.*, 2009	
P. umsongensis		Kwon *et al.*, 2003	*P. jessenii* subgroup
P. vancouverensis		Mohn *et al.*, 1999	*P. jessenii* subgroup
P. veronii		Elomari *et al.*, 1996	*P. fluorescens* subgroup
P. vesicularis	*Brevundimonas vesicularis*	Galarneault & Leifson, 1964	
		Segers *et al.*, 1994	
P. viridiflava		Dowson, 1939	*P. syringae* group
P. vranovensis		Tvrzová *et al.*, 2006	
P. woodsii	*Burkholderia andropogoni*	Stevens, 1925	
		Coenye *et al.*, 2001	
P. xanthomarina		Romanenko *et al.*, 2005b	*P. stutzeri* group
P. xiamenensis		Lai & Shao, 2008	
P. xinjiangensis		Liu *et al.*, 2009a	

Despite these efforts *Pseudomonas* taxonomy is still far from satisfying. Some species are still classified as members of the genus although it is generally known among *Pseudomonas* taxonomists that these species should be transferred to other taxonomic units. 16S rRNA gene sequencing clearly indicates *Pseudomonas flectens* should be transferred to the genus *Tatumella*, *Pseudomonas halophila* to *Halovibrio*, *Halospina* or *Salicoli* (further taxonomic work is needed to determine its exact affiliation), *Pseudomonas cissicola* to the genus *Xanthomonas*; *Pseudomonas pictorum*, *Pseudomonas beteli*, *Pseudomonas hibiscicola* and *Pseudomonas geniculata* to the genus *Stenotrophomonas*; *Pseudomonas boreopolis* to the genus *Xylella* and *Pseudomonas carboxydohydrogena* to *Afipia* or *Oligotropha* (again further work is needed to fully elucidate its taxonomic position). Furthermore, the All-Species Living Tree, showing 16S rRNA gene sequences for all type strains described at the time of writing (http://www.arb-silva.de/fileadmin/silva_databases/living_tree/LTP_release_106/LTPs106_SSU_tree.pdf), clearly points out that the cluster formed by members of the genus *Pseudomonas* as it is known now is far from homogeneous (hereby neglecting the obviously wrongly classified species mentioned above). Species of the genera *Azotobacter*, *Serpens*, *Thermoleophilum*, *Azorhizophilus*, *Azotobacter* and *Azomonas* are dispersed in between members of the genus *Pseudomonas*. Additionally, this All-Species Living Tree shows both the intrageneric clusters as indicated for the first time by Moore and co-workers (1996), one situated around the *P. fluorescens* type strain and one around the *P. aeruginosa* type strain, but also shows a third intrageneric cluster harbouring the following species: *P. anguilliseptica, P. azotifigens, P. balearica, P. borbori, P. caeni, P. cuatrocienegasensis, P. guinea, P. indica, P. marincola, P. pachastrellae, P. pelagia, P. peli, P. pertucinogena, P. pohangensis, P. sabulinigri, P. segetis, P. taeanensis, P. thermotolerans, P. xiamenensis* and *P. xinjiangensis* clustering with *Azomonas* and *Azotobacter* species. These species were either not included by Mulet and co-workers (2010) or

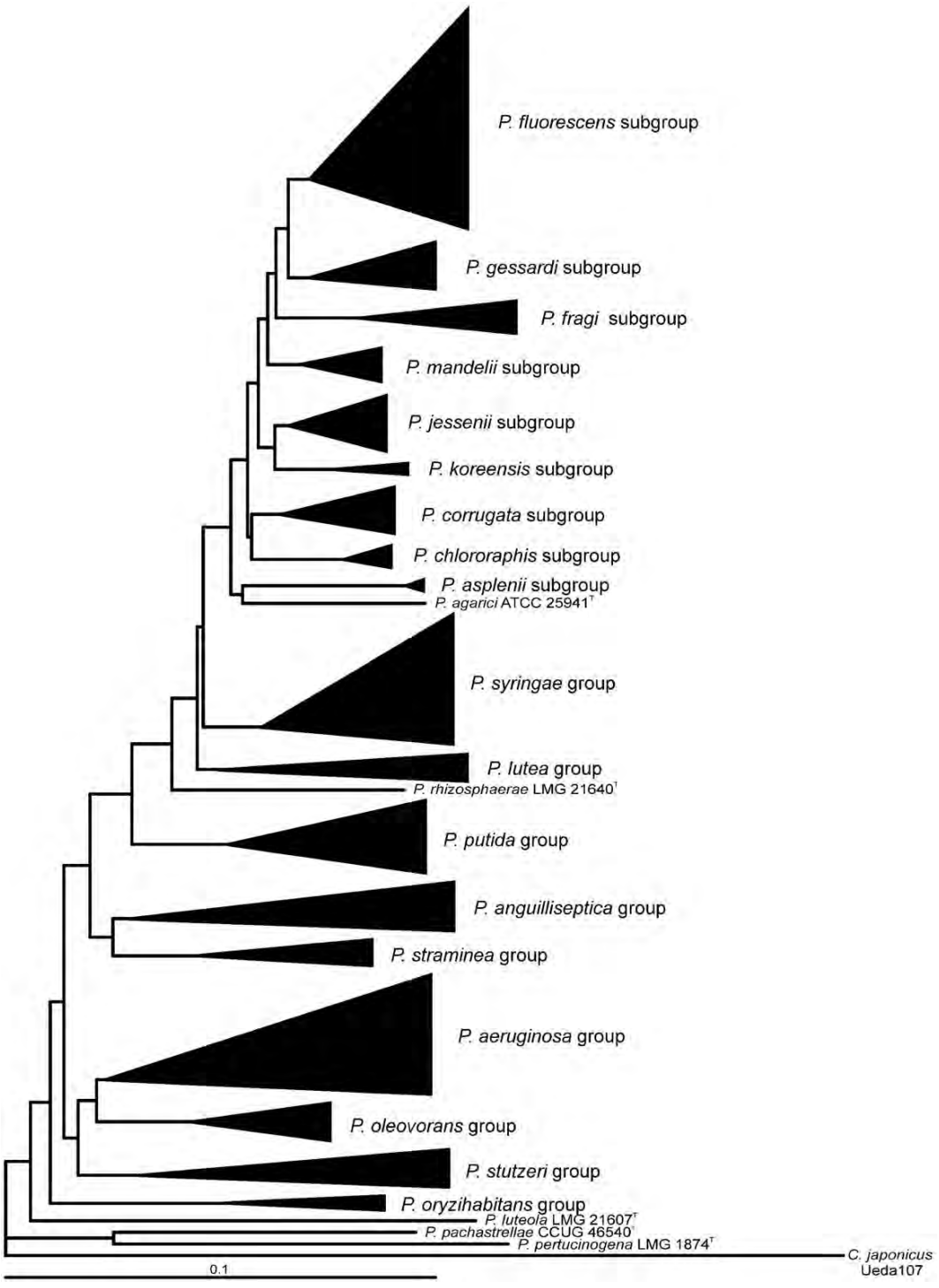

Figure 3.5: Schematic phylogenetic tree of 107 *Pseudomonas* type strains based on the concatenated analysis of 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes. Distance matrix was calculated by the Jukes-Cantor method. Dendrogram was generated by neighbour-joining. *Cellvibrio japonicum* Ueda107 was used as outgroup. The bar indicates sequence divergence (Mulet *et al.*, 2010).

assigned to the *P. anguilliseptica* group, the *P. aeruginosa* group or the *P. stutzeri* group.

These observations clearly indicate that the present standing of the genus *Pseudomonas* in taxonomy is far from being satisfactory, and the large phenotypic and genotypic diversity of its members is not very helpful in this prospect. As early as 1986, Molin and Ternström wondered if restricting the genus to members of rRNA group 1 was sufficient to harbor this diverse group of species, and considered splitting the genus further into several genera might be a workable solution. Whether or not the genus is to be splitted will probably largely depend on further evolution in taxonomical science (e.g. consensus about the species definition, outcome of large-scale genome studies) and how taxonomy will fit into the needs of the large scientific community.

3.4.2 Identification of pseudomonads

Phenotypic characterization of pseudomonads relies on a number of classical tests, such as colony and cell morphology, growth characteristics (temperature-, %NaCl-, pH-optimum), flagella type, utilization of carbon sources and antibiotic sensitivity (Palleroni, 2005). Commercial kits are available with multiple miniaturized phenotypic tests, and these are routinely used in laboratories all over the world, as they are quick, easy-to-use and reliable. However, for the pseudomonads, specialized kits such as API 20NE (bioMérieux) are not reliable for identification onto the species level as these tests are often database-dependant, meaning if a species is not included in the database, it can't be identified. This implies these kits are useful for characterization but not for identification of *Pseudomonas* species (Peix *et al.*, 2009). A striking example of misidentification of *Pseudomonas* species using commercial test kits (API 20NE) in the dairy industry was demonstrated by Marchand and co-workers (2008). These authors clearly indicated that the presence of *P. fluorescens* in raw milk was largely overestimated due to misidentification based on API 20NE solely, in contrast to the polyphasic identification approach they used, indicating *P. fragi* and *P. lundensis* as the predominant spoilage organisms in raw milk.

The applicability of the chemotaxonomic methods FAME and phospholipid analysis has been studied by Vancanneyt and co-workers (1996a) who indicated these techniques supported the bigger groups (rRNA homology groups, intrageneric clusters and subgroups) but mostly failed to differentiate onto the species level. The same authors (Vancanneyt *et al.*, 1996b) studied the usability of SDS-PAGE analysis, which showed a higher resolution and thus provided a good identification tool for *Pseudomonas* species. However, some important drawbacks of this technique are *i*) the need for a curated database containing up-to-date profiles for each species, *ii*) the standardized growth conditions that are required to obtain comparable profiles and *iii*) the non-interchangeability of profiles between different laboratories. The polyamine and polar lipid composition of pseudomonads have also been analyzed but again proved insufficient for differentiation onto the species level (Auling *et al.*, 1991; Camara *et al.*, 2007). Another, rather unusual but powerful phenotypic technique, was first applied by Meyer and co-workers (2002), who showed the siderophore system types could be used for identification of *Pseudomonas* strains onto the species level, a tool they called siderotyping.

Nonetheless some of these phenotypic techniques approved very useful for differentiating *Pseudomonas* species, most attention is given to genotypic methods by taxonomists, and in particular to MLSA schemes, most likely because the resolution of gene sequence analysis by far exceeds the resolution of any phenotypic technique used thus far (Peix *et al.*, 2009).

The key molecule in bacterial phylogeny, the 16S rRNA gene, has also been used for *Pseudomonas*

taxonomy as described in §3.4.1, however its resolution proved to be insufficient to differentiate onto the species level within some subgeneric groups. Therefore, several MLSA schemes have been developed but one should keep in mind, these MLSA schemes do have their shortcomings, as discussed above. As for now however, the MLSA scheme proposed by Mulet and co-workers (2010) is an encouraging initiative to resolve *Pseudomonas* taxonomy based on gene sequence analysis. Additionally to the 16S rRNA gene, it includes sequences of three house-keeping genes, *rpoD*, *gyrB* and *rpoB*, each showing a resolution three to eight times higher than 16S rRNA gene sequences. Furthermore, this MLSA scheme is connected with a comprehensive database, (http://www.uib.es/microbiologiaBD/Welcome.html), available online and providing a valuable additive to analyze *Pseudomonas* species (Bennasar *et al.*, 2010). The joined efforts of researchers working in the field of *Pseudomonas* taxonomy might result in a thorough MLSA scheme, including type strains and several other non type reference strains and supported by DDH values.

Whole genome sequencing is limited in the taxonomy of *Pseudomonas* with only 24 strains being sequenced, representing 9 different species.

3.5 Taxonomy of the genus *Bacillus*

The genus *Bacillus* was proposed in 1872 by Cohn, and is the type genus of the family *Bacillaceae* within the order *Bacillales*, class *Bacilli* and phylum *Firmicutes*. Members of the genus *Bacillus* are usually described as 'aerobic, endospore-forming, Gram-positive rod-shaped organisms'. Due to this vague definition, the genus *Bacillus* developed as a dumping ground for all bacteria more or less complying with this definition, an evolution parallel to that of the genus *Pseudomonas*. Even more, several species have been allocated to this genus despite lacking one of the distinctive characteristics, e.g. *Bacillus horti* (Yumoto *et al.*, 1998), *Bacillus oleronius* (Kuhnigk *et al.*, 1995) and *Bacillus azotoformans* (Pichinoty *et al.*, 1983), all staining Gram-negative, and *Bacillus infernus* (Boone *et al.*, 1995) being a strict anaerobic non-sporeformer (Fritze, 2002). This led to a very heterogeneous genus comprising metabolically versatile bacteria. Members are mainly soil inhabitants, but due to their spore-forming capacity they are able to survive and persist in many different environments.

Again parallel to the genus *Pseudomonas*, the genus *Bacillus* harbors some species that are of particular interest to the industrial and clinical world. Industrially produced enzymes are mainly of *Bacillus* origin and are widely applied in technical and food industries. For instance, *Bacillus clausii* and *Bacillus licheniformis* are the main producers of protease and amylase, respectively (Outtrup & Jørgensen, 2002). Some *Bacillus* species are used in agriculture for their plant growth promoting capacities (e.g. *Bacillus pumilus, Bacillus subtilis*) (Chanway, 2002) and other *Bacillus* species are pathogenic to insects (e.g. *Bacillus thuringiensis*) or to animals (e.g. *Bacillus cereus* and *Bacillus anthracis*). Some members of the genus are also implicated in dairy spoilage as extensively discussed above.

3.5.1 History of *Bacillus* taxonomy

The genus *Bacillus* was established in 1872 by Cohn to accommodate *Vibrio subtilis* (1835), now *Bacillus subtilis*, the type species of the genus, and '*Bacteridium*' (1864), an organism associated with the anthrax disease (Berkeley, 2002). Since then, the genus grew rapidly with a peak of 146 species reported in the fifth edition of Bergey's Manual in 1939. However, Gordon attributed

this exponential growth of *Bacillus* species to the fact that many new species were named and described without extensive comparative study with existing species (Gordon, 1973). A first attempt to clarify the classification of the genus *Bacillus* was made by Clark (1937) and Smith and co-workers (1946) who recognized three subgroups within the genus, mainly based on spore (oval, cylindrical or round spores) and sporangium (swollen or not) morphology. Later, an extensive phenotype-based study of Gordon on more than 1000 *Bacillus* strains, reduced the number of species to 33 in the sixth edition and further to 25 in the seventh edition of Bergey's Manual. However, the number of species steadily increased again later on, and *Bacillus* taxonomy was far from satisfactory to its users. This was mainly due to the enormous heterogeneity of the genus, as illustrated by the %G + C range which exceeded 30% while 10% is considered an acceptable range for delineating a genus.

It was suggested by numerous authors (Gibson & Gordon, 1974; Logan & Berkeley, 1984) that the genus should be split in at least five genera, but none of these proposals could be carried out confidently at that time. Until 1991, when Ash and co-workers (Ash *et al.*, 1991) performed the first elaborate genotype-based study in this genus by analyzing 16S rRNA gene sequences of over 50 *Bacillus* species and this study formed the basis for splitting the genus into several new genera. They established five phylogenetically distinct groups, assigned rRNA groups 1 to 5. The first rRNA group was considered *Bacillus sensu stricto*, harboring the type species *Bacillus subtilis*, and another 27 species. The second rRNA group consisted of *Bacillus sphaericus, Bacillus fusiformis, Bacillus globisporus, Bacillus insolitus, Bacillus pasteurii, Bacillus psychrophilus* and *Sporosarcina ureae*. Two members of this group were later on assigned to the novel genus *Lysinibacillus*, and are now known as *L. sphaericus* and *L. fusiformis* (Ahmed *et al.,* 2007b). *Bacillus globisporus* (Larkin & Stokes, 1967)*, Bacillus pasteurii* (Chester, 1898) and *Bacillus psychrophilus (*Nakamura, 1984a*)* have all been transferred to the genus *Sporosarcina* (Yoon *et al.,* 2001b) of which *S. ureae*, also clustering within that second rRNA group, is the type species (Kluyver & van Niel, 1936). This second rRNA group is somewhat special in that it groups *Bacillus* species with *Sporosarcina* species (that show spherical spores), and non spore-forming genera such as *Kurthia*, *Caryophanon* and *Filibacter*. rRNA group 3 harbors 10 species, that later have all been transferred to the novel genus *Paenibacillus* (Ash *et al.*, 1993), while the two members of the fourth rRNA group – *Bacillus brevis* and *Bacillus laterosporus* – have been transferred to the novel genus *Brevibacillus* (Shida *et al*., 1996). Members of the last and fifth rRNA group, comprising *Bacillus kaustophilus* (Priest *et al.*, 1989), *Bacillus stearothermophilus* (Donk, 1920) and *Bacillus thermoglucosidasius* (Suzuki *et al*., 1983), have been transferred to the genus *Geobacillus* (Nazina *et al.,* 2001).

Other genera that were proposed to accommodate former *Bacillus* species are *Virgibacillus* (Heyndrickx *et al*., 1998), "*Salibacillus*" (Wainø *et al*., 1999), *Alkalibacillus* (Jeon *et al.,* 2005), *Salimicrobium* (Yoon *et al.*, 2007), *Alicyclobacillus* (Wisotzkey *et al.,* 1992), *Aneurinibacillus* (Shida *et al*., 1996), "*Marinibacillus"* (Yoon *et al.,* 2001c), *Ureibacillus* (Fortina *et al.,* 2001), *Viridibacillus* (Albert *et al*., 2007), *Solibacillus* (Krishnamurthi *et al*., 2009) and *Pullulanibacillus* (Hatayama *et al*., 2006). In 2003, the genus "*Salibacillus'* has merged with *Virgibacillus* (Heyrman *et al*., 2003a) and "*Marinibacillus"* merged with *Jeotgalibacillus* (Yoon *et al.,* 2010). The genera *Gracilibacillus* (Wainø *et al*., 1999), *Rummeliibacillus* (Vaishampayan *et al.,* 2009) and *Sporolactobacillus* (Kitahara & Suzuki, 1963) also harbored former *Bacillus* species, but were not established for that particular purpose. Following Ash and co-workers (1991), Nielsen and co-workers (1995) established a sixth rRNA group to accomodate alkaliphilic endospore-formers, clustering in the neighborhood of *B. alcalophilus*, but members of this group have not 'yet' been assigned to another genus. Additionally, about 40 genera exist that harbor aerobic endospore-forming species

not previously assigned to the genus *Bacillus*. A comprehensive overview of all genera of aerobic endospore formers can be found in Logan and Halket (2011). The re-organization of the entire genus *Bacillus* up to now is represented in Table 3.5, listing the up-to-date names for all validly published species. A phylogenetic tree based on 16S rRNA gene sequences of all these species was constructed, and is represented in Figure 3.6. Accession numbers for the 16S rRNA gene sequences used, and corresponding type strain numbers are given in Table 3.5, as well as the group name the species were assigned to, based on 16S rRNA gene sequence analysis. Groups that were established by Ash and co-workers (1991) are indicated in red, and the group established by Nielsen and co-workers (1995) is indicated in green.

Table 3.5: Current classification of the bacilli. Abbreviations of culture collections are gathered under abbreviations. The current classification is only given when different from the previous name. Group letters (A-P) used in this table correlate with the groups observed in Figure 3.6.

			Figure 3.6		
previous name	**current classification**	**references**	**group**	**strain number**	**acc. number**
B. acidiceler		Peak *et al.*, 2007	J	CBD 119^T	DQ374637
B. acidicola		Albert *et al.*, 2005	E	105-2^T	AF547209
B. acidiproducens		Jung *et al.*, 2009	D	SL213^T	EF379274
B. acidocaldarius	*Alicyclobacillus acidocaldarius*	Darland & Brock, 1971 Wisotzkey *et al.*, 1992	rRNA 4	DSM 446^T	AJ496806
B. acidoterrestris	*Alicyclobacillus acidoterrestris*	Deinhard *et al.*, 1987 Wisotzkey *et al.*, 1992	rRNA 4	ATCC 49025^T	AB042057
B. aeolius		Gugliandolo *et al.*, 2003		4-1^T	AJ504797
B. aerius		Shivaji *et al.*, 2006	E	24K^T	AJ831843
B. aerophilus		Shivaji *et al.*, 2006	E	28K^T	AJ831844
B. agaradhaerens		Nielsen *et al.*, 1995	P	DSM 8721^T	X76445
B. agri	*Brevibacillus agri*	Nakamura, 1993 Shida *et al.*, 1996	rRNA 4	DSM 6348^T	AB112716
B. aidingensis		Xue *et al.*, 2008	N	17-5^T	DQ504377
B. akibai		Nogi *et al.*, 2005	L	1139^T	AB043858
B. alcalophilus		Vedder, 1934	L	DSM 485^T	X76436
B. algicola		Ivanova *et al.*, 2004	L	KMM 3737^T	AY228462
B. alginolyticus	*Paenibacillus alginolyticus*	Nakamura, 1987 Shida *et al.* 1997	rRNA 3	DSM 5050^T	AB073362
B. alkalidiazotrophicus	*Anaerobacillus alkalidiazotrophicus*	Sorokin *et al.*, 2008a Zavarzina *et al.*, 2009	L	MS 6^T	EU143680
B. alkalinitrilicus		Sorokin *et al.*, 2008b	L	ANL-iso4^T	EF422411
B. alkalitelluris		Lee *et al.*, 2008	J	BA288^T	AY829448
B. altitudinis		Shivaji *et al.*, 2006	E	41KF2b^T	AJ831842
B. alveayuensis		Bae *et al.*, 2005	rRNA 5	TM1^T	AY605232
B. alvei	*Paenibacillus alvei*	Cheshire & Cheyne, 1885 Ash *et al.*, 1993	rRNA 3	DSM 29^T	AJ320491
B. amyloliquefaciens		Priest *et al.*, 1987	E	ATCC 23350^T	X60605
B. amylolyticus	*Paenibacillus amylolyticus*	Nakamura, 1984c Ash *et al.*, 1993	rRNA 3	NRRL NRS-290^T	D85396
B. aneurinilyticus	*Aneurinibacillus aneurinilyticus*	Shida *et al.*, 1994b Shida *et al.*, 1996	rRNA 4	DSM 5562^T	X94194
B. anthracis		Cohn, 1872	C	ATCC 14578^T	AB190217
B. aquimaris		Yoon *et al.*, 2003b	A	TF-12^T	AF483625
B. arenosi	*Viridibacillus arenosi*	Heyrman *et al.*, 2005b Albert *et al.*, 2007	rRNA 2	LMG 22166^T	AJ627212
B. arseniciselenatis	*Anaerobacillus arseniciselenatis*	Switzer Blum *et al.*, 1998 Zavarzina *et al.*, 2009	L	E1H^T	AF064705
B. arsenicus		Shivaji *et al.*, 2005	O	Con a/3^T	AJ606700
B. arvi	*Viridibacillus arvi*	Heyrman *et al.*, 2005b Albert *et al.*, 2007	rRNA 2	LMG 22165^T	AJ627211
B. aryabhattai		Shivaji *et al.*, 2009	B	B8W22^T	EF114313
B. asahii		Yumoto *et al.*, 2004	J	FERM BP-4493^T	AB109209
B. atrophaeus		Nakamura, 1989	E	JCM 9070^T	AB021181
B. aurantiacus		Borsodi *et al.*, 2008	P	K1-5^T	AJ605773
B. axarquiensis	*Bacillus mojavensis*	Ruiz-García *et al.*, 2005b Wang *et al.*, 2007b	E	IFO 15718^T	AB021191
B. azotofixans	*Paenibacillus durus*	Seldin *et al.*, 1984 Ash *et al.*, 1993 Collins *et al.*, 1994	rRNA 3	ATCC 35681^T	X60608
B. azotoformans		Pichinoty *et al.*, 1983		NBRC 15712^T	AB363732
B. badius		Batchelor, 1919	I	ATCC 14574^T	X77790
B. barbaricus		Täubel *et al.*, 2003	O	V2-BIII-A2^T	AJ422145
B. bataviensis		Heyrman *et al.*, 2004	J	LMG 21833^T	AJ542508
B. beijingensis		Qiu *et al.*, 2009	K	ge10^T	EF371374
B. benzoevorans		Pichinoty *et al.*, 1984	G	NCIMB 12555^T	X60611
B. beveridgei		Baesman *et al.*, 2009	P	MLTeJB^T	FJ825145

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previous name	current classification	references	group	strain number	acc. number
B. bogoriensis		Vargas *et al.*, 2005	L	LBB3^T	AY376312
B. boroniphilus		Ahmed *et al.*, 2007a	K	T-15Z^T	AB198719
B. borstelensis	*Brevibacillus borstelensis*	Shida *et al.*, 1995 Shida *et al.*, 1996	rRNA 4	DSM 6347^T	AB112721
B. brevis	*Brevibacillus brevis*	Migula, 1900 Shida *et al.*, 1996	rRNA 4	NBRC 15304^T	AB271756
B. butanolivorans		Kuisiene *et al.*, 2008	J	K9^T	EF206294
B. canaveralius		Newcombe *et al.*, 2009	H	KSC_SF8b^T	DQ870688
B. carboniphilus		Fujita *et al.*, 1996	E	JCM 9731^T	AB021182
B. cecembensis		Reddy *et al.*, 2008	rRNA 2	PN 5^T	AM773821
B. cellulosilyticus		Nogi *et al.*, 2005	P	DSM 2522^T	CP002394
B. centrosporus	*Brevibacillus centrosporus*	Nakamura, 1993 Shida *et al.*, 1996	rRNA 4	NRRL NRS-664^T	D78458
B. cereus		Frankland & Frankland, 1887	C	ATCC 14579^T	AE016877
B. chagannorensis		Carrasco *et al.*, 2007	P	CG-15^T	AM492159
B. chitinolyticus	*Paenibacillus chitinolyticus*	Kuroshima *et al.*, 1996 Lee *et al.*, 2004	rRNA 3	HSCC 596^T	AB045100
B. chondroitinus	*Paenibacillus chondroitinus*	Nakamura, 1987 Shida *et al.*, 1997	rRNA 3	DSM 5051^T	D82064
B. choshinensis	*Brevibacillus choshinensis*	Takagi *et al.*, 1993 Shida *et al.*, 1996	rRNA 4	DSM 8552^T	AB112713
B. chungangensis		Cho *et al.*, 2010	I	CAU 348^T	FJ514932
B. cibi		Yoon *et al.*, 2005a	F	JG-30^T	AY550276
B. circulans		Jordan, 1890	G	ATCC 4513^T	AY724690
B. clarkii		Nielsen *et al.*, 1995	P	DSM 8720^T	X76444
B. clausii		Nielsen *et al.*, 1995	L	DSM 8716^T	X76440
B. coagulans		Hammer, 1915	D	NBRC 12583^T	AB271752
B. coahuilensis		Cerritos *et al.*, 2008	A	m4-4^T	EF014452
B. cohnii		Spanka & Fritze, 1993	J	DSM 6307^T	X76437
B. curdlanolyticus	*Paenibacillus curdlanolyticus*	Kanzawa *et al.*, 1995 Shida *et al.*, 1997	rRNA 3	DSM 10247^T	AB073202
B. cycloheptanicus	*Alicyclobacillus cycloheptanicus*	Deinhard *et al.*, 1987 Wisotzkey *et al.*, 1992	rRNA 4	DSM 4006^T	AB042059
B. decisifrondis		Zhang *et al.*, 2007	rRNA 2	E5HC-32^T	DQ465405
B. decolorationis		Heyrman *et al.*, 2003b	O	LMG 19507^T	AJ315075
B. dipsosauri	*Gracilibacillus dipsosauri*	Lawson *et al.*, 1996 Wainø *et al.*, 1999	L	DSM 11125^T	AB101591
B. drentensis		Heyrman *et al.*, 2004	J	LMG 21831^T	AJ542506
B. edaphicus	*Paenibacillus edaphicus*	Shelobolina *et al.*, 1998 Hu *et al.*, 2010	rRNA 3	T7^T	AF006076
B. ehimensis	*Paenibacillus ehimensis*	Kuroshima *et al.*, 1996 Lee *et al.*, 2004	rRNA 3	KCTC 3748^T	AY116665
B. endophyticus		Reva *et al.*, 2002		2D^T	AF295302
B. farraginis		Scheldeman *et al.*, 2004	I	R-6540^T	AY443036
B. fastidiosus		den Dooren de Jong, 1929	J	DSM 91^T	X60615
B. firmus		Bredemann & Werner, 1933	G	IAM 12464^T	D16268
B. flexus		Priest *et al.*, 1989	B	IFO 15715^T	AB021185
B. foraminis		Tiago *et al.*, 2006	K	CV53^T	AJ717382
B. fordii		Scheldeman *et al.*, 2004	I	R-7190^T	AY443039
B. formosus	*Brevibacillus formosus*	Shida *et al.*, 1995 Shida *et al.*, 1996	rRNA 4	DSM 9885^T	AB112712
B. fortis		Scheldeman *et al.*, 2004	I	R-6514^T	AY443038
B. fumarioli		Logan *et al.*, 2000	J	LMG 17489^T	AJ250056
B. funiculus		Ajithkumar *et al.*, 2002		NAF001^T	AB049195
B. fusiformis	*Lysinibacillus fusiformis*	Priest *et al.*, 1989 Ahmed *et al.*, 2007b	rRNA 2	NRS-350^T	AF169537
B. galactophilus	*Brevibacillus agri*	Takagi *et al.*, 1993 Shida *et al.*, 1994a Shida *et al.*, 1996	rRNA 4	DSM 6348^T	AB112716
B. galactosidilyticus		Heyndrickx *et al.*, 2004	I	LMG 17892^T	AJ535638
B. galliciensis		Balcázar *et al.*, 2010	J	BFLP-1^T	FM162181
B. gelatini		De Clerck *et al.*, 2004	O	LMG 21880^T	AJ551329
B. gibsonii		Nielsen *et al.*, 1995	L	DSM 8722^T	X76446
B. ginsengi		Qiu *et al.*, 2009	K	ge14^T	EF371375
B. ginsengihumi		Ten *et al.*, 2006b		Gsoil 114^T	AB245378
B. globisporus subsp. globisporus	*Sporosarcina globispora*	Larkin & Stokes, 1967 Yoon *et al.*, 2001b	rRNA 2	DSM 4^T	X68415
B. globisporus subsp. marinus	*Jeotgalibacillus marinus*	Rüger & Richter, 1979 Rüger, 1983 Yoon *et al.*, 2001b Yoon *et al.*, 2010	G	DSM 1297^T	AJ237708
B. glucanolyticus	*Paenibacillus glucanolyticus*	Alexander & Priest, 1989 Shida *et al.*, 1997	rRNA 3	DSM 5162^T	AB073189
B. gordonae	*Paenibacillus validus*	Pichinoty *et al.*, 1986 Ash *et al.*, 1993 Heyndrickx *et al.*, 1995	rRNA 3	ATCC 29948^T	X60617
B. halmapalus		Nielsen *et al.*, 1995	J	DSM 8723^T	X76447
B. haloalkaliphilus	*Alkalibacillus haloalkaliphilus*	Fritze, 1996 Jeon *et al.*, 2005	M	DSM 5271^T	AJ238041
B. halochares		Pappa *et al.*, 2010	N	MSS4^T	AM982516
B. halodenitrificans	*Virgibacillus halodenitrificans*	Denariaz *et al.*, 1989 Yoon *et al.*, 2004b	M	DSM 10037^T	AY543169
B. halodurans		Nielsen *et al.*, 1995	L	DSM 497^T	AJ302709

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previous name	current classification	references	group	strain number	acc. number
B. halophilus	*Salimicrobium halophilum*	Ventosa *et al.*, 1989 Yoon *et al.*, 2007	M	DSM 4771^T	AJ243920
B. hemicellulosilyticus		Nogi *et al.*, 2005	L	C-11^T	AB043846
B. herbersteinensis		Wieser *et al.*, 2005	J	D-1,5a^T	AJ781029
B. horikoshii		Nielsen *et al.*, 1995	J	DSM 8719^T	AB043865
B. horneckiae		Vaishampayan *et al.*, 2010	A	1P01SC^T	EU861362
B. horti		Yumoto *et al.*, 1998	O	K13^T	D87035
B. humi		Heyrman *et al.*, 2005b		LMG 22167^T	AJ627210
B. hwajinpoensis		Yoon *et al.*, 2004a	L	SW-72^T	AF541966
B. idriensis		Ko *et al.*, 2006	F	SMC 4352-2^T	AY904033
B. indicus		Suresh *et al.*, 2004	F	Sd/3^T	AJ583158
B. infantis		Ko *et al.*, 2006	H	SMC 4352-1^T	AY904032
B. infernus		Boone *et al.*, 1995		TH-23^T	U20385
B. insolitus	*Psychrobacillus insolitus*	Larkin & Stokes, 1967 Krishnamurthi *et al.*, 2010	rRNA 2	DSM 5^T	AM980508
B. isabeliae		Albuquerque *et al.*, 2008		CVS-8^T	AM503357
B. isronensis		Shivaji *et al.*, 2009	rRNA 2	B3W22^T	EF114311
B. jeotgali		Yoon *et al.*, 2001a	K	YKJ-10^T	AF221061
B. kaustophilus	*Geobacillus kaustophilus*	Priest *et al.*, 1989 Nazina *et al.*, 2001	rRNA 5	NCIMB 8547^T	X60618
B. kobensis	*Paenibacillus kobensis*	Kanzawa *et al.*, 1995 Shida *et al.*, 1997	rRNA 3	DSM 10249^T	AB073363
B. koreensis		Lim *et al.*, 2006b		BR030^T	AY667496
B. korlensis		Zhang *et al.*, 2009	G	ZLC-26^T	EU603328
B. kribbensis		Lim *et al.*, 2007	H	BT080^T	DQ280367
B. krulwichiae		Yumoto *et al.*, 2003	L	AM31D^T	AB086897
B. laevolacticus	*Sporolactobacillus laevolacticus*	Andersch *et al.*, 1994 Hatayama *et al.*, 2006	D	IAM 12321^T	D16270
B. larvae	*Paenibacillus larvae*	Ash *et al.*, 1993 Heyndrickx *et al.*, 1996a Genersch *et al.*, 2006	rRNA 3	DSM 7030^T	AY530294
B. laterosporus	*Brevibacillus laterosporus*	Laubach, 1916 Shida *et al.*, 1996	rRNA 4	IAM 12465^T	D16271
B. lautus	*Paenibacillus lautus*	Nakamura, 1984c Heyndrickx *et al.*, 1996b	rRNA 3	JCM 9073^T	AB073188
B. lehensis		Ghosh *et al.*, 2007	L	MLB2^T	AY793550
B. lentimorbus	*Paenibacillus lentimorbus*	Dutky, 1940 Pettersson *et al.*, 1999	rRNA 3	ATCC 14707^T	AB073199
B. lentus		Gibson, 1935	I	NCIMB 8773^T	AB021189
B. licheniformis		Chester, 1901	E	ATCC 14580^T	CP000002
B. litoralis		Yoon & Oh, 2005	J	SW-211^T	AY608605
B. luciferensis		Logan *et al.*, 2002	J	LMG 18422^T	AJ419629
B. macauensis		Zhang *et al.*, 2006	O	ZFKHF-1^T	AY373018
B. macerans	*Paenibacillus macerans*	Schardinger, 1905 Ash *et al.*, 1993	rRNA 3	IAM 12467^T	AB073196
B. macquariensis	*Paenibacillus macquariensis*	Marshall & Ohye, 1966 Ash *et al.*, 1993	rRNA 3	NCTC 10419^T	X60625
B. macyae		Santini *et al.*, 2004	L	JMM-4^T	AY032601
B. malacitensis	*Bacillus mojavensis*	Ruiz-García *et al.*, 2005b Wang *et al.*, 2007b	E	IFO 15718^T	AB021191
B. mannanilyticus		Nogi *et al.*, 2005	O	AM-001^T	AB043864
B. marinus	*Jeotgalibacillus marinus*	Rüger, 1983 Yoon *et al.*, 2001c Yoon *et al.*, 2010	G	DSM 1297^T	AJ237708
B. marisflavi		Yoon *et al.*, 2003b	A	TF-11^T	AF483624
B. marismortui	*Virgibacillus marismortui*	Arahal *et al.*, 1999 Arahal *et al.,* 2000 Heyrman *et al.*, 2003a	M	123^T	AJ009793
B. marmarensis		Denizci *et al.*, 2010	L	GMBE 72^T	EU621902
B. massiliensis		Glazunova *et al.*, 2006	rRNA 2	4400831^T	AY677116
B. megaterium		de Bary, 1884	B	IAM 13418^T	D16273
B. methanolicus		Arfman *et al.*, 1992	K	NCIMB 13113^T	AB112727
B. methylotrophicus		Madhaiyan *et al.*, 2010	E	CBMB205^T	EU194897
B. migulanus	*Aneurinibacillus migulanus*	Takagi *et al.*, 1993 Shida *et al.*, 1996	rRNA 4	DSM 2895^T	X94195
B. mojavensis		Roberts *et al.*, 1994	E	IFO 15718^T	AB021191
B. mucilaginosus	*Paenibacillus mucilaginosus*	Avakyan *et al.*, 1986 Hu *et al.*, 2010	rRNA 3	1480D^T	AF006077
B. muralis		Heyrman *et al.*, 2005a	J	LMG 20238^T	AJ316309
B. murimartini		Borchert *et al.*, 2007	L	LMG 21005^T	AJ316316
B. mycoides		Flügge, 1886	C	ATCC 6462^T	AB021192
B. naganoensis	*Pullulanibacillus naganoensis*	Tomimura *et al.*, 1990 Hatayama *et al.,* 2006	O	ATCC 53909^T	AB021193
B. nanhaiensis		Chen *et al.*, 2011	O	JSM 082006^T	GU477780
B. nealsonii		Venkateswaran *et al.*, 2003	G	DSM 15077^T	EU656111
B. neidei	*Viridibacillus neidei*	Nakamura *et al.*, 2002 Albert *et al.*, 2007	rRNA 2	BD-87^T	AF169520
B. neizhouensis		Chen *et al.*, 2009	P	JSM 071004^T	EU925618
B. niabensis		Kwon *et al.*, 2007	J	4T19^T	AY998119
B. niacini		Nagel & Andreesen, 1991	J	IFO 15566^T	AB021194
B. novalis		Heyrman *et al.*, 2004	J	LMG 21837^T	AJ542512
B. oceanisediminis		Zhang *et al.*, 2010	G	H2^T	GQ292772
B. odysseyi		La Duc *et al.*, 2004	rRNA 2	34hs-1^T	AF526913

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previous name	current classification	references	group	strain number	acc. number
B. okhensis		Nowlan *et al.*, 2006	L	ATCC BAA-1137^T	DQ026060
B. okuhidensis		Li *et al.*, 2002	L	GTC 854^T	AB047684
B. oleronius		Kuhnigk *et al.*, 1995	D	ATCC 700005^T	AY988598
B. oshimensis		Yumoto *et al.*, 2005	L	K11^T	AB188090
B. pabuli	*Paenibacillus pabuli*	Nakamura, 1984c Ash *et al.*, 1993	rRNA 3	HSCC 492^T	AB045094
B. pallidus	*Aeribacillus pallidus*	Scholz *et al.*, 1988 Banat *et al.*, 2004 Miñana-Galbis *et al.*, 2010	rRNA 5	DSM 3670^T	Z26930
B. pallidus	*Falsibacillus pallidus*	Zhou *et al.*, 2008 Zhou *et al.*, 2009	I	CW 7^T	EU364818
B. panaciterrae		Ten *et al.*, 2006a		Gsoil 1517^T	AB245380
B. pantothenticus	*Virgibacillus pantothenticus*	Proom & Knight, 1950 Heyndrickx *et al.*, 1998	M	IAM 11061^T	D16275
B. parabrevis	*Brevibacillus parabrevis*	Takagi *et al.*, 1993 Shida *et al.*, 1996	rRNA 4	IFO 12334^T	AB112714
B. pasteurii	*Sporosarcina pasteurii*	Chester, 1898 Yoon *et al.*, 2001b	rRNA 2	NCIMB 8841^T	X60631
B. patagoniensis		Olivera *et al.*, 2005	L	PAT 05^T	AY258614
B. peoriae	*Paenibacillus peoriae*	Montefusco *et al.*, 1993 Heyndrickx *et al.*, 1996b	rRNA 3	DSM 8320^T	AJ320494
B. persepolensis		Amoozegar *et al.*, 2009	N	HS-136^T	FM244839
B. plakortidis		Borchert *et al.*, 2007	L	P203^T	AJ880003
B. pocheonensis		Ten *et al.*, 2007	J	Gsoil420^T	AB245377
B. polygoni		Aino *et al.*, 2008	P	YN-1^T	AB292819
B. polymyxa	*Paenibacillus polymyxa*	Macé, 1889 Ash *et al.*, 1993	rRNA 3	IAM 13419^T	D16276
B. popilliae	*Paenibacillus popilliae*	Dutky, 1940 Pettersson *et al.*, 1999	rRNA 3	ATCC 14706^T	AB073198
B. pseudalcaliphilus		Nielsen *et al.*,1995	L	DSM 8725^T	X76449
B. pseudofirmus		Nielsen *et al.*, 1995	L	DSM 8715^T	X76439
B. pseudomycoides		Nakamura, 1998	C	NRRL B-617^T	AF013121
B. psychrodurans	*Psychrobacillus psychrodurans*	Abd El-Rahman *et al.*, 2002 Krishnamurthi *et al.*, 2010	rRNA 2	DSM 11713^T	AJ277984
B. psychrophilus	*Sporosarcina psychrophila*	Nakamura, 1984a Yoon *et al.*, 2001b	rRNA 2	IAM 12468^T	D16277
B. psychrosaccharolyticus		Priest *et al.*, 1989	J	ATCC 23296^T	AB021195
B. psychrotolerans	*Psychrobacillus psychrotolerans*	Abd El-Rahman *et al.*, 2002 Krishnamurthi *et al.*, 2010	rRNA 2	DSM 11706^T	AJ277983
B. pulvifaciens	*Paenibacillus larvae*	Nakamura, 1984b Ash *et al.*, 1993 Heyndrickx *et al.*, 1996a Genersch *et al.*, 2006	rRNA 3	DSM 3615^T	AY530295
B. pumilus		Meyer & Gottheil, 1901	E	ATCC 7061^T	AY876289
B. pycnus	*Rummeliibacillus pycnus*	Nakamura *et al.*, 2002 Vaishampayan *et al.*, 2009	rRNA 2	NBRC 101231^T	AB271739
B. qingdaonensis		Wang *et al.*, 2007c	N	CM1^T	DQ115802
B. reuszeri	*Brevibacillus reuszeri*	Shida *et al.*, 1995 Shida *et al.*, 1996	rRNA 4	DSM 9887^T	AB112715
B. rigui		Baik *et al.*, 2010	O	WPCB074^T	EU939689
B. ruris		Heyndrickx *et al.*, 2005	I	LMG 22866^T	AJ535639
B. safensis		Satomi *et al.*, 2006	E	FO-36b^T	AF234854
B. salarius		Lim *et al.*, 2006c	N	BH169^T	AY667494
B. salexigens	*Virgibacillus salexigens*	Garabito *et al.*, 1997 Wainø *et al.*, 1999 Heyrman *et al.*, 2003a	M	C20-Mo^T	Y11603
B. saliphilus		Romano *et al.*, 2005a	P	6AG^T	AJ493660
B. schlegelii		Schenk & Aragno, 1979	rRNA 4	ATCC 43741^T	AB042060
B. selenatarsenatis		Yamamura *et al.*, 2007	K	SF-1^T	AB262082
B. selenitireducens		Switzer Blum *et al.*, 1998	P	MLS10^T	AF064704
B. seohaeanensis		Lee *et al.*, 2006	A	BH724^T	AY667495
B. shackletonii		Logan *et al.*, 2004		LMG 18435^T	AJ250318
B. siamensis		Sumpavapol *et al.*, 2010	E	BD-A10^T	GQ281299
B. silvestris	*Solibacillus silvestris*	Rheims *et al.*, 1999 Krishnamurthi *et al.*, 2009	rRNA 2	HR3-23^T	AJ006086
B. simplex		Priest *et al.*, 1989	J	DSM 1321^T	AJ439078
B. siralis		Pettersson *et al.*, 2000	G	171544^T	AF071856
B. smithii		Nakamura *et al.*, 1988	K	DSM 4216^T	Z26935
B. soli		Heyrman *et al.*, 2004	J	LMG 21838^T	AJ542513
B. solisalsi		Liu *et al.*, 2009b	O	YC1^T	EU046268
B. sonorensis		Palmisano *et al.*, 2001	E	NRRL B-23154^T	AF302118
B. sphaericus	*Lysinibacillus sphaericus*	Meyer & Neide, 1904 Ahmed *et al.*, 2007b	rRNA 2	NRRL B-23268^T	AF169495
B. sporothermodurans		Pettersson *et al.*, 1996	G	M215^T	U49078
B. stearothermophilus	*Geobacillus stearothermophilus*	Donk, 1920 Nazina *et al.*, 2001	rRNA 5	IFO 12550^T	AB021196
B. stratosphericus		Shivaji *et al.*, 2006	E	41KF2a^T	AJ831841
B. subterraneus		Kanso *et al.*, 2002	K	COOI3B^T	AY672638
B. subtilis subsp. inaquosorum		Rooney *et al.*, 2009	E	NRRL B-23052^T	EU138467
B. subtilis subsp. spizizenii		Nakamura *et al.*, 1999	E	NRRL B-23049^T	AF074970
B. subtilis subsp. subtilis		Cohn, 1872	E	DSM 10^T	AJ276351
B. taeanensis		Lim *et al.*, 2006a	L	BH030017^T	AY603978
B. tequilensis		Gatson *et al.*, 2006	E	ATCC BAA-819^T	HQ223107

continued on next page . . .

previous name	current classification	references	group	strain number	acc. number
B. thermantarcticus		Nicolaus *et al.*, 1996	rRNA 5	DSM 9572^T	FR749957
B. thermoaerophilus	*Aneurinibacillus thermoaerophilus*	Meier-Stauffer *et al.*, 1996 Heyndrickx *et al.*, 1997	rRNA 4	DSM 10154^T	X94196
B. thermoamylovorans		Combet-Blanc *et al.*, 1995	rRNA 5	CNCM I-1378^T	L27478
B. thermocatenulatus	*Geobacillus thermocatenulatus*	Golovacheva *et al.*, 1991 Nazina *et al.*, 2001	rRNA 5	BGSC 93^T	AY608935
B. thermocloacae		Demharter & Hensel, 1989		DSM 5250^T	Z26939
B. thermodenitrificans	*Geobacillus thermodenitrificans*	Manachini *et al.*, 2000 Nazina *et al.*, 2001	rRNA 5	BGSC 94^T	AY608961
B. thermoglucosidasius	*Geobacillus thermoglucosidasius*	Suzuki *et al.*, 1983 Nazina *et al.*, 2001	rRNA 5	BGSC 95^T	AY608981
B. thermoleovorans	*Geobacillus thermoleovorans*	Zarilla & Perry, 1987 Nazina *et al.*, 2001	rRNA 5	DSM 5366^T	Z26923
B. thermoruber	*Brevibacillus thermoruber*	Manachini *et al.*, 1985 Shida *et al.*, 1996	rRNA 4	DSM 7064^T	Z26921
B. thermosphaericus	*Ureibacillus thermosphaericus*	Andersson *et al.*, 1995a Fortina *et al.*, 2001	rRNA 2	DSM 10633^T	AB101594
B. thiaminolyticus	*Paenibacillus thiaminolyticus*	Nakamura, 1990 Shida *et al.*, 1997	rRNA 3	IFO 15656^T	AB073197
B. thioparans		Pérez-Ibarra *et al.*, 2007	K	BMP-1^T	DQ371431
B. thuringiensis		Berliner, 1915	C	IAM 12077^T	D16281
B. trypoxylicola		Aizawa *et al.*, 2010	L	SU1^T	AB434284
B. tusciae		Bonjour & Aragno, 1985	rRNA 4	IFO 15312^T	AB042062
B. validus	*Paenibacillus validus*	Nakamura, 1984c Ash *et al.*, 1993	rRNA 3	JCM 9077^T	AB073203
B. vallismortis		Roberts *et al.*, 1996	E	DSM 11031^T	AB021198
B. vedderi		Agnew *et al.*, 1995	P	JaH^T	Z48306
B. velezensis	*Bacillus amyloliquefaciens*	Ruiz-García *et al.*, 2005a Wang *et al.*, 2008	E	ATCC 23350^T	X60605
B. vietnamensis		Noguchi *et al.*, 2004	A	15-1^T	AB099708
B. vireti		Heyrman *et al.*, 2004	J	LMG 21834^T	AJ542509
B. vulcani	*Geobacillus vulcani*	Caccamo *et al.*, 2000 Nazina *et al.*, 2004	rRNA 5	3S-1^T	AJ293805
B. wakoensis		Nogi *et al.*, 2005	L	N1^T	AB043851
B. weihenstephanensis		Lechner *et al.*, 1998	C	DSM 11821^T	AB021199

As already stated by Stackebrandt and Swiderski (2002), the phylogenetic heterogeneity within rRNA group 1 is huge, and when selected species are included in larger databases, they often do not cluster together, which is also the case here, with members of rRNA group 1 being dispersed among different groups. To enhance visibility of the tree (Figure 3.6), the *Bacillus* species not allocated to rRNA groups 2 to 6 were clustered in species groups; some of these are well established in *Bacillus* taxonomy, such as the *Bacillus cereus* and *Bacillus subtilis* groups, indicated in blue; but others have been 'created' by the author, indicated in black, and names of these groups are based on the 'oldest' validly described species. Additionally, species groups harboring species that have been assigned to other genera are indicated in grey. This phylogenetic tree reveals *Bacillus* taxonomy is far from being elucidated. The earlier established rRNA groups remain, but *Bacillus* species are randomly dispersed among these groups. The heterogeneity of the genus is also shown by various groups harboring species that were later on assigned to other genera. Furthermore, this tree only includes species that once have been assigned to the genus *Bacillus*; if other closely related genera / species would be included, the tree would render even more complex, and heterogeneity of the genus *Bacillus* even more obvious.

It is clear from this picture that, although considerable efforts have already been made in resolving *Bacillus* taxonomy, the genus still needs a thorough revision. A possible first step could be an extension of the study of Ash and co-workers (1991), by dividing the genus into several large rRNA groups, each group probably representing at least a separate genus status. However, division of the genus *Bacillus* into several groups could be rather complicated. As for the pseudomonads, one can already imagine (based on descriptions of established species and genera of aerobic endospore formers) that there will be no distinctive phenotypic characteristics that unequivocally distinguish *Bacillus* s.l. species from one group from those belonging to other groups, and this is indeed confirmed by Stackebrandt and Swiderski (2002). Furthermore, many species have been established based on single strains, which not only hampers the definition of

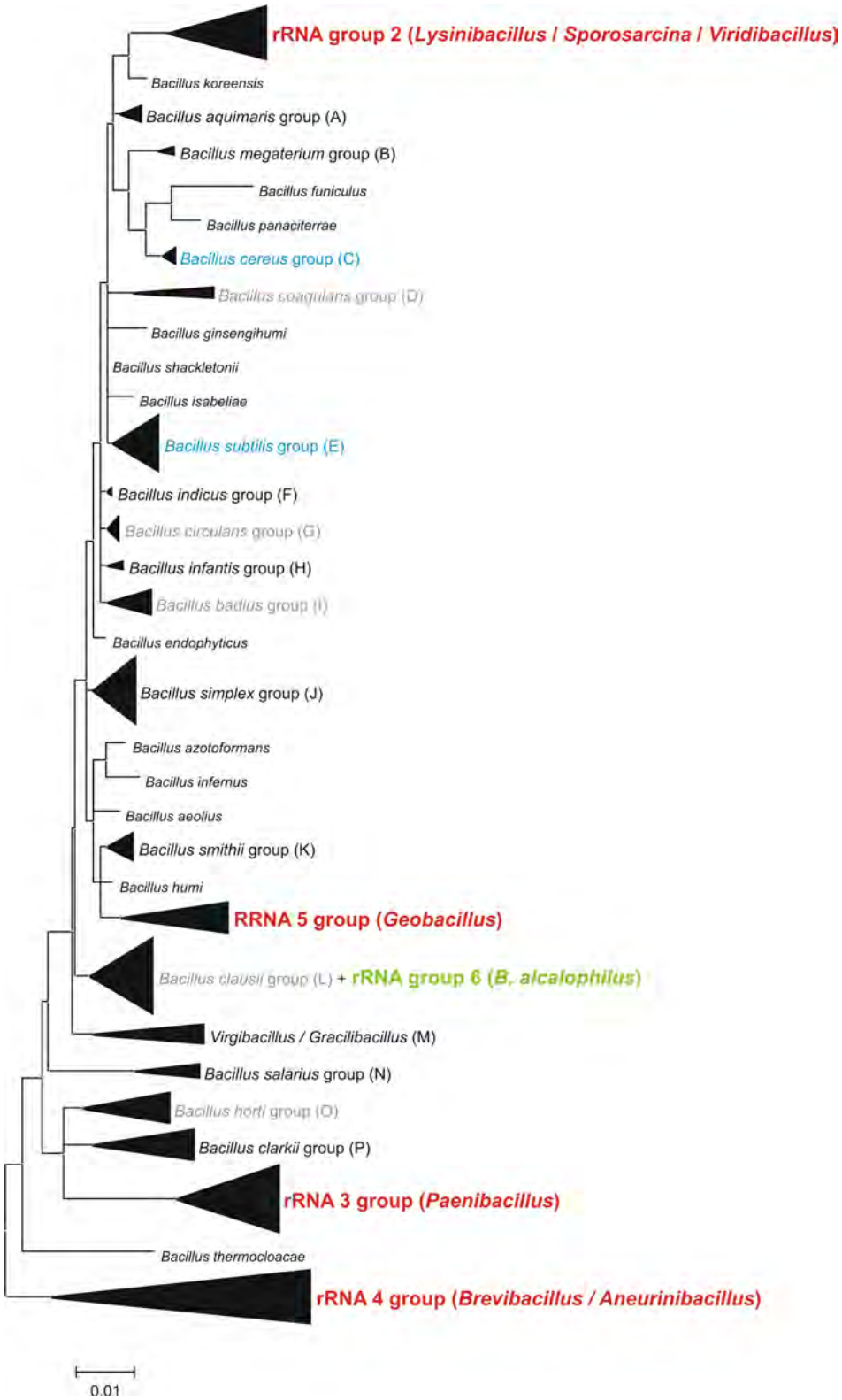


Figure 3.6: Maximum likelihood tree of all validly published *Bacillus* species based on 16S rRNA gene sequences. Bar, 0.01 substitutions per nucleotide position. Accession numbers for all sequences can be found in Table 3.5.

the intraspecies phenotypic diversity but also hinders establishment of their phylogenetic position within the general framework of bacilli. Since the year 2000, 131 *Bacillus* species have been described, and for 77 of those species, description was based on a single isolate, which is almost 60%! (An interesting detail in this prospect is that of all species descriptions based on multiple strains, since the year 2000, 30% is on behalf of the triad research groups De Vos / Heyndrickx / Logan.) Another obstacle in marking 'genus' groups refers to the lack of guidelines to delineate the genus taxon in bacterial taxonomy, in order to allow assignment of species at this level.

3.5.2 Identification of bacilli

Classification of *Bacillus* strains and closely related members has proven to be very complex, and the current situation is far from satisfactory as mentioned above. This of course implies that identification suffers from the same difficulties as classification, namely *i*) rapid expansion of the number of novel species, often poorly described and/or based on very few strains, *ii*) transfer of species to other genera, *iii*) the lack of clear distinctive phenotypic properties to differentiate between species and genera harboring aerobic spore-forming species and *iv*) allocation of phenotypically completely aberrant species, such as anaerobes (*B. infernus*), Gram-negatives (*B. horti*), coccoid cells (*B. saliphilus*) and non spore-formers (*B. subterraneus*). This emphasizes the need for a polyphasic approach when identifying bacilli, in order to obtain a more complete characterization which should help in allocating the strains to their corresponding taxon.

Phenotypic characterization of bacilli relied on the classical tests as described by Gordon and co-workers (1973) but these tests are laborious, suffer from poor interlaboratory resolution, and are time-consuming. A valuable alternative was found in the application of miniaturized biochemical tests, the API systems (bioMérieux), namely API 50CHB and API 20E (Logan & Berkeley, 1984), as well as the VITEK system (bioMérieux) and Biolog plates. These systems are able to differentiate a wide range of bacilli, but fail to distinguish between phenotypically highly similar organisms, for instance members of the *B. subtilis* group. As recommended by Logan and co-workers (2009), some standard phenotypic tests that should be applied when investigating *Bacillus* strains are: cell, colony and sporangial morphology, Gram-reaction, motility, presence of storage inclusions and parasporal bodies, temperature growth range, pH growth range, % salinity growth range, catalase, oxidase, oxygen requirements, hydrolysis of gelatin, casein and starch, citrate utilization and acid production from D-glucose. As reported by Kaneda (1977), the chemotaxonomic method FAME is very useful in differentiating *Bacillus* s.l. species from other taxa due to a distinctive fatty acid composition (preferentially branched chain fatty acids are incorporated) (Kämpfer, 2002). FAME even proved to be useful on a higher taxonomic level, sometimes even onto species level (Coorevits *et al*., 2008; Scheldeman *et al*., 2005; Vaerewijck *et al*., 2001), and the corresponding MIDI-identification often proved to be supportive if similarity scores above 0.7 were reached (Coorevits *et al*., 2008). Disadvantages however are *i*) the need for standardization, impeding the identification of organisms that require special growth conditions, and *ii*) the need for an up-to-date database. So, FAME appears to be very useful in describing *Bacillus* organisms, rather than in identifying them. Other chemotaxonomic techniques could be used for classification and identification of *Bacillus* species, such as SDS-PAGE, matrix assisted laser desorption / ionization-time-of-flight mass spectrometry, polar lipid analysis, quinone content and cell wall composition. The latter three are highly specific for *Bacillus* and thus considered essential when describing new genera and highly recommended when describing novel species (Logan *et al.*, 2009).

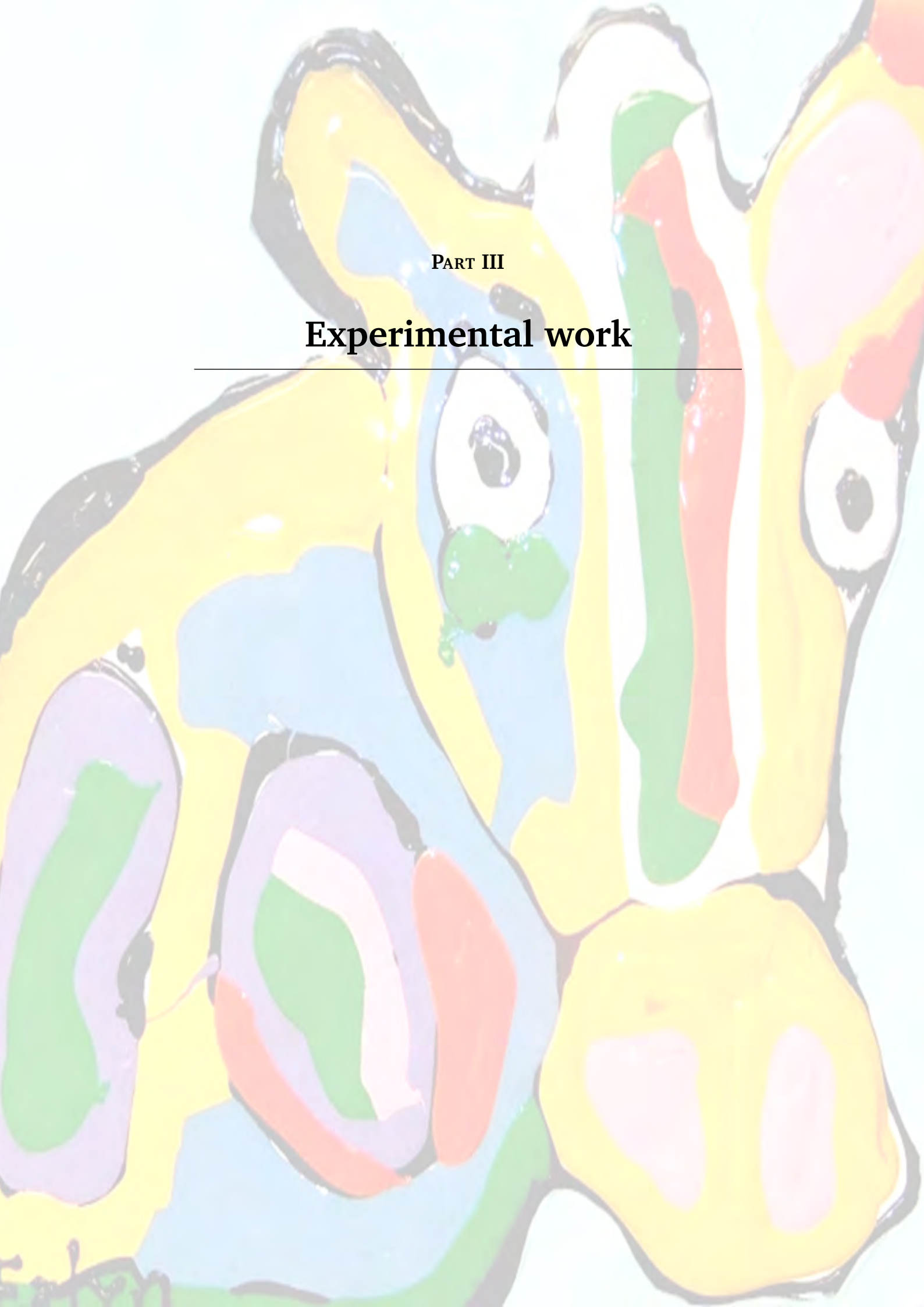
As for the genotypic methods, the same could be said about *Bacillus* as already stated for *Pseu-*

domonas: the 16S rRNA gene is again the key molecule, but often lacks sufficient resolution. For some subgroups, the applicability of other genes has already been investigated and validated such as the *gyrB* (coding for the gyrase β-subunit) gene to differentiate members of the *Bacillus subtilis* group (Wang *et al.*, 2007a). An overview of possible biomarker genes for bacilli, based on developed Multi Locus Sequence Typing (MLST) schemes, is represented in Table 3.6

Unfortunately, for now, developed schemes are limited to certain subgroups, and it is to be expected more MLSA schemes will be developed to cover a broader range of *Bacillus* species. Ideally, one MLSA scheme could be developed to cover the whole genus but due to huge intrageneric diversity, this is very unlikely. Probably, 16S rRNA gene sequencing will define to what subgroup the organisms belong to and a further identification onto a finer taxonomic level could then be achieved by applying a subgroup specific MLSA-scheme. Whole genome sequencing is limited in the taxonomy of *Bacillus* with only 43 strains being sequenced, representing 17 different species.

gene	protein	function	study
gyrB	DNA gyrase β subunit	introduction of neg. supercoils into bact. chromosomes during replication	Wang *et al.*, 2007a
glpF	glycerol uptake facilitator protein	catalysis of transmembrane diffusion of glycerol	Priest *et al.*, 2004
gmk	guanylate kinase	catalysis of the ATP-dependent phosphorylation of GMP	Priest *et al.*, 2004
ilvD	dihydroxyacid dehydratase	cleavage of carbon-oxygen bonds	Priest *et al.*, 2004
pta	phosphate acetyltransferase	acetyl-CoA and phosphate to CoA and acetyl-phosphate	Priest *et al.*, 2004
pur	phosphoribosylaminoimidazole carboxamide formyltransferase	10-formyltetrahydrofolate and AICAR to tetrahydrofolate and FAICAR	Priest *et al.*, 2004
pycA	pyruvate carboxylase	irreversible carboxylation of pyruvate to oxaloacetate	Priest *et al.*, 2004
tpi	triphosphate isomerase	reversible interconversion between triphosphate isomers	Priest *et al.*, 2004
clpC	caseinolytic protease C	degradation of casein	Sorokin *et al.*, 2006
dinB	DNA polymerase IV	synthesis of DNA	Sorokin *et al.*, 2006
gdpD	glycerophosphodiester phosphodiesterase	glycerophosphodiester in alcohol and glycerol phosphate	Sorokin *et al.*, 2006
panC	pantothenate synthetase	converts pantoate and β-alanine to pantothenate	Sorokin *et al.*, 2006

Table 3.6: Overview of genes other than 16S rRNA used for resolving *Bacillus* taxonomy; the encoded protein, its function and the study the gene has been used in are given.



PART III

Experimental work

Chapter 5 has been redrafted from

1. De Jonghe, V., **Coorevits, A.**, Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2011b). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl Environ Microbiol* **77**, 460-470.

Chapter 6 has been redrafted from

2. **Coorevits, A.**, De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126-140.

3. **Coorevits, A.**, Logan, N.A., Dinsdale, A.E., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961.

4. De Jonghe, V., **Coorevits, A.**, Vandroemme, J., Heyrman, J., Herman, L., De Vos, P. & Heyndrickx, M. (2008). Intraspecific genotypic diversity of *Bacillus* species from raw milk. *Int Dairy J* **18**, 496 - 505.

5. De Jonghe V., **Coorevits, A.**, De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., De Vos, P. & Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *Int J Food Microbiol* **136**, 318-325.

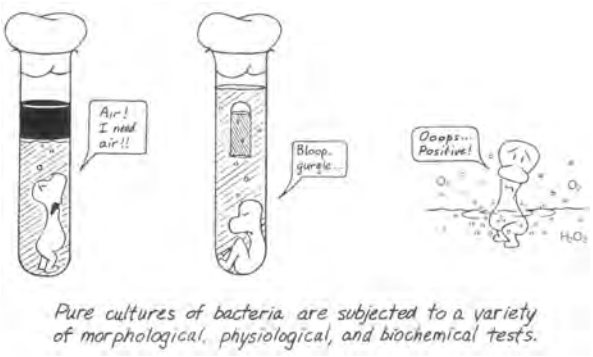
Chapter 7 has been redrafted from

6. Dinsdale A.E., Halket G., **Coorevits** A., Van Landschoot A., Busse H.-J., De Vos P. & Logan N.A. (2011). Emended descriptions of *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*. *Int J Syst Evol Microbiol* **61**, 1802-1810.

7. **Coorevits A,** Dinsdale A.E., Halket G., Lebbe L., De Vos P., Van Landschoot A. & Logan N.A (2011b). Taxonomic revision of the genus *Geobacillus*: emendation of *Geobacillus*, *G. stearothermophilus*, *G. jurassicus, G. toebii*, *G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly "*thermoglucosidasius*"); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus*; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. *Int J Syst Evol Microbiol*, in press (doi.101099/ijs.0.030346-0).

CHAPTER 4

Materials and methods



This work was the result of collaboration with two other laboratories. On the one hand, the work on microbial diversity of milk was performed in cooperation with Dr. Valerie De Jonghe and Prof. Dr. Marc Heyndrickx from the ILVO (Institute for Agricultural and Fisheries Research). On the other hand, the elaborate taxonomic work on bacilli was performed in cooperation with Prof. Niall Logan, Dr. Anna Dinsdale and Dr. Gillian Halket from the Glasgow Caledonian University. The techniques described in detail in this chapter are the techniques performed by the author.

4.1 Fatty acid methyl ester analysis (FAME)

Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P., & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126-140. 'Bacterial cultures used for FAME-extraction were grown as described by Scheldeman *et al.* (2002). FAME-extraction and analysis was performed as described by Vancanneyt *et al.* (1996a). A preliminary identification of the bacteria, based on their FAME-profiles, was obtained using the TSBA database (version 5.0) of the Microbial Identification System software (MIDI; Microbial ID, USA). A similarity index of ≥ 0.8 (on a scale 0 to 1.0) was considered as a reliable FAME-based identification. The data were transferred to the BioNumerics 4.6 software (Applied Maths, Belgium) and numerically analyzed (Dawyndt *et al.*, 2006). To obtain a first grouping of the isolates, a Canberra metric distance matrix was calculated from all FAME profiles. The distance matrix was applied in a cluster analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Initial group margins were chosen at 0.7 Canberra metric distance'.

De Jonghe, V., **Coorevits, A.**, Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2011b). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl Environ Microbiol* **77**, 460-470. 'FAME extraction and analysis were performed as described by Vancanneyt *et al.* (1996a). A preliminar identification of bacteria based on their FAME profiles, was obtained using the TSBA database (version 5.0) of the MIDI-software program (MIDI microbial ID system)'.

4.1.1 Introduction

Cell membranes consist of a phospholipid bilayer with proteins embedded in them. Phospholipids are typically constructed of a polar head, and two hydrophobic fatty acid tails (Figure 4.1).

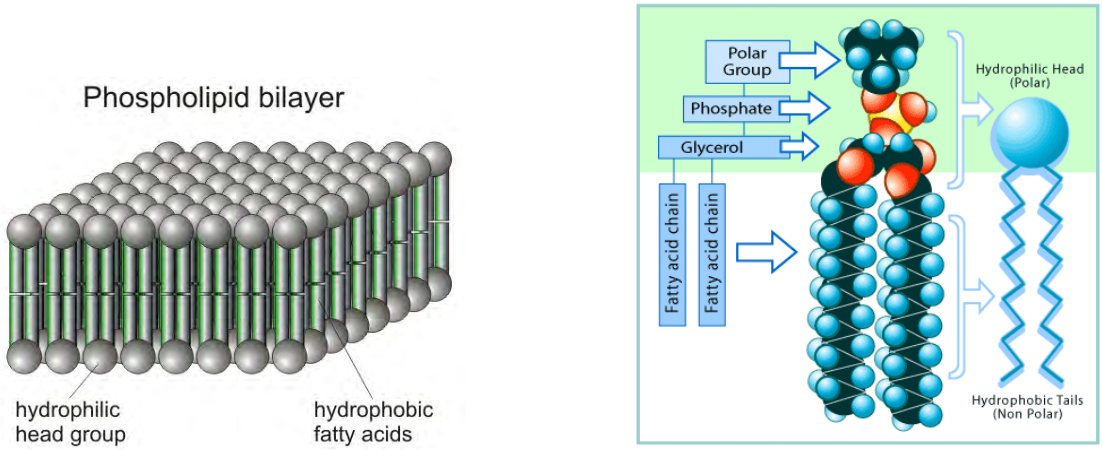


Figure 4.1: Structure of a phospholipid bilayer (left) and detailed view of a phospholipid (right) (Quizlet, 2011).

The composition of the fatty acids of bacterial membranes is highly variable and depends on environmental conditions. Standardization of growth temperature, growth medium, and growth time are thus essential parameters in FAME analysis.

4.1.2 Method description

Cells were harvested after growth under standardized conditions. Fatty acids were subsequently extracted and the extracts were analyzed using a gas chromatograph (GC).

4.1.2.1 Cultivation of bacteria

All cells were grown on TSBA-medium, with the following composition: 30 g Trypticase Soy Broth (Becton Dickinson) and 15 g granulated agar (Becton Dickinson) in 1 L of distilled water. This medium was prepared by dissolving all components while gently heating and stirring. When all constituents were completely dissolved (visible by a clear appearance of the solution), the medium was autoclaved for 15 minutes at 121°C.

Ideally, growth temperature was 28°C and incubation time was 24 hours. Unfortunately, not all bacteria included in this study were able to grow in these conditions. Therefore, sometimes deviant growth temperature (52°C) and incubation time (48h) was applied. The use of other parameters than those predefined hampers reliable identification through comparison of profiles with the commercially available MIDI database. However, it allows grouping of bacteria grown under exactly the same conditions.

Bacteria were inoculated twice on TSBA for 24 hours, the first time in a dilution streak pattern, the second time in a specific quadrant streak pattern as represented in Figure 4.2. The overlap area between the second and third quadrant was harvested and allowed for a rough standardization

of physiological age of the cultures. However, when harvesting cells from the overlap area did not result in enough biomass (app. 40 mg), cells from the remaining areas or even from multiple plates were harvested. Again, this hampered reliable identification.

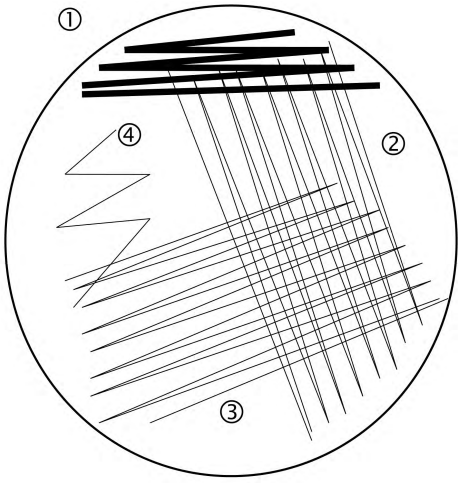


Figure 4.2: Quadrant streak method.

4.1.2.2 Extraction of fatty acids

A schematic overview of the extraction procedure is represented in Figure 4.3. Cells were harvested with a plastic loop and transferred to a glass tube, from where the four-step extraction procedure started. In a first saponification step, 1 mL of a strong methanolic base (45 g NaOH in 300 mL methanol/water, v/v, 1/1) was added to the cells, and this solution in combination with heat (30 min at 100°C) killed and lysed the cells. Fatty acids were cleaved from the cell lipids and converted to their sodium salts. The second step involved methylation of the fatty acids by adding 2 mL methylation reagent (325 mL 6N hydrochloric acid, 275 mL methanol) and incubation for 10 minutes at 80°C. Methylation converted the fatty acids (as sodium salts) to fatty acid methyl esters which increased the volatility of the fatty acids for GC analysis. During a third step, fatty acids were removed from the aqueous phase into an organic phase with a liquid-liquid extraction procedure. This was achieved by adding 1.25 mL extraction solvent (200 mL hexane, 200 mL methyl-tertiary butyl ether) to the tubes and stirring for 10 minutes. The aqueous phase was then removed and in a last washing step residual reagents and free fatty acids were cleared away by adding 3 mL base wash solution (10.8 g NaOH in 900 mL water). The organic phase was then transferred to GC-vials and capped. These vials could be stored at -20°C or immediately analyzed on the gas chromatograph. If stored at -20°C, residual water could be visualized as it crystallized in the solution. These water crystals had to be removed to prevent damage to the GC-column.

4.1.2.3 Gas chromatography analysis

Analysis of fatty acids was performed using a Hewlett-Packard 5890A Gas Chromatograph, with flame ionization detector (FID). An injector needle picked up 2 μL from the sample that had been placed in the sample loading block, and injected it in the injection block. The needle was then washed several times with the extraction solvent solution before picking up a next sample. The

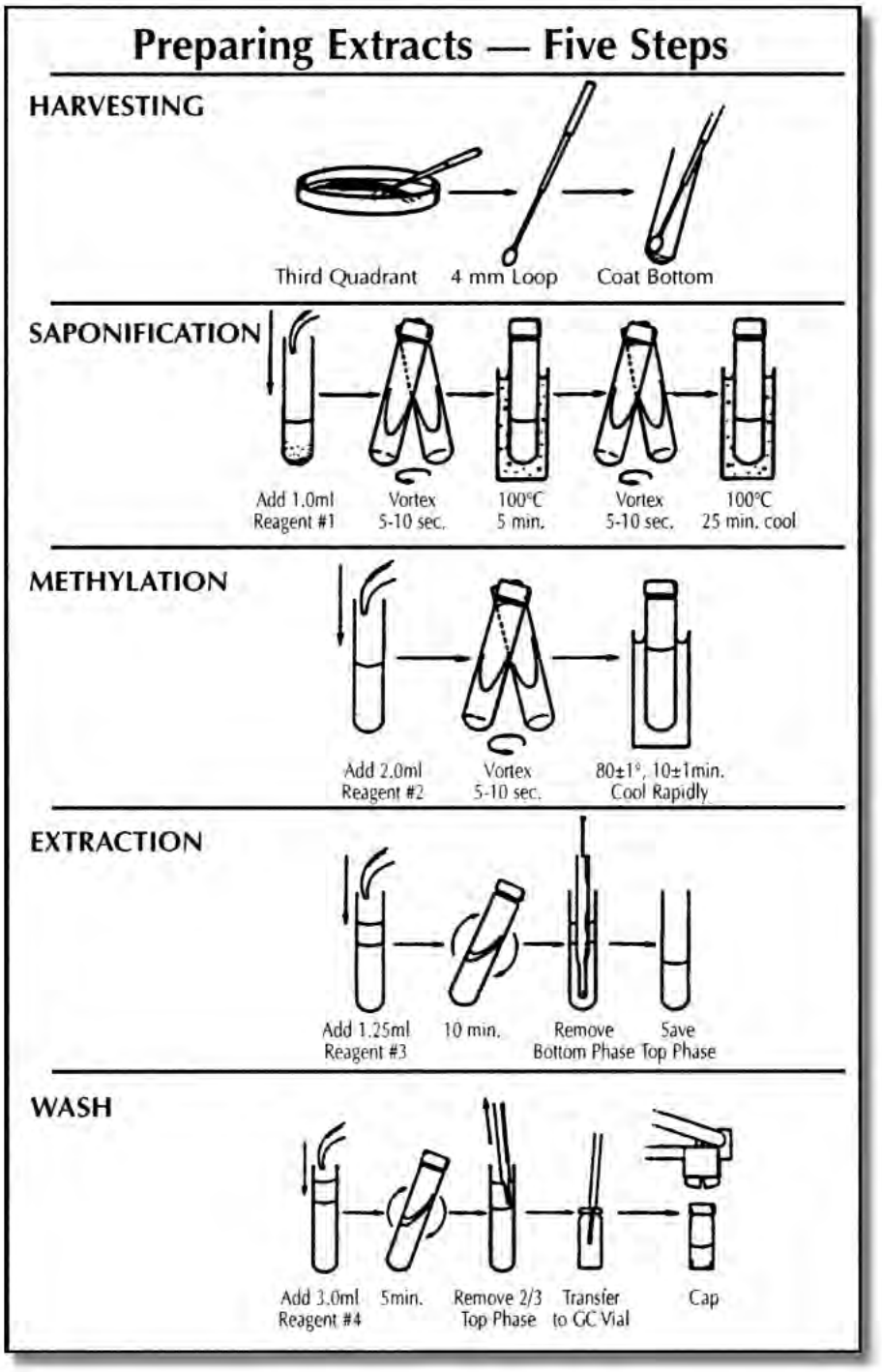


Figure 4.3: Schematic representation of fatty acid extraction procedure (Sasser, 1990).

FAME extract was carried through the capillary column (25 m × 0.2 mm methyl silicone fused column) by hydrogen gas, and the column was heated from 170 to 270°C during a run of 30 minutes (a 5°C increase every minute). Fatty acid methyl esters were separated based on polarity and size. The FID at a temperature of 300°C burned the carbon atoms of the esters, resulting in a signal that was printed as a peak. After every ten samples a calibration mixture was loaded consisting of known fatty acids, which allowed the user to link the retention times of out-coming peaks with corresponding fatty acids.

4.1.2.4 Computer analysis of profiles

The obtained chromatogram could then be compared with the commercial chromatogram library of MIDI (Sherlock Microbial Identification Systems (MIS), version 3.0 / Library TSBA 50, version 5.0). This library contains mean chromatograms for all taxa that have been recorded. Depending on the degree of similarity between profiles (the query profile and the database profile), a genus- and species identification was given, together with a similarity index. This similarity index ranged from 0 to 1 and was kind of a statistical score to visualize how good both profiles matched. A similarity score above 0.6 was generally regarded as a reliable identification while similarity scores lower than 0.01 resulted in no identification at all. Identifications could further be confirmed by qualitatively and quantitatively comparing with an in-house database containing over 70000 fatty acid profiles, also known as FAME FATAL (Fast Automatic Tracking And Loading).

The software BioNumerics version 5.1 was used to perform numerical analysis of fatty acid profiles. Basically, all peak patterns (= chromatograms) involved in the analysis were compared with each other, and this comparison resulted in a similarity score that was then recorded in a similarity matrix. To compare two curve profiles, multiple points on those curves were extracted and distances between these corresponding points were calculated (represented by the two-arrowed lines in Figure 4.4).

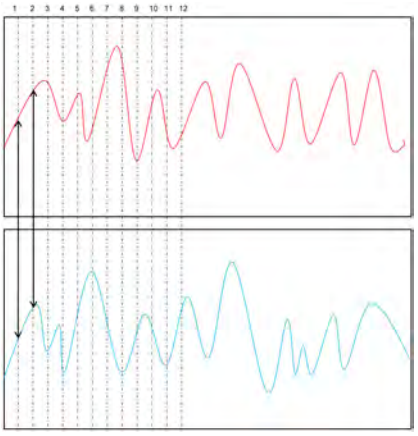


Figure 4.4: Comparison of two curve profiles.

There are various ways of calculating the distance between those two points, represented by various correlation coefficients available. A correlation value close to 1 means both profiles correlate almost perfectly, while a correlation value near 0 indicates no correlation between both profiles. The correlation coefficient used in this study was the Canberra metric correlation because often

closely related bacteria were compared with each other and the Canberra metric allows differentiation between highly similar profiles by highlighting characters with low values (near 0). A second step in numerical analysis was to convert the obtained similarity matrix into a dendrogram, in other words to obtain a grouping of similar profiles. This was mostly done by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) whereby two entries sharing the highest similarity score in the similarity matrix were grouped first. Then the third highest match was grouped alongside, and further on to the fourth, fifth and so on, until all entries got a place in the dendrogram. That way, highly similar profiles were grouped with each other.

4.2 Polar lipid analysis

Coorevits, A., Dinsdale, A.E., Heyrman, J., Schumann, P., Van Landschoot, A., Logan, N.A. & De Vos, P. (2011a). *Lysinibacillus macroides* sp. nov., nom. rev. *Int J Syst Evol Microbiol*, doi 10.1099/ijs.0.027995-0. 'Cell material was obtained after growth on TSA (Oxoid) for 24h at 28°C. Polar lipids were subsequently extracted and separated by using two-dimensional thin-layer chromatography according to Tindall (1990a, b). The total lipid profiles were visualised by spraying with molybdatophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates) and α-naphthol (specific for sugars).'

4.2.1 Introduction

Polar lipids are main compounds of the cell membrane (Figure 4.1). They consist of a polar head, and variable fatty acid chains, that were studied in FAME analysis. Different types of polar lipids exist, one of which is represented in Figure 4.5.

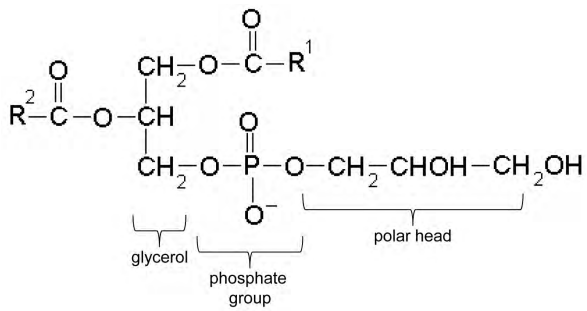


Figure 4.5: Structure of phosphatidylglycerol; R: fatty acid alkyl chain.

Composition of the phospholipids is also dependant of environmental conditions, and as for fatty acids, standardized growth conditions should be applied.

4.2.2 Method description

Cells were harvested after growth under standardized conditions, and lyophilized. After lyophilisation, polar lipids were extracted using a chloroform/methanol extraction procedure, and separated by thin layer chromatography (TLC) on silica plates. In a last step, the lipids were visualized by spraying with specific dyes.

4.2.2.1 Cultivation of bacteria

All cells in this study were grown on TSA-medium (Oxoid) for 24 hours at 28°C or 52°C. Most important is that all strains that were to be compared for their polar lipid profile, were grown under exactly the same conditions, but these conditions didn't need to be universal for all analyses, i.e. cultivation parameters are standardized but not fixed. There was no need to apply universal growth conditions as is the case for FAME, because there was no commercial identification database linked that could be used to identify profiles. Approximately 1 g of biomass was required in order to obtain a detectable amount of polar lipids. The biomass was harvested in 12 mL Falcon tubes and sealed with hydrophobic cotton wadding for lyophilization. These tubes could be stored at -20°C.

4.2.2.2 Lyophilisation of biomass

Extraction of polar lipids can start from fresh, frozen or freeze-dried material. However, freeze-dried material was preferred, because presence of water might have interfered with the chloroform/methanol extraction procedure, and thus could have hampered a good extraction of polar lipids. The lyophilized biomass did not need to be resuscitated, so no lyoprotectants were added. Lyophilization was carried out with a FT Dura-Top Microprocessor consisting of 2 units, the bulk tray driver and the corrosion resistant freeze dryer unit. The program applied is graphically represented in Figure 4.6, showing the temperature/time/pressure profile of a lyophilization run, with the red line (control) representing the sample line.

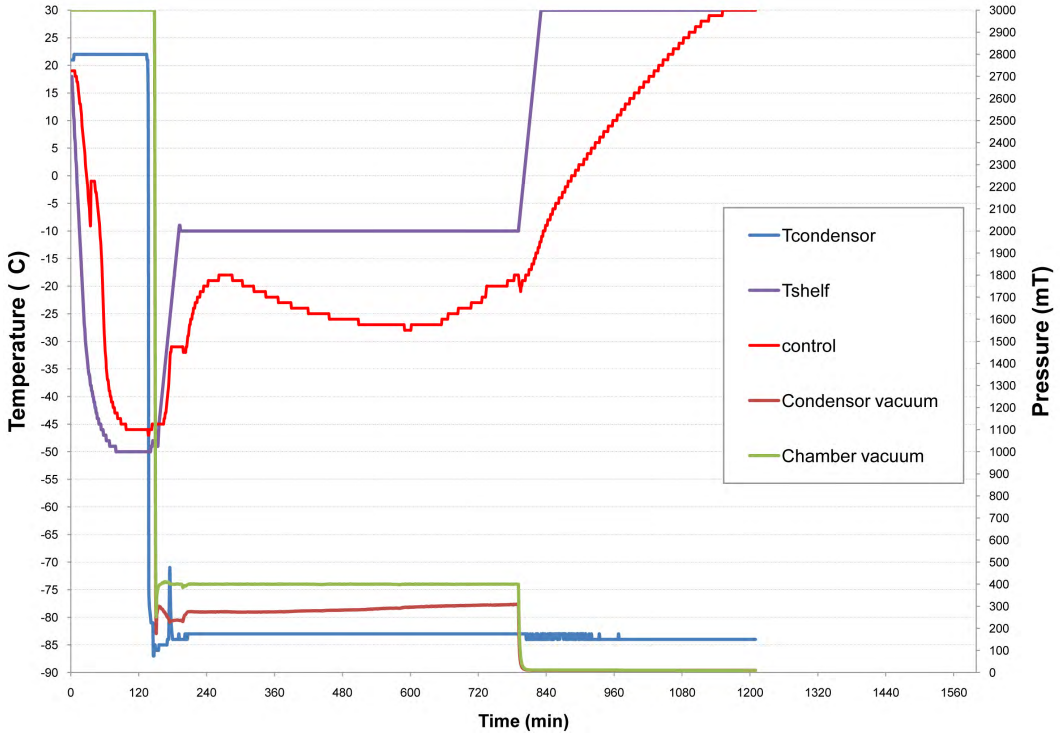


Figure 4.6: Example of a lyo-run.

4.2.2.3 Polar lipid extraction

Extraction started by adding 2 mL 0.3% NaCl, 5 mL methanol and 2.5 mL chloroform to 100 mg freeze-dried biomass in a brown pyrex bottle (brown bottles were used to protect the extract from light to prevent oxidation). The bottle was gassed with nitrogen gas to create an oxygen-free atmosphere in the bottle, again to prevent oxidation of lipids. The bottle was sealed with Teflon-lined screw caps, and heated for 15 minutes at 80°C. After heating, the extract was allowed to cool to room temperature, while it was continuously shaken on a magnetic stirring plate. A centrifugation step in glass centrifugation tubes was performed (10 min. at 3000 rpm) to get rid of cell debris. The supernatant was then decanted into 5 mL chloroform/0.3% NaCl (1/1, v/v) in a brown pyrex bottle, and gently stirred. This shifted the ratios of chloroform/water/methanol, so that a biphasic system was obtained, which became apparent after a second centrifugation step (5 min. at 3000 rpm). The upper aqueous phase contained sugars and salts, and was separated from the lower organic phase containing dissolved lipids by a protein layer. The lower chloroform phase was separated from the upper phase and taken to dryness under a stream of nitrogen gas. The dried lipid material was then redissolved in 250 μL chloroform/methanol (2/1, v/v) and extracts could be stored at -20°C for at least up to one year.

4.2.2.4 Thin layer chromatography

The lipid solution (app. 10 μL) was then used to spot thin layer silica plates (10 $\times$ 10 cm), and was developed in two directions in saturated chromatography chambers. In the first dimension a chloroform/methanol/water (65/24/4, v/v/v) solution was used and in the second dimension a chloroform:methanol:acetic acid:water (80/12/15/4, v/v/v/v) solution was used. Between the first and second dimension, plates were allowed to dry for at least 20 minutes.

4.2.2.5 Detection of lipids

Lipids were visualized by spraying with specific dyes. One dye, molybdatophosphoric acid, stained all lipids grey on a yellowish green background, while the other dyes were specific for functional groups. Ninhydrin-reagens specifically stained aminolipids red, while molybdenum blue spray reagens stained phosphor groups blue and α-naphthol reagens stained glycolipids purple on a brown background. For all dyes, except molybdenum blue, plates had to be heated for 5 minutes at 110°C to visualize the spots. Spots on the common dye plate were identified by comparing spot position and form on the other plates, and thus pointing the functional groups for each lipid present. For example, a lipid that was visible on the molybdenum blue sprayed plate, and on the α-naphthol sprayed plate was identified as a glycophospholipid.

4.3 rep-PCR

Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126–140. 'Bacterial genomic DNA was extracted from pure cultures as described by Heyndrickx *et al.* (1996a).'

Coorevits, A., Logan, N., Dinsdale, A., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961. 'Total genomic DNA for 16S rRNA gene sequencing and DNA fingerprinting was extracted as described by Coorevits *et al.* (2008). A fingerprint pattern of all isolates was generated using a repetitive sequence based PCR with a $(GTG)_5$-primer (Heyrman *et al*., 2005c).'

De Jonghe, V., **Coorevits, A.,** Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2011b). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl Environ Microbiol* **77**, 460–470. 'DNA from all isolates (n = 779) was obtained through simple alkaline lysis, and repetitive sequence-based PCR analysis with BOX primers was performed (Heyrman *et al*., 2005c). To obtain a first grouping of the isolates, a Pearson correlation based distance matrix was calculated from all BOX-patterns, and the distance matrix was applied in a cluster analysis using UPGMA.'

4.3.1 Introduction

Rep-PCR is a genotypic fingerprinting technique that allows rapid grouping of bacteria into the species, sometimes even strain level. It is based on the presence of repetitive DNA sequences in the genome, where primers anneal and a polymerase chain reaction (PCR) is carried out starting from those repetitive elements. The PCR-products obtained this way, are then separated by agarose gel electrophoresis and patterns are subsequently analysed using BioNumerics software version 5.1.

4.3.2 Method description

The method includes *i*) DNA-extraction, *ii*) rep-PCR, *iii*) gel electrophoresis and *iv*) numerical analysis of profiles.

4.3.2.1 DNA-extraction

Two methods were used for DNA-extraction. One was the Pitcher DNA extraction method, mostly applied for Gram-positive bacilli, while the other was simple alkaline lysis, mostly applied for Gram-negative pseudomonads. When performing rep-PCR on DNA obtained from alkaline lysis, it was important to start with fresh material, because multiple freeze/thawing cycles influenced banding patterns of the strains.

Alkaline lysis

This method was fully described by Coenye *et al.* (2002) as follows. 'DNA from each isolate was prepared by heating one colony at 95°C for 15 minutes in 20 μL lysis buffer containing 0.25% (wt/vol) sodium dodecyl sulphate (SDS) and 0.05 M NaOH. Following lysis, 180 μL of distilled water was added, and DNA solutions were stored at 4°C. Only one minor difference for the alkaline lyses performed in this work was that after adding distilled water, tubes were

centrifuged (5 min. at 13000 rpm), and 180 μL of supernatant was transferred to a new tube, in order to get rid of cell debris. Tubes were stored at -20°C.

Pitcher DNA extraction

This method was more time consuming but resulted in a relatively large amount of high quality DNA, that could be stored for years at -20°C. It is based on the extraction procedure as described by Pitcher *et al.* (1989) with some minor modifications. Cells were harvested (app. 20 mg) after growth on TSA-plates (Oxoid) for 24 hours at 28°C or 52°C, and suspended in 500 μL resuspension buffer (0.15 M NaCl, 0.01 M EDTA in MQ-water) to wash the cells and get rid of exopolysaccharides. After centrifugation for 2 minutes at 13000 rpm, supernatant was discarded and cells were resuspended in 100 μL lysozyme-solution (50 mg/mL lysozyme in 1 × TE-solution; TE-solution: 1 mM EDTA, 10 mM Tris-HCl, pH 8), and incubated for one hour at 37°C. The lysozyme added disrupted the Gram-positive cell wall; logically this step was skipped when working with Gram-negatives. Subsequently, 500 μL GES-reagens (600 g/L guanidiumthiocyanate, 200 mL/L 0.5 M EDTA, 10 g/L sarkosyl in MQ-water) was added to lyse the cells. To enhance precipitation of released proteins NH_4Ac-solution (7.5 M; 578.1 g/L NH_4OAc in MQ-water) was added to the lysed cell solution. After that, 500 μL chloroform / isoamylalchol (24/1, v/v) was added and thoroughly shaken to obtain a homogenous mixture. A centrifugation step of 20 minutes at 11000 rpm resulted in a three-phase system with the nucleid acids in the upper aqueous phase, and lipids in the lower chloroform phase, separated by a protein layer. The upper phase was transferred to a new tube (app. 700 μL), and 378 μL isopropanol was added to precipitate the nucleic acids, which was visible as a white fluffy cloud after gently shaking the tubes. A centrifugation step of 10 minutes at 13000 rpm resulted in a white pellet of nucleic acids. The supernatant was discarded and the pellet was washed three times with 70% ethanol. After that, the pellet was air-dried until all the ethanol was removed, and dissolved in 100 μL 1 × TE-solution for at least 24 hours. To get rid of RNA, a RNase step was performed by adding RNase-solution (200 mg RNase, 0.8766 g NaCl in 100 mL MQ-water) and incubating for one hour at 37°C. Quality and quantity control check of the obtained DNA was performed by OD-measurement and agarose gel electrophoresis.

4.3.2.2 PCR reaction

Three types of rep-PCR were frequently applied, namely REP-, BOX- and $(GTG)_5$-PCR, all making use of different primers. However, other components of the PCR-reaction were similar and are listed in Table 4.1.

The PCR-temperature program consisted of an initial denaturation step (7 min. at 95°C), followed by 30 cycles of denaturation (1 min. at 94°C), annealing (1 min. at T_A) and elongation (8 min. at 65°C) and finalized with an elongation step (16 min. at 65°C). In Table 4.2, an overview of the primer sequences and annealing temperatures (T_A) is given.

4.3.2.3 Agarose gel electrophoresis

Fragments obtained by rep-PCR were then separated by agarose gel electrophoresis. This occurred under highly standardized conditions to obtain reproducible patterns. The electrophoresis was performed in freshly made 1 × TAE-buffer (Bio-Rad), and same buffer was used to make the

component	amount per 25 μL reaction
5 × Gitschier buffer[1]	5 μL
bovine serum albumine (20 mg/mL)	0.2 μL
DMSO[2] 100%	2.5 μL
MQ water	12.65 μL
for BOX and $(GTG)_5$	13.65 μL
dNTP's (25 mM each)	1.25 μL
rep-primer 1	1 μL
rep-primer 2 (not for BOX and $(GTG)_5$)	1 μL
Taq DNA polymerase (5U/μL)	0.4 μL

Table 4.1: Components of rep-PCR reaction.
[1]Gitschier buffer: 16.6 mL 1 M $(NH_4)_2SO_4$, 67 mL 1M Tris pH 8.8, 6.7 mL 1M $MgCl_2$, 1.3 mL 1/100 dilution of 0.5 M EDTA pH 8.8, 2.08 mL β-mercaptoethanol.
[2]DMSO: dimethylsulfoxide.

primer	sequence	T_A(°C)
REP 1	5'-IIIICGICGICATCIGGC-3'	40
REP 2	5'-ICGICTTATCIGGCCTAC-3'	40
BOX	5'-CTACGGCAAGGCGACGCTGACG-3'	52
$(GTG)_5$	5'-GTGGTGGTGGTGGTG-3'	40

Table 4.2: Primer sequences with corresponding annealing-temperature (T_A).

1.5% agarose gels. The evaporation losses during heating of the gel were compensated for by re-adding the evaporated amount with MQ-water. The electrophoresis was run for 960 minutes at 55V under cooled conditions (4°C). Molecular rulers were loaded on the gel on well-defined lanes to allow normalisation of the gel during further computer processing. DNA bands were visualized after staining with Ethidium Bromide under UV-light, and a digital picture was taken (tiff-file).

4.3.2.4 Numerical analysis of rep-patterns

The tiff-files of rep-profiles were further processed with BioNumerics version 5.1. In a first step, different gel lanes were defined on the gels. In a second step densitometric curves were defined, as the software 'translates' every banding pattern into a curve pattern (represented in Figure 4.7). The more intense a band appears on the gel, the higher the peak of the curve. This intensity difference was also used to differentiate the bands from the background colour. Spot removal was based on the aberrant form of the spots.

These curves could later on be used for calculating similarity scores in exactly the same manner as explained for fatty acid numerical analysis. In a third step, the gels were normalised by comparing the molecular rulers loaded on the gel to the internal molecular ruler of the database. This ensures that all gels within the same database could be compared with each other. In a fourth and final step, curves could again be 'translated' to bands in order to allowed band-based comparison approaches, rather than curve-based. However, in this study, all profiles were compared based on the curves, and the Pearson correlation coefficient was applied to calculate similarities between profiles. This eventually resulted in a dendrogram of similar profiles clustered together with each other.

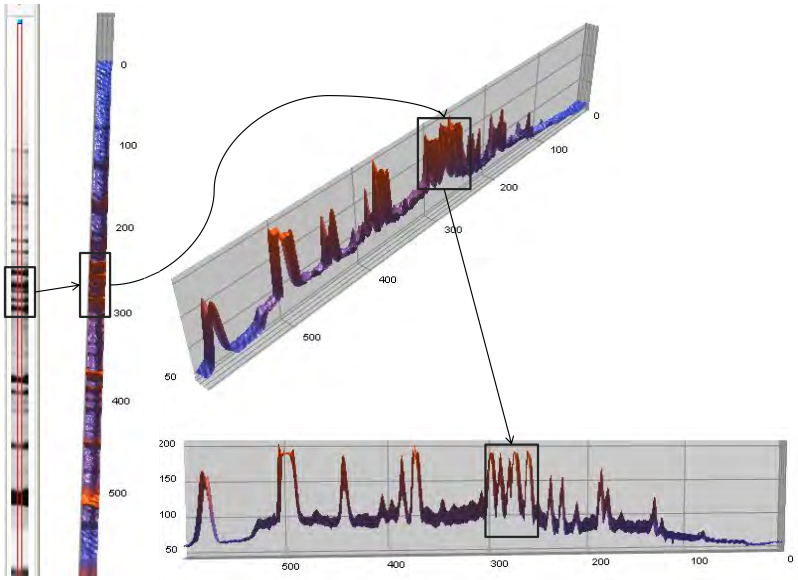


Figure 4.7: Visualization of the "translation" of a rep-banding pattern into a curve.

4.4 Sequence analysis

Coorevits, A., Logan, N., Dinsdale, A., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961. 'The nearly complete 16S rRNA gene sequences of all isolates were generated as described by Heyrman and Swings (2001). Sequencing products were purified with the 'BigDye® XTerminator Purification Kit' (Applied Biosystems) according to manufacturer's instructions using sequential pipetting and a MixMate (Eppendorf) shaking device. Sequences were assembled using the BioNumerics 5.1 software (Applied Maths, Belgium) and the fifty most closely related organisms were appraised using the online FASTA tool of EMBL (http://www.ebi.ac.uk/fasta33/). FASTA results indicated these isolates were members of the genus *Bacillus*. Both phylogenetic trees were based on almost complete 16S rRNA gene sequences and were constructed by aligning all sequences using ClustalX (Thompson *et al.*, 1997), and trimming the overhangs. The jModelTest 0.1.1 program (Posada, 2008) was then applied to the data sets to determine the best fit evolutionary model. Maximum likelihood analyses were performed using PhyML (Guindon & Gascuel, 2003) by applying the parameters determined by jModeltest. aLRT (approximate Likelihood Ratio Test) values were calculated to assess the reliability of the clusters (Anisimova & Gascuel, 2006). Additional maximum parsimony and neighbor joining analyses have been performed using MEGA4 (Tamura *et al.,* 2007).'

Coorevits, A., Dinsdale, A.E., Heyrman, J., Schumann, P., Van Landschoot, A., Logan, N.A. & De Vos, P. (2011b). Taxonomic revision of the genus *Geobacillus*: emendation of *Geobacillus*, *G. stearothermophilus*, *G. jurassicus*, *G. toebii*, *G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly "*thermoglucosidasius*"); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus*; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. *Int J Syst Evol Microbiol*, in press (doi 10.1099/ijs.0.030346-0). 'Pairwise similarity values (S_p) were calculated using the arb software (Ludwig *et al.*, 2004). Phylogenetic trees based on these 16S rRNA gene sequences were constructed by aligning all sequences based on the integrated aligner of the arb software (Ludwig *et al.*, 2004). Refinement of the alignment was obtained by applying the bacterial position variability filter integrated in arb, resulting in a final alignment of 1402 positions. The alignment was then exported to MEGA5 software (Tamura *et al.*, 2011) to construct a neighbor joining, maximum likelihood and maximum parsimony tree. The jModelTest 0.1.1 program (Posada, 2008) was applied to the data set to determine the best fit evolutionary model and the resulting parameters, namely the GTR evolutionary model with 0.581 as the proportion of invariable sites value and a gamma shape value of 0.418, were applied for tree construction where appropriate. Bootstrap analysis based on 1000 replicates was performed on all three types of trees to assess the reliability of the clusters.'

4.4.1 Introduction

Sequence analysis appeared to be a rather complex part of the analyses, for which multiple software tools are available. Some of these have been tested for their applicability, which accounts for the various ways of representing data throughout this study.

4.4.2 Method description

Sequence analysis first involved the generation of the sequences through *i*) DNA extraction, *ii*) PCR-amplification of the desired gene, *iii*) purification of the amplicon, *iv*) sequencing PCR and *v*) assembly of all partial gene sequences (contigs) to obtain the full sequence (as sequencing by the Sanger method is restricted in length to maximum 1000 nucleotides). In a second step these sequences needed to be analyzed, through comparison with other sequences.

4.4.3 Generation of sequences

In a first step genomic DNA was obtained using one of the above mentioned DNA-extraction procedures. In a second step, the sequence of interest was amplified with sequence-specific primers. All amplification primers that were used in this study are listed in Table 4.5, together with the sequence target. The PCR-mixtures used are specified in Table 4.3, and the PCR temperature programs applied are indicated in Table 4.6.

		16S rRNA	***rpoB***	***rpoD***	***gyrB***
component	**amount**	**amount (if changed)**			
10 × PCR-buffer	2.5 μL				
dNTP's (2 mM each)	2.5 μL				
primer 1 (10 μM)	0.25 μL				
primer 2 (10 μM)	0.25 μL				
Taq pol. (1U/μL)	0.25 μL		0.5 μL	2.5 μL	2.5 μL
MQ	18.25 μL		18.0 μL	16.0 μL	16.0 μL
DNA (OD-1 or AL)	1 μL				

Table 4.3: Overview of PCR mixtures.

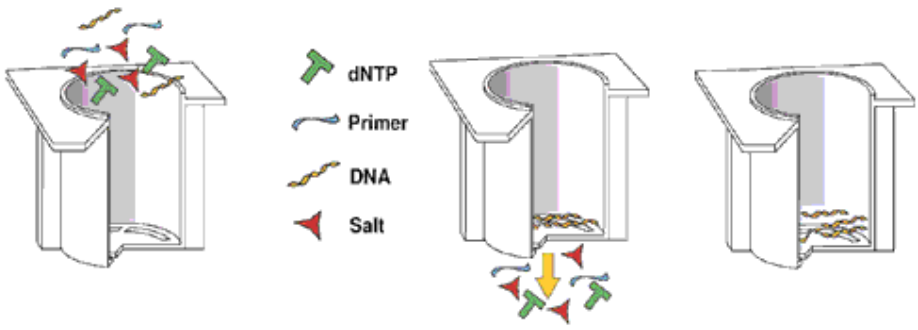


Figure 4.8: Purification of the amplification reaction through filtering (Clontech, 2011).

If the amplification reaction was successful, bands of the expected weight could be observed on an agarose gel. The amplification product was then purified from the remaining components of the PCR-mix through a filtration step as visualized in Figure 4.8.

The amplicon was then resuspended in a specific amount of MQ dependant of the amount of amplicon and this was derived from the intensity of the band on the agarose gel compared to band intensities of a standard. The amplicon was then used as template for the sequencing reactions, with primers annealing on specific parts of the amplified sequence. Mostly, the outer parts of the amplicon were targeted, but for 16S rRNA gene sequences, internal sequence primers were also used, to obtain sufficient overlap between different contigs of the gene. Sequencing primers are listed in Table 4.4.

primer	sequence	target	position	reference
gamma	5'-ACT GCT GCC TCC CGT AGG AG-3'	16S rRNA	358-339	
BKL1	5'-GTA TTA CCG CGG CTG CTG GCA-3'	16S rRNA	536-516	
3	5'-GTT GCG CTC GTT GCG GGA CT-3'	16S rRNA	1112-1093	
*3	5'-AGT CCC GCA ACG AGC GCA AC-3'	16S rRNA	1093-1112	
*O	5'-AAC TCA AAG GAA TTG ACG G-3'	16S rRNA	908-926	
*R	5'-GCT ACA CAC GTG CTA CAA TG-3'	16S rRNA	1222-1241	
PD	5'-CAG CAG CCG CGG TAA TAC-3'	16S rRNA	536-519	
*gamma	5'-CTC CTA CGG GAG GCA GCA GT-3'	16S rRNA	339-358	
LAPS	5'-TGGCCGAGAACCAGTTCCGCGT-3'	*rpoB*	1531-1552	Tayeb *et al.*, 2005
LAPS-27	5'-CGGCTTCGTCCAGCTTGTTCAG-3'	*rpoB*	2760-2739	Tayeb *et al.*, 2005
rpoBF'	5'-CAGTTCATGGACCAGAACAACCCG-3'	*rpoB*	1552-1575	this study
rpoBR'	5'-ACGCTGGTTGATGCAGGTGTTC-3'	*rpoB*	2298-2277	this study
UP-1S	5'-GAAGTCATCATGACCGTTCTGCA-3'	*gyrB*	273-295	Yamamoto & Harayama, 1995
UP-2SR	5'-AGCAGGGTACGGATGTGCGAGCC-3'	*gyrB*	1506-1484	Yamamoto & Harayama, 1995
70Fs	5'-ACGACTGACCCGGTACGCATGTA-3'	*rpoD*	300-323	Yamamoto & Harayama, 1998b
70Rs	5'-ATAGAAATAACCAGACGTAAGTT-3'	*rpoD*	1149-1127	Yamamoto & Harayama, 1998b

Table 4.4: Overview of sequencing primers used in this study, the gene of interest, its position and literature reference.

The PCR-mixture consisted for all genes of a 4 μL solution (containing 1.857 μL 5 $\times$ sequencing buffer, 1.857 μL MQ-water, 0.286 μL BigDye®) and 3 μL primer (4 μM). 3 μL of amplicon was added to the mixture and subjected to a PCR temperature program of denaturation at 96°C for 15 s, annealing at 35°C for 1 s and elongation at 60°C for 4 min. For *rpoB* gene sequencing with the primers rpoBF' and rpoBR', the annealing temperature was raised to 50°C for some strains to avoid sequencing of aspecific amplification products. This program was repeated 30 times. Sequencing reactions were performed following the Sanger-method and resulting products were purified with the 'BigDye® XTerminator Purification Kit' (Applied Biosystems) according to manufacturer's instructions using sequential pipetting and a MixMate (Eppendorf) shaking device. Sequences were analysed with the Applied Biosystems *3130xl* genetic analyzer, and resulting contigs were assembled using BioNumerics 5.1 software.

4.4.3.1 Analyzing sequences

Analysis of sequences usually involved *i*) comparison of the query sequence with publicly available sequences through BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and FASTA search (http://www.ebi.ac.uk/Tools/sss/fasta/) tools and *ii*) phylogenetic analysis of the query

primer	sequence	target	position	reference
pA	5'-AGA GTT TGA TCC TGG CTC AG-3'	16S rRNA	8-27	
pH	5'-AAG GAG GTG ATC CAG CCG CA-3'	16S rRNA	1541-1522	
ARI-CT	5'-C TGG CTC AGG AC/TG AAC GCT G-3'	16S rRNA	19-38	
MH1	5'-A GTT TGA TCC TGG CTC AG-3'	16S rRNA	10-27	
MH2	5'-T ACC TTG TTA CGA CTT CAC CCC A-3'	16S rRNA	1507-1485	
LAPS	5'-TGGCCGAGAACCAGTTCCGCGT-3'	*rpoB*	1531-1552	Tayeb *et al.*, 2005
LAPS-27	5'-CGGCTTCGTCCAGCTTGTTCAG-3'	*rpoB*	2760-2739	Tayeb *et al.*, 2005
UP-1	5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3'	*gyrB*	273-313	Yamamoto & Harayama, 1995
UP-2R	5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3'	*gyrB*	1527-1484	Yamamoto & Harayama, 1995
70F	5'-ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT-3'	*rpoD*	300-344	Yamamoto & Harayama, 1998b
70R	5'-ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT-3'	*rpoD*	1149-1106	Yamamoto & Harayama, 1998b

Table 4.5: Overview of amplification primers used in this study, the gene of interest, its position and literature reference.

			16S rRNA		***rpoB***		***rpoD***		***gyrB***
initial denaturation			5 min. at 95°C		1 min. 30 s at 94°C		5 min. at 94°C		5 min. at 94°C
cycles	denaturation	×3	1 min. at 95°C	×40	10 s at 94°C	×30	1 min. at 94°C	×30	1 min. at 94°C
	annealing		2 min. 15 s at 55°C		20 s at 45°C / 50°C		45 s at 59°C		1 min. at 60°C
	elongation		1 min. 15 s at 72°C		50 s at 72°C		2 min. at 72°C		2 min. at 72°C
	denaturation	× 30	35 s at 95°C						
	annealing		1 min. 15 s at 55°C						
	elongation		1 min. 15 s at 72°C						
final elongation			7 min. at 72°C		5 min. at 72°C		7 min. at 72°C		7 min. at 72°C
hold			8 at 4°C		8 at 4°C		8 at 4°C		8 at 4°C

Table 4.6: Overview of PCR temperature programs.

sequence to define its position within a set of representative sequences. A short overview of software tools used for phylogenetic analysis in this study will be discussed here.

arb (Ludwig *et al.*, 2004)

Free software tool that allows aligning rRNA gene sequences based on the structural features of rRNA, which results in more accurate and biological meaningful alignments (Kumar *et al.*, 2006). The alignment procedure performed by arb is an incremental procedure, meaning new sequences are aligned against a so-called 'seed alignment', implemented in the software and based on validated rRNA secondary structure models.

The arb software is often regarded as not user-friendly (working in a Linux environment) but is for now probably the best software tool for aligning rRNA gene sequences. Furthermore, once used working with arb, it approves extremely flexible, as shortly exemplified with the filter options. rRNA gene sequences typically exhibit highly conserved and more variable regions, a feature making these genes well suited for phylogenetic analyses. Alignments based on the secondary structure in arb can be refined by applying filters to eliminate the noise of the alignment, i.e. those parts of the alignment where the position of nucleotides is uncertain or in other words, where sequence variability is extremely high, and thus not informative. The degree of conservation is evaluated in arb by a gradient from 0 to Z (0-1-2-...-9-A-B-...-Z), with 0 being the least conserved and Z being highly conserved. This enables the arb-user to eliminate the uninformative parts of an alignment by selecting only positions that, for instance, show a degree of conservation of a least 9.

MEGA5 software (Tamura *et al.*, 2011)

The MEGA5 software is extremely useful when analyzing protein encoding genes. It allows alignment of sequences based on the amino acid sequences, and then switch back to the nucleotide sequences, or continue with the amino acid sequences for further tree construction. Alignment based on amino acid sequences prevents the introduction of gaps in the middle of a codon, as this is a non-sense event in nature, and thus very unlikely to have occurred.

jModeltest (Posada, 2008)

When analyzing sequences, and calculating similarities that later on define branch lengths in the phylogenetic tree, different approaches can be used, reflected by different models. For instance, the simplest model (the Jukes Cantor model) assumes that the probability that one nucleotide changes into another nucleotide is equal for any nucleotide. The Kimura-2 parameter model, however, takes into account that transitions are much more likely to occur than transversions, and does not assume all changes are equally likely to happen. The jModeltest program is a tool to carry out statistical selection of best-fit models of nucleotide substitution for a given set of sequences.

PhyML (Guindon & Gascuel, 2003)

This software tool allows the construction of maximum likelihood (ML) trees based on aligned sequences via a website interface (http://www.atgc-montpellier.fr/). ML trees are mostly

considered the best method of choice, because the method seeks the tree that makes the data most likely. This is contrary to Maximum Parsimony (MP) method which is based on the assumption that the most likely tree is the one that requires the fewest number of changes. Neighbor joining (NJ) tree is a distance method tree, meaning calculations for tree construction are not based on the data themselves, but on a similarity matrix derived from the data, and this extra step automatically involves loss of data. PhyML was mostly used in the beginning of this study but has recently been implemented in arb and MEGA5.

4.5 DNA-DNA hybridizations

Coorevits, A., Logan, N., Dinsdale, A., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961. 'For this purpose, approximately 1 g biomass was harvested from TSA plates, and DNA was purified as described by Logan *et al*. (2000). DNA-DNA hybridization was performed using a modification of the microplate method of Ezaki *et al*. (1989) as described by Willems *et al.* (2001). A hybridization temperature of 32°C (calculated with correction for the presence of 50% formamide) was used.'

4.5.1 Introduction

This technique is currently accepted as the gold standard in prokaryotic taxonomy, and measures total genomic relatedness between 2 strains, using microwell plates. Strains that share at least 70% DNA relatedness are assumed to belong to the same species.

4.5.2 Method description

DNA-DNA hybridization experiments started with the extraction of DNA from the strains that needed to be hybridized. Sufficient DNA should be harvested of high quality, therefore other extraction procedures were used than the ones described above. After DNA-extraction, DNA was fixed onto the microwell plates, and subsequently fragmented biotin-labeled probe-DNA of the strain to be hybridized with, was added to the fixed DNA. Microplates were read by a luminescence reader, and data were analyzed to calculate homology values.

4.5.2.1 DNA-extraction procedure

Two different extraction methods have been used in this study. One is based on the method of Pitcher *et al.* (1989) and has already partly been described for small-scale DNA extraction (§4.3.2.1). The other one is the CTAB-extraction procedure that will be outlined below.

Large-scale Pitcher DNA-extraction method

The first steps in the procedure were identical to the small-scale DNA extraction procedure, except that bigger amounts of reagents were used. However, the addition of RNase after incubation of DNA in 1 × TE-buffer for at least 24 hours, was followed by a second chloroform extraction step, after which the DNA was again precipitated with isopropanol and dissolved in 1 × TE-buffer after drying. Often, an additional proteinase step was required to get rid of proteins that precipitated along with the nucleic acids, especially when working with Gram-positives.

Briefly, the large-scale DNA extraction procedure was performed by suspending 1 g of biomass in 15 mL resuspension buffer. After a centrifugation step (5 min. at 11000 rpm), the supernatant was discarded and pellet was redissolved in 3 mL lysozyme-solution and 60 μL RNase solution (10 mg/mL), and incubated for one hour at 37°C for Gram-positives. For Gram-negatives the pellet was redissolved in 3 mL 1 × TE-buffer. 15 mL of GES-solution was added, and carefully mixed. Then 7.5 mL NH_4Ac was added and again gently mixed. This was followed by adding 10 mL chloroform/isoamylalcohol to the tubes, and tubes were thoroughly shaken to obtain a homogenous one-phase system. After centrifugation (20 min. at 9000 rpm) the upper phase was transferred to a beaker, and 13.5 mL isopropanol was added. The precipitated DNA was collected on a glass rod, washed three times with 70% ethanol and air-dried. The DNA was allowed to dissolve in 1 × TE-buffer for at least 24 hours. If protein contamination was observed, 50 μL of proteinase K (20 mg/mL) was added, and the sample was incubated at 45-55°C for at least 30 minutes. To get rid of the proteins and the proteinase, an additional chloroform extraction step was performed, by adding 560 μL sodium-acetate (0.3 M), and 3.5 mL chloroform/isoamylalcohol. After centrifugation (20 min. at 9000 rpm), the upper phase was transferred to an empty tube, 50 μL RNase was added, and incubated for one hour at 37°C. Again, 3.5 mL chloroform/isoamylalcohol was added and after centrifugation (20 min. at 9000 rpm), the upper phase was transferred to a beaker, and 3.3 mL isopropanol was added to precipitate the DNA. This DNA was collected on a glass rod, and washed three times. After drying, the DNA was dissolved in 1 mL 0.1 × SSC-solution (10 mL/L 10 × SSC-solution: 1.5 M NaCl, 0.15 M citric acid monohydrate, 0.45 M NaOH in MQ-water).

CTAB DNA-extraction method

This method was only performed on Gram-positive strains when DNA-extraction with the large-scale Pitcher method failed. CTAB ($(C_{16}H_{33})N(CH_3)_3Br$) hexadecyl-trimethyl-ammonium bromide) is a cationic surfactant used in DNA-extraction procedures as a buffer solution that helps in solubilizing molecules by dissociating aggregates and unfolding proteins. Approximately 1 to 2 g of biomass was dissolved in 10 mL resuspension buffer, and centrifuged for 10 min. at 7000 rpm. After centrifugation, the pellet was resuspended in 7.5 mL lysozyme-solution and incubated for 2 hours at 37°C. Then, 400 μL RNase solution (10 mg/mL) was added, as well as 2 mL 10% SDS, and 100 μL proteinase K. After incubation at 65°C for 1 hour, 3 mL NaCl-solution was added (5 M) and 2 mL CTAB/NaCl (0.70 M NaCl and 0.27 M CTAB in MQ-water). This was again incubated at 65°C for 20 minutes. 10 mL chloroform/isoamylalcohol was added and tubes were thoroughly shaken to obtain a homogenous one-phase system. After centrifugation (30 min. at 9000 rpm), the upper phase was transferred to a glass beaker, and 8.4 mL isopropanol was added. The subsequent steps in the procedure were identical to those from the large-scale Pitcher DNA-extraction method.

4.5.2.2 Fixation of DNA

Fixing DNA in microwell plates started by preparing DNA-solutions of OD_{260} between 4 and 16, ideally around 10. This concentration needed to be stable, meaning the same OD should be obtained during multiple measurements spread over a couple of days. This could only be achieved when the DNA was completely dissolved, and no protein or RNA-contamination was present. The stabilized OD_{260}-10 solution was diluted with 0.1 × SSC to obtain a final OD_{260}-2 solution, and this solution was heated for 10 min at 100°C to denature the DNA. Following denaturation,

samples were quickly cooled on ice, and then further diluted to OD_{260}-0.2 by adding sufficient amount MgPBS (100 mL/L 10 × PBS, 0.1 M $MgCl_2.6H_2O$; 10 × PBS: 1.37 M NaCl, 0.027 M KCl, 0.032 M $Na_2HPO_4.12H_2O$, 0.0147 M KH_2PO_4 in MQ-water)-solution. The MgPBS was added to ensure non-covalent fixation of the DNA to the polystyrene wells. Each well on the microwell plate was then filled with 100 μL of the DNA-MgPBS-solution. Each reaction was performed in quadruplate, so four wells were filled for each reaction. Filled plates were sealed with plastic and incubated for at least 4 hours at 30°C. After incubation, the solution was discarded, and plates were dried for 15 min. at 52°C. Dried plates could be stored in a dry place at room temperature. The fixation procedure is schematically represented in Figure 4.9.

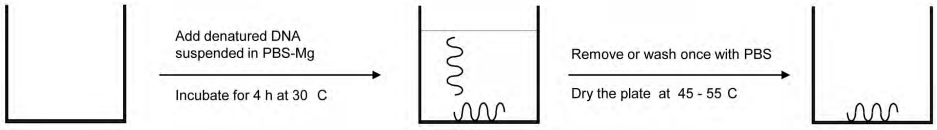


Figure 4.9: Schematic representation of the fixation procedure.

4.5.2.3 Hybridisation

Approximately 10 μL of the OD_{260}-10 DNA solution was needed for a probe. To this volume, exactly the same amount of photobiotine was added, and probes were incubated for 30 minutes under a mercury lamp (400 W). Probes were kept on ice during this incubation step. Meanwhile, the wells containing fixed DNA were filled with 200 μL prehybridization solution (200 mL 10 × SSC, 100 mL 50 × Denhardt solution, 10 mL denatured salmon sperm DNA, 500 mL formamide in 1L MQ-water). The bovine serum albumin and salmon sperm in this prehybridization solution prevented non-specific binding of bacterial DNA with polystyrene walls of the microwell plate. Plate was covered with a plastic seal and incubated at the appropriate hybridisation temperature (T_H) for at least 30 minutes. The T_H was calculated with the following formula:

$$T_H = (0.51 \times \%GC + 47) - 36^\circ C$$

with 36°C being the correction for working under less stringent conditions in the presence of 50% formamide. For most bacilli, the optimal T_H was around 37°C, and for pseudomonads around 45°C. After photochemical labelling of the probes with photobiotine, the remaining unbound photobiotine was discarded during a butanol extraction procedure. 185 μL Tris-HCl (pH 9.0) and 200 μL 1-butanol was added to the probes, the solution was mixed and centrifuged for 2 min. at 10000 rpm. After centrifugation, the upper 1-butanol phase was discarded and the lower phase was again washed with 200 μL of 1-butanol. After a second centrifugation step, the upper phase was carefully removed, and the purified probe was subjected to fragmentation and denaturation by sonication (30 s) and heating (10 min. at 100°C). After cooling on ice, probes were mixed with hybridization solution (25 g/L dextran sulphate in prehybridisation solution). The prehybridisation solution from the microwell plates was discarded and wells were now filled with the hybridisation solution (100 μL) mixed with the probes. Plates were again sealed with plastic foil and incubated for 3 hours at the appropriate T_H. After incubation, microwell plates were washed with 1 × SSC, and 100 μL of streptavidin-β-galactosidase solution (0.05 g bovine serum albumin, 1 mL PBS 10 ×, 9 mL MQ-water, 10 μL β-galactosidase) was added. Plates were covered with preheated lids, and incubated for 10 min at 37°C, to allow the streptavidin

to bind with the biotin. The streptavidin-β-galactosidase solution was then discarded and 100 μL 4-methylumbelliferyl-(β-D-galactoside) solution was added to each well. Plates were again covered with a preheated seal and incubated for 45 minutes at 37°C. Microplates were read with the luminescence reader after 0, 15, 30 and 45 minutes of incubation. Based on these values, DNA-relatedness values were determined between strains. The hybridisation procedure is schematically represented in Figure 4.10.

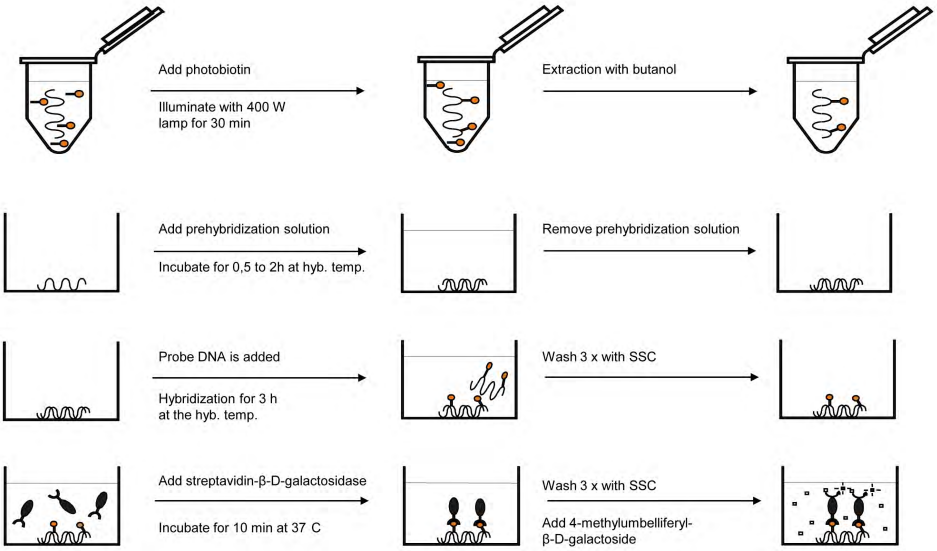


Figure 4.10: Schematic representation of the hybridisation procedure.

4.6 Short overview of other techniques

These techniques have either not been performed by the author, or are commercially available kits, that don't need any further explanation than a reference to the manufacturer's instructions.

4.6.1 %GC

Coorevits, A., Logan, N., Dinsdale, A., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961. 'G + C contents were determined by HPLC (Mesbah *et al.,* 1989), using further specifications given by Logan *et al.* (2000)'.

Genomic DNA is denatured by heating for 10 minutes at 100°C and immediately cooled on ice. This denaturation step makes it an appropriate substrate for the P1 nuclease, that targets single-stranded DNA and cuts it to its respective nucleotides. Nucleotides are converted to corresponding nucleosides by adding alkaline phosphatase because nucleosides are less polar and thus easier to separate on a reversed-phase high pressure liquid chromatography column. %GC is calculated from the ratio T/dG.

4.6.2 API / Biolog

These kits are commercially available and are thus applied according to the manufacturer's instructions. The only alteration implies the incubation in plastic bags for strains that grew optimally at temperatures above 50°C to prevent evaporation.

4.6.3 DGGE

DGGE (Denaturating Gradient Gel Electrophoresis) involves electrophoresis of PCR-fragments (mostly a part of the 16S rRNA gene, not larger than 500 bp) in a denaturing electrophoretic system. The acrylamide gels used contain a gradient of two DNA-denaturing agents, formamide and urea, causing the double stranded DNA to become single stranded during electrophoresis. Top of the gels have a low gradient of denaturing agents but this steadily increases moving to the bottom of the gel. DNA-fragments thus melt during their migration through the gel, and move slower until they stop migrating. However complete denaturation is prevented by a GC-clamp at one end of the fragments. Because of differences in stability between GC- and AT-bounds, AT-rich DNA fragments will denature faster and move at a much slower pace compared to GC-rich DNA fragments. The fragments are thus separated through the gel not based on size (because all fragments have the same size) but based on nucleotide composition. DGGE is often used in culture independent studies to map the bacterial diversity in a sample of interest, e.g. milk.

CHAPTER 5

Pseudomonads in the dairy industry

This diversity study focused on the psychrotrophic aerobic bacterial fraction, that benefits from the prolonged cold storage of raw milk prior to processing. The dominant *Pseudomonas* microbiota was identified as members of the *Pseudomonas fluorescens* group, *Pseudomonas lundensis* and *Pseudomonas fragi*. It was shown these species have an important proteolytic and lipolytic spoilage potential, and *P. fragi* and *P. lundensis* were predominantly isolated at the end of the dairy chain which indicates these organisms may be controlled by adequate cooling and rapid processing at the dairy plant. Suboptimal cooling conditions greatly favored growth of these pseudomonads, and of some as yet undescribed strains with considerable spoilage potential. Strains assigned to the *Pseudomonas fluorescens* group could often not be identified onto the species level for two reasons. Firstly, the complex taxonomic situation of this group hampers reliable species identification. Secondly, several strains probably represent as yet undescribed species, the so-called 'likes' throughout the study. Additionally, the applicability of a recently proposed MLSA scheme to identify *Pseudomonas* strains was validated, but preliminar results indicate there are some discrepancies between different taxonomic markers used in this scheme, and identification onto the species level remains problematic.

5.1 Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk

The refrigerated storage of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of psychrotolerant bacteria. These bacteria, mainly belonging to the genus *Pseudomonas*, are capable of producing thermoresistant extracellular proteases and lipases, which can cause spoilage and structural defects in pasteurized and ultra high temperature treated milk (products). To map the influence of refrigerated storage on the growth of these pseudomonads, milk samples were taken after the first milking turn and incubated lab-scale at temperatures simulating optimal and suboptimal pre-processing storage conditions. The outgrowth of *Pseudomonas* members was monitored over time by means of cultivation-independent DGGE. Isolates were identified by a polyphasic approach and characterized for their spoilage potential. These incubations revealed that outgrowth of *Pseudomonas* members occurred from the beginning of the dairy chain (farm tank) under both optimal and suboptimal storage conditions. An even greater risk for outgrowth as indicated by a vast increase of about 2 log cfu per mL raw milk existed downstream in the chain, especially when raw milk was stored under suboptimal conditions. This difference in *Pseudomonas* outgrowth between optimal and suboptimal storage already became statistically significant from within the farm tank. The predominant taxa were identified as *Pseudomonas fragi, Pseudomonas lundensis* and belonging to the *Pseudomonas fluorescens / Pseudomonas gessardii* species complex. These taxa show an important spoilage potential as determined on differential media for proteolysis and lipolysis.

5.1.1 Introduction

Psychrotolerant bacteria have become more important for the shelf life of heat treated dairy products because of the development of these bacteria during prolonged refrigerated storage of raw milk on the farm and at the dairy plant. In an effort to reduce the total aerobic plate count of raw milk, a lower storage temperature (1 - 4°C) is upheld, leading to the perception that raw milk could be stored for a longer period before further processing. However, the combination of a longer storage time and a lower temperature creates a selective advantage for psychrotolerant bacteria, especially *Pseudomonas* members, that enter raw milk via biofilms in the milk tanks, contaminated water and soil (Cousin, 1982; Simões *et al.*, 2009). These pseudomonads are able to outgrow other bacteria such as members of *Aeromonas*, *Listeria*, *Staphylococcus*, *Enterococcus* and the family *Enterobacteriaceae*, thus becoming the predominant microbiota in raw milk (Sørhaug & Stepaniak, 1997) counting up to 70 - 90% of the psychrotrophic raw milk microbiota (Adams *et al.*, 1975). Even though they are easily inactivated through pasteurization or UHT-treatment, their heat resistant enzymes persist upon processing of the milk (Chen *et al.*, 2003a).

A persisting problem for unravelling the exact nature of the spoilage microbiota, is the unresolved taxonomic situation of the genus *Pseudomonas*. *Pseudomonas* members are still often identified based on phenotypic characteristics, a methodology that became outdated since the general introduction of molecular DNA methodologies. However, a clear-cut phylogenetically based identification approach for *Pseudomonas* members is not available yet. Even recent studies therefore still rely on phenotypic methods for routine identification of isolates (Flint & Hartley, 1996; Wiedmann *et al.*, 2000; Dogan & Boor, 2003; Munsch-Alatossava & Alatossava, 2006).

This study aims at a better understanding of the outgrowth of *Pseudomonas* species throughout

the dairy chain (farm tank - transport - dairy plant) under optimal and suboptimal cooling conditions as well as assessing the qualitative species composition in the stored raw milk through a polyphasic identification approach. Furthermore, it combines the use of cultivation, spoilage potential characterization and non-cultivation monitoring of the psychrotolerant bacteria in raw milk to better assess the shelf life risks in the end product.

5.1.2 Materials and methods

Preface: most of the experimental work was performed by Dr. De Jonghe at ILVO, and a detailed description of this work, among which the culture-indepedent DGGE-approach can be found in the Materials and Methods section of De Jonghe and co-workers (2011b). However the experimental setup and isolation procedure of the lab-scale simulation will be outlined here, as well as the polyphasic approach used to identify all isolates, as main focus in this thesis is on the identification of pseudomonads.

Simulation of the cold dairy chain and sampling. Three independent simulations (s1, s2 and s3) were set up that imitate pre-processing conditions at two temperature extremes that represent optimally and suboptimally cooled storage conditions. Milk samples used for the simulations were constituted by mixing equal volumes of raw milk samples from a number (n) of different farms (s1: $n = 1$, s2: $n = 8$ and s3: $n = 7$) collected from the farm bulk tank after the first milking turn. The 600 mL samples were incubated in a water bath, of which the temperature was regulated with a cryostat. A smaller bottle with 300 mL of the same mixed milk sample was used for temperature registration with the Ellab Tracksense® PRO Basic logger system (Ellab Inc., Centennial, CO, USA). An overview of the different experimental conditions during the various simulations is summarized in Table 5.1.

To simulate the storage at the farm – where in theory an optimal resident temperature of 3.5°C is envisaged - the milk mixtures were heated up twice a day (morning and afternoon with 8 hours in between) to imitate the increased temperature due to milking peaks (= warming up of the tank milk when fresh milk enters the tank) being 6°C and 10°C for optimal and suboptimal conditions, respectively (Figure 5.1).

The conditions for these milking-linked temperatures were extracted from data obtained in 205 Belgian dairy farms. The stored farm milk is collected after 2 to 3 days (4 days were allowed to simulate extreme conditions) and was stored again within 8 hours after collection for maximum 24 hours in an industrial tank before processing at the dairy plant. The temperature regime during the simulation of transport and storage at the dairy factory was a consensus decided upon by an expert panel with people from the dairy industry. During these ca. six-day simulation experiments, 14 milk samples for microbial analysis (RM1 → RM14; Table 5.1) were collected at regular time intervals for (i) total aerobic plate counting (TAPC) by pour plating serial dilutions on PCA (Oxoid) with incubation at 30°C for 3 days and for (ii) presumptive psychrotolerant *Pseudomonas* counting (PC) by streaking serial dilutions on a selective medium for *Pseudomonas* that contains cetrimide (10 mgL^{-1}), fucidin (10 mgL^{-1}) and cephalosporin (50 mgL^{-1}) (CFC-agar) (Oxoid) with incubation at 22°C for 4 days.

Isolation. *Pseudomonas* isolates were picked from CFC-agar at 4 sampling occasions: (i) at the beginning (RM 1) and (ii) end of the simulation of farm bulk tank (RM 8), (iii) at the end of simulation of the transport (RM 11) and (iv) at the end of simulation of storage at the dairy plant prior to processing (RM 14). Where possible, 30 isolates (constituting 10-20% of the total amount

	simulated storage mode		
parameter	**farm milk tank**	**transport**	**industrial milk tank**
temperature			
optimal	3.5°C	6°C	6°C
suboptimal	6°C	10°C	10°C
milking peak temperature			
optimal	6°C		
suboptimal	10°C		
duration	96 h	8 h	24 h
use of stirring	yes	no	yes
sampling	2×*	3×	3×
sampling time			
RM1	t_0		
RM2	t_0 + 8 h		
RM3	t_0 + 24 h		
RM4	t_0 + 32 h		
RM5	t_0 + 48 h		
RM6	t_0 + 56 h		
RM7	t_0 + 72 h		
RM8	t_0 + 80 h		
RM9		t_0 + 96 h	
RM10		t_0 + 100 h	
RM11		t_0 + 104 h	
RM12			t_0 + 120 h
RM13			t_0 + 124 h
RM14			t_0 + 128 h

Table 5.1: Simulation of the dairy chain from the farm milk tank, transport and storage at the industrial milk tank on a 0.6 litre scale. Conditions were based on data from 205 Belgian dairy farms and advice from an expert panel from the dairy industry. t0: time = zero. * Samples were taken prior to simulation of the milking peak.

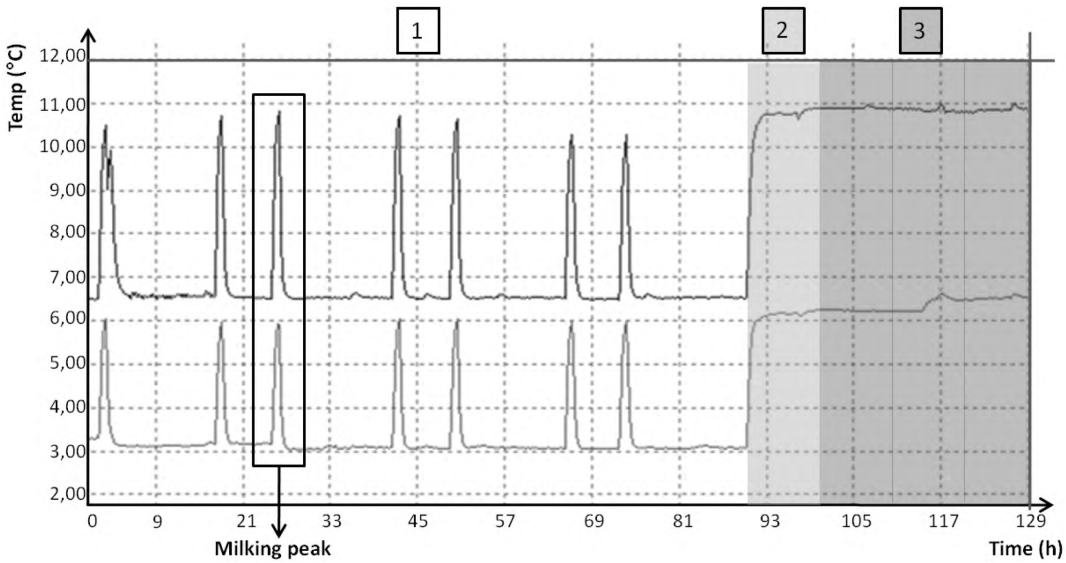


Figure 5.1: Temperature profile of cold milk chain simulation. Stage 1 is storage at the dairy farm bulk tank (RM1 to RM8), stage 2 represents transport (RM9 to RM11) and stage 3 storage at the dairy plant (RM12 to RM14).

of colonies) were randomly picked from the same dilution plate at each isolation point. Isolates were subsequently stored in the R- (Research) collection of the Laboratory of Microbiology (LMG; Ghent University) at -80°C under cryoprotection. For simulation 3, not only CFC-agar was used as an isolation medium for pseudomonads, but also MPCA (Milk Plate Count Agar, Oxoid). Only Gram-negative strains (tested with the KOH string test) were retained from MPCA (n = 114) and stored in the R-collection. All isolates are listed in the table in Appendix A.

Polyphasic identification. DNA from all isolates (n = 779) was obtained through simple alkaline lysis, and repetitive sequence-based PCR analysis with BOX primers was performed (Heyrman *et al.,* 2005c). To obtain a first grouping of the isolates, a Pearson correlation based distance matrix was calculated from all BOX-patterns, and the distance matrix was applied in a cluster analysis using UPGMA. Groups were visually delineated, and for each group, representatives were chosen and further analysed with FAME analysis and sequencing of the 16S rRNA and *rpoB* genes. A preliminary identification of the bacteria, based on their FAME profiles, was obtained using the TSBA database (version 5.0) of the MIDI software (MIDI, Microbial ID, USA). Only those representatives belonging to the genus *Pseudomonas* - according to FAME analysis - were further considered for sequencing of the 16S rRNA and *rpoB* genes. Sequencing of the *rpoB* gene was executed as described by Tayeb and co-workers (2005). However, for several isolates, no *rpoB*-amplicon could be obtained following the author's instructions. For those isolates, new primers were designed targeting the *rpoB* gene: rpoBF' and rpoBR' aligning on positions 1552 and 2298 of the *rpoB* gene sequence of *Pseudomonas aeruginosa* UCBPP-PA14 (CP000438). A species allocation based on *rpoB*-sequences was obtained by comparing (UPGMA, NJ algorithm) the representative sequences with publicly available sequences of *Pseudomonas* type strains. All sequences were deposited in EMBL under the accession numbers: FN650710 to FN650746 (16S rRNA gene sequences) and FN650748 to FN650791 (*rpoB* sequences). Some representative isolates were even more thoroughly identified by applying the MLSA-scheme provided by Mulet and co-workers (2010) but this approach will be extensively discussed in §5.2. Wherever appropriate, the updated identification is indicated in this section already.

Screening for spoilage potential. Differential media were used as screening tool for proteolytic and lipolytic spoilage potential for all isolates (n = 779) as described by De Jonghe and co-workers (2010). The inoculated media were incubated at 22°C for 72h and display of enzymatic activity (a clear halo around the colony) was checked daily. Per batch of inoculated plates, the diameter of the halo was determined to assess the degree of activity: strong (d > average halo in the same batch), intermediate (d < average halo in the same batch) or no activity (absence of halo).

5.1.3 Results

Sampling and bacterial counts. The TAPC remained fairly stable in the farm tank even under suboptimal storage conditions. An outgrowth to approximately 10^5 cfu mL^{-1} was visible only after 4 days of storage at the farm. The outgrowth of bacteria was observed during transport and storage at the dairy plant to approximately 10^6 and 10^8 cfu mL^{-1} for optimally and suboptimally cooled raw milk, respectively. However, *Pseudomonas* members started growing already within the farm tank and showed an enhanced outgrowth under suboptimal storage conditions of approximately 1 log cfu mL^{-1} at the end of storage in the farm tank compared to the optimal conditions of storage. Further downstream in the simulation of the dairy chain, the difference between optimal and suboptimal storage conditions became even bigger (2 log cfu mL^{-1}) in the

case of suboptimal storage for both TAPC and PC reaching levels of 10^6 and 10^8 cfu mL^{-1} in optimally and suboptimally stored raw milk, respectively.

Polyphasic identification. A BOX-pattern could be generated for 684 out of 779 presumptive *Pseudomonas* isolates (87.8%) from the three dairy chain simulations. A grouping of these fingerprints is represented in Figure 5.2.

This dendrogram shows that 573 isolates (73.5%) were grouped into 23 BOX-clusters, and 111 isolates appeared separately. The strains were polyphasically identified as shown in Appendix A. Identification was mainly based on the *rpoB* gene sequence, which has recently been described as a useful taxonomic marker in the genus *Pseudomonas* (Tayeb *et al*., 2005). The majority of the isolates in the BOX-clusters belonged to the *Pseudomonas fluorescens* group with isolates very similar to *P. gessardii* (clusters J, L, N and O) that were only isolated in simulation 2 and to *Pseudomonas fluorescens* (clusters C, D, E, F, G and H), the so called *P. gessardii*-like and *P. fluorescens*-like clusters. The annotation '-like' stands for probably novel species of which the *rpoB* gene sequences showed high similarity (app. 98%) with those of *P. fluorescens* or *P. gessardii* type strains *rpoB* gene sequences as demonstrated in Figure 5.3. However, a further identification approach (§5.2) indicated members of both species groups, *P. fluorescens* and *P. gessardii*, can't reliably be distinguished from one another based on *rpoB* gene sequencing, and it was thus preferred to further refer to these isolates as members of the *P. fluorescens / P. gessardii* species complex.

Two other major groups (Figure 5.2) were preliminarily identified as *Pseudomonas lundensis* (clusters T, U and V) and *Pseudomonas fragi* (clusters R and S). Members of one (smaller) cluster W were identified as *P. fragi*-like and cluster M was identified as *P. gessardii* (Figure 5.3). Cluster W was later allocated to *P. fragi* and not *P. fragi*-like (§5.2). Four other clusters probably represent as yet undescribed species within *Pseudomonas* (clusters A, B, I and K) as they could not be identified onto the species level. Strains of cluster A and B could be allocated to the *P. fragi* species group. In the table in Appendix A, the absolute numbers of isolates, picked from the different isolation points throughout the simulation experiment under both storage conditions, are given for each taxon (Figure 5.2). *P. fragi* isolates were isolated markedly more frequently at the end of simulation of the dairy chain as well as *P. gessardii*-like 2, later identified as *P. proteolytica*, and *Pseudomonas* sp. 3 (respectively 55.5%, 50% and 50% of all isolates in that taxon). *Stenotrophomonas* sp. and *Delftia* sp., however, were only isolated at the very beginning of the dairy chain simulation (respectively 96% and 100% of all isolates in that taxon). Isolates identified as *P. proteolytica* (*P. gessardii*-like 2 in Figure 5.3) and *P. fragi* group members (A & B) were isolated markedly more frequently under suboptimal storage conditions (respectively 65%, 100% and 87.5% of all isolates in that taxon) as opposed to members of the *P. fluorescens / P. gessardii* species complex (clusters M, C & D) that were picked up in much higher numbers in raw milk stored under optimal conditions (respectively 69%, 83% and 75% of all isolates in these taxa). In general, a different microbial diversity was isolated under suboptimal storage conditions.

The cultivation independent DGGE-approach revealed pseudomonads were the dominant microbiota at the end of the cold chain of raw milk, as they were represented by the most intense bands in the DGGE assay. Members of the *P. fluorescens / P. gessardii* species complex and *P. lundensis* could be detected in each of the 3 simulations in both optimally and suboptimally stored milk. *P. fragi* was detected in optimally stored milk from simulation 3 and in suboptimally cooled milk from simulations 2 and 3, however, no *P. fragi* isolates were obtained from simulation 2. An unknown *Pseudomonas* sp. was detected in optimally and suboptimally stored milk from simulation 1, although it was only isolated from suboptimally stored raw milk. Two fragments were

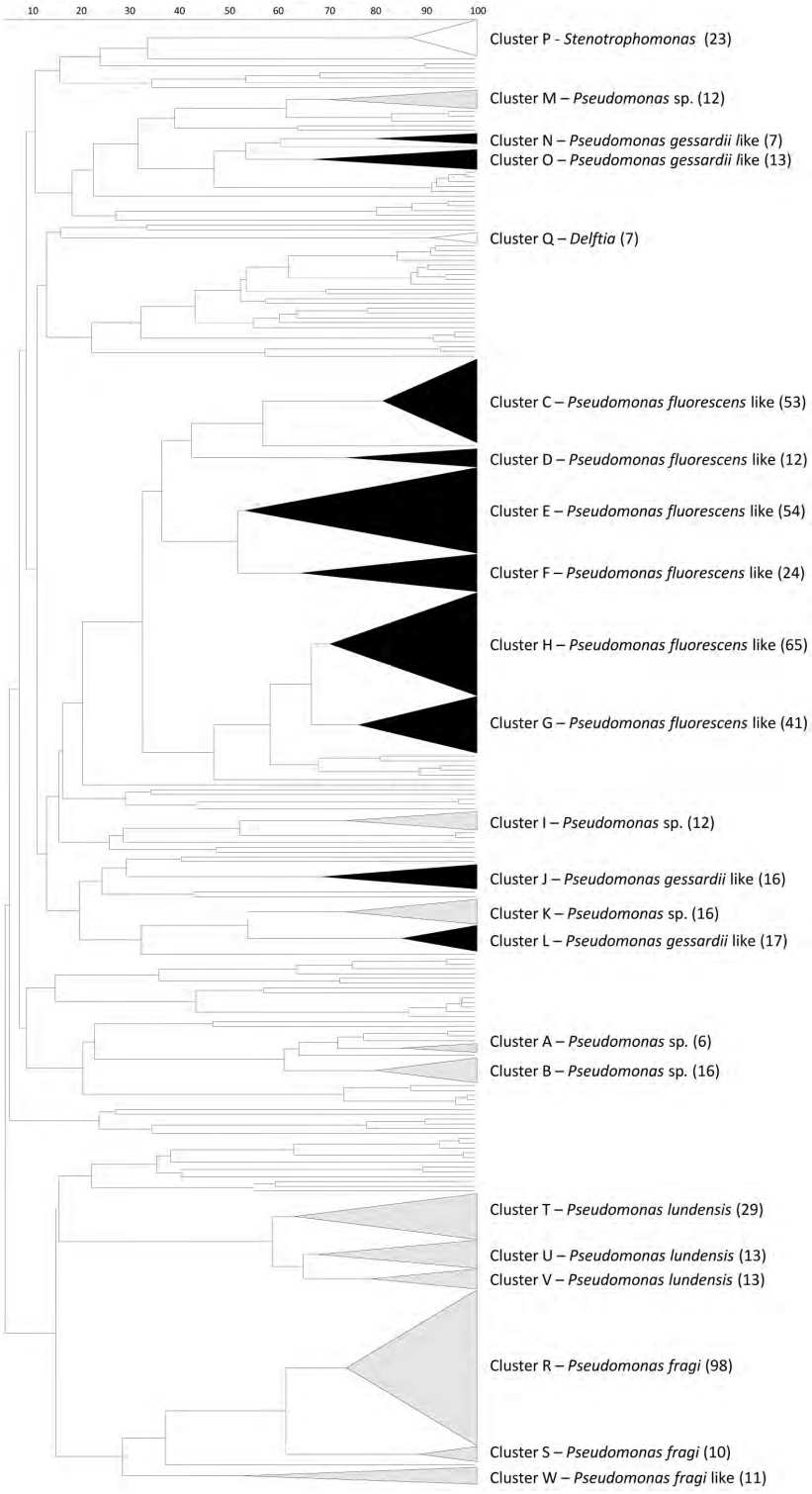


Figure 5.2: BOX-PCR dendrogram of the isolates obtained from three simulations of the dairy chain. Clusters representing similar BOX-profiles are marked with a letter. The number of isolates in each cluster is mentioned between parentheses. Clusters belonging to the *P. fluorescens* group are visualized in black, and other clusters are shown in grey.

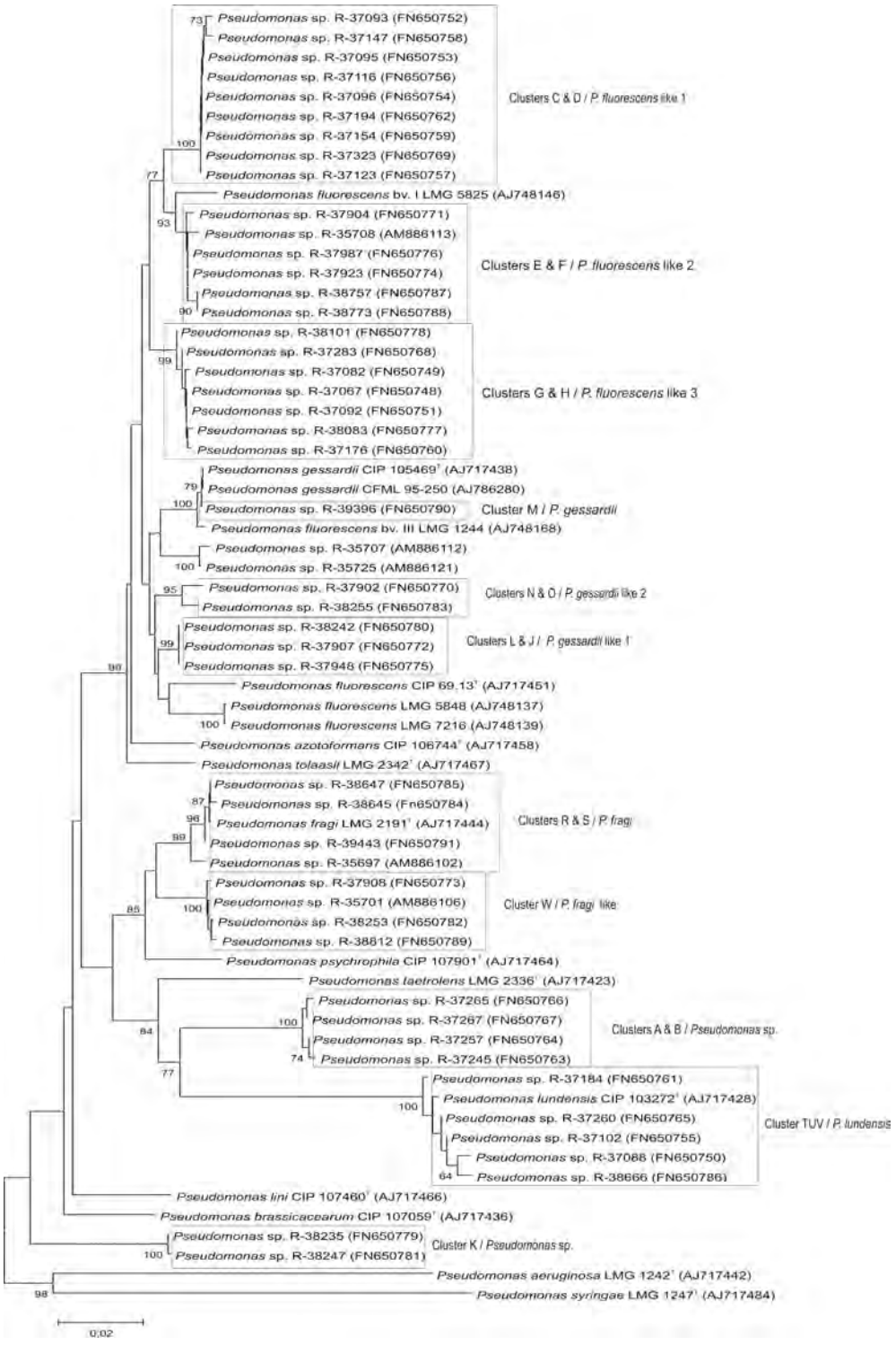


Figure 5.3: Neighbor joining tree based on *rpoB* sequences of the milk isolates and closest relatives. The unrooted tree was constructed using the MEGA software version 4.0 (Tamura *et al.*, 2007); bootstraps (%) are based on 1000 replications. Scale bar, 0.02 substitutions/site. The BOX-cluster from which the representative isolates originate are als given.

identified as *Acinetobacter* species and those fragments were more typical of suboptimally cooled raw milk at the end of the simulation of the dairy chain. The outgrowth of *Pseudomonas* species reached detectable numbers at the end of the simulation of storage at the farm tank for suboptimally cooled raw milk (RM8) and at the start of simulation of transport for optimally stored raw milk (RM9), i.e. when *Pseudomonas* count reached approximately 10^5 cfu mL^{-1}.

Screening for spoilage potential *Pseudomonas* isolates were also screened for their proteolytic and lipolytic spoilage potential using a similar approach as for the aerobic spore-formers by streak-plating on differential media. Results of this analysis are represented in the table in Appendix A. Members of the *P. fluorescens / P. gessardii* species complex demonstrated lipolytic and proteolytic spoilage potential as opposed to *P. lundensis* and *P. proteolytica* which demonstrated mainly proteolytic activity and *P. fragi* which showed only an important lipolytic activity. *Stenotrophomonas* sp. also showed an important spoilage potential, but this can be largely ignored as these strains were not growing out under the simulated storage conditions.

5.1.4 Discussion

This study aimed at a better understanding of the outgrowth of *Pseudomonas* members in raw milk. To achieve this, we (i) determined which storage conditions favor or minimize this outgrowth and (ii) thoroughly identified and characterized the isolated strains to assess their enzymatic spoilage potential in heat-treated milk.

From a simulation of different raw milk storage conditions, it was observed that the farmer's efforts to lower the total colony count of raw milk by cooling the raw milk to approximately 4°C or less, seem to pay off, since the TAPC remained stable under optimal storage conditions in the farm tank. Still, such low temperatures are not always achieved. Surprisingly, suboptimal storage of raw milk at the farm did not appear to have a great effect on TAPC as long as the raw milk was not stored longer than three days at the farm. In Belgium, it is mandatory for the dairy companies to collect the raw milk every two to three days, thereby controlling total aerobic microbiota as shown in our simulation experiments.

However, psychrotolerant bacteria are not so much hampered by low storage temperatures (Lafarge *et al.*, 2004) with *Pseudomonas* members making up 90% of total psychrotolerant microbiota of raw milk (Champagne *et al.*, 1994; Dogan & Boor, 2003). In this study, members of *Stenotrophomonas* and *Delftia acidovorans* were isolated only at the beginning of the simulation of the dairy chain, meaning that these species either cannot grow out under refrigerated storage conditions or that they are overgrown by the better adapted *Pseudomonas* species. Further downstream in the simulation of the dairy chain - transport and storage at the dairy plant - the outgrowth of both pseudomonads and total microbiota continues, resulting in a striking difference of 2 log cfu mL^{-1} between optimal and suboptimal storage. TAPC and PC reached the same level at the end of the simulation of the dairy chain (10^6 and 10^8 cfu mL^{-1} for optimally and suboptimally cooled milk, respectively), indicating that cold storage of raw milk selects for the outgrowth of the *Pseudomonas* microbiota.

A culture-independant molecular approach, DGGE, allowed to confirm that the pseudomonads, and specifically members of the *P. fluorescens / P. gessardii* species complex, *P. lundensis* and *P. fragi* are the only psychrotolerant bacteria able to grow out in the cooled raw milk. DGGE and cultivation monitoring proved to be complementary approaches as some *Pseudomonas* species were detected with the DGGE assay that could not be isolated in a particular simulation or storage

condition. DGGE-monitoring allowed to visualize that the final predominant *Pseudomonas* species composition is already formed in the milk tanker during transport under suboptimal cooling conditions, whereas under optimal cooling conditions, this is formed in the dairy plant tank. This means that under suboptimal storage conditions, the bacterial population can be more active in producing spoilage enzymes which may have a more pronounced effect on the spoilage potential of the end product. The culture-dependent identification approach also indicated members of the *P. fluorescens / P. gessardii* species complex, *P. fragi* and *P. lundensis* as the dominant *Pseudomonas* microbiota. Within the first two taxa several strain clusters could be observed that very likely represent novel species. Moreover, three taxa, identified as *Pseudomonas* sp., may also represent novel species within the genus *Pseudomonas*. In general, the retrieved dominant microbiota from this study largely extends earlier work by Marchand and co-workers (2009a) who identified some of these species (or species groups) as the predominant proteolytic spoilers isolated from raw milk. Further novel species allocations must be verified by more in depth taxonomic studies comprising also DNA-DNA hybridization experiments.

The dominant *Pseudomonas* microbiota that were established in this study also shows an important proteolytic and lipolytic spoilage potential. Not only the general outgrowth of members of known *Pseudomonas* species represents a potential danger for milk spoilage but also the isolation of some presumptive novel species under suboptimal storage conditions indicates that these conditions favor the development of a larger diversity of *Pseudomonas* microbiota with possible spoilage potential. These new insights make it more difficult to assess the spoilage potential of processed dairy products made from such milk. It is not clear from our simulation experiments whether members of *Acinetobacter* species indicate a possible risk of a still more complex microbiota with proteolytic and lipolytic traits (Hantsis-Zacharov & Halpern, 2007). The presence of *Acinetobacter* in particular also poses a safety issue as for instance *Acinetobacter haemolyticus* is able to produce Shiga toxin (Grotiuz *et al.,* 2006).

In the third simulation experiment, not only the *Pseudomonas*-specific medium CFC-agar was used for isolation, but also the more general MPCA medium (only the Gram-negative isolates were taken into consideration) to test whether members of other important taxa might have been missed on CFC agar. Surprisingly, the general diversity of *Pseudomonas* members retrieved from MPCA was less than from CFC-agar, and comprised mainly *P. fragi* and to a lesser extent *Pseudomonas* sp. 2, *P. lundensis* and members of the *P. fluorescens / P. gessardii* species complex (Appendix A). This may indicate that these bacteria are better equipped to compete with other raw milk microbiota during cultivation. It also indicates the important bias which can be introduced by the use of general growth media.

To our knowledge, this is the first study that has attempted to monitor (on a laboratory scale) the outgrowth of *Pseudomonas* microbiota throughout the first part of the dairy chain. A striking result is that *P. fragi* and to a lesser extent *P. lundensis* were predominantly isolated at the end of the simulation of the dairy chain. This might indicate that these organisms may be controlled by adequate cooling and/or rapid processing at the dairy plant. This study further shows that minimizing the outgrowth of spoilage microbiota must be the result of contributions in every step in the dairy chain, and control of raw milk quality should not be restricted to the farm tank level. It is highly recommended to reduce storage time of raw milk prior to processing to a minimum and to keep storage temperature as low as possible (preferentially 3.5°C or lower) throughout the dairy chain.

5.1.5 Conclusions

The use of refrigerated conditions throughout milk processing in order to maintain a safe product has created a specific niche in which the psychrotolerant spoilage microbiota can thrive. The implications associated with the stringency of this cold storage were studied by comparing the *Pseudomonas* microbiota that can grow out under optimal and suboptimal conditions. It appeared that prolonged storage under suboptimal conditions indeed significantly affected the growth rate of the *Pseudomonas* strains, resulting in a 2 log cfu mL^{-1} difference compared with optimal storage before processing. This study demonstrated that the combined use of cultivation, spoilage potential characterization and non-cultivation monitoring of the psychrotolerant bacteria in raw milk helps to better assess the shelf life risks in the end product.

5.2 Further identification of *Pseudomonas fluorescens* group isolates obtained from raw cow's milk

The *Pseudomonas fluorescens* group is a complex species group within the genus *Pseudomonas,* harboring more than 50 *Pseudomonas* species including *Pseudomonas fluorescens*. Differentiation between members of this group is difficult, mainly due to insufficient resolution of the 16S rRNA gene and broad intraspecific phenotypic diversity. Indeed, all species within this group share at least 97.0% 16S rRNA gene sequence similarity, a cut-off value generally regarded as the species delineation value (Coenye *et al*., 2002). The *P. fluorescens* group has been further subdivided into several subgroups, namely the *P. fluorescens*, the *P. gessardii*, the *P. fragi*, the *P. mandelii*, the *P. jessenii*, the *P. koreensis*, the *P. chlororaphis*, the *P. asplenii* and the *P. corrugata* subgroup (Table 5.3). This grouping, as proposed by Mulet and co-workers (2010), was based on a MLSA scheme taking into account 4 gene sequences, namely 16S rRNA, *rpoB*, *rpoD* and *gyrB*.

5.2.1 Identification approach

The study on *Pseudomonas* diversity in raw milk (§5.1) revealed a considerable part of all isolates (81.4%) could be attributed to the *P. fluorescens* group based on 16S rRNA gene sequence analysis. A further identification was obtained by comparing the *rpoB* gene sequence of representative isolates with *rpoB* gene sequences of *P. fluorescens* group type strains. This approach was based on the study of Tayeb and co-workers (2005), stating the *rpoB* gene sequence is a valuable taxonomic marker within the genus *Pseudomonas*.

However, during the course of the study on *Pseudomonas* milk isolates (§5.1), Mulet and coworkers (2010) described a MLSA-scheme for pseudomonad identification, based on 4 different genes among which the *rpoB* gene, and it was expected this approach would result in an even better identification of the *Pseudomonas* isolates retrieved from raw cow's milk. Indeed, it is known that identification methods based on the DNA sequence of a single gene are prone to stochastic genetic variation, horizontal gene transfer and recombination (Gevers *et al.*, 2005). The use of DNA sequences from multiple genes buffers these effects, and MLSA schemes based on at least four different genes are thus generally regarded as more reliable identification approaches (McTaggart *et al*., 2010). The 4 gene sequences, 16S rRNA, *rpoB*, *gyrB* and *rpoD*, were generated for some milk isolate representatives from the study presented in §5.1 and from a study performed

by Marchand and co-workers (2008), that screened the proteolytic spoilage potential of pseudomonads in raw milk. Additionally, several other representative strains (not types) within the *P. fluorescens* group were included for analysis in an attempt to reveal the intraspecies variability and better assess the identification of milk isolates (Table 5.2). Indeed, MLSA ideally utilizes a large number of strains that are representative of the diversity within the genus or genus group as large numbers of strains produce robust species clusters, which in turn increase confident species identification (Gevers *et al.*, 2005; Hanage *et al.*, 2006; McTaggart *et al.*, 2010).

Visualisation of the applicability of the different gene sequences (taxonomic markers) was performed using Taxongap software (Slabbinck *et al.*, 2008). In short, the software provides a graphical output of the heterogeneity and separability of operational taxonomic units (OTUs) as light and dark grey horizontal bars, respectively. OTUs in MLSA studies are mostly species, but in this study of *Pseudomonas* members, OTUs are species groups. If heterogeneity within an OTU exceeds its separability from other OTUs, the defined taxonomic marker is probably not the best choice as it fails to differentiate the OTU's from one another. The larger the gap between heterogeneity and separability, i.e. the taxongap, the better the applicability of the taxonomic marker.

5.2.2 Materials and methods

DNA of representative strains (listed in Table 5.2) was obtained through simple alkaline lysis (Coenye *et al.*, 2002). The nearly complete 16S rRNA gene sequences of all isolates were generated as described by Heyrman and Swings (2001). *rpoB*, *rpoD* and *gyrB* gene sequences were amplified as described by Mulet and co-workers(2010) (see also Chapter 4). Sequencing products were purified with the 'BigDye® XTerminator Purification Kit' (Applied Biosystems) according to manufacturer's instructions using sequential pipetting and a MixMate (Eppendorf) shaking device. Sequences were assembled using the BioNumerics 5.1 software (Applied Maths, Belgium). All sequences have been deposited in EMBL and accession numbers for all strains under study are shown in Table 5.2.

Taxongap analysis (Slabbinck *et al.*, 2008) was performed on type strains within the *Pseudomonas fluorescens* group to assess the reliability of the *rpoB* gene identification compared to other genes from the MLSA scheme proposed by Mulet and co-workers (2010). All accession numbers included for analysis are shown in Table 5.3. Species included for each species group are also indicated.

DNA-DNA hybridization experiments were performed at a hybridization temperature of 45°C (Coorevits *et al.*, 2011c).

Phylogenetic analyses were performed as follows. The 16S rRNA gene sequences were aligned using the arb software (Ludwig *et al.*, 2004) with the integrated arb aligner based on the secondary structures of the 16S rRNA gene. Aligned sequences were exported applying the position variability filter for bacteria (integrated in the software) and re-imported in the MEGA5 software. Overhangs were trimmed resulting in a final alignment of 1337 positions. A Maximum Likelihood tree was constructed applying the Jukes-Cantor substitution model, and bootstrap analysis was performed based on 1000 replications. The three other genes were aligned based on amino acid sequences using the MEGA5 software (Tamura *et al.*, 2011). Overhangs were trimmed resulting in final alignments of 915 positions for *rpoB* gene sequences, 717 positions for *rpoD* gene sequences and 798 for *gyrB* gene sequences. Maximum likelihood trees were constructed ap-

		accession number		
species name	**strain number**	**16S rRNA**	***rpoB***	***rpoD***
Pseudomonas sp.	R-35712	AM886077	AM886117	HE586476
Pseudomonas sp.	R-38242	HE586384	FN650780	HE586491
Pseudomonas sp.	R-35700	AM886091	AM886105	HE586470
Pseudomonas sp.	R-35716	AM886081	AM886101	HE586478
Pseudomonas sp.	R-35699	AM886074	AM886104	HE586469
Pseudomonas sp.	R-35708	AM886099	AM886113	HE586474
Pseudomonas sp.	R-38757	FN650742	FN650787	HE586494
Pseudomonas sp.	R-37283	FN650726	FN650768	HE586486
Pseudomonas sp.	R-37194	FN650720	FN650762	HE586485
Pseudomonas sp.	R-37095	FN650713	FN650753	HE586484
Pseudomonas sp.	R-38101	FN650734	FN650778	HE586490
Pseudomonas sp.	R-38083	FN650733	FN650777	HE586489
Pseudomonas sp.	R-35704	AM886095	AM886109	HE586471
Pseudomonas sp.	R-35698	AM886073	AM886103	HE586468
Pseudomonas sp.	R-35707	AM886098	AM886112	HE586473
Pseudomonas sp.	R-35725	AM886090	AM886121	HE586483
Pseudomonas sp.	R-35720	AM886085	AM886125	HE586482
Pseudomonas sp.	R-38255	FN650737	FN650783	HE586492
Pseudomonas sp.	R-37948	FN650732	FN650775	HE586488
Pseudomonas sp.	R-35715	AM886080	AM886120	HE586477
Pseudomonas sp.	R-35718	AM886083	AM886123	HE586480
Pseudomonas sp.	R-35719	AM886084	AM886124	HE586481
Pseudomonas sp.	R-35706	AM886097	AM886111	HE586472
Pseudomonas sp.	R-35717	AM886082	AM886122	HE586479
Pseudomonas sp.	R-35697	AM886072	AM886102	HE586467
Pseudomonas sp.	R-38645	FN650739	FN650784	HE586493
Pseudomonas sp.	R-37908	HE586385	FN650773	HE586487
Pseudomonas sp.	R-35709	AM886100	AM886114	HE586475
Pseudomonas aeruginosa	LMG 1242^{T}	X06684	AJ717442	AJ633568
Pseudomonas antarctica	LMG 23832	HE586386	HE586403	HE586453
Pseudomonas asplenii	LMG 5147	HE586387	AJ748150	AB039594
Pseudomonas chlororaphis subsp. *aureofaciens*	LMG 5832	HE586388	AJ748162	HE586461
Pseudomonas corrugata	LMG 5036	HE586389	HE586404	HE586454
Pseudomonas corrugata	LMG 1276	HE586390	AJ748164	HE586438
Pseudomonas fluorescens	LMG 5849	GU198113	HE586405	HE586463
Pseudomonas fluorescens	LMG 7207	GU198115	HE586406	HE586465
Pseudomonas fluorescens	LMG 7220	GU198116	HE586407	HE586466
Pseudomonas fluorescens	LMG 5848	GU198112	AJ748137	HE586462
Pseudomonas fluorescens	LMG 2189	GU198103	HE586408	HE586450
Pseudomonas fluorescens	LMG 1799	GU198102	HE586409	HE586448
Pseudomonas fluorescens - BV I	LMG 5830	GU198109	AJ748152	HE586459
Pseudomonas fluorescens - BV I	LMG 14562	HE586392	HE586410	HE586439
Pseudomonas fluorescens - BV I	LMG 14571	GU198119	HE586411	HE586441
Pseudomonas fluorescens - BV I	LMG 14566	GU198118	HE586412	HE586440
Pseudomonas fluorescens - BV I	LMG 14673	GU198123	HE586413	HE586443
Pseudomonas fluorescens - BV I	LMG 5829	GU198108	HE586414	HE586458
Pseudomonas fluorescens - BV III	LMG 14573	GU198120	HE586415	AB039531
Pseudomonas fluorescens - BV III	LMG 14576	GU198121	HE586416	HE586442
Pseudomonas fluorescens - BV III	LMG 5822	GU198106	HE586417	HE586457
Pseudomonas fluorescens - BV III	LMG 14674	GU198124	HE586418	HE586444
Pseudomonas fluorescens - BV III	LMG 5831	GU198110	HE586419	HE586460
Pseudomonas fluorescens - BV III	LMG 1244	GU198101	AJ748168	AB039532
Pseudomonas fluorescens - BV III	LMG 5938	GU198114	AJ748170	HE586464
Pseudomonas fluorescens - BV IV	LMG 5168	GU198105	AJ748167	HE586456
Pseudomonas fluorescens - BV IV	LMG 5939	AJ308306	AJ748135	AB039529
Pseudomonas fluorescens - BV V	LMG 14675	GU198125	HE586420	HE586445
Pseudomonas fluorescens - BV V	LMG 14677	GU198127	HE586421	HE586447
Pseudomonas fluorescens - BV V	LMG 14676	GU198126	HE586422	HE586446
Pseudomonas fluorescens - BV V	LMG 14577	GU198122	HE586423	AB039533
Pseudomonas fluorescens - BV V	LMG 5167	GU198104	AJ748158	HE586455
Pseudomonas marginalis - BV II	LMG 14572	HE586391	HE586424	AB039536
Pseudomonas marginalis pv. *alfalfae*	LMG 5039	HE586393	HE586425	AB039570
Pseudomonas marginalis pv. *alfalfae*	LMG 2214	HE586394	AJ748151	AB039569
Pseudomonas marginalis pv. *marginalis*	LMG 1243	HE586395	HE586426	AB039567
Pseudomonas marginalis pv. *marginalis*	LMG 2210	AJ308309	AJ717486	FN554482
Pseudomonas marginalis pv. *pastinacae*	LMG 2238	HE586396	HE586427	AB039576
Pseudomonas tolaasii	LMG 12211	HE586398	HE586429	HE586434
Pseudomonas tolaasii	LMG 12213	HE586399	HE586430	HE586435
Pseudomonas tolaasii	LMG 12215	HE586400	HE586431	HE586436
Pseudomonas tolaasii	LMG 2339	AF320992	HE586432	HE586452
Pseudomonas tolaasii	LMG 2829	AF320986	AJ748173	to be assigned

Table 5.2: Overview of all milk isolates with tentative identification (species name), strain number, and accession number for 16S rRNA, *rpoB* and *rpoD* gene sequences.

species name	accession number 16S rRNA	*rpoB*	*rpoD*	*gyrB*	subgroup
Pseudomonas asplenii^{T}	AB021397	AJ717432	AB039593	AB039455	*P. asplenii* subgroup
Pseudomonas fuscovaginae^{T}	FJ483519	AJ717433	FN554467	FN554185	*P. asplenii* subgroup
Pseudomonas chlororaphis subsp. *aurantiaca*^{T}	DQ682655	AJ717421	FN554452	FN554171	*P. chlororaphis* subgroup
Pseudomonas chlororaphis subsp. *aureofaciens*^{T}	AY509898	FJ652689	FN554453	FN554172	*P. chlororaphis* subgroup
Pseudomonas chlororaphis subsp. *chlororaphis*^{T}	Z76673	FJ652691	AB039549	FJ652718	*P. chlororaphis* subgroup
Pseudomonas brassicacearum^{T}	AF100321	AJ717436	AM084334	AM084675	*P. corrugata* subgroup
Pseudomonas corrugata^{T}	D84012	AJ717487	AB039566	AB039460	*P. corrugata* subgroup
Pseudomonas kilonensis^{T}	AJ292426	AJ717472	—	—	*P. corrugata* subgroup
Pseudomonas thivervalensis^{T}	AF100323	AM084680	AM084338	AM084679	*P. corrugata* subgroup
Pseudomonas antarctica^{T}	AJ537601	FN554727	FN554450	FN554169	*P. fluorescens* subgroup
Pseudomonas azotoformans^{T}	D84009	AJ717458	AB039547	AB039411	*P. fluorescens* subgroup
Pseudomonas cedrina subsp. *cedrina*^{T}	AF064461	AJ717424	FN554459	FN554178	*P. fluorescens* subgroup
Pseudomonas cedrina subsp. *fulgida*^{T}	AJ492830	HE586401	HE586449	—	*P. fluorescens* subgroup
Pseudomonas costantinii^{T}	AF374472	FN554732	FN554461	FN554180	*P. fluorescens* subgroup
Pseudomonas extremorientalis^{T}	AF405328	FN554733	FN554464	FN554182	*P. fluorescens* subgroup
Pseudomonas fluorescens^{T}	D84013	AJ717451	AB039545	D86016	*P. fluorescens* subgroup
Pseudomonas grimontii^{T}	AF268029	AJ717439	FN554470	FN554188	*P. fluorescens* subgroup
Pseudomonas libanensis^{T}	AF057645	AJ717454	FN554477	FN554195	*P. fluorescens* subgroup
Pseudomonas lurida^{T}	AJ581999	HE586402	HE586451	—	*P. fluorescens* subgroup
Pseudomonas marginalis^{T}	Z76663	AJ717425	AB039575	AB039448	*P. fluorescens* subgroup
Pseudomonas orientalis^{T}	AF064457	AJ717434	FN554493	FN554209	*P. fluorescens* subgroup
Pseudomonas palleroniana^{T}	AY091527	FN554747	FN554497	FN554213	*P. fluorescens* subgroup
Pseudomonas panacis^{T}	AY787208	FN554748	FN554498	FN554214	*P. fluorescens* subgroup
Pseudomonas poae^{T}	AJ492829	FN554751	FN554504	FN554219	*P. fluorescens* subgroup
Pseudomonas rhodesiae^{T}	AF064459	AJ717431	FN554511	FN554225	*P. fluorescens* subgroup
Pseudomonas salomonii^{T}	AY091528	FN554756	FN554512	FN554226	*P. fluorescens* subgroup
Pseudomonas simiae^{T}	AJ936933	FN554757	FN554513	FN554227	*P. fluorescens* subgroup
Pseudomonas synxantha^{T}	D84025	AJ717420	AB039550	AB039415	*P. fluorescens* subgroup
Pseudomonas tolaasii^{T}	AF255336	AJ717467	FN645158	FN645137	*P. fluorescens* subgroup
Pseudomonas trivialis^{T}	AJ492831	FN554762	FN554515	FN554230	*P. fluorescens* subgroup
Pseudomonas veronii^{T}	AF064460	AJ717445	FN554518	FN554233	*P. fluorescens* subgroup
Pseudomonas fragi^{T}	AF094733	AJ717444	FN554466	FN554184	*P. fragi* subgroup
Pseudomonas lundensis^{T}	AB021395	AJ717428	FN554479	FN554197	*P. fragi* subgroup
Pseudomonas psychrophila^{T}	AB041885	AJ717464	FN554506	FN554221	*P. fragi* subgroup
Pseudomonas taetrolens^{T}	D84027	AJ717423	AB039523	AB039412	*P. fragi* subgroup
Pseudomonas brenneri^{T}	AF268968	AJ717482	FN554457	FN554176	*P. gessardii* subgroup
Pseudomonas gessardii^{T}	AF074384	AJ717438	FN554468	FN554186	*P. gessardii* subgroup
Pseudomonas meridiana^{T}	AJ537602	FN554740	HE586433	HE586495	*P. gessardii* subgroup
Pseudomonas mucidolens^{T}	D84017	AJ717427	AB039546	AB039409	*P. gessardii* subgroup
Pseudomonas proteolytica^{T}	AJ537603	FN554752	FN554505	FN554220	*P. gessardii* subgroup
Pseudomonas jessenii^{T}	AF068259	AJ717447	FN554473	FN554191	*P. jessenii* subgroup
Pseudomonas mohnii^{T}	AM293567	FN554741	FN554487	AM293561	*P. jessenii* subgroup
Pseudomonas moorei^{T}	AM293566	FN554742	FN554489	AM29560	*P. jessenii* subgroup
Pseudomonas reinekei^{T}	AM293565	FN554754	FN554508	AM293559	*P. jessenii* subgroup
Pseudomonas umsongensis^{T}	AF468450	FN554763	FN554516	FN554231	*P. jessenii* subgroup
Pseudomonas vancouverensis^{T}	AJ011507	AJ717473	FN554517	FN554232	*P. jessenii* subgroup
Pseudomonas koreensis^{T}	AF468452	FN554737	FN554476	FN554194	*P. koreensis* subgroup
Pseudomonas moraviensis^{T}	AY970952	FN554743	FN554490	FN554206	*P. koreensis* subgroup
Pseudomonas frederiksbergensis^{T}	FR750403	AJ717465	AM084335	AM084676	*P. mandelii* subgroup
Pseudomonas lini^{T}	AY035996	AJ717466	FN554478	FN554196	*P. mandelii* subgroup
Pseudomonas mandelii^{T}	AF058286	AJ717435	FN554482	FN554200	*P. mandelii* subgroup
Pseudomonas migulae^{T}	AF074383	AJ717446	FN554486	FN554204	*P. mandelii* subgroup

Table 5.3: Overview of gene sequences for *Pseudomonas fluorescens* type strains used in Taxongap analysis.

plying the Jukes-Cantor substitution model (with complete deletion of gaps/missing data), and bootstrap analysis was performed based on 1000 replications. The obtained pairwise similarity matrices were used for Taxongap analysis. Alignments of the 4 gene sequences were concatenated (3767 positions) using the Seaview v4 software (Gouy *et al.*, 2010).

5.2.3 Results and discussion

Validation of *rpoB* gene sequences for pseudomonad identification. The initial approach used in §5.1, namely *rpoB* gene sequencing for identification of *Pseudomonas* milk isolates, was evaluated by comparing the *rpoB* gene sequence phylogeny with the *Pseudomonas* phylogeny based on concatenated 16S rRNA, *rpoB*, *rpoD* and *gyrB* gene sequences (Mulet *et al.*, 2010), with respect to the *P. fluorescens* species subgroups. *P. fluorescens* species subgroups were represented by the type strain sequences of species they harbor (Table 5.3). Results of the phylogenetic analyses are represented in a graphical overview generated with Taxongap software (Figure 5.4, biomarkers conc. and *rpoB*), and individual trees are represented in Figures 5.6 and 5.7.

rpoB gene phylogeny did show considerable discrepancies compared to the concatenated 16S rRNA-*rpoB*-*rpoD*-*gyrB* gene tree phylogeny. Only three *Pseudomonas* species subclusters were recovered (supported by high bootstrap values) when using *rpoB* gene sequences alone, namely the *P. chlororaphis*, the *P. koreensis* and the *P. asplenii* subcluster. All other subclusters could not unequivocally be distinguished from one another because species intermingled between different subclusters or because intrasubgroup variability was too high compared to intersubgroup variability (which is particularly true for the *P. fragi* subgroup). This is clearly shown in the Taxongap graphic (Figure 5.4), with light grey bars, representing subgroup heterogeneity, largely extending the dark grey bars, representing subgroup separability, for the subgroups *P. fluorescens*, *P. gessardii*, *P. jessenii*, *P. mandelii, P. fragi* and *P. corrugata*. Members of the *P. gessardii* and the *P. fluorescens* subgroups could not be distinguished based on *rpoB* gene sequencing. One species assigned to the *P. gessardii* subgroup, *P. mucidolens*, intermingled with species of the *P. mandelii* subgroup. Additionally, the *P. jessenii* and *P. corrugata* subgroups were not observed as monophyletic groups, but their species were scattered throughout the tree.

These observations clearly indicate that *rpoB* gene phylogeny does not resemble *Pseudomonas* phylogeny as deduced from concatenated 16S rRNA-*rpoB*-*rpoD*-*gyrB* gene sequences. Nonetheless, *rpoB* gene sequencing might still be useful as an identification tool for pseudomonads, as shown in the study of Marchand and co-workers (2008) where *Pseudomonas* milk isolates were identified as *P. fragi* and *P. lundensis* species based on *rpoB* gene sequencing, and confirmed with DNA-DNA hybridization values. Identification becomes rather problematic when no high similarity values can be found between *rpoB* gene sequences from the isolate and the type strains of any described *Pseudomonas* species because the closest relatives retrieved by *rpoB* gene sequencing are not necessarily the 'true' closest relatives. Unfortunately, there is no percent similarity cut-off value for *rpoB* gene sequences that indicates whether the unknown isolate can confidently be allocated to a known *Pseudomonas* species or not (comparable with the 97% cut-off value for 16S rRNA gene sequences). A possible reason for this could be that the proposal of using *rpoB* as an identification tool was mainly based on sequences of type strains only (Tayeb *et al.*, 2005), thus neglecting the intraspecies variability, and missing the opportunity for determining a cut-off value. Nonetheless, even if more strains per species were included, it is far from sure this would result in one threshold value for species delineation, as it is known intraspecies genetic diversity generally varies between species. This is the main reason that species clusters in MLSA schemes

are based on visual inspection rather than on an arbitrary percent similarity value (Gevers *et al.*, 2005, McTaggart *et al.*, 2010).

Validation of the MLSA scheme proposed by Mulet and co-workers (2010) for pseudomonad identification. The proposed MLSA-scheme looks very promising with a subdivision in intra-generic groups, species groups and species subgroups, as already observed with 16S rRNA gene sequence analysis and other house-keeping genes (Moore *et al.*, 1996; Yamamoto & Harayama, 1998a, b; Anzai *et al.*, 2000; Hilario *et al.*, 2004). Nonetheless, the large amount of discrepancies between *rpoB* gene sequenced based phylogeny and the MLSA-scheme based phylogeny was somewhat surprising because, after all, *rpoB* is one of the biomarkers used in the MLSA scheme, and one would expect a high degree of similarity between both phylogenies which was clearly not the case. For that reason, it was decided to evaluate the four biomarkers individually for their applicability in representing *Pseudomonas* phylogeny. This was again achieved by constructing phylogenetic trees for each gene and for the concatenated sequence (Figures 5.6-5.10) and these analyses were summarized in a graphical overview using Taxongap software (Figure 5.4).

A first quick look at this graphical representation of data already indicates two striking aspects of the MLSA-scheme being *i*) that the resolution of the different biomarkers, represented by dark grey bars, is highest for the *rpoD* gene, followed by *gyrB*, *rpoB* and 16S rRNA genes and *ii*) the subgroup differentiation based on the MLSA-scheme largely represents the *rpoD* phylogeny but is often not supported by the other three genes. Looking at the data more in detail, only the *P. chlororaphis*, *P. fragi*, *P. asplenii* and *P. koreensis* subgroups are supported by all four individual biomarkers, and can thus confidently be regarded as representing 'real' closely related species. The positions of these subgroups compared to the other subgroups depend on the biomarker used, so no conclusions can be drawn about the phylogenetic relation between the different subgroups (Figures 5.6-5.10). The *P. corrugata* subgroup is supported by 16S rRNA, *rpoD* and *gyrB* but not by *rpoB*, indicating this subgroup also harbors 'real' closely related species, and that the type strain of *P. corrugata* obtained an aberrant allele for the *rpoB* locus. For these subgroups supported by three or four genes, it can confidently be assumed their members are indeed more related to each other than to other members of the *P. fluorescens* group and this was confirmed by DNA-relatedness values obtained from literature (Table 5.4; references are given in Table 3.4).

The *P. mandelii* subgroup was only observed in *rpoD* and *rpoB* gene phylogeny, and the *P. gessardii* subgroup was only observed in *rpoD* and *gyrB* gene phylogeny. As these groups are only supported by two out of four biomarkers, it remains unresolved whether these groups indeed harbor most closely related species or not. The *P. fluorescens* and *P. jessenii* subgroups are only supported by *rpoD* gene phylogeny, again questioning the 'true' close relationship between the species these subgroups harbor. Indeed, some of these groupings were not supported by DNA-relatedness values (Table 5.4). DNA-relatedness values reported for *P. meridiana* and *P. orientalis* (64%), and *P. gessardii* and *P. grimontii* (53%) were high, indicating a close relationship between both species couples, which is contrary to their split in two different species subgroups.

MLSA schemes are usually developed to overcome the insufficient resolution often observed for 16S rRNA gene sequences, which is certainly the case for members of the *Pseudomonas fluorescens* group, all sharing at least 97.0% 16S rRNA gene sequence similarity, and they often proved useful for that purpose (Stackebrandt *et al.*, 2002; Kuhnert & Korczak, 2006). However, this does not automatically implies that these schemes can be used to deduce phylogenetic relationships (as claimed by Mulet *et al.*, 2010) between organisms. Nonetheless, Zeigler (2003) proposed several candidate genes of which the similarities could be used in a formula to confidently predict whole

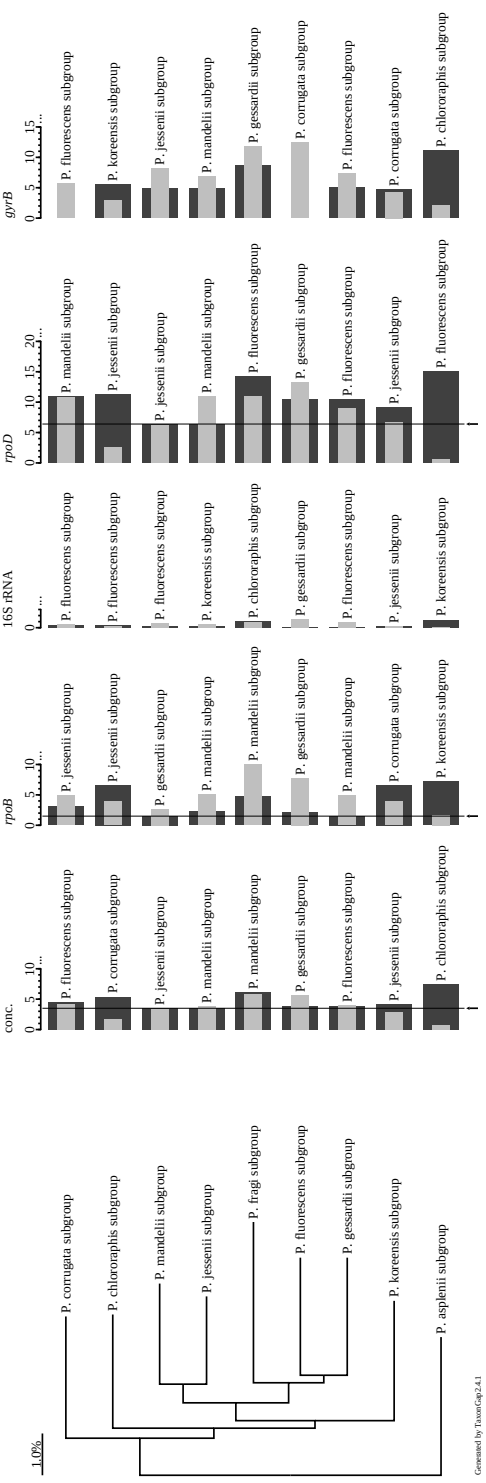


Figure 5.4: Taxonomic resolution of 4 house-keeping genes (16S rRNA, *rpoB*, *rpoD* and *gyrB*) and the concatenated sequence (conc.) used for *Pseudomonas* phylogeny determination, represented using Taxongap software v.4.2.1 (Slabbinck *et al.*, 2008). *Pseudomonas fluorescens* subgroups are represented at the left side of the graphic, in the phylogenetic tree, and the ability for each biomarker to differentiate these subgroups from one another is represented at the right side of the graphic. Heterogeneity within subgroups is represented as light grey horizontal bars, while subgroup separability is represented as dark grey horizontal bars. Next to the bars, the closest related subgroup is given. If length of the light grey bar extends length of the dark grey bar, subgroup differentiation is not possible. The greater the length of the dark grey bars, the better the resolution of the gene. All sequences used for this analysis, as well as the members of the different species subgroups are listed in Table 5.3.

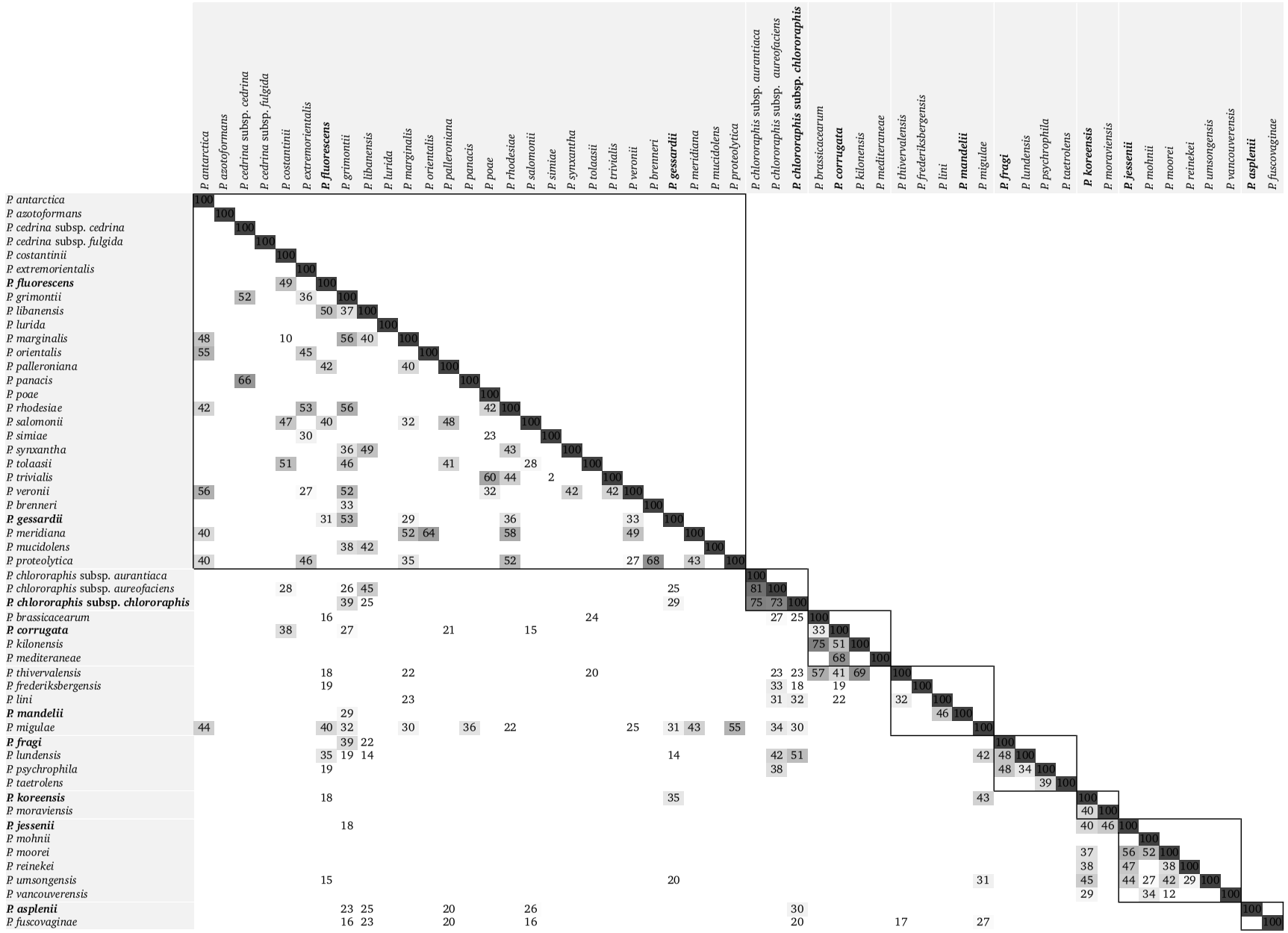


Table 5.4: DNA-relatedness values between *Pseudomonas fluorescens* species. A more intense grey colorization is indicative for higher similarity values. Species subgroups have been delineated as defined by Mulet *et al.* (2010). The name bearer of the subgroup is indicated in bold.

genome relatedness and MLSA schemes based on such genes could be used to deduce phylogenetic relationships, and even to predict whole genome relatedness. It appears the biomarkers chosen by Mulet and co-workers (2010) do not fully comply to the criteria for being considered such a candidate gene. It can be concluded this MLSA-scheme is a valuable tool for identification purposes, but does have difficulties in determining the phylogenetic relationships between some members of the *P. fluorescens* species group. Comparable to *rpoB* gene based identification this implicates identification is very likely reliable when high similarity values are obtained, but unresolved for lower similarity values, and closest relatives in the MLSA-scheme are not necessarily the 'real' closest relatives.

Implications for identification of *Pseudomonas* milk isolates. These findings most likely will have an impact on the identification of *Pseudomonas* milk isolates in §5.1 and Marchand and co-workers (2008). Taxongap analysis clearly indicated the *rpoB* gene is unfortunately the worse taxonomic marker of all four taxonomic markers presented (Figure 5.4), with striking examples being *P. corrugata*, grouping separately from the other members of the *P. corrugata* group, and *P. mucidolens,* grouping in between members of the *P. mandelii* group, while it is a member of the *P. gessardi* subgroup. This does not necessarily mean *rpoB* is a bad taxonomic marker for species identification, but it implicates allocating an isolate to a certain species group based on this gene alone could be unreliable. Therefore, the initial identification approach was validated by sequencing almost complete 16S rRNA and *rpoD* gene sequences additionally to the already obtained *rpoB* gene sequences. Furthermore, several other non-type *Pseudomonas fluorescens* strains were included to obtain a more robust clustering of species, which in turn increases confidence in species identification (Gevers *et al.*, 2005; Hanage *et al.*, 2006). All strains included for analysis are given in Table 5.2. Unfortunately, no *gyrB* gene sequences could be obtained for any of the milk isolates, and this gene was thus discarded for further analysis. A possible explanation could be that primers for the MLSA-scheme were designed based on type strain sequences only, thus neglecting a large amount of the strain diversity present.

Phylogenetic analysis of all three genes simultaneously for all milk isolates, type and non-type strains within the *P. fluorescens* complex (listed in Tables 5.2 and 5.3) resulted in a tree as represented in Figure 5.5. Before discussing the identification of milk isolates some problems encountered during the development of MLSA-schemes for *Pseudomonas* taxonomy, and now clearly illustrated with this tree, will be dealt with.

First, it should be noted this tree is based on three genes, while the MLSA-scheme proposed by Mulet and co-workers (2010) is complemented with *gyrB* gene sequences. Although this might explain the aberrant position of *P. reinekei*^{T} in the tree, it does not account for the aberrant position of *P. migulae*^{T}, because when looking at *gyrB* gene sequence phylogeny only (Figure 5.10), *P. migulae*^{T} is grouped alongside the *P. fragi* subgroup instead of with the *P. mandelii* subgroup, indicating addition of the *gyrB* gene sequence to the other 3 gene sequences would not result in a monophyletic clustering of the *P. mandelii* subgroup in the tree represented in Figure 5.5. A possible other explanation might be that the subgroups proposed by Mulet and co-workers (2010) are based on type strain sequences only, thus neglecting the intraspecies diversity and hampering robust and reliable clustering of species groups and subgroups. All other subgroups are recovered in the tree, but sometimes not supported by high bootstrap values.

Secondly, it is obvious that many non type strains are misidentified, and this has been confirmed for some of these strains by DNA-DNA hybridization experiments. For instance, strains LMG 14576 and LMG 14674, denoted *P. fluorescens* bv. III are most probably species of *P. proteolytica*,

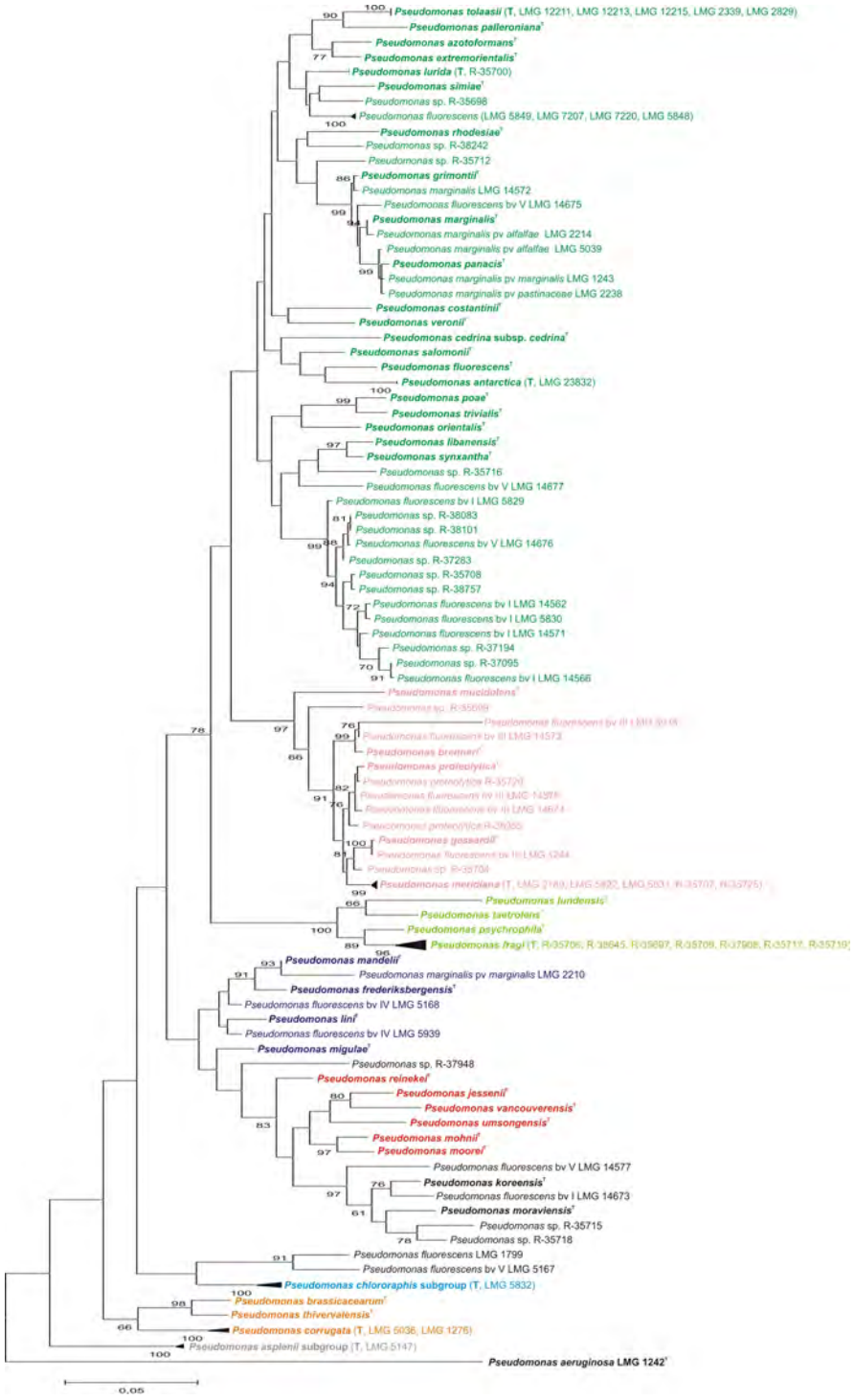


Figure 5.5: Phylogenetic tree based on 16S rRNA, *rpoB* and *rpoD* gene sequences for representative milk isolates, type strains within the *P. fluorescens* complex, and additional strains belonging to the *P. fluorescens* complex according to their name given in BRC's. The subgroups as proposed by Mulet *et al.* (2010) are indicated with colors. Type strains (or groups harboring the type strain) are indicated in bold. Bootstrap values above 65% are given at the branch points. Bar, 0.05 substitutions per nucleotide position.

while LMG 5938 and LMG 14573, also denoted *P. fluorescens* bv. III, are species of *P. brenneri,* which has been confirmed by DNA-relatedness values above 70% with *P. brenneri*^{T} (99.8% and 94.6%, respectively) and below 40% with the type strain of *P. fluorescens*^{T} (34.4% and 35.4%, respectively). The absence of a set of representative strains (non types) that have been reliably identified, seriously hampers any attempt to resolve the complex situation in *Pseudomonas* taxonomy. Additionally, it is known for MLSA-schemes that defining a cut-off value for species delineation is difficult or even useless due to varying intraspecies gene sequence diversity. *Pseudomonas* taxonomists are thus doomed to move in circles, unless they perform an enormous amount of laborious time-consuming DDH-experiments to overcome this taxonomical deadlock.

A third difficulty, coinciding with the second one, is that the species *P. fluorescens* is a very heterogeneous group of strains representing different biovars (I to V), also called biotypes (A to G), and displaying a rather atypical type strain. Biovar V is known to be very heterogeneous and its members are scattered throughout the tree (Figure 5.5) over various *P. fluorescens* complex subgroups (e.g. LMG 14577 in the *P. koreensis* subgroup and LMG 5167 in the vicinity of the *P. chlororaphis* subgroup). Biovar III strains cluster in the *P. gessardii* subgroup and most likely should be attributed to other species (e.g. LMG 14573 and LMG 5938 to *P. brenneri*), while biovar IV strains can be found in the *P. mandelii* subgroup. Biovar I includes the type strain LMG 1794^{T} but within this biovar a large diversity can be observed, and again it is very likely this biovar itself also represents different species.

Keeping all these obstacles in the back of our minds, what can we conclude for the identification of milk isolates? It has already been discussed above that the proposed MLSA-scheme (Mulet *et al.*, 2010) does not necessarily reflects 'true' phylogenies of the pseudomonads. This means assigning an isolate to a species subgroup, does not implicate members of this subgroup are its 'true' closest relatives. Therefore, it was preferred to allocate the isolates to species complexes if a reliable identification could not be obtained. A summary of the final identification of the milk isolates is represented in Table 5.5.

Only 12 strains (44.4%) could reliably be assigned to a described *Pseudomonas* species. R-35720 and R-38255 were identified as *P. proteolytica*, a species first recovered from cyanobacterial mat samples collected from various water bodies in Antarctica (Reddy *et al.*, 2004). *P. proteolytica* strain R-38255 was a representative of cluster O in §5.1 (Figure 5.2), consisting of isolates that were almost all retrieved from suboptimally cooled milk at the end of the dairy chain. These isolates show strong proteolytic activity and intermediate lipolytic activity. R-35725 and R-35707 could be allocated to *Pseudomonas meridiana*, a species also recovered from cyanobacterial mat samples in Antarctica (Reddy *et al.*, 2004). R-35700 was assigned to *Pseudomonas lurida*, a fluorescent species associated with the phyllosphere of grasses (Behrendt *et al.*, 2007).

Unfortunately, the analysis of an additional gene did not help in resolving the phylogenetic position of the other strains. Two strains (R-35715 and R-35718) belong to the *P. koreensis* subgroup but DNA-DNA hybridization experiments are needed to determine whether these strains represent a novel species within this group or not. One strain, R-37948, could not be assigned to a subgroup level and was thus designated '*Pseudomonas* sp.' while all the other strains belonged to the *P. fluorescens* / *P. gessardii* complex, and these strains are representative for 241 isolates out of a total of 574 analysed, which is 42%.

The allocation of strains R-38645, R-35706, R-35697, R-35719, R-35717, R-37908 and R-35709 to the species *Pseudomonas fragi* was confirmed through DNA-DNA hybridization experiments as described by Marchand and co-workers (2008). Intraspecies variability for this group of strains

species name[1]	strain number	cluster	consensus identification
Pseudomonas sp.	R-35715 (W17a)		*P. koreensis* subgroup 1
Pseudomonas sp.	R-35718 (W17c)		*P. koreensis* subgroup 1
Pseudomonas gessardii like 1	R-37948	L (17)	*Pseudomonas* sp.
Pseudomonas fluorescens like 2	R-38757	E (53)	*P. fluorescens - P. gessardii* complex 1
Pseudomonas sp.	R-35708 (Z34b)		*P. fluorescens - P. gessardii* complex 1
Pseudomonas fluorescens like 3	R-38083	G (41)	*P. fluorescens - P. gessardii* complex 2
Pseudomonas fluorescens like 3	R-38101		*P. fluorescens - P. gessardii* complex 2
Pseudomonas fluorescens like 3	R-37283	H (65)	*P. fluorescens - P. gessardii* complex 2
Pseudomonas fluorescens like 1	R-37194	D (12)	*P. fluorescens - P. gessardii* complex 3
Pseudomonas fluorescens like 1	R-37095	C (53)	*P. fluorescens - P. gessardii* complex 3
Pseudomonas sp.	R-35704 (Z22b)		*P. fluorescens - P. gessardii* complex
Pseudomonas sp.	R-35725 (Z38b)		*P. meridiana*
Pseudomonas sp.	R-35707 (Z34a)		*P. meridiana*
Pseudomonas sp.	R-35720 (W51e)		*P. proteolytica*
Pseudomonas gessardii like 2	R-38255	O (13)	*P. proteolytica*
Pseudomonas gessardii like 1	R-38242	J (16)	*P. fluorescens - P. gessardii* complex
Pseudomonas sp.	R-35716 (W38a)		*P. fluorescens - P. gessardii* complex
Pseudomonas sp.	R-35698 (W15a)		*P. fluorescens - P. gessardii* complex
Pseudomonas sp.	R-35700 (W2a)		*P. lurida*
Pseudomonas sp.	R-35712 (W30a)		*P. fluorescens - P. gessardii* complex
Pseudomonas sp.	R-35699 (W31b)		*P. fluorescens - P. gessardii* complex
Pseudomonas fragi	R-38645	R (98)	*P. fragi*
Pseudomonas fragi	R-35706 (Z53a)		*P. fragi*
Pseudomonas fragi	R-35697 (W29a)		*P. fragi*
Pseudomonas fragi	R-35719 (W51a)		*P. fragi*
Pseudomonas fragi	R-35717 (W12d)		*P. fragi*
Pseudomonas fragi like	R-37908	W (11)	*P. fragi*
Pseudomonas fragi	R-35709 (Z41b)		*P. fragi*

Table 5.5: Identification of milk isolates based on 16S rRNA-rpoB-rpoD gene sequences. For the strains from Marchand *et al.*, (2008), the original strain number is given in parentheses next to the R-number assigned. In the column 'cluster', it is indicated for which BOX-cluster in §5.1 (Figure 5.2) these strains are representative, and the number of isolates assigned to each of these BOX-clusters is indicated between brackets.
[1]Species name based on the identification obtained by Marchand *et al.* (2008) and De Jonghe *et al.* (2011).

ranged for 16S rRNA gene sequences from 99.5 to 100%, for *rpoB* gene sequences from 97.5 to 100% and for *rpoD* gene sequences from 96.4% to 100%. However, these species boundary values as determined for the *P. fragi* species and confirmed by DNA-DNA hybridization experiments could not be expanded to other species. For instance, *P. synxantha* and *P. libanensis* showed a *rpoD* sequence similarity of 98.4% but are clearly separate species with a DNA-relatedness value of 49% (Dabboussi *et al.*, 1999a).

5.2.4 Conclusions

Pseudomonas taxonomy is known for its complexicity and unfortunately the above presented analyses again confirm this fact, but do not offer a quick ready-to-use solution. In short, development of MLSA-schemes within this genus suffers from *i*) the size of the genus, making it a tedious, time-consuming job to generate gene sequences for all species type strains and representative non type strains, *ii*) the lack of a representative set of reliably identified non type strains, hampering the formation of robust and reliable species clusters, and *iii*) the limited amount of strains with known whole genome sequence, which could help in determining good biomarkers that represent the true phylogeny of the genus. The efforts made by Mulet and co-workers (2010) in creating a MLSA-scheme might be valuable for identification purposes, but fail to determine phylogenetic relations between pseudomonads. This would not be such a problem, as resolving the phylogeny of a genus does not necessarily needs to be accomplished through MLSA-schemes. However, because no non type strains have been included in the scheme, no species clusters could be delineated, only species subgroups. This makes it extremely difficult to determine whether an unknown belongs to a certain species or not, and thus hampers reliable identification too.

Acknowledgements

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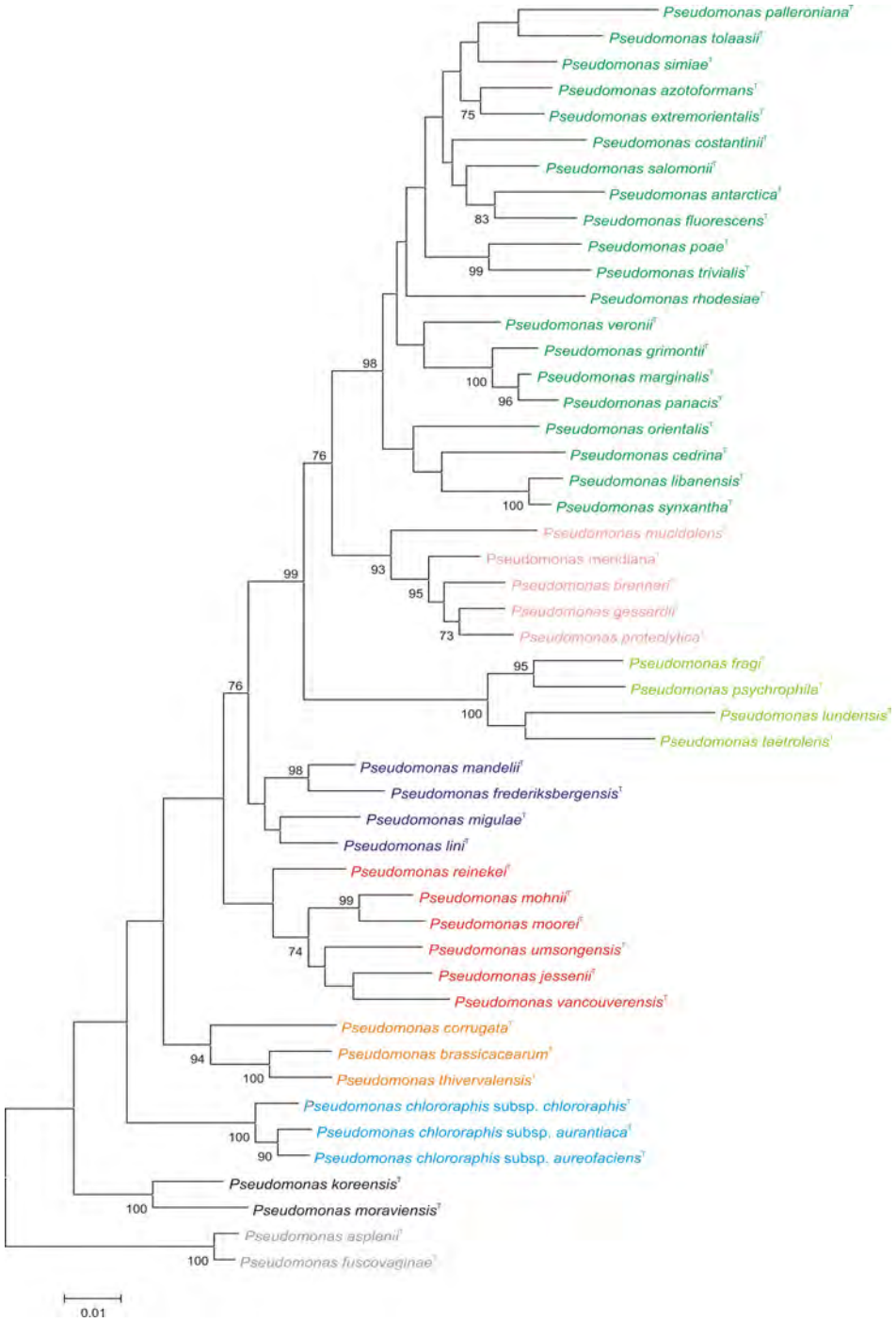

Figure 5.6: ML phylogenetic tree of *Pseudomonas fluorescens* group type strains based on the concatenated gene sequences. Members of different species subgroups were assigned different colours. Grey: *P. asplenii* subgroup; blue: *P. chlororaphis* subgroup; orange: *P. corrugata* subgroup; dark green: *P. fluorescens* subgroup; light green: *P. fragi* subgroup; pink: *P. gessardii* subgroup; red: *P. jessenii* subgroup; black: *P. koreensis* subgroup; purple: *P. mandelii* subgroup. Bootstrap values above 70% are given at the branch points. Bar, 0.01 substitutions per nucleotide position.

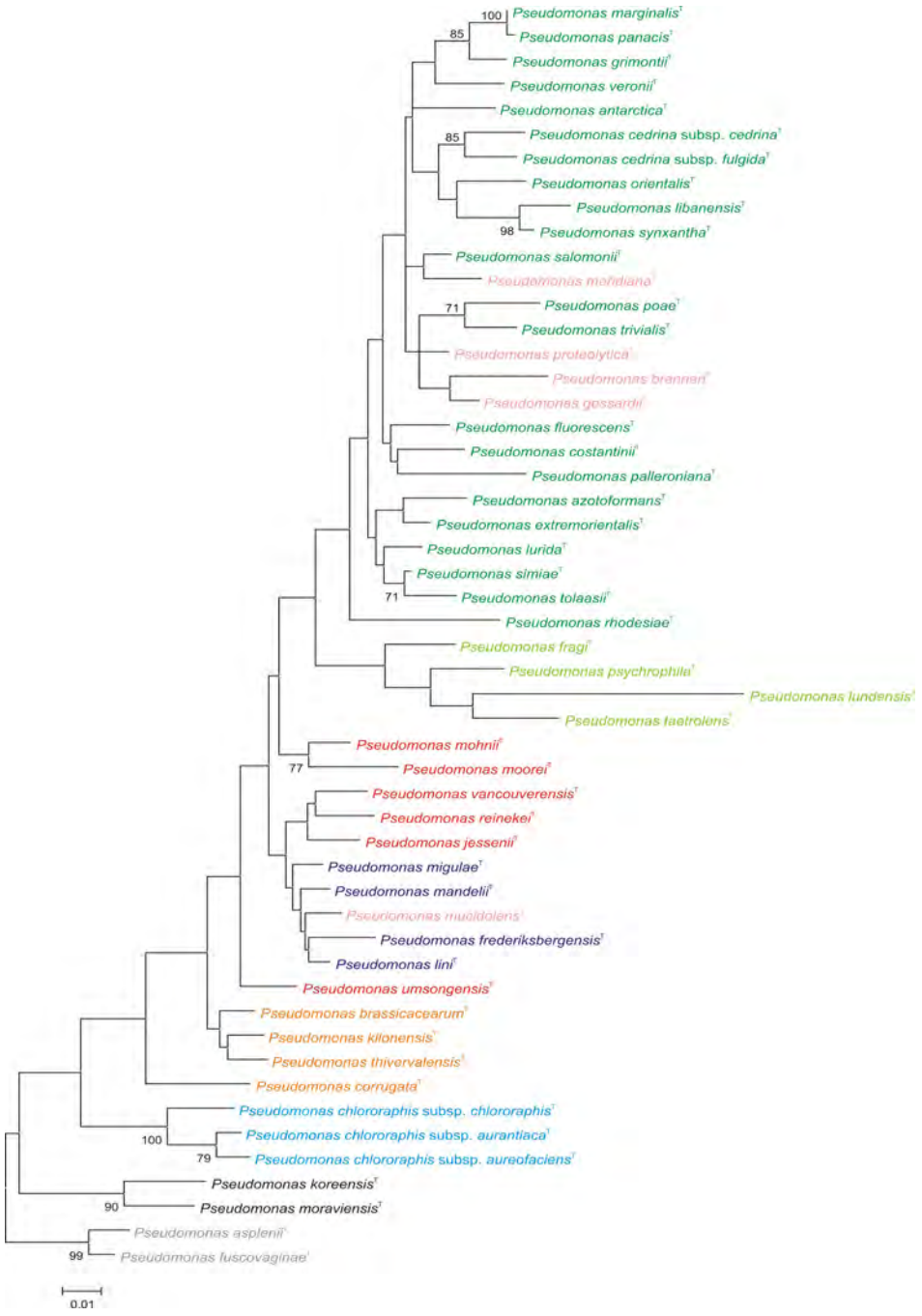

Figure 5.7: ML phylogenetic tree of *Pseudomonas fluorescens* group type strains based on *rpoB* gene sequences. Members of different species subgroups were assigned different colours. Grey: *P. asplenii* subgroup; blue: *P. chlororaphis* subgroup; orange: *P. corrugata* subgroup; dark green: *P. fluorescens* subgroup; light green: *P. fragi* subgroup; pink: *P. gessardii* subgroup; red: *P. jessenii* subgroup; black: *P. koreensis* subgroup; purple: *P. mandelii* subgroup. Bootstrap values above 70% are given at the branch points. Bar, 0.01 substitutions per nucleotide position.

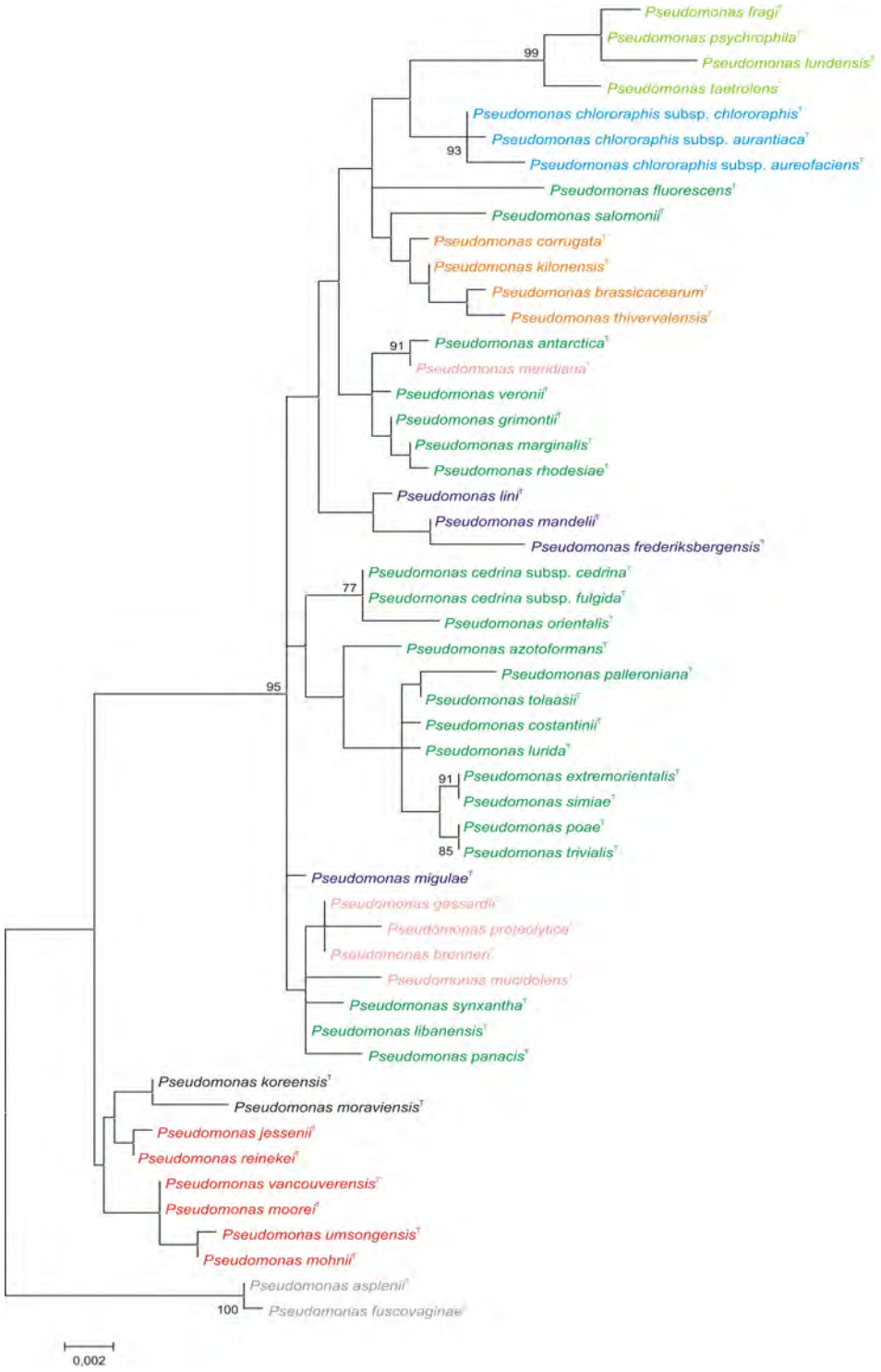

Figure 5.8: ML phylogenetic tree of *Pseudomonas fluorescens* group type strains based on 16S rRNA gene sequences. Members of different species subgroups were assigned different colours. Grey: *P. asplenii* subgroup; blue: *P. chlororaphis* subgroup; orange: *P. corrugata* subgroup; dark green: *P. fluorescens* subgroup; light green: *P. fragi* subgroup; pink: *P. gessardii* subgroup; red: *P. jessenii* subgroup; black: *P. koreensis* subgroup; purple: *P. mandelii* subgroup. Bootstrap values above 70% are given at the branch points. Bar, 0.002 substitutions per nucleotide position.

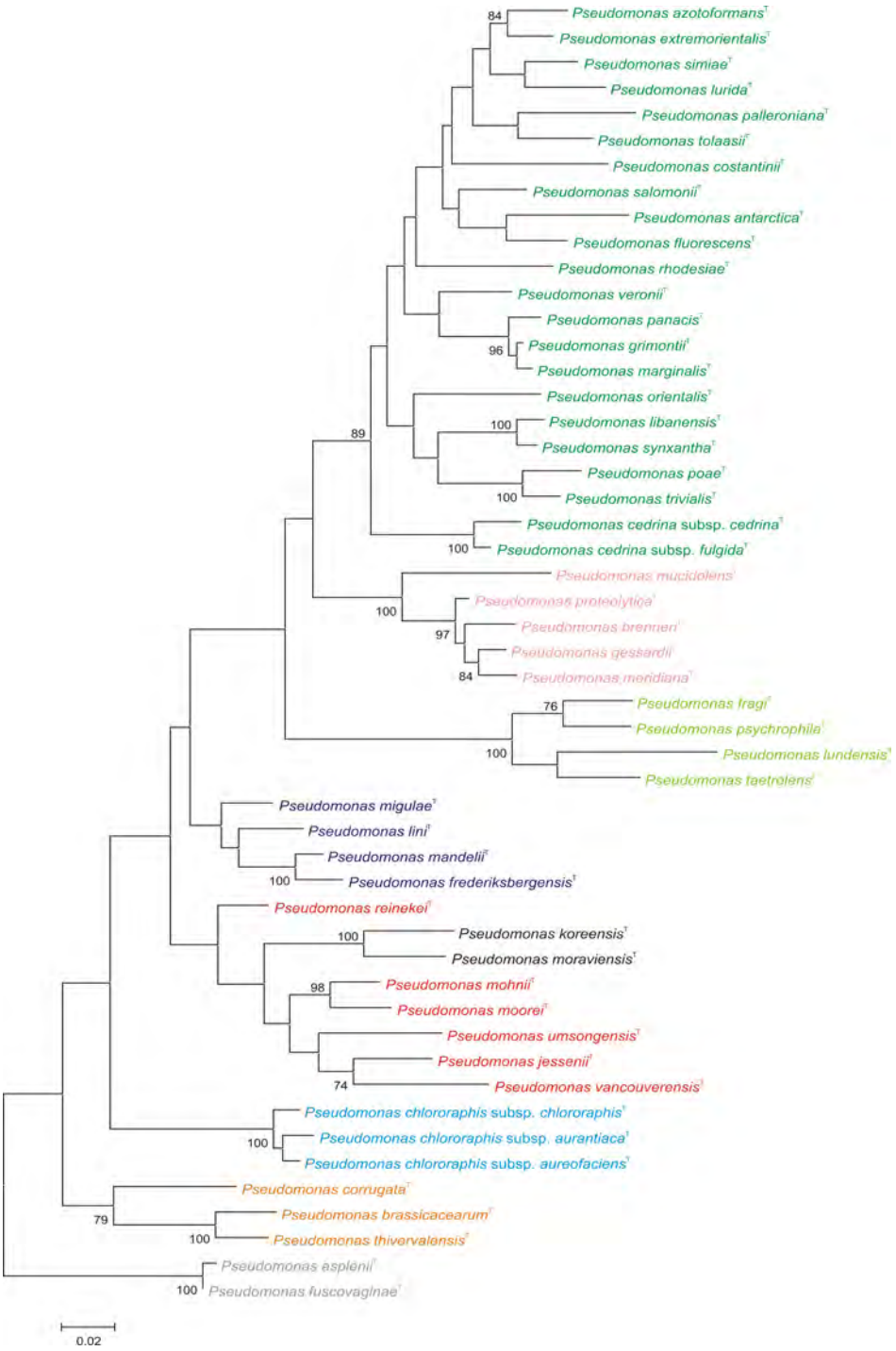

Figure 5.9: ML phylogenetic tree of *Pseudomonas fluorescens* group type strains based on *rpoD* gene sequences. Members of different species subgroups were assigned different colours. Grey: *P. asplenii* subgroup; blue: *P. chlororaphis* subgroup; orange: *P. corrugata* subgroup; dark green: *P. fluorescens* subgroup; light green: *P. fragi* subgroup; pink: *P. gessardii* subgroup; red: *P. jessenii* subgroup; black: *P. koreensis* subgroup; purple: *P. mandelii* subgroup. Bootstrap values above 70% are given at the branch points. Bar, 0.02 substitutions per nucleotide position.

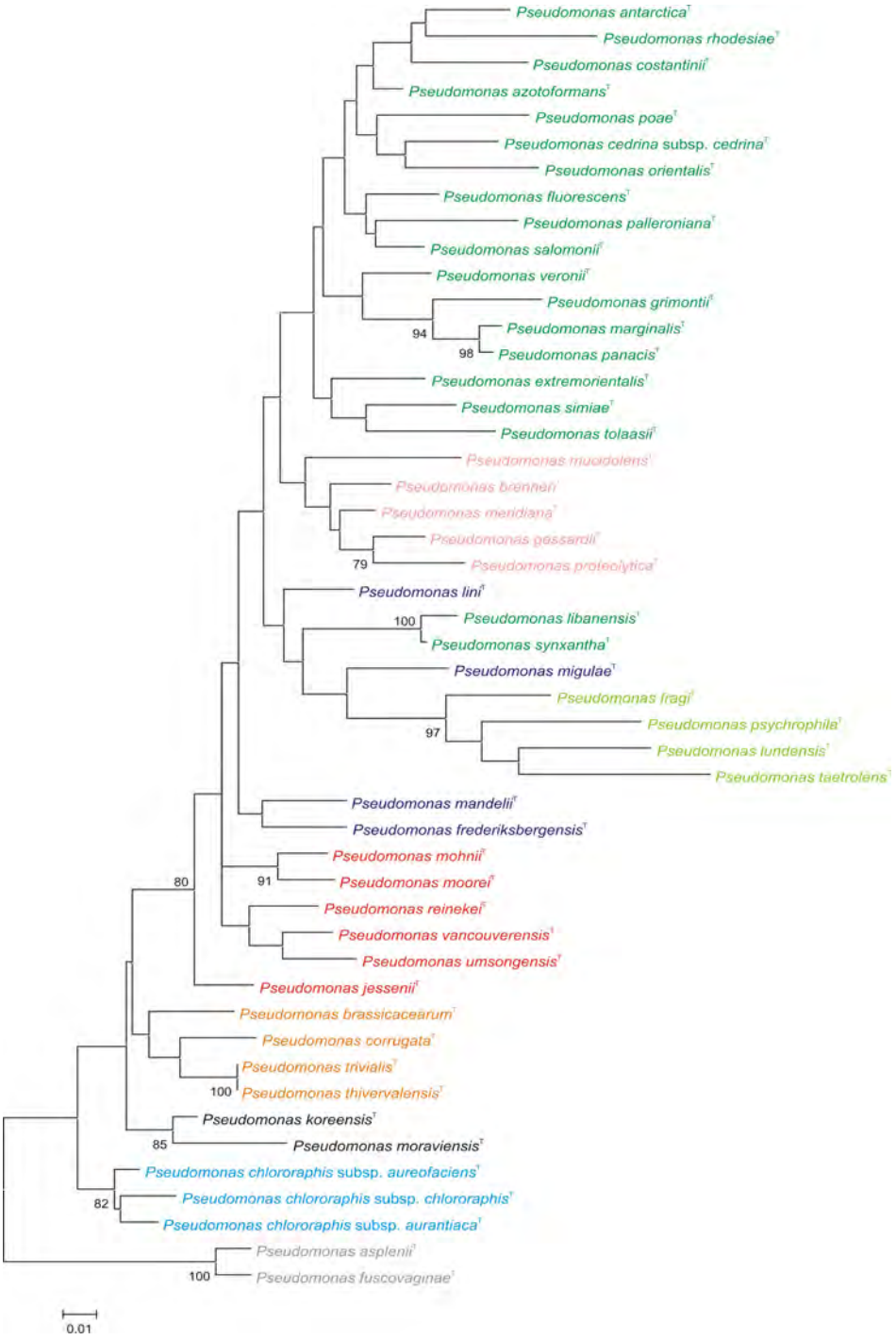

Figure 5.10: ML phylogenetic tree of *Pseudomonas fluorescens* group type strains based on *gyrB* gene sequences. Members of different species subgroups were assigned different colours. Grey: *P. asplenii* subgroup; blue: *P. chlororaphis* subgroup; orange: *P. corrugata* subgroup; dark green: *P. fluorescens* subgroup; light green: *P. fragi* subgroup; pink: *P. gessardii* subgroup; red: *P. jessenii* subgroup; black: *P. koreensis* subgroup; purple: *P. mandelii* subgroup. Bootstrap values above 70% are given at the branch points. Bar, 0.01 substitutions per nucleotide position.

CHAPTER 6

Bacilli in the dairy industry

In this study focus was on diversity of the aerobic endospore-formers. Isolates were subjected to fatty acid methyl ester (FAME) analysis, and representatives of FAME-groups were further identified on the basis of 16S rRNA gene sequencing and rep-fingerprinting. The predominant species were *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus circulans* and members of the *Bacillus cereus* group, as confirmed by other studies focusing on the diversity of aerobic spore-formers in milk. However, this study was the first to demonstrate the presence of representatives of the genus *Oceanobacillus*, *Bacillus clausii*, *Lysinibacillus massiliensis*, *Paenibacillus odorifer*, *Solibacillus silvestris*, *Bacillus simplex* and *Paenibacillus lautus*. This indicates the importance of a polyphasic identification approach. Furthermore, a considerable fraction of spore-forming isolates (7%) probably represent as yet undescribed species, of which some show proteolytic and lipolytic actitivity, indicating their spoilage potential. One group of these isolates was further investigated and described as the novel species *Bacillus thermolactis*. It was also shown that differences in feeding and housing strategy at the farm (e.g. conventional versus organic dairy farming) did influence the bacterial quality of raw milk in terms of aerobic spore content and bacterial diversity and implies the need for further investigation to exactly point out the contamination sources. Study on the impact of these isolates on milk quality and safety indicated members of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Lysinibacillus fusiformis*, *Bacillus amyloliquefaciens* and *Paenibacillus polylmyxa* showed strong to intermediate proteolytic activity and members of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens* showed lipolytic activity. The ability to reduce nitrate to nitrite was shown for *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Lysinibacillus sphaericus* and *Bacillus clausii*, implicating these species might cause structural defects during cheese production. Even more, for *Paenibacillus polymyxa*, this effect is fortified due to gas production during fermentation.

6.1 Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms

Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass. It is hypothesised that differences in feeding and housing strategies of cows may influence the microbial quality of milk. This assumption was investigated through comparison of the aerobic spore-forming flora in milk from organic and conventional dairy farms.

Laboratory pasteurized milk samples from five conventional and five organic dairy farms, sampled in late summer/autumn and in winter, were plated on a standard medium and two differential media, one screening for phospholipolytic and the other for proteolytic activity of bacteria. 927 isolates were obtained of which 898 could be screened via fatty acid methyl ester analysis. Representative isolates were further analyzed using 16S rRNA gene sequencing and $(GTG)_5$-PCR.

The majority of aerobic spore-formers in milk belonged to the genus *Bacillus* and showed at least 97% 16S rRNA gene sequence similarity with type strains of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus circulans*, *Bacillus subtilis* and with type strains of species belonging to the *Bacillus cereus* group. About 7% of all isolates may belong to possibly new spore-forming taxa. Although the overall diversity of aerobic spore-forming bacteria in milk from organic versus conventional dairy farms was highly similar, some differences between both were observed: i) a relatively higher number of thermotolerant organisms in milk from conventional dairy farms compared to organic farms (41.2% vs. 25.9%), and ii) a relatively higher numbers of *B. cereus* group organisms in milk from organic (81.3%) and *Ureibacillus thermosphaericus* in milk from conventional (85.7%) dairy farms.

One of these differences, the higher occurrence of *B. cereus* group organisms in milk from organic dairy farms, may be linked to differences in housing strategy between the two types of dairy farming. However, no plausible clarification was found for the relatively higher number of thermotolerant organisms and the higher occurrence of *U. thermosphaericus* in milk from conventional dairy farms. Possibly this is due to differences in feeding strategy but no decisive indications were yet found to support this assumption.

6.1.1 Introduction

The heat resistant nature of the spores of *Bacillus* species implies that it is especially important to prevent initial contamination of raw milk at the dairy farm, with soil, grass, faeces, feed and milking equipment being possible contamination sources. A better insight into the factors that influence the introduction of spores into raw milk is crucial to guarantee quality and safety of consumption milk and milk products.

Earlier studies have revealed that the composition of the *Bacillus* microbiota in raw milk exhibits seasonal differences (Sutherland & Murdoch, 1994). For example, *Bacillus cereus*, one of the most important species for the milk industry due to its possible psychrotolerance and toxin production (Van Netten *et al.,* 1990), exhibits high spore counts in milk in summer (Christiansson *et al.*, 1999; Philips & Griffiths, 1986). Housing of cows is equally important: soil has been identified as a major source of *B. cereus* spores and spore counts were significantly higher in milk of cows grazing outside than in milk of housed cows (Slaghuis *et al.,* 1997). Also, feed such as silage and feed concentrate have been reported to contain high levels of aerobic spores (Slaghuis *et al.,* 1997; Vaerewijck *et al.*, 2001). A relation between aerobic spore populations in contaminated

silage and raw milk already has been demonstrated by te Giffel and co-workers (2002). Moreover, because feed concentrate often contains cheap, exotic agricultural (waste) products, it is a potential source of new, unknown bacterial spore-formers, possibly introducing new properties of resistance, spoilage or health risk.

It is obvious that the operational management on dairy farms, which determines many of the mentioned factors, will influence the aerobic spore-forming flora of raw milk. A study by Ellis and co-workers (2007) shows that dairy cow cleanliness is influenced by housing strategy as cows became dirtier in the transition from summer grazing to winter housing, and this was especially true for cows on conventional farms. Dairy cow cleanliness is determined using a validated scoring system, with four anatomical areas taken into account: the flanks, the hind legs, the whole tail and the udder. Knowing that soiling of the udder and teats is one of the most important contamination sources of milk for spores (Waes, 1976), the study by Ellis and co-workers (2007) supports the hypothesis that housing strategy might influence microbial milk quality. Furthermore, Ward and co-workers (2002) found that faecal consistency is also correlated with cow cleanliness. Cows producing large quantities of loose faeces are generally dirtier than cows producing firm faeces. This faecal consistency in turn reflects cow nutrition and digestion, adding extra support to the hypothesis that feed strategy might influence the microbial milk quality, not only by composition of the feed but also by its influence on faecal consistency.

In this study, the aerobic spore-forming flora of raw milk, produced at five conventional and five organic dairy farms, was compared during two seasons, late summer/autumn and winter. The most important differences between organic and conventional dairy farming are, as already mentioned, feed and housing strategy. While many conventional dairy farmers use commercial feed concentrate, which usually contains imported agricultural products such as soy, organic farmers feed locally cultivated crops. Also, the grazing-surface per cow on organic farms is usually higher, and cubicles are often bigger. Comparison of the aerobic spore-forming flora in raw milk of conventional and organic dairy farming may thus reveal the operational factors influencing this flora.

6.1.2 Materials and methods

Questionnaire. Farm management data were collected by interview questionnaire with the herd owners of the two types of dairy farming, during late summer/autumn and winter. Special attention was given to the feeding and housing strategy, in order to reveal the expected operational differences between conventional and organic dairy farming.

Sampling. Raw milk samples (100 mL) were aseptically collected from the farm cooling tank of five conventional and five organic dairy farms at geographically different locations in the region of Flanders (Belgium) during a late summer/autumn and a winter campaign in 2004/2005. The samples were heated for 10 minutes at 80°C to select for spore-forming bacteria. Of each preheated milk sample, 80 mL was subjected to chemical extraction, as previously described (Herman *et al.*, 1997). The resultant pellet was then resuspended in 8 mL Ringer solution (Oxoid).

Enumeration and isolation. To determine total aerobic spore counts (TASCs), 500 μL of each preheated milk sample was streaked out on Brain Heart Infusion (BHI) agar plates (Oxoid) supplemented with filter-sterilized vitamin B_{12} (1 mg/L) (Sigma, USA), and incubated at 37°C for 72h. Spore-forming bacteria were isolated from three different media, all supplemented with vitamin B_{12}(1 mg/L): (i) BHI, (ii) Nutrient Agar (Oxoid) supplemented with 8% Egg Yolk emulsion

(Oxoid) and 1% NaCl (Merck, USA) (SNA) and (iii) Plate Count Agar (Oxoid) supplemented with 4% Skim Milk Powder (Oxoid) (SSM). The latter two are differential media, and similarly to the differential media used for screening for spoilage potential of the pseudomonads, these media indicate a characteristic of bacteria by colour change, formation of a halo, or dense deposit zone. In this case, phospholipolytic activity was indicated by a dense deposit zone around the colony on SNA-medium and proteolytic activity was visualized by a clear halo surrounding the colony in the white coloured SSM-medium. To isolate spore-formers, appropriate dilutions from each sample were streaked on these three different media, starting from 100 μL of preheated raw milk as well as from 100 μL of the suspension solution obtained after chemical extraction of the preheated milk. Plates were incubated at 20°C, 37°C and 55°C for 72h to select for psychrotolerant, mesophilic and thermotolerant aerobic spore-formers, respectively. All visibly different colonies on BHI were picked and purified by sub-culturing. On the differential media (SNA and SSM), only visibly different colonies exhibiting the inquired enzymatic activity were sub-cultured. Pure bacterial cultures were stored at –80°C in the Research collection (R-collection) of the Laboratory of Microbiology, Ghent.

Polyphasic identification approach. Bacterial cultures were subjected to FAME analysis and a preliminary identification was obtained using the TSBA database (version 5.0) of the MIS software (MIDI; Microbial ID, USA). A similarity score of >0.8 was considered as a reliable FAME-based identification. All isolates were grouped based on similarities in their FAME-profiles. Initial group margins were chosen at 0.7 Canberra metric distance. Of each cluster obtained this way, several isolates were further identified using 16S rRNA gene sequence analysis. A partial 16S rRNA gene sequence, comprising of the first 400 – 500 base pairs of the gene, a region consisting of hypervariable parts within the genus *Bacillus* (Goto *et al.*, 2000), was generated for 181 isolates. For each partial sequence, a species allocation was obtained in two steps: (i) using the online FASTA tool of EMBL, the 50 most closely related organisms in the EMBL sequence database were appraised; (ii) the 16S rRNA gene sequences of type strains of all species retrieved in the FASTA search were collected and compared to the query sequence using the BioNumerics 4.6 software. Arbitrarily, isolates were assumed to belong to a given species if 16S rRNA gene sequence similarity values of the partial query sequence and the type strain sequence of that given species, exceeded 97%. Nucleotide accession numbers for all sequences obtained in this study are listed in the table in Appendix B. Some species within the *Bacillus subtilis* group (Wang *et al.*, 2007a), could not easily be distinguished based on fatty acid pattern and 16S rRNA gene sequencing. Therefore, $(GTG)_5$-fingerprinting patterns were obtained as an additional tool to aid in isolate identification on the species level.

Growth temperature analysis. All isolates obtained at 20°C were plated on TSA (Oxoid) and incubated at 7°C during seven days, giving an indication of psychrotolerance. Growth was evaluated after 24h, 48h, 72h and one week. Isolates were considered potentially psychrotolerant (hereafter denominated as psychrotolerant) when growth occurred within seven days.

Statistical analysis. Total aerobic spore counts (TASCs) were transformed to $\log_{10}$ counts before statistical analysis. As the distribution of colony forming units in Petri dishes follows a Poisson series, the significant differences ($P < 0.05$) between counts obtained from the two farm types and the two isolation periods, were tested using generalized linear models (GLMs) with Poisson regression analysis. In case the numbers were below the detection limit, the detection limit itself was used in the calculations (thus inherently assuming an overestimation for these values). The number of isolates obtained from the two farm types and the two isolation periods were tested using the same approach. Here, groups of isolates obtained on BHI at the three isolation temper-

atures were included. Noncentral Confidence Interval (CI) estimation was used to calculate the CI at the 95% confidence level to determine the relevance of differences between isolates from organic and conventional farms and for the percentage of growth after seven days at 7°C. All analyses were performed in Statistica 8.0 (Statsoft Inc., Tulsa, Okla).

6.1.3 Results

Questionnaire. All herd owners were questioned about their housing and feeding strategy during late summer/autumn and winter. Organic farmers fed their cows locally cultivated crops such as maize, trefoil, barley, wheat, grass, carrots, turnip and chicory, in general supplemented with salt, minerals and seaweed calcium. This was done during both seasons, although the diet was less diverse during winter. Conventional farmers fed their cows maize, grass and wheat, supplemented with feed concentrate containing components including or derived from soy, rape, coconut, citrus and cacao during both seasons. Also, differences in housing strategy were clearly shown by this interview. In winter season, cows were mostly stabled day and night for both dairy farm types. Only at one organic farm cows grazed outside during the day. In late summer/autumn, cows from all organic dairy farms grazed outside during the day and mostly at night too (at only two of those farms, the cows were stabled at night). In contrast, cows were housed indoor both day and night at two out of five conventional dairy farms in late summer/autumn, and during the night at another farm.

Total Aerobic Spore Counts. An overview of TASCs for each raw milk sample is given in Table 6.1. TASCs ranged from undetected (< 2 spores mL^{-1}) to > 2 $\log_{10}$ spores per mL, with a median value of 28 spores per mL. GLM indicated no significant difference between counts obtained from preheated raw milk of conventional dairy farms compared to organic farms ($P > 0.05$) and between counts obtained during the late summer/autumn period compared to the winter period ($P > 0.05$). A trend was however observed with higher counts during the late summer/autumn period for conventional dairy farms.

	organic dairy farms					**conventional dairy farms**				
	1	2	3	4	5	6	7	8	9	10
late summer/autumn	63	36	7	28	16	14	126	134	144	ND[2]
winter	28	10	<2[1]	60	<2[1]	6	58	<2[1]	18	32

Table 6.1: Total Aerobic spore counts (TASCs) for each raw milk sample. Results are represented in spores per mL.
[1] Not detected, counts below the detection limit of 2 spores per mL;
[2] Not determined.

A polyphasic approach to identify aerobic spore-forming bacteria. Out of 927 isolates obtained, 898 could be analyzed by the standardized FAME method. The remaining 29 isolates failed to grow under the prescribed culturing conditions for FAME analysis, and were not further identified. Hundred and four isolates belonged to non spore-forming genera *Staphylococcus* and *Micrococcus* and - to a lesser degree - *Rothia*, *Kocuria*, *Pseudomonas* and *Moraxella*. Isolation of these non-spore-forming bacteria from the heat-treated samples may be explained by a more abundant presence of these non-spore-formers in raw milk as compared to spore-formers, resulting in a small, though significant surviving population after pasteurisation. Indeed, cocci are known to possess a higher degree of heat resistance than other non-spore-forming microorganisms, probably due to their ability to form clumps (Hammer & Trout, 1924). However, the

most probable explanation for this high number of non spore-formers is the pasteurization procedure itself, which was performed on whole milk during laboratory pasteurization, but in industry is performed on the cream and skim milk fraction separately. The cream fraction in milk very likely protects contaminants during the applied heating procedures, which is the main reason why - for industrial purpose - the cream fraction is separated from the skim milk fraction before pasteurization and subjected to a more severe heat treatment.

16S rRNA gene sequence data were used to redefine the initial FAME groups - set at 0.7 Canberra metric - resulting in a dendrogram consisting of 793 isolates in 18 clusters (A-R) and 12 ungrouped (Figure 6.1; Appendix B).

In order to improve the visual presentation, two heterogeneous clusters (C and K) were each represented as one entity. Nevertheless, a more detailed description of these two clusters is necessary. All 17 isolates in cluster C (Figure 6.2) required 16S rRNA gene sequence data for identification, as similarity coefficients of the MIDI-identifications were insufficient (< 0.8).

Cluster C proved to be a heterogeneous group representing three different genera, *Bacillus*, *Paenibacillus* and *Ornithinibacillus*, and comprising rarely isolated species such as *Bacillus galactosidilyticus* and *Paenibacillus odorifer*. Nevertheless, two subclusters could be distinguished, one containing four isolates with at least 98.4% 16S rRNA gene sequence similarity with the type strain of *Paenibacillus lactis* and another subcluster with five isolates, of which four probably represent an as yet undescribed species within the *Paenibacillus* genus.

The 39 isolates in cluster K (Figure 6.3) were subdivided in ten subclusters and four ungrouped isolates and also required 16S rRNA gene sequencing for a more accurate identification. Four different genera were represented, *Bacillus, Brevibacillus, Lysinibacillus* and *Sporosarcina*, with most isolates belonging to three species, *Bacillus silvestris*, (now *Solibacillus silvestris*; Krishnamurthi *et al.*, 2009) *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus*. Apart from the heterogeneous composition, cluster K stood out because of thirteen isolates probably representing four as yet undescribed species, closely related to aforementioned species in this cluster.

Almost 70% of all other isolates ($n = 509$) appeared to belong to the *Bacillus subtilis* group, harbouring *B. subtilis*, *B. licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus* and *B. pumilus* (Wang *et al.*, 2007a), and were grouped in six FAME clusters (D, E, F, G, H and L). Identification onto the species level for all isolates within these clusters was obtained as follows. When similarity scores exceeded 0.8, the MIDI-identification was accepted as such. This assumption was based on the work of Vaerewijck *et al.* (2001), which shows that species within the *B. subtilis* group can be differentiated from each other based on FAME patterns. The MIDI identification was confirmed or improved by i) colony morphology, ii) $(GTG)_5$-PCR when available (50% of the isolates were screened) (De Jonghe *et al.*, 2008) and iii) 16S rRNA gene sequence analysis. Special care was required for *B. amyloliquefaciens* as isolates belonging to this species could be distinguished from the other species within the *B. subtilis* group based on FAME patterns, but were nevertheless misidentified as *Paenibacillus macerans* by the commercial MIDI-system with similarity scores around 0.7.

The described approach allowed reliable species identification of 97.5% of all isolates in clusters D (*B. licheniformis*), E (*B. subtilis*), F (*B. licheniformis* or *B. subtilis*), G (*B. pumilus*), H (*B. amyloliquefaciens*) and L (*B. licheniformis*). For only 9 isolates no unambiguous identification could be obtained. *Paenibacillus polymyxa* (cluster A), *Bacillus oleronius* (cluster B), *Bacillus badius* (cluster I), *Bacillus clausii* (cluster J), *Ureibacillus thermosphaericus* (cluster M), *Bacillus circulans* (cluster O) and *Bacillus thermoamylovorans* (cluster Q) were each represented by clearly delineated

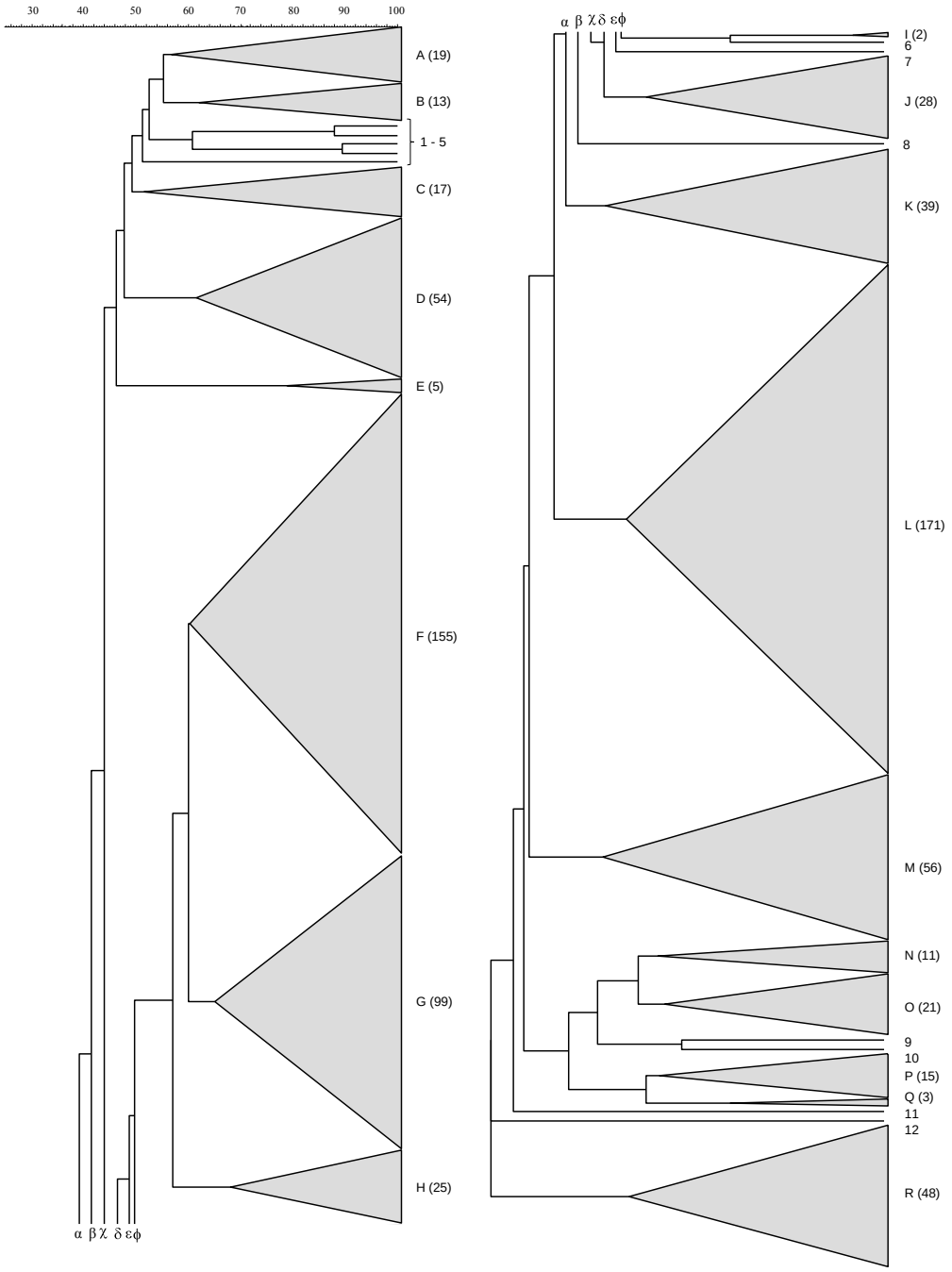

Figure 6.1: Dendrogram of all isolates based on FAME-patterns (Canberra, UPGMA). Names of clusters were assigned based on 16S rRNA gene sequence identifications. Number of isolates between brackets. A: *Paenibacillus polymyxa*; B: *Bacillus oleronius*; C: Diverse 1; D: *Bacillus licheniformis*; E: *Bacillus subtilis*; F: *Bacillus licheniformis* / *Bacillus subtilis* group; G: *Bacillus pumilus*; H: *Bacillus amyloliquefaciens*; I: *Bacillus badius*; J: *Bacillus clausii*; K: Diverse 2; L: *Bacillus licheniformis*; M: *Ureibacillus thermosphaericus*; N: *Oceanobacillus* sp. 2; O: *Bacillus circulans*; P: *Bacillus* sp. 4; Q: *Bacillus thermoamylovorans*; R: *Bacillus cereus* group 1: 98.3% *Paenibacillus terrae*; 2: *Paenibacillus* sp. 4; 3: 99.9% *Bacillus simplex*; 4: 99.6% *Bacillus licheniformis*; 5: 98.4% *Paenibacillus lactis*; 6: *Bacillus* sp. 2; 7: 99.5% *Bacillus licheniformis*; 8: 99.9% *Ureibacillus thermosphaericus*; 9: *Ornithinibacillus* sp.; 10: 100% *Oceanobacillus profundus*; 11: *Brevibacillus* sp.; 12: 99.1% *Bacillus licheniformis*. (Percentage 16S rRNA gene sequence similarity with the type strain of a given species is represented for the ungrouped isolates.)

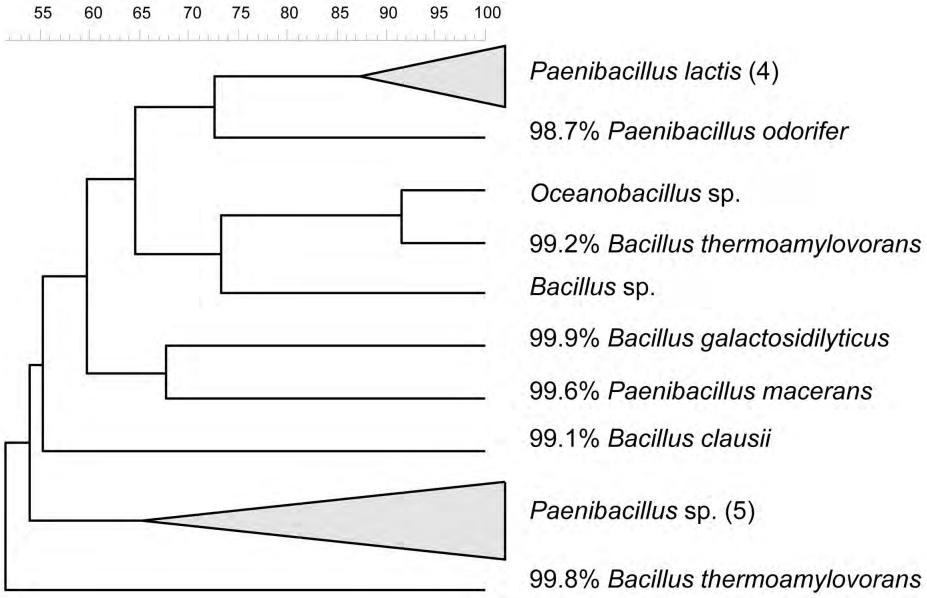

Figure 6.2: Detail of cluster C (Figure 6.1). Grouping based on FAME-patterns, identification based on 16S rRNA gene sequence data. Number of isolates between brackets. Percentage 16S rRNA gene sequence similarity with the type strain of a given species is represented.

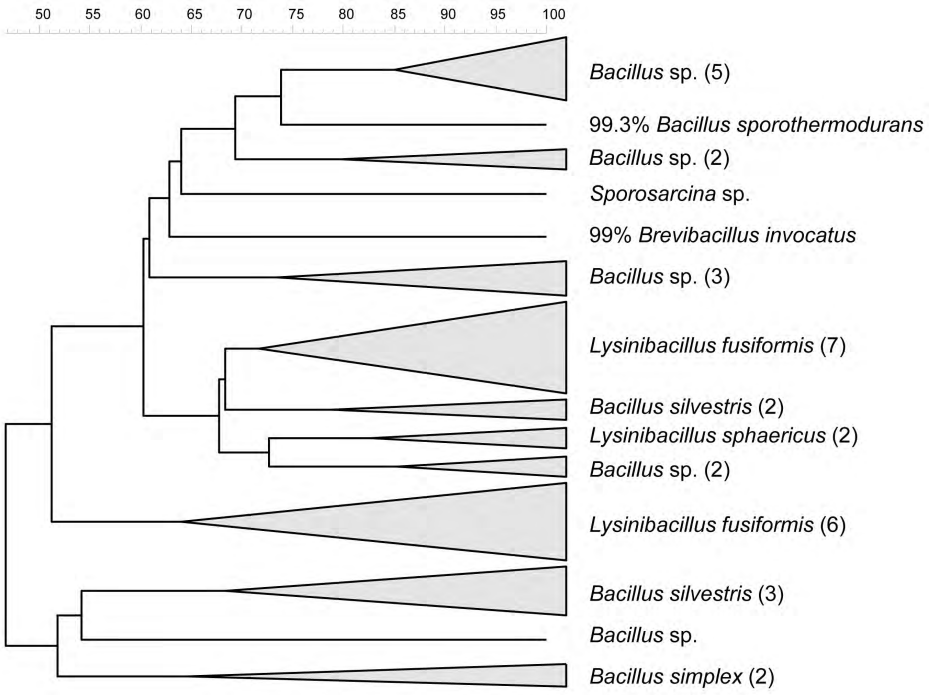

Figure 6.3: Detail of cluster K (Figure 6.1). Grouping based on FAME-patterns, identification based on 16S rRNA gene sequence data. Number of isolates between brackets. Percentage 16S rRNA gene sequence similarity with the type strain of a given species is represented.

groups of isolates. The isolates of two remaining clusters, N and P, probably represent undescribed species within the genera *Oceanobacillus* and *Bacillus*, respectively. Isolates belonging to the *Bacillus cereus* group (cluster R) were not further identified, as other techniques than 16S rRNA gene sequence analysis are required to distinguish different species belonging to this group (Rasko *et al.,* 2005). Until now, six species are described in the *B. cereus* group, namely *B. cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus anthracis* and *Bacillus weihenstephanensis*.

Influence of different isolation and farm parameters on the aerobic spore-forming flora from milk. Isolates in this study were attributed to 27 different species representing genera *Bacillus, Paenibacillus, Oceanobacillus, Brevibacillus, Lysinibacillus, Ureibacillus, Ornithinibacillus* and *Sporosarcina*. About 7% of all isolates probably represent as yet undescribed species within the genera *Bacillus*, *Paenibacillus*, *Brevibacillus, Oceanobacillus, Ornithinibacillus* and *Sporosarcina*. An overview of all data is available in Tables 6.5 – 6.8 at the end of this section. For each isolate, the identification based on FAME and 16S rRNA gene sequence analysis is given, together with isolation and farm parameters (isolation temperature, medium, season and type of dairy farm). BHI-medium showed the highest morphological diversity of colonies and yielded the majority of isolates (71.2%).

Only two species, *Paenibacillus cookii* (n = 1), *Paenibacillus terrae* (n = 2), and two possibly new species belonging to the genera *Paenibacillus* (n = 1) and *Brevibacillus* (n = 2), were not isolated from BHI. It should however be noted that from SNA and SSM only visibly different colonies exhibiting the enzymatic activity were picked up, hence resulting in a lesser diverse collection. Isolates selected from SNA were later identified as *B. licheniformis* (n = 14), *P. cookii* (n = 1) and members of the *B. cereus* group (n = 15).

Twenty-five percent of all isolates were obtained from SSM medium and were mainly attributed to *B. licheniformis* (n = 101), the *B. cereus* group (n = 17), *B. pumilus* (n = 40), *B. subtilis* (n = 14), *B. amyloliquefaciens* (n = 8) and *P. polymyxa* (n = 10). Isolates identified as *B. licheniformis* and *B. pumilus* were ubiquitous, being isolated from raw milk from all farms included in this study. Isolates possibly belonging to as yet undescribed species were also retrieved from each farm, except from organic farm 3. At this farm, only 25 isolates were obtained from all media at the three different incubation temperatures, indicating a generally less diverse aerobic spore-forming flora, as only visibly different colonies were picked up. Furthermore, Table 6.1 reveals that TASCs of farm 3 were rather low compared to all other dairy farms, during late summer/autumn and winter. Other species frequently occurring in raw milk (isolated from at least 7 farms) were members of the *B. cereus* group, *B. amyloliquefaciens*, *B. clausii*, *B. subtilis* and *U. thermosphaericus*.

The influence of seasonal variation and type of dairy farm on the aerobic spore-forming microbiota in raw milk was investigated by incorporating only those isolates obtained from BHI in order to prevent possible multiple counts of the same strain. Isolates obtained at the three isolation temperatures were included. A generalized overview of all isolates retrieved from BHI, per isolation parameter, is given in Table 6.2. For those isolates that could not be identified onto the species level only a genus name was assigned. Confidence intervals were calculated for the percentage of isolates obtained at either organic dairy farms or at conventional dairy farms on the total number of isolates for species that were represented by at least ten isolates. One exception is *P. lactis* (less than 10 isolates), of which the CI indicates the observed difference is statistically significant. A large variability in numbers of isolates can be noticed, indicating that the farm sampled also plays a major role. Statistical analysis using GLM of the total number of isolates showed

a significant difference for the farm type ($P < 0.01$), but not for the isolation period ($P > 0.05$). In total, more isolates were obtained when sampling from conventional dairy farms (56.3%, CI 52.1 – 60.4%) than from organic dairy farms (43.7%, CI 39.6 – 47.9 %).

Considering the thermotolerant spore-formers only (isolated at 55°C), the seasonal effect depended on the farm type, as illustrated in Figure 6.4. More isolates were obtained in the late summer/autumn period in conventional dairy farms ($P < 0.001$). This was not observed for organic farms ($P > 0.05$). In the winter period, no significant difference ($P > 0.05$) between number of thermotolerant isolates from conventional vs. organic dairy farms was observed. For *U. thermosphaericus*, mostly isolated at 55°C (47 out of 56 isolates) and representing a quarter (24.10%) of all isolates obtained at 55°C, more isolates were obtained in the late summer/autumn period on conventional dairy farms ($P = 0.002$). Considering isolations at 20°C, more isolates were obtained in the winter than in the late summer/autumn period ($P < 0.001$) (Figure 6.4). For isolations at 37°C, the opposite was true ($P < 0.01$). This was observed both in conventional and organic dairy farms (Figure 6.4). Members of *B. amyloliquefaciens* were isolated at both 37°C (63.16%) and 20°C (remainder), and were almost exclusively obtained in the winter period (89.5%, CI 66.9% - 98.7%) ($P < 0.01$).

Members of the *B. cereus* group were isolated as frequent at 20°C as at 37°C and were obtained in 87.50% (CI 61.7% - 98.5%) of the cases in the late summer/autumn period ($P = 0.01$). *B. cereus* isolates were also more frequently (81.3%, CI 54.4% - 96.0%) obtained in milk from organic dairy farms ($P < 0.05$). The exclusive occurrence of *P. lactis* (only isolated at 37°C) in milk from conventional dairy farms is noticeable. Also significant, although less obvious, was the higher abundance of all isolates identified as *B. licheniformis* (57.1%, CI 50.7% – 63.3%) in milk from conventional dairy farms. Isolates possibly belonging to as yet undescribed species were mostly isolated at 37°C (58.0%) and at 55°C (30.0%). These were more abundantly isolated from milk from conventional dairy farms (64.0%, CI 49.2% - 77.1% of isolates) ($P = 0.05$) and during the late summer months (66.0%, CI 51.2% - 78.8%) ($P < 0.05$).

Growth temperature analysis. 66.7% (CI 56.0% - 76.3%) of all isolates obtained from BHI at the isolation temperature of 20°C also grew at 7°C after seven days and were considered to be psychrotolerant (Table 6.3). The isolate belonging to *Brevibacillus invocatus* and the isolate belonging to *Bacillus* sp. 5 did not grow at 7°C. Psychrotolerant growth capacity for isolates belonging to *B. licheniformis*, *B. amyloliquefaciens*, *B. clausii*, *B. circulans* and *B. subtilis* was strain-specific. It is striking that 18.9% (CI 11.4% - 28.5%) of all isolates obtained at 20°C from BHI were able to grow abundantly after 24h, indicating the potential of these isolates to spoil dairy products stored at refrigerator temperatures.

6.1.4 Discussion

Aim of this study was to compare the diversity of aerobic spore-forming bacteria in milks from conventional and organic dairy farms. In addition, the influence of seasonal variation on the aerobic spore-forming microbiota in milk was investigated and psychrotolerance of isolates was evaluated. For this purpose, a polyphasic taxonomic approach was applied, which has become indispensable for a reliable identification to the species level within the taxonomically complex group of aerobic spore-forming micro-organisms.

Average spore counts observed in this study ($< 10^2$ spores per mL) are rather low compared to reported spore counts in previous studies, where averages vary between 1 and 10^3 spores per

		organic dairy farms									conventional dairy farms								
		late summer/autumn			winter			total			late summer/autumn			winter			total		
identification	n	20°C	37°C	55°C	20°C	37°C	55°C	n	%	CI	20°C	37°C	55°C	20°C	37°C	55°C	n	%	CI
B. licheniformis	247		30	25	10	15	26	106	42.9	36.7% - 49.3%	1	30	51	10	22	27	141	57.1	50.7% - 63.3%
B. pumilus	61	2	14		14	8		38	62.3	49.0% - 74.4%		9		9	5		23	37.7	25.6% - 51.0%
U. thermosphaericus	56			2		1	5	8	14.3	6.4% - 26.2%			35		8	5	48	85.7	73.8% - 93.6%
B. clausii	28		8		5	2		15	53.6	33.9% - 72.5%		3		4	6		13	46.4	27.5% - 66.1%
B. circulans	20	1	1		2	6		10	50.0	27.2% - 72.8%		6		3	1		10	50.0	27.2% - 72.8%
B. amyloliquefaciens	19		1		3	4		8	42.1	20.3% - 66.5%		1		4	6		11	57.9	33.5% - 79.8%
B. cereus group	16	5	6		1	1		13	81.3	54.4% - 96.0%	2	1					3	18.8	4.1% - 45.7%
Bacillus sp. 4	15			1			3	4	26.7	7.8% - 55.1%			8			3	11	73.3	44.5% - 92.2%
B. oleronius	13		3			5		8	61.5	31.6% - 86.1%		1			4		5	38.5	13.9% - 68.4%
L. fusiformis	10		1			2		3	30.0	6.7% - 65.3%	2	3		1	1		7	70.0	34.8% - 93.3%
Oceanobacillus sp. 2	9				1			1	11.1			8					8	88.9	
B. subtilis	8		3		1	3		7	87.5					1			1	12.5	
P. lactis	7							0	0	0% - 40.9%		3			4		7	100	59.0% - 100%
B. silvestris	6		2					2	33.3		3	1					4	66.7	
B. thermoamylovorans	5						2	2	40.0			1				2	3	60.0	
Bacillus sp. 5	5							0	0.0			3		1	1		5	100.0	
Bacillus sp. 6	5				1			1	20.0			1		2	1		4	80.0	
B. simplex	4	1				1		2	50.0		2						2	50.0	
Paenibacillus sp. 1	4		2			1		3	75.0			1					1	25.0	
P. polymyxa	3							0	0			1			2		3	100	
B. badius	2		1					1	50.0			1					1	50.0	
P. lautus	2				1			1	50.0			1					1	50.0	
Bacillus sp. 3	2		2					2	100								0	0	
Ornithinibacillus sp.	2		1					1	50.0			1					1	50.0	
Oceanobacillus sp. 1	2		2					2	100								0	0	
L. sphaericus	1		1					1	100								0	0	
B. galactosidilyticus	1		1					1	100							0	0		
B. massiliensis	1		1					1	100								0	0	
B. sporothermodurans	1							0	0			1					1	100	
Br. invocatus	1							0	0					1			1	100	
O. profundus	1		1					1	100								0	0	
P. macerans	1							0	0						1		1	100	
P. odorifer	1					1		1	100								0	0	
Bacillus sp. 1	1		1					1	100								0	0	
Bacillus sp. 2	1		1					1	100								0	0	
Bacillus sp. 7	1							0	0						1		1	100	
Paenibacillus sp. 2	1		1					1	100								0	0	
Paenibacillus sp. 3	1					1		1	100								0	0	
Sporosarcina sp.	1							0	0					1			1	100	
total	**565**	9	84	28	39	51	36	247	43.7	39.6% - 47.9%	10	77	94	37	63	37	318	56.3	52.1% - 60.4%

Table 6.2: Overview of all isolates obtained from BHI per isolation parameter (isolation temperature, dairy farm type, isolation season). Identification of isolates is based on FAME and 16S rRNA gene sequence analysis. Isolates that could not be identified onto the species level are named on the genus level. Results are represented in absolute numbers. For each dairy farm type, an additional column is given with values in percentage per species. CI are calculated for those species that are represented by at least ten isolates and for *P. lactis*. Used abbreviations: *B.* = *Bacillus, U.* = *Ureibacillus, L.* = *Lysinibacillus, P.* = *Paenibacillus, Br.* = *Brevibacillus, O.* = *Oceanobacillus*, n = number of isolates, Tot. = Total, CI = Confidence Interval.

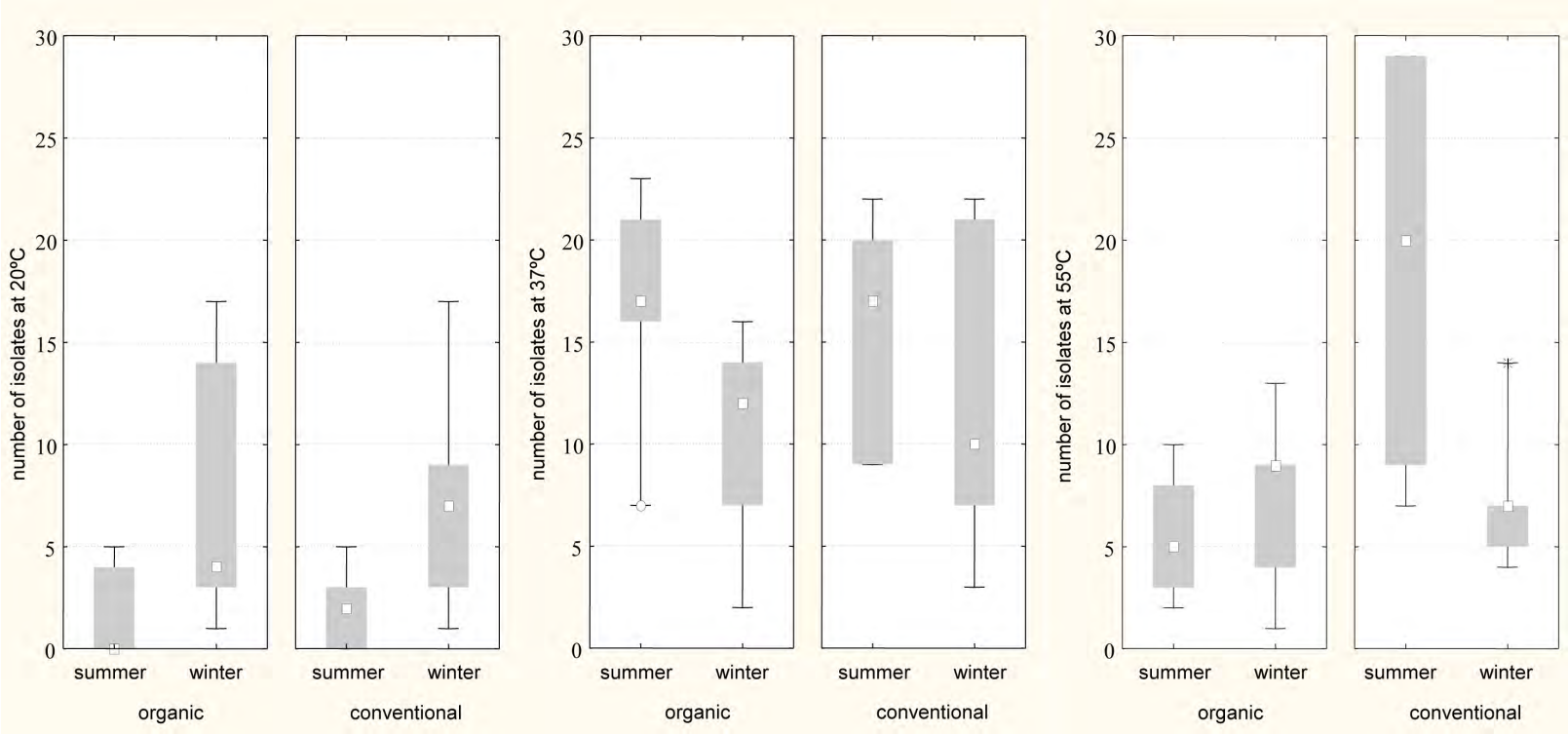

Figure 6.4: BOX plots representing the number of isolates obtained by isolation at 20°C, 37°C and 55°C for organic and conventional dairy farms during the late summer/autumn versus winter period. The median is shown by the square in the box. The boundaries of the box represent the 25^{th} and 75^{th} percentiles. Whiskers above and below the box indicate the 10^{th} and 90^{th} percentile. Outliers are shown as symbols.

		incubation time					
species name	*n*	**24h**	**48h**	**72h**	**7 days**	**% (7 d)**	**CI**
B. pumilus	24	3	6	8	24	100%	85.8% - 100%
B. licheniformis	21	1	1	1	3	14.2%	3.1% - 36.3%
B. clausii	9	2	2	2	3	33.3%	7.5% - 70.1%
B. cereus	8	4	6	6	8	100%	63.1% - 100%
B. circulans	6			1	4	66%	22.3% - 95.7%
B. amyloliquefaciens	5		1	1	3	60%	
S. silvestris	3	1	3	3	3	100%	
B. simplex	3	2	3	3	3	100%	
Bacillus sp. 6	3	2	2	2	3	100%	
B. subtilis	2	1	1	1	1	50%	
L. fusiformis	2	1	1	1	2	100%	
Bacillus sp. 5	1						
Brevibacillus invocatus	1						
Oceanobacillus sp. 2	1		1	1	1	100%	
P. lautus	1				1	100%	
Sporosarcina sp.	1		1	1	1	100%	
all isolates	90	17	30	31	60		
%		18.9%	33.3%	34.4%	66.7%		
CI		11.4 – 28.5%	23.7 – 44.1%	24.7 – 45.2%	56.0 – 76.3%		

Table 6.3: Psychrotrophic growth capacities of all isolates obtained from BHI at the isolation temperature of 20°C. Growth was evaluated at 7°C after 24h, 48h, 72h and 7 days. Results are represented in absolute numbers of strains. An additional column with percentages of strains growing after 7 days is given together with CI if appropriate. n = number of isolates, CI = Confidence Interval.

mL (Waes, 1976; Crielly *et al*., 1994; te Giffel *et al*., 1995; Eneroth *et al*., 1998; McGuiggan *et al*., 2002). It should be noted though, that hygiene on dairy farms has increased further since these previous studies, as indicated by a continuous drop of the total colony count in raw milk, which may have its effect on the total spore count as well. Higher spore counts were observed during late summer/autumn in conventional dairy farms. This would confirm earlier reports which attribute the spore increase in late summer/autumn to lower udder hygiene and dietary changes (Christiansson *et al*., 1999; Lukasova *et al*., 2001; Ellis *et al*., 2007). However, other studies report the opposite, i.e. higher spore counts during winter, attributed to the housing of cows (Crielly *et al.*, 1994; Sutherland & Murdoch, 1994).

Seasonal variation was observed for three species, namely *U. thermosphaericus* and the *B. cereus* group mostly isolated during late summer/autumn, and *B. amyloliquefaciens* almost exclusively isolated in the winter period. The predominance of *B. cereus* in milk during late summer/autumn is confirmed by other studies (Crielly *et al*., 1994; Christiansson *et al*., 1999), but to our knowledge there are no reports on seasonal variation for the other two species. It is thought that grazing of cows might influence the *B. cereus* group concentration in milk, soil being the main contamination source then. A reduction of *B. cereus* group spore concentration could be achieved during the grazing period if the soil contamination of teats could be minimized and teat cleaning optimized. During winter months, feed and bedding are considered as the main contamination sources as the cows are housed indoor in that period (te Giffel *et al*., 1995; Vissers *et al*., 2007). In contrast to studies of Phillips and Griffiths (1986) and Sutherland and Murdoch (1994), a higher amount of psychrotolerant strains during the winter campaign was observed in this study. McGuiggan and co-workers (2002), on the other hand, reported no seasonal variations in psychrotolerant spore counts. A possible explanation for this discrepancy has not been demonstrated yet, but may be related to different farm practices in different countries.

The more frequent isolation of members of the *B. cereus* group at the sampled organic dairy farms can be linked to the observed seasonal variation. As already stated, the main contamination source for the *B. cereus* group is soil to which cows are more exposed while pasturing during summer. Indeed, it has been shown that milk from cows housed indoor during summer has less chance of becoming contaminated with *B. cereus* spores (Slaghuis *et al.,* 1997). The interview questionnaire at all farms revealed that two out of five conventional dairy farmers housed their cows indoors during summer (by day and night), while none of the organic dairy farmers housed their cows during summer (by day). This could possibly explain the more frequent isolation of *B. cereus* group organisms in milk from organic farms.

No decisive explanation could be found for the higher occurrence of thermotolerant isolates, especially *U. thermosphaericus*, during the late summer/autumn period, and to a lesser extent *B. licheniformis,* in milk from conventional dairy farms compared to organic dairy farms. These differences might possibly be correlated with dietary differences between the two types of dairy farming. The diet of cows from Belgian organic dairy farms mainly consists of organic feed composed of agricultural ingredients from local organic farming, while cows at conventional dairy farms are fed conventional crops, usually grain, supplemented with vitamins, minerals and feed concentrate. The main contamination source of thermotolerant isolates could be commercial concentrate feed, as it is often composed of pelleted (hence heat-treated) and/or imported tropical (waste) ingredients. It was shown previously that on average 20% of the aerobic spore-forming isolates from feed concentrate samples were thermotolerant of which several belonged to *B. licheniformis* (Vaerewijck *et al*., 2001). The differences in diet could also be the explanation for the higher amount of unidentified isolates in milk from conventional dairy farms compared to

milk from organic dairy farms (67.3% vs. 32.7%). But possibly other sources exist for particular species such as *U. thermosphaericus* in conventional dairy farming; up to now, this species has only been occasionally isolated from the teat cups in the milking installation (Scheldeman *et al.*, 2005). It has indeed been proposed recently that the composition of a microbial community in milk can vary according to factors other than feeding (Callon *et al.*, 2007).

Most isolates obtained after incubation at 37°C were identified as *B. licheniformis*, *B. pumilus*, *B. clausii* and *B. subtilis*, indicating the predominance of these species in raw milk, as largely confirmed by previous studies, except for *B. clausii* (Philips & Griffiths, 1986; Sutherland & Murdoch, 1994; Tatzel *et al.*, 1994; Lukasova *et al.*, 2001). This study is the first to report on the frequent isolation of *B. clausii* in raw milk. Vaerewijck *et al.* (2001) isolated *B. clausii* strains from feed concentrate samples, but not from raw milk. Detection of *B. clausii* in raw milk provides extra support for feed as an important contamination source of raw milk for spores.

All isolates obtained at 55°C were identified as *B. licheniformis*, *U. thermosphaericus*, *B. thermoamylovorans* and a possible novel *Bacillus* species closely related to the latter. These four species have also been isolated from milk, fodder, green crop and milking equipment by Scheldeman and co-workers (2005) after a heat treatment of 30 min at 100°C, selecting for spore-formers with a potential high heat resistance, and using a similar polyphasic identification approach. Indeed, the temperature at which spores are inactivated has been shown to correlate with the optimal growth temperature of the vegetative cells (Ward *et al.*, 2002). The presence of these potentially highly heat resistant spore-formers in milk is considered undesirable since they may represent a risk for survival of UHT-treatments and thus the non-achievement of commercial sterility requirements (Scheldeman *et al.* 2005). Also, numerous isolates identified as *B. licheniformis* and one isolate identified as *U. thermosphaericus* were retrieved from SNA and SSM, indicating their potential to produce harmful proteolytic and/or phospholipolytic enzymes.

Psychrotolerant spore-forming strains are a major concern to the dairy industry as they are able to survive pasteurization and subsequently grow at the storage temperature of milk (4 - 7°C) (Meer *et al.*, 1991; te Giffel *et al.*, 1995). The shelf life of pasteurized milk and derivatives mainly depends on the presence and spoilage potentials of these psychrotolerant spore-formers. *B. cereus* is a well known and extensively studied pathogen and spoiler in the dairy industry, producing toxins and/or harmful enzymes (Christiansson *et al.*, 1999; Rowan & Anderson, 1998; Stenfors *et al.*, 2007). Although *B. licheniformis*, *B. subtilis* and *B. pumilus* are generally regarded as mesophilic (Pacova *et al.*, 2003), the ability of a fraction of their isolates to grow at 7°C is shown here. The dairy industry itself probably selects for these psychrotolerant strains due to prolonged storage times at 4 - 7°C of raw milk before the heating step (Meer *et al.*, 1991). The latter three species have also occasionally been linked to incidents of food-borne illnesses (Drobniewski, 1993). Isolates of *B. circulans, B. licheniformis, B. subtilis* and *P. polymyxa* have been found to produce toxins (Griffiths, 1990). Hemolytic, phospholipolytic and proteolytic activity has already been detected in *B. subtilis* strains isolated from cured sausages (Matarante *et al.*, 2004). It is obvious that these psychrotolerant spore-formers pose problems to the dairy industry regarding milk quality and safety.

To achieve a correct identification, in a first step, the isolates were submitted to FAME analysis which is a rapid and cheap method to identify bacteria (Stead *et al.,* 1991). It has been shown by Kaneda (1977) that *Bacillus* s.l. is characterized by a distinctive fatty acid composition, enabling straightforward discrimination between these aerobic spore-formers and other contaminants in milk. Various studies (Scheldeman *et al.,* 2005; Vaerewijck *et al.*, 2001) report on the use of FAME

within the *Bacillus* s.l. group. Cluster analysis of the FAME profiles yielded distinct groups of which representatives were further analyzed with additional techniques to confirm or contradict MIDI-identifications. Thus, it could be established that MIDI-identifications are generally to be trusted if similarity scores are higher than 0.7 - 0.8. An exception was *B. amyloliquefaciens*, denominated as *P. macerans* by the MIDI-identification system (similarity scores around 0.7), but rebutted by 16S rRNA gene sequence analysis and $(GTG)_5$-PCR. In contrast to other non spore-forming contaminants, isolates identified as *Staphylococcus* with both FAME data and 16S rRNA gene sequencing, formed a distinctive subgroup within the *Bacillus* s.l. group. Moreover, several isolates within the mainly thermotolerant group L were initially identified, albeit poorly (MIDI-score < 0.7) as members of *Staphylococcus* by FAME, while later 16S rRNA gene sequencing eventually identified these isolates as belonging to the *B. subtilis* group. Rilfors and co-workers (1978) showed that at elevated growth temperatures the fatty acid composition of bacterial cell membranes is limited to certain specific types to prevent the membrane from being to fluid and leaky, which was confirmed in our study. The major fatty acids of mesophilic isolates identified as *B. licheniformis* were anteiso-$C_{15:0}$ (36.7%), iso-$C_{15:0}$ (30.1%), anteiso-$C_{17:0}$ (11.9%) and iso-$C_{17:0}$ (7.8%). Iso-$C_{16:0}$ (3.8%), $C_{16:0}$ (3.7%), iso-$C_{17:0}$ ω10c (1.4%) and $C_{14:0}$ (0.8%) were also detected. At elevated growth temperature, the same major fatty acids were represented, but in different ratios. Thermotolerant isolates identified as *B. licheniformis* mainly contained iso-$C_{15:0}$ (49.0%), iso-$C_{17:0}$ (21.0%), anteiso-$C_{15:0}$ (13.4%) and anteiso-$C_{17:0}$ (7.2%). This change in fatty acid composition at higher growth temperatures could explain both the subdivision of *B. licheniformis* isolates in a thermotolerant group (L) and two non-thermotolerant groups (D and F), and the unreliable MIDI-identification of the former. 16S rRNA gene sequence analysis was conducted on representatives of the groups initially delineated by FAME. Heterogeneous clusters (C and K) were more extensively analyzed by sequencing the 16S rRNA gene of all involved isolates. In the *B. subtilis* group, colony morphology and $(GTG)_5$-profiles were used to confirm or repudiate MIDI-identifications, as 16S rRNA gene sequencing of this large group of isolates would be too expensive and moreover exhibit only a limited discrimination. The identification approach used in this study turned out to be very useful for the large amount of isolates.

The overall diversity of the dominant aerobic spore-forming microbiota observed in this study is quite similar to those of former studies, with *B. licheniformis* as the most abundantly isolated species, followed by *B. pumilus*, the *B. cereus* group, *B. subtilis* and *B. circulans* (Crielly *et al.*, 1994; Sutherland & Murdoch, 1994; Tatzel *et al.*, 1994; Cosentino *et al.*, 1997). To our knowledge however, it is the first time that the presence of representatives of the genus *Oceanobacillus* is reported in milk. The related genus *Ornithinibacillus* has been reported in pasteurized milk recently (Mayr *et al.*, 2006). Also the occurrence of *B. clausii, B. massiliensis, P. odorifer, Solibacillus silvestris, B. simplex, P. lautus* and *B. simplex* in raw milk has not been reported before to our knowledge. This indicates the importance of a polyphasic identification approach in studying the microbial diversity of raw milk. Furthermore, a considerable fraction (7%) of spore-forming isolates probably represent about 14 as yet undescribed species, of which some, belonging to the genera *Brevibacillus* and *Paenibacillus,* show putative proteolytic activity indicating the spoilage potential of these unknown isolates. Reliable identification and determination of the spoilage potentials of *Bacillus* species (in particular psychrotolerant strains) is of major significance to the dairy industry. This paper is the first to show in detail that housing and feeding strategies affect the bacterial quality of raw milk in terms of aerobic spore content and diversity and implies the need for further investigation to exactly point the contamination sources. Moreover, as changes in farm operational management will affect the aerobic spore-forming microbiota in milk, possibly

even select for as yet unknown species, special alertness on microbial milk quality is required.

6.1.5 Addendum: update on the *Bacillus* isolates identification

In the above study, several strains were assigned to the genus level, and were expected to represent novel species within *Bacillus* s.l. Additional phenotypic and genotypic work has been performed on some of these strains for further identification. A state-of-the art is given in Table 6.4.

ID (Coorevits *et al*., 2008)	**R-number**	**update on the ID (2011)**
Bacillus sp. 1	26208	*Bacillus sp. nov.*
Bacillus sp. 2	26048	*Bacillus sp.*
Bacillus sp. 3	27276, 27275	*Rummeliibacillus stabekisii*
Bacillus sp. 4	26358, 31288, 26955, 26953, 26949, 26954, 26950, 33857, 28160, 28193, 33367, 33520, 33378, 33550, 33858	*Bacillus thermolactis*[1]
Bacillus sp. 5	30915, 31319	*Lysinibacillus* sp. A
	27357, 28348, 27368, 30998	*Lysinibacillus* sp. B
Bacillus sp. 6	31189, 31190, 31320, 31037	*Bacillus sp.*
Bacillus sp. 7	30914	*Brevibacillus borstelensis*
Brevibacillus sp. 1	28542, 28324	*Brevibacillus* sp.
Sporosarcina sp. 1	31323	*Sporosarcina newyorkensis*[1]
Paenibacillus sp. 1	27422, 27420, 28338, 30631	*Paenibacillus* sp. nov.
Paenibacillus sp. 2	27413	*Paenibacillus* sp.
Paenibacillus sp. 3	30653	*Paenibacillus* sp. nov.
Paenibacillus sp. 4	31009	*Paenibacillus* sp.
Oceanobacillus sp. 1	26277, 27028	*Ornithinibacillus bavariensis*
Oceanobacillus sp. 2	27401, 27402	*Oceanobacillus* sp. nov.
Oceanobacillus sp. 3	27606, 27610, 27612, 28520, 28519, 27633, 27626, 34296, 31213	*Oceanobacillus sojae*

Table 6.4: Update on identification of strains previously not assigned to the species level from study of Coorevits *et al*., 2008. [1] Described species in Coorevits *et al*., 2011c and Wolfgang *et al*., 2011.

For the single strain species, no additional experiments have been performed yet, as the description of a novel species is preferably based on more than one strain. Strain R-26208 (*Bacillus* sp. 1) showed highest similarity with the type strain of *B. lentus*, namely 96.6% 16S rRNA gene sequence similarity. This means DNA-DNA relatedness determination experiments are not necessary to unequivocally assign this species to a novel species within the genus *Bacillus*. Strain R-26048 (*Bacillus* sp. 2) showed highest 16S rRNA gene sequence similarities (97.5%) with members of the *Bacillus cereus* group (Rasko *et al*., 2005) species and DNA-relatedness experiments should be performed to determine whether it can be assigned to a new species or not within that species group. Strain R-30914 (*Bacillus* sp. 7) probably represents a strain of *Brevibacillus borstelensis*, sharing 99.9% 16S rRNA gene sequence similarity with the corresponding type strain. Strain R-27413 (*Paenibacillus* sp. 2) shared 99.6% 16S rRNA gene sequence similarity with the type strain of *Paenibacillus residui*, and strain R-31009 (Paenibacillus sp. 4) shared 98.35% 16S rRNA gene sequence similarity with the type strain of *Paenibacillus terrae*. Again, DNA-relatedness experiments should clarify if these strains represent separate species within the genus *Paenibacillus* or

not. Strain R-30653 (*Paenibacillus* sp. 3) definitely represents a novel species as it shares only 94.5% 16S rRNA gene sequence similarity with its closest relative *Paenibacillus terrigena*.

Strain R-31323 (*Sporosarcina* sp.) had been allocated to the genus *Sporosarcina*, and has now been described as *Sporosarcina newyorkensis*, in collaboration with the Wadsworth Center, New York State Department of Health (Wolfgang *et al.*, 2011) who recovered the same species from clinical samples in the state of New York.

Other species were represented by at least 2 strains, and some of these groups were studied more in detail. Strains R-27276 and R-27275 (*Bacillus* sp. 3) most likely represent species of *Rummeliibacillus stabekisii*, a novel species described in 2009 by Vaishampayan and co-workers (2009) as 16S rRNA gene sequence similarities with the type strain are 99.7%. Strains belonging to this species were recovered from the floor of a spacecraft assembly clean room (USA), a field scale composter (Japan) and an unknown source. Strains previously assigned to *Bacillus* sp. 5 most likely represent two novel species within the genus *Lysinibacillus*, with strains R-30915 and R-31319 sharing 97.6% 16S rRNA gene sequence similarity with the *L. sinduriensis* type strain, and strains R-27357, R-28348 and R-27368 sharing 98.5% 16S rRNA gene sequence similarity. However, as 16S rRNA gene similarity values exceed 97.0% DNA-relatedness experiments should be performed to further confirm this. Five strains previously assigned to *Bacillus* sp. 6 (strains R-31189, R-31190, R-31320, R-31037 and R-27341) also most likely represent a novel species, within the genus *Bacillus,* showing 97.6% 16S rRNA gene sequence similarity with *Bacillus acidicola* but again, DNA-relatedness experiments should be performed to further confirm this. The same could be said about strains R-28542 and R-28324 (*Brevibacillus* sp.), sharing 98.3% 16S rRNA gene sequence similarity with *Brevibacillus thermoruber.* Four strains (*Paenibacillus* sp. 1; R-27422, R-27420, R-28338 and R-30631) represent a novel species within the genus *Paenibacillus*, sharing 96.8% 16S rRNA gene sequence similarity with the type strain of *Paenibacillus fonticola.*

Strains R-26277 and R-27028 (*Oceanobacillus* sp. 1) were assigned to *Ornithinibacillus bavariensis* based on DNA-relatedness experiments, and members previously assigned to *Oceanobacillus* sp. 3 (R-27606, R-27610, R-27612, R-28520, R-28519, R-27633, R-27620, R-34296 and R-31213) most likely represent the recently described species *Oceanobacillus sojae*, sharing 99.8% 16S rRNA gene sequence similarities, although not confirmed by DNA-relatedness experiments. *Oceanobacillus sojae* was first described in 2009 to harbor a strain recovered from the bottom of a mold fermenter used in soy sauce production process (Tominaga *et al.*, 2009). Two remaining *Oceanobacillus* strains R-27401 and R-27402 showed identical $(GTG)_5$-profiles, and although sharing 99.1% 16S rRNA gene sequence similarity with *Oceanobacillus profundus*, clearly represented a separate species within the genus *Oceanobacillus*, confirmed with DNA-DNA hybridization experiments. Strains previously assigned to *Bacillus* sp. 4 (R-26358, R-31288, R-26955, R-26953, R-26949, R-26954, R-26950, R-33857, R-28160, R-28193, R-33367, R-33520, R-33378, R-33550 and R-33858) have been described as the novel species *Bacillus thermolactis*, represented in §6.2. Only ten out of 15 isolates were included in this description due to growth problems with the remaining 5 isolates (R-33550, R-26949, R-33858, R-26953 and R-33857).

			organic				
			farm 1	**farm 2**	**farm 3**	**farm 4**	**farm 5**
late summer/autumn	BHI	20°C	*B. cereus* group (3) *B. simplex* (1)	*B. cereus* group (2) *B. circulans* (1) *B. pumilus* (2)			
		37°C	*B. amyloliquefaciens* (1) *B. badius* (1) *B. cereus* group (4) *B. clausii* (1) *B. circulans* (1) *B. galactosidilyticus* (1) *B. licheniformis* (3) *B. oleronius* (1) *B. pumilus* (3) *S. silvestris* (2) *B. subtilis* (1) *Bacillus* sp. 1 (1) *Bacillus* sp. 2 (1) *L. fusiformis* (1) *L. sphaericus* (1)	*B. cereus* group (1) *B. clausii* (4) *B. pumilus* (3) *B. licheniformis* (6) *Bacillus* sp. 3 (2) *Ornithinibacillus* sp. (1)	*B. oleronius* (1) *B. licheniformis* (6)	*B. clausii* (3) *B. licheniformis* (6) *B. oleronius* (1) *B. pumilus* (6) *B. subtilis* (2) *Oceanobacillus* sp. 1 (2) *O. profundus* (1)	*B. cereus* group (1) *B. licheniformis* (9) *B. massiliensis* (1) *B. pumilus* (2) *Paenibacillus* sp. 1 (2) *Paenibacillus* sp. 2 (1)
		55°C	*B. licheniformis* (3)	*B. licheniformis* (8) *Bacillus* sp. 4 (1) *U. thermosphaericus* (1)	*B. licheniformis* (1) *U. thermosphaericus* (1)	*B. licheniformis* (8)	*B. licheniformis* (5)
	SNA	20°C	*B. cereus* group (4)	*B. cereus* group (3)			
		37°C	*B. cereus* group (4) *B. licheniformis* (1) *P. cookii* (1)	*B. cereus* group (2)		*B. cereus* group (1) *B. licheniformis* (1)	
		55°C	*B. licheniformis* (2)				
	SSM	20°C	*B. cereus* group (3)	*B. cereus* group (2)			
		37°C	*B. cereus* group (6) *B. pumilus* (2) *B. licheniformis* (4)	*B. cereus* group (4) *B. licheniformis* (3) *B. pumilus* (1) *B. subtilis* (1) *L. fusiformis* (1)	*B. licheniformis* (1)	*B. licheniformis* (5)	*B. cereus* group (1) *B. licheniformis* (5)
		55°C	*B. licheniformis* (2)			*B. licheniformis* (2)	*B. licheniformis* (1)

Table 6.5: Overview of all isolates per season (late summer/autumn vs. winter), per isolation temperature (20, 37 or 55°C), per medium (BHI, SNA or SSM) and per dairy farm (organic (farm 1 - 5) vs. conventional dairy farms (farm 6 - 10)). Identifications are based on FAME and 16S rRNA gene sequence analysis. Isolates that are representatives of possibly yet undescribed species are identified onto the genus level. Number of isolates per species is represented between brackets. Used abbreviations: *B.*= *Bacillus*, *Br.*= *Brevibacillus*, *L.*= *Lysinibacillus*, *P.*= *Paenibacillus*, *O.*= *Oceanobacillus*, *U.*= *Ureibacillus*.

			conventional				
			farm 6	**farm 7**	**farm 8**	**farm 9**	**farm 10**
late summer/autumn	BHI	20°C		*B. cereus* group (2) *B. silvestris* (2) *B. simplex* (1)	*S. silvestris* (1) *L. fusiformis* (2)	*B. licheniformis* (1) *B. simplex* (1)	
		37°C	*B. licheniformis* (5) *B. pumilus* (1) *L. fusiformis* (2) *Ornithinibacillus* sp. (1)	*B. circulans* (3) *B. licheniformis* (8) *B. oleronius* (1) *L. fusiformis* (1) *Oceanobacillus* sp. 2 (5) *P. lautus* (1) *P. polymyxa* (1)	*B. amyloliquefaciens* (1) *B. cereus* group (1) *B. circulans* (1) *B. licheniformis* (6) *B. pumilus* (2) *B. sporothermodurans* (1) *Bacillus* sp. 6 (1) *Oceanobacillus* sp. 2 (3) *P. lactis* (1)	*B. badius* (1) *B. circulans* (2) *B. clausii* (2) *B. licheniformis* (7) *B. pumilus* (3) *B. silvestris* (1) *B. thermoamylovorans* (1) *Bacillus* sp. 5 (3) *P. lactis* (1) *Paenibacillus* sp. 1 (1)	*B. clausii* (1) *B. licheniformis* (4) *B. pumilus* (3) *P. lactis* (1)
		55°C	*B. licheniformis* (13) *U. thermosphaericus* (7)	*B. licheniformis* (11) *Bacillus* sp. 4 (6) *U. thermosphaericus* (12)	*B. licheniformis* (14) *Bacillus* sp. 4 (1) *U. thermosphaericus* (14)	*B. licheniformis* (6) *Bacillus* sp. 4 (1) *U. thermosphaericus* (2)	*B. licheniformis* (7)
	SNA	20°C					
		37°C					
		55°C		*B. licheniformis* (1)			*B. licheniformis* (4)
	SSM	20°C					
		37°C	*B. licheniformis* (3)	*B. licheniformis* (5) *B. subtilis* (1) *B. pumilus* (1) *P. terrae* (1)	*B. licheniformis* (5) *B. pumilus* (5) *P. polymyxa* (1)	*B. licheniformis* (2) *B. pumilus* (1)	*B. cereus* group (1) *B. licheniformis* (2) *B. pumilus* (1) *B. subtilis* (1)
		55°C	*B. licheniformis* (4)	*B. licheniformis* (6) *Brevibacillus* sp. (1)	*B. licheniformis* (1) *Brevibacillus* sp. (1)	*B. licheniformis* (2) *U. thermosphaericus* (1)	*B. licheniformis* (5)

Table 6.6: Overview of all isolates per season (late summer/autumn vs. winter), per isolation temperature (20, 37 or 55°C), per medium (BHI, SNA or SSM) and per dairy farm (organic (farm 1 - 5) vs. conventional dairy farms (farm 6 - 10)). Identifications are based on FAME and 16S rRNA gene sequence analysis. Isolates that are representatives of possibly yet undescribed species are identified onto the genus level. Number of isolates per species is represented between brackets. Used abbreviations: *B.*= *Bacillus*, *Br.*= *Brevibacillus*, *L.*= *Lysinibacillus*, *P.*= *Paenibacillus*, *O.*= *Oceanobacillus*, *U.*= *Ureibacillus*.

			organic				
			farm 1	**farm 2**	**farm 3**	**farm 4**	**farm 5**
winter	**BHI**	**20°C**	*B. amyloliquefaciens* (1) *B. circulans* (2) *B. clausii* (1) *B. licheniformis* (4) *B. pumilus* (7) *Bacillus* sp. 6 (1) *P. lautus* (1)	*B. clausii* (1) *B. licheniformis* (1) *B. subtilis* (1) *Oceanobacillus* sp. 2 (1)	*B. amyloliquefaciens* (1) *B. pumilus* (2)	*B. amyloliquefaciens* (1) *B. clausii* (3) *B. licheniformis* (5) *B. pumilus* (5)	*B. cereus* group (1)
		37°C	*B. circulans* (5) *B. licheniformis* (3) *B. oleronius* (3) *B. pumilus* (1) *L. fusiformis* (2) *P. odorifer* (1) *Paenibacillus* sp. 1 (1)	*B. amyloliquefaciens* (1) *B. cereus* group (1) *B. circulans* (1) *B. clausii* (1) *B. licheniformis* (4) *B. oleronius* (1) *B. pumilus* (2) *Paenibacillus* sp. 3 (1)	*B. amyloliquefaciens* (2) *B. pumilus* (2) *B. subtilis* (2) *B. simplex* (1)	*B. amyloliquefaciens* (1) *B. clausii* (1) *B. licheniformis* (7) *B. oleronius* (1) *B. pumilus* (2) *B. subtilis* (1) *U. thermosphaericus* (1)	*B. licheniformis* (1) *B. pumilus* (1)
		55°C	*B. licheniformis* (7) *U. thermosphaericus* (2)	*B. licheniformis* (6) *B. thermoamylovorans* (2) *Bacillus* sp. 4 (1)	*B. licheniformis* (1)	*B. licheniformis* (8) *Bacillus* sp. 4 (2) *U. thermosphaericus* (3)	*B. licheniformis* (4)
	SNA	**20°C**					
		37°C					
		55°C				*B. licheniformis* (2)	
	SSM	**20°C**	*B. circulans* (1) *B. pumilus* (2) *P. polymyxa* (1)	*B. pumilus* (1)		*B. amyloliquefaciens* (3) *B. pumilus* (4) *B. subtilis* (1)	*B. pumilus* (2)
		37°C	*B. licheniformis* (2) *B. pumilus* (6) *P. polymyxa* (1) *P. terrae* (1)	*B. licheniformis* (5) *B. pumilus* (1)	*B. licheniformis* (2) *B. pumilus* (1) *P. polymyxa* (1)	*B. amyloliquefaciens* (1) *B. licheniformis* (8) *B. subtilis* (1)	*B. licheniformis* (2) *B. pumilus* (1)
		55°C				*B. licheniformis* (3)	

Table 6.7: Overview of all isolates per season (late summer/autumn vs. winter), per isolation temperature (20, 37 or 55°C), per medium (BHI, SNA or SSM) and per dairy farm (organic (farm 1 - 5) vs. conventional dairy farms (farm 6 - 10)). Identifications are based on FAME and 16S rRNA gene sequence analysis. Isolates that are representatives of possibly yet undescribed species are identified onto the genus level. Number of isolates per species is represented between brackets. Used abbreviations: *B.*= *Bacillus*, *Br.*= *Brevibacillus*, *L.*= *Lysinibacillus*, *P.*= *Paenibacillus*, *O.*= *Oceanobacillus*, *U.*= *Ureibacillus*.

			conventional				
			farm 6	**farm 7**	**farm 8**	**farm 9**	**farm 10**
winter	**BHI**	**20°C**	*B. pumilus* (1)	*B. amyloliquefaciens* (4) *B. clausii* (1) *B. licheniformis* (7) *B. pumilus* (2) *B. subtilis* (1) *Bacillus* sp. 6 (1) *L. fusiformis* (1)	*B. circulans* (1) *B. clausii* (1) *B. pumilus* (2) *Bacillus* sp. 5 (1) *Bacillus* sp. 6 (1) *Sporosarcina* sp. (1)	*B. clausii* (1) *B. licheniformis* (2)	*B. circulans* (2) *B. clausii* (1) *B. licheniformis* (1) *B. pumilus* (4) *Br. invocatus* (1)
		37°C	*B. licheniformis* (2) *B. pumilus* (1)	*B. amyloliquefaciens* (6) *B. clausii* (4) *B. licheniformis* (8) *B. pumilus* (3)	*B. licheniformis* (3) *B. pumilus* (1) *P. lactis* (3)	*B. clausii* (1) *B. licheniformis* (7) *B. oleronius* (4) *Bacillus* sp. 6 (1) *P. lactis* (1) *U. thermosphaericus* (8)	*B. circulans* (1) *B. clausii* (1) *B. licheniformis* (2) *Bacillus* sp. 5 (1) *Bacillus* sp. 7 (1) *L. fusiformis* (1) *P. macerans* (1) *P. polymyxa* (2)
		55°C	*B. licheniformis* (4)	*B. licheniformis* (12) *B. thermoamylovorans* (1) *U. thermosphaericus* (1)	*B. licheniformis* (7)	*Bacillus* sp. 4 (1) *U. thermosphaericus* (4)	*B. licheniformis* (4) *B. thermoamylovorans* (1) *Bacillus* sp. 4 (2)
	SNA	**20°C**				*B. cereus* group (1)	
		37°C				*B. licheniformis* (1)	
		55°C					*B. licheniformis* (2)
	SSM	**20°C**		*B. amyloliquefaciens* (2) *B. licheniformis* (1) *B. pumilus* (2) *B. subtilis* (2) *P. polymyxa* (1)	*B. licheniformis* (1) *B. pumilus* (1) *L. fusiformis* (1)	*B. pumilus* (1) *P. polymyxa* (1)	*B. amyloliquefaciens* (1) *B. pumilus* (1) *P. polymyxa* (1) *Paenibacillus* sp. 4 (1)
		37°C	*B. licheniformis* (2) *B. pumilus* (2)	*B. licheniformis* (7) *B. pumilus* (1) *B. subtilis* (6) *P. polymyxa* (2)	*B. amyloliquefaciens* (1) *B. licheniformis* (3) *B. pumilus* (1) *B. subtilis* (1)	*B. licheniformis* (2) *L. sphaericus* (1) *P. polymyxa* (1)	*B. licheniformis* (5) *B. pumilus* (1)
		55°C					

Table 6.8: Overview of all isolates per season (late summer/autumn vs. winter), per isolation temperature (20, 37 or 55°C), per medium (BHI, SNA or SSM) and per dairy farm (organic (farm 1 - 5) vs. conventional dairy farms (farm 6 - 10)). Identifications are based on FAME and 16S rRNA gene sequence analysis. Isolates that are representatives of possibly yet undescribed species are identified onto the genus level. Number of isolates per species is represented between brackets. Used abbreviations: *B.*= *Bacillus*, *Br.*= *Brevibacillus*, *L.*= *Lysinibacillus*, *P.*= *Paenibacillus*, *O.*= *Oceanobacillus*, *U.*= *Ureibacillus*.

6.2 Description of a novel *Bacillus* species recovered from raw cow's milk in Belgium, *Bacillus thermolactis*, and emended description of the closely related *Bacillus thermoamylovorans*

A polyphasic taxonomic study was performed on 22 thermotolerant, aerobic, endospore-forming bacteria from dairy environments. Seventeen isolates were retrieved from raw milk, one from a filter cloth and four were from grass, straw and milking equipment. These four isolates (R-6546, R-7499, R-7764 and R-7440) were identified as *Bacillus thermoamylovorans* based on DNA/DNA hybridisations (values above 70% with LMG 18084^T, the type strain of *Bacillus thermoamylovorans*) but showed discrepancies in characteristics with the original species description, so an emended description of this species is given. According to 16S rRNA gene sequence analysis and DNA/DNA hybridisation experiments, the remaining 18 isolates (R-6488^T, R-28193, R-6491, R-6492, R-7336, R-33367, R-6486, R-6770, R-31288, R-28160, R-26358, R-7632, R-26955, R-26950, R-33520, R-6484, R-26954 and R-7165) represented one single species, most closely related to *Bacillus thermoamylovorans* (93.9% 16S rRNA gene sequence similarity), for which the name *Bacillus thermolactis* is proposed. Cells were Gram-positive, facultatively anaerobic, endospore-forming rods that grew optimally at 40 – 50°C. The cell wall peptidoglycan type of strain R-6488^T, the proposed type strain, was A1γ based on *meso*-diaminopimelic acid. Major fatty acids of the strains were $C_{16:0}$ (28.0%), iso-$C_{16:0}$ (12.1%) and iso-$C_{15:0}$ (12.0%). MK-7 was the predominant menaquinone, and major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and some unidentified phospholipids. DNA G + C content was 35.0 mol%. Phenotypic properties allowed discrimination from other thermotolerant *Bacillus* species and supported the description of the novel *Bacillus thermolactis* species with strain R-6488^T (= LMG 25569^T = DSM 23332^T) as the proposed type strain.

6.2.1 Introduction

Two previous studies (Scheldeman *et al.*, 2005; Coorevits *et al.*, 2008) focusing on the diversity of (highly) heat resistant endospore-forming bacteria in milk from Belgian dairy farms, collected 22 isolates that could not unequivocally be identified onto the species level. Coorevits and coworkers (2008) assigned their isolates to Bacillus sp. 4 (cluster P in Figure 6.1). On the basis of partial 16S rRNA gene sequencing, these isolates appeared to represent *Bacillus thermoamylovorans*, and a novel species of *Bacillus*. *Bacillus thermoamylovorans* was firstly isolated in 1995 from a palm wine sample in Senegal (Combet-Blanc *et al.,* 1995). It's description — based on a single isolate — was a non spore-forming, moderately thermophilic, facultatively anaerobic, Gram-positive rod. Following further characterization of the isolates using a polyphasic taxonomic approach as recommended by Logan *et al.* (2009), an emended description of *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995) is given as discrepancies with the original species description were observed, and the species *Bacillus thermolactis* sp. nov., is proposed.

6.2.2 Experimental work

All twenty-two isolates originated from dairy farm environments, more specifically from raw milk samples, straw, grass, milking equipment and a filter cloth, and were retrieved during a summer and a winter isolation campaign at Belgian dairy farms. Samples obtained during the first study

(Scheldeman *et al.*, 2005) were heat-treated (30 minutes at 100°C) to select for highly heat resistant spore-formers specifically, while samples obtained during the later study (Coorevits *et al.*, 2008) were heat treated (10 minutes at 80°C) to select for all spore-formers. Samples were incubated at 55°C for 24 hours to select for thermotolerant spore-formers. All isolates were further subcultured on BHI, supplemented with vitamin B_{12} (1 mg mL^{-1}) at 55°C. The isolates, their source and isolation conditions are listed in Table 6.9.

		isolation parameters			
strain n°	**other designations**	**source**	**period**	**heat treatment**	**study**
***Bacillus thermolactis* sp. nov.**					
R-6484		filter cloth	winter	30 min @ 100°C	A
R-6486		raw milk	winter	30 min @ 100°C	A
R-6488^T	LMG 25569^T; DSM 23332^T	raw milk	winter	30 min @ 100°C	A
R-7165		raw milk	winter	30 min @ 100°C	A
R-6491		raw milk	winter	30 min @ 100°C	A
R-6492		raw milk	winter	30 min @ 100°C	A
R-6770		raw milk	winter	30 min @ 100°C	A
R-7336		raw milk	winter	30 min @ 100°C	A
R-7632		raw milk	winter	30 min @ 100°C	A
R-33520		raw milk	winter	10 min @ 80°C	B
R-28193		raw milk	summer	10 min @ 80°C	B
R-31288		raw milk	winter	10 min @ 80°C	B
R-26955		raw milk	summer	10 min @ 80°C	B
R-33378		raw milk	winter	10 min @ 80°C	B
R-26358		raw milk	summer	10 min @ 80°C	B
R-33367		raw milk	winter	10 min @ 80°C	B
R-26954		raw milk	summer	10 min @ 80°C	B
R-26950		raw milk	summer	10 min @ 80°C	B
R-28160		raw milk	summer	10 min @ 80°C	B
Bacillus thermoamylovorans					
R-7499		straw	winter	30 min @ 100°C	A
R-6546		grass	winter	30 min @ 100°C	A
R-7764		milking equipment	winter	30 min @ 100°C	A
R-7440		milking equipment	winter	30 min @ 100°C	A

Table 6.9: Overview of all isolates and their corresponding isolation conditions; for all isolates, the isolation location is Flanders, the isolation medium is BHI + vitamin B_{12}. A: study of Scheldeman *et al.*,(2005), B: study of Coorevits *et al.*, (2008)

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Total genomic DNA for 16S rRNA gene sequencing and DNA fingerprinting was extracted (Coorevits *et al.*, 2008) and the nearly complete 16S rRNA gene sequences of all isolates were generated as described by Heyrman and Swings (2001). Sequencing products were purified with the 'BigDye® XTerminator Purification Kit' (Applied Biosystems) according to manufacturer's instructions using sequential pipetting and a MixMate (Eppendorf) shaking device. Sequences were assembled using the BioNumerics 5.1 software (Applied Maths, Belgium) and the fifty most closely related organisms were appraised using the online FASTA tool of EMBL (http://www.ebi.ac.uk/fasta33/). FASTA results indicated these isolates were members of the genus *Bacillus*. A phylogenetic tree harboring all validly described species within *Bacillus* at the time of writing confirmed the position of strain R-6488^T within the genus with *Bacillus ther-*

moamylovorans as its closest relative at 93,9% 16S rRNA gene sequence similarity (Figure 6.5). A detailed view of part of this tree, with all isolates and their closest relatives included is represented in Figure 6.6. Strain R-33378 was not included in the tree because length of its partial 16S rRNA gene sequence was not sufficient for reliable tree reconstruction. Both phylogenetic trees were based on almost complete 16S rRNA gene sequences and were constructed by aligning all sequences using ClustalX (Thompson *et al.*, 1997), and trimming the overhangs. The jModelTest 0.1.1 program (Posada, 2008) was then applied to the data sets to determine the best fit evolutionary model. Maximum likelihood analyses were performed using PhyML (Guindon & Gascuel, 2003) by applying the parameters determined by jModeltest. aLRT (approximate Likelihood Ratio Test) values were calculated to assess the reliability of the clusters (Anisimova & Gascuel, 2006). Additional maximum parsimony and neighbor joining analyses have been performed using MEGA4 (Tamura *et al.,* 2007) for the tree represented in Figure 6.6. Resulting phylogenetic trees are shown in Figures 6.9 & 6.10, respectively, (and support the maximum likelihood analysis.

One group of milk isolates (strains R-33520, R-6488^T, R-6770, R-6491, R-7336, R-31288, R-7632, R-7165, R-28193, R-6492, R-6484, R-33367, R-26954, R-26950, R-26955, R-6486, R-26358 and R-28160) showed less than 0.7% variability in 16S rRNA gene sequences with each other and with two *Bacillus* sp. strains TAT105 and TAT112, representing two patented microorganisms. The closest relative to this group of strains was the type strain of *Bacillus thermoamylovorans* LMG 18084^T, showing 93.9% 16S rRNA gene sequence similarity with strain R-6488^T. These values indicated the above mentioned isolates probably represented a novel species within the genus *Bacillus*. 16S rRNA gene sequences of strains R-7499, R-7440, R-6546 and R-7764 showed at least 99.2% similarity with each other and with the type strain of *Bacillus thermoamylovorans* LMG 18084^T, indicating these isolates probably belonged to this species. Surprisingly, two strains, WSBC20060 and WSBC20059 assigned to *Bacillus circulans*, also clustered in the vicinity of the *Bacillus thermoamylovorans* group. However, no further information could be found about these strains, and it was assumed these strains were probably wrongly identified, and most likely should be allocated to *Bacillus thermoamylovorans*.

As prescribed by the minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.,* 2009), a fingerprint pattern of all isolates was generated using a repetitive sequence based PCR with a $(GTG)_5$-primer (Heyrman *et al.*, 2005c). This allowed to group the isolates into manageable clusters of similar strains (Heyrman *et al.*, 2004). Five unique fingerprint patterns could be distinguished as shown in Figure 6.7, and were assigned groups A - E (supported by a 90% cut-off value).

For each group, representative strains were chosen for DNA-DNA hybridization experiments, namely strains R-33367, R-7499, R-7440, R-6488^T and R-6484 (indicated in bold in Figure 6.7). Indeed, it is generally recommended and accepted that strains with a DNA-DNA relatedness value below 70%, or with 16S rRNA gene sequence dissimilarity above 3% are considered as belonging to a separate species. Yet, bacterial strains with a difference in 16S rRNA gene sequence of less than 3% cannot be allocated to the same species without support from DNA-DNA hybridization experiments (Stackebrandt & Ebers, 2006). For this purpose, approximately 1 g biomass from the representative strains as well as from the type strain LMG 18084^T of *Bacillus thermoamylovorans,* was harvested from TSA plates, and DNA was purified as described by Logan and co-workers (2000). DNA-DNA hybridization was performed using a modification of the microplate method of Ezaki and co-workers (1989) (Willems *et al.*, 2001). A hybridization temperature of 32°C (calculated with correction for the presence of 50% formamide) was used. DNA-DNA relatedness

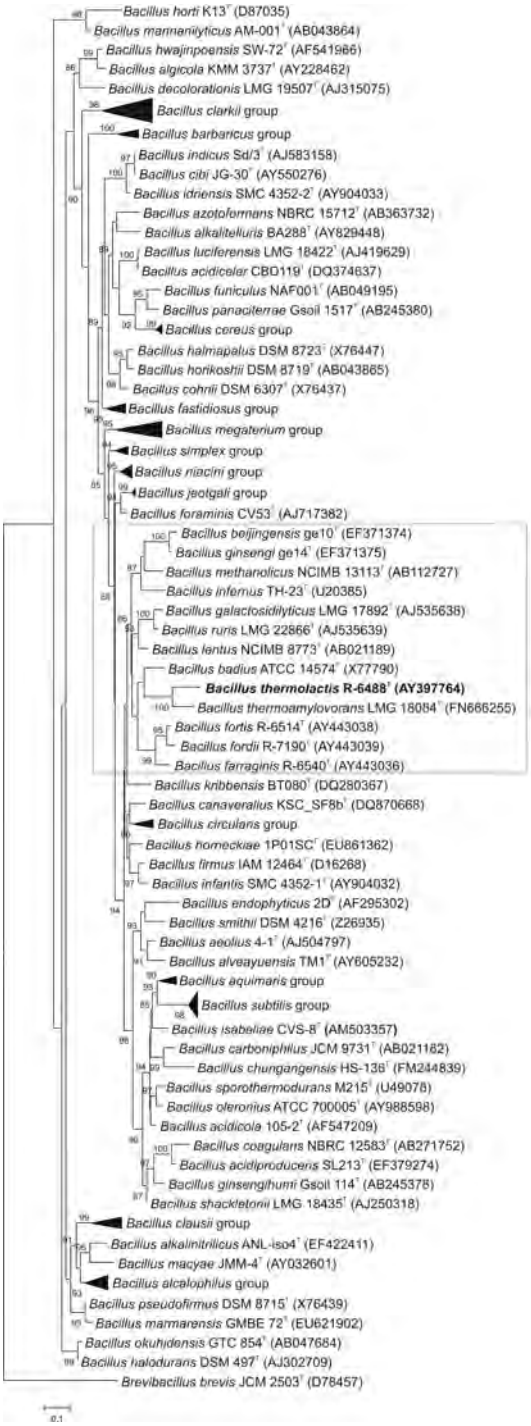


Figure 6.6

Figure 6.5: ML phylogenetic tree of all validly described species within the genus *Bacillus* at the time of writing (# 159), based on almost complete 16S rRNA gene sequences (1461 characters). aLRT values (at least 85%) are shown at the branch points. Species are represented by species name, strain number and accession number of the 16S rRNA gene sequence between brackets. The 16S rRNA gene sequence of *Brevibacillus brevis* JCM 2503^{T} (D78457) was used as an outgroup to root the tree. The proposed novel species *Bacillus thermolactis* is indicated in bold. Bar: 0.1 substitutions per nucleotide position.

Figure 6.5 (continued): ***Bacillus clarkii* group**: *B. taeanensis* BH030017^T (AY603978), *B. neizhouensis* JSM 071004^T (EU925618), *B. agaradhaerens* DSM 8721^T (X76645), *B. saliphilus* 6AG^T (AJ493660), *B. chagannorensis* CG-15^T (AM492159), *B. vedderi* JaH^T (Z48306), *B. aurantiacus* K1-5^T (AJ605773), *B. cellulosilyticus* N4^T (AB043852), *B. clarkii* DSM 8720^T (X76444), *B. polygoni* YN-1^T (AB292819), *B. persepolensis* HS-136^T (FM244839), *B. salarius* BH169^T (AY667494), *B. aidingensis* 17-5^T (DQ504377), *B. halochares* MSS4^T (AM982516) and *B. qingdaonensis* CM1^T (DQ115802); ***Bacillus barbaricus* group**: *B. gelatini* LMG 21880T (AJ551329), *B. macauensis* ZFHKF-1T (AY373018), *B. solisalsi* YC1T (EU046268), *B. barbaricus* V2-BIII-A2T (AJ422145)and *B. arsenicus* Con a/3T (AJ606700); ***Bacillus cereus* group**: *B. cereus* NCDO 1771^T (X55060), *B. thuringiensis* IAM 12077^T (D16281), *B. pseudomycoides* NRRL B-617^T (AF013121), *B. mycoides* ATCC 6462^T (AB021192), *B. weihenstephanensis* DSM 11821^T (AB021199) and *B. anthracis* ATCC 14578^T (AB190217); ***Bacillus fastidiosus* group**: *B. fastidiosus* DSM 91^T (X60615), *B. humi* LMG 22167^T (AJ627210), *B. litoralis* SW-211^T (AY608605), *B. niabensis* 4T19^T (AY998119), *B. galliciensis* BFLP-1^T (FM162181), *B. herbersteinensis* D-1,5a^T (AJ781029)*;* ***Bacillus megaterium* group**: *B. psychrotolerans* DSM 11706^T (AJ277983), *B. psychrodurans* DSM 11713^T (AJ277984), *B. insolitus* DSM 5^T (AM980508), *B. isronensis* B3W22^T (EF114311), *B. cecembensis* PN 5^T (AM773821), *B. decisifrondis* E5HC-32^T (DQ465405), *B. koreensis* BR030^T (AY667496), *B. megaterium* IAM 13418^T (D16273), *B. aryabhattai* B8W22^T (EF114313), and *B. flexus* IFO 15715^T (AB021185); ***Bacillus simplex* group**: *B. simplex* DSM 1321^T (AJ439078), *B. muralis* LMG 20238^T (AJ316309), *B. butanolivorans* K9^T (EF206294), *B. psychrosaccharolyticus* ATCC 23296^T (AB021195) and *B. asahii* FERM BP-4493^T (AB109209); ***Bacillus niacini* group**: *B. fumarioli* LMG 17489^T (AJ250056), *B. pocheonensis* Gsoil 420^T (AB245377), *B. niacini* IFO 15566^T (AB021194), *B. novalis* LMG 21837^T (AJ542512), *B. vireti* LMG 21834^T (AJ542509), *B. soli* LMG 21838^T (AJ542513), *B. bataviensis* LMG 21833^T (AJ542508) and *B. drentensis* LMG 21831^T (AJ542506); ***Bacillus jeotgali* group**: *B. boroniphilus* T-15Z^T (AB198719), *B. jeotgali* YKJ-10^T (AF221061), *B. selenatarsenatis* SF-1^T (AB262082), *B. thioparans* BMP-1^T (DQ371431) and *B. subterraneus* COOI3B^T (AY672638). ***Bacillus circulans* group**: *B. benzoevorans* NCIMB 12555^T (X60611), *B. siralis* 171544^T (AF071856), *B. circulans* ATCC 4513^T (AY043084), *B. nealsonii* DSM 15077^T (EU656111) and *B. korlensis* ZLC-26^T (EU603328); ***Bacillus aquimaris* group**: *B. seohaeanensis* BH724^T (AY667495), *B. marisflavi* TF-11^T (AF483624), *B. coahuilensis* m4-4^T (EF014452), *B. vietnamensis* 15-1^T (AB099708) and *B. aquimaris* TF-12^T (AF483625); **Bacillus subtilis group**: *B. tequilensis* 10b^T (AY197613), *B. safensis* FO-36b^T (AF234854), *B. pumilus* ATCC 7061^T (AY876289), *B. altitudinis* 41KF2b^T (AJ831842), *B. stratosphericus* 41KF2a^T (AJ831841), *B. aerophilus* 28K^T (AJ831844), *B. subtilis* subsp. *subtilis* DSM 10^T (AJ276351), *B. subtilis* subsp. *inaquosorum* NRRL B-23052^T (EU138467), *B. subtilis* subsp. *spizizenii* NRRL B-23049^T (AF074970), *B. amyloliquefaciens* ATCC 23350^T (X60605), *B. sonorensis* NRRL B-23154^T (AF302118), *B. licheniformis* DSM 13^T (X68416), *B. atrophaeus* JCM 9070^T (AB021181), *B. mojavensis* IFO 15718^T (AB021191), *B. vallismortis* DSM 11031^T (AB021198) and *B. aerius* 24K^T (AJ831843); ***Bacillus clausii* group**: *B. oshimensis* K11^T (AB188090), *B. lehensis* MLB2^T (AY793550), *B. patagoniensis* PAT 05^T (AY258614), *B. clausii* DSM 8716^T (X76440), *B. plakortidis* P203^T (AJ880003), *B. murimartini* LMG 21005^T (AJ316316) and *B. gibsonii* DSM 8722^T (X76446); and ***Bacillus alcalophilus* group**: *B. wakoensis* N1^T (AB043851), *B. okhensis* ATCC BAA-1137^T (DQ026060), *B. krulwichiae* AM31D^T (AB086897), *B. akibai* 1139^T (AB043858), *B. pseudalcaliphilus* DSM 8725^T (X76449), *B. trypoxylicola* SU1^T (AB434284), *B. alcalophilus* DSM 485^T (X76436), *B. bogoriensis* LBB3^T (AY376312) and *B. hemicellulosilyticus* C-11^T (AB043846).

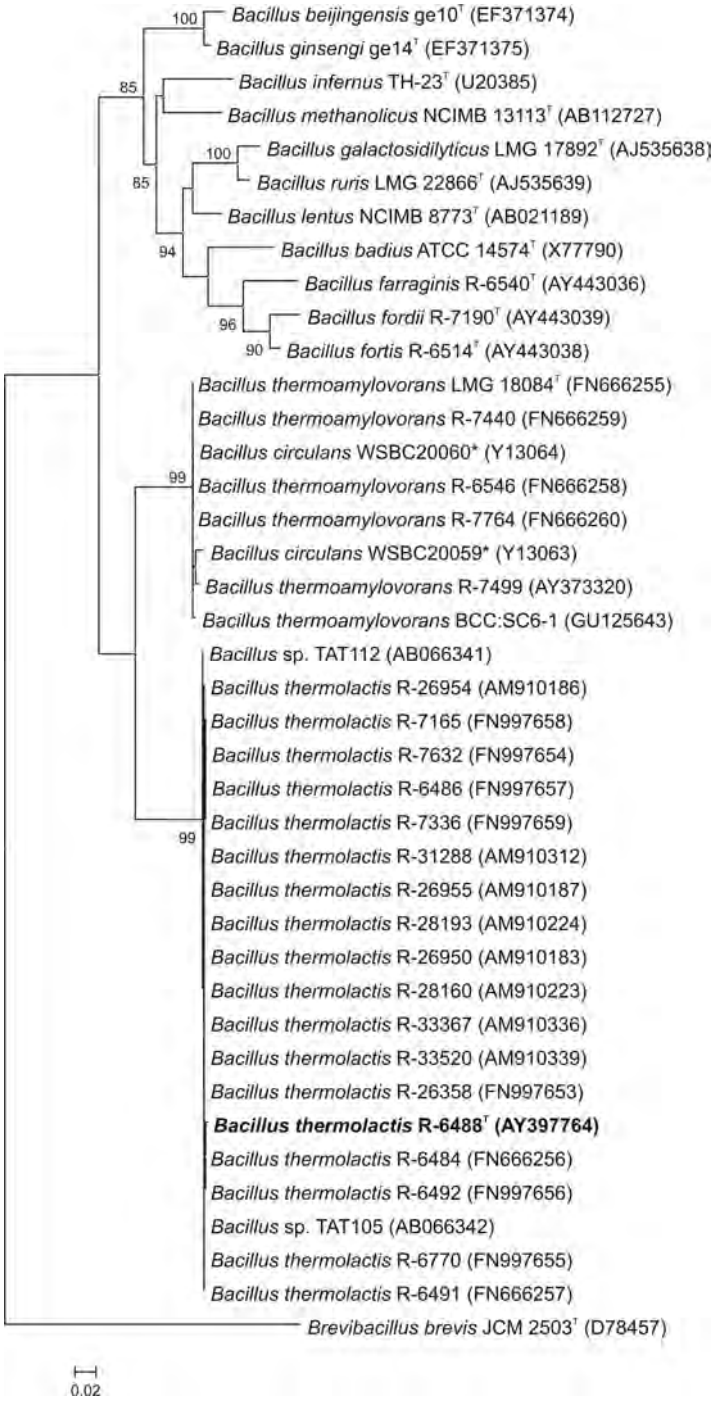


Figure 6.6: ML phylogenetic tree of the milk isolates with their closest relatives based on almost complete 16S rRNA gene sequences (1486 characters). aLRT values (at least 85%) are shown at the branch points. Species are represented by species name, strain number and accession number of the 16S rRNA gene sequence between brackets. The 16S rRNA gene sequence of *Brevibacillus brevis* JCM 2503^T (D78457) was used as an outgroup to root the tree. The *Bacillus thermolactis* type strain R-6488^T is indicated in bold. *: these strains were misnamed and should be allocated to *Bacillus thermoamylovorans*. Bar, 0.02 substitutions per nucleotide position.

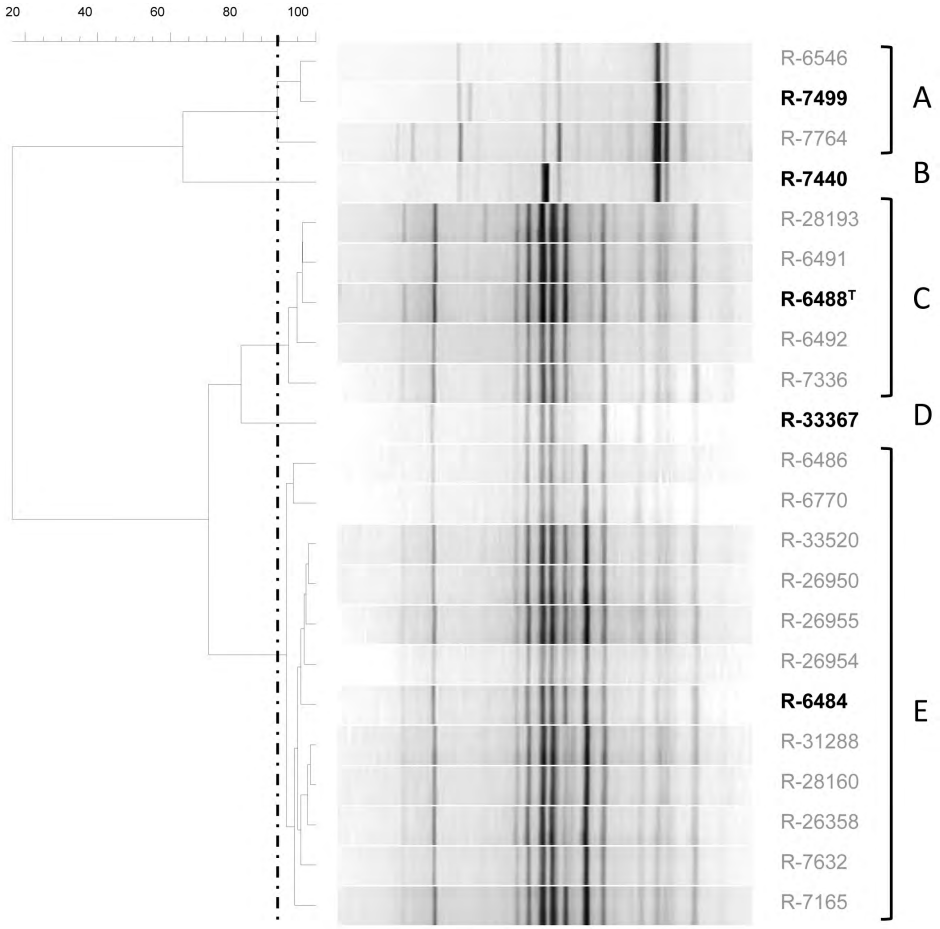


Figure 6.7: Grouping of normalized $(GTG)_5$ patterns of 22 *Bacillus* isolates from dairy environments in a dendrogram based on UPGMA clustering of Pearson similarity coefficients.

confirmed 16S rRNA gene sequence analyses, with strains R-6488^T, R-6484 and R-33367 representing a novel species, and strains R-7499 and R-7440 belonging to *Bacillus thermoamylovorans*. An overview of DNA-DNA relatedness values between the strains is given in Table 6.10. G + C contents of strains R-6488^T and LMG 18084^T were 35.0 mol% and 37.0 mol%, respectively, as determined by HPLC (Mesbah *et al.,* 1989; Logan *et al.*, 2000).

	$(GTG)_5$-group	R-7499	R-7440	R-6488^T	R-33367	R-6484	LMG 18084^T
R-7499	A	**100**					
R-7440	B	97.2 ± 12.8	**100**				
R-6488^T	C	18.2 ± 0.4	20.2 ± 0.8	**100**			
R-33367	D	21.2 ± 1.3	24.3 ± 1.6	100.0 ± 7.7	**100**		
R-6484	E	20.2 ± 0.7	26.0 ± 4.3	99.5 ± 0.5	98.1 ± 0.3	**100**	
LMG 18084^T		98.1 ± 6.5	92.4 ± 7.5	27.7 ± 7.7	20.9 ± 2.9	28.1 ± 1.9	**100**

Table 6.10: DNA-DNA relatedness values among representative strains of the dairy isolates and *Bacillus thermoamylovorans* LMG 18084^T.

For phenotypic analysis, type strains of the following thermotolerant species were also included: *Bacillus thermoamylovorans* LMG 18084^T, *Bacillus fumarioli* LMG 17489^T, *Bacillus circulans* LMG 13261^T, *Bacillus coagulans* LMG 6326^T and *Bacillus smithii* LMG 12526^T. *Bacillus fumarioli* LMG 17489^T was grown on *Bacillus fumarioli* agar (BFA) at pH 5.5 and 50°C (Logan *et al.*, 2000); all other strains were grown on TSA for 24 hours at 50°C. Cellular morphology and motility were investigated by phase contrast microscopy at 1000 × magnification, and cells were Gram stained. Sporangial morphologies were studied in cultures grown for several days at 50°C on TSA containing 5 mg mL^{-1} $MnSO_4$. Temperature, pH and salt tolerance ranges for growth were determined using the methods given by Logan and De Vos (2009). *Bacillus fumarioli* LMG 17489^T was characterized using API20E and API50CHB kits at pH 6 as described by Logan and co-workers (2000); biochemical characteristics for other strains were tested using API 20E and API50CHB kits, according to manufacturer's instructions but with incubation at 50°C; kits were incubated within loosely closed plastic bags in order to maintain humidity. Characteristics differentiating between the species are shown in Table 6.11.

Whole cell hydrolysates (4N HCl, 100°C, 16 hours) of strains LMG 18084^T and R-6488^T were subjected to thin layer chromatography on cellulose plates using the solvent system of Rhuland and co-workers (1955). *Meso*-diaminopimelic acid (*meso*-DAP) was found as the diagnostic diamino acid, and this has only been reported for peptidoglycan type A1γ and for three variations of peptidoglycan type A4γ; however these variations of peptidoglycan type A4γ have been found so far exclusively in members of the genera *Brachybacterium, Dermabacter* and *Devriesia*. It was clear from 16S rRNA gene sequence data that a close relationship with these three genera could be excluded, thus, it was concluded that LMG 18084^T and R-6488^T showed the peptidoglycan type A1γ. The presence of *meso*-DAP is a characteristic typical for members of the genus *Bacillus* (Schleifer & Kandler, 1972). Menaquinones for both type strains LMG 18084^T and R-6488^T were analysed as described by Groth and co-workers (1996). The major menaquinone was MK-7 for both strains, and strain R-6488^T also showed trace amounts of MK-8 (1%). The type species of the genus *Bacillus*, i.e. *Bacillus subtilis* subsp. *subtilis*, also contains a quinone system with MK-7 predominant (Collins & Jones, 1981), again supporting the attribution of R-6488^T and related isolates to the genus *Bacillus*. Polar lipids were extracted from 100 mg of freeze dried cell material using a chloroform/methanol/0.3% aqueous NaCl mixture (1/2/0.8; v/v/v) (modified after Bligh & Dyer, 1959). The extraction solvent was stirred overnight and the cell debris pelleted

by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1/1/0.9 (v/v/v). Polar lipids were separated by two dimensional silica gel thin layer chromatography (Machery Nagel art. n° 818135). The first direction was developed in chloroform/methanol/water (65/25/4; v/v/v), and the second in chloroform/methanol/acetic acid/water (80/12/15/4; v/v/v/v). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (alpha-glycols), Dragendorff (quaternary nitrogen), and alpha-naphthol-sulphuric acid (glycolipids) (Tindall *et al.*, 2007).

Polar lipid patterns of strains R-6488^T and LMG 18084^T were quite similar, complex patterns, showing diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and four to five unidentified phospholipids (PL's) as the major components (Figure 6.8). Phosphatidylethanolamine (PE) was detected in strain LMG 18084^T but not in strain R-6488^T. These profiles are somewhat similar with the polar lipid profile of *Bacillus subtilis* subsp. *subtilis* strain DSM10^T (Kämpfer *et al.*, 2006), with DPG and PG as the major polar lipids; furthermore PE has been detected (also observed in strain LMG 18084^T) and three unidentified glycolipids (also observed in strain R-6488^T).

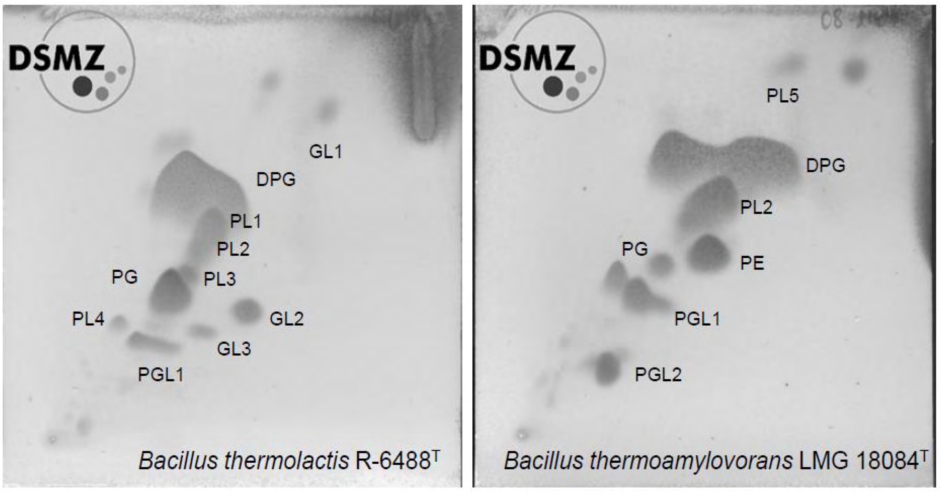


Figure 6.8: Polar lipid patterns for strains R-6488^T (left) and LMG 18084^T (right). Plates have been stained with 5% molybdatophosphoric acid to show all lipids. DPG: diphosphatidylglycerol; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; PL1-PL5: phospholipids; GL1-GL3: glycolipids; PGL1-PGL2: phosphoglycolipids.

For FAME analysis, strains were pre-cultured, then incubated for exactly 48 hours at 52°C on TSBA. A loopfull of well grown cells was harvested and fatty acid methyl esters were prepared and extracted according to the standardized protocol of the Microbial Identification System (MIS; Microbial ID; Inc.). All strains exhibited typical fatty acid profiles for the genus *Bacillus*, with a lot of branched chain components (Kaneda, 1977). Kämpfer (1994) specified fatty acid profiles of members of the genus *Bacillus*, containing large amounts of anteiso-$C_{15:0}$ (26 - 60%) and iso-$C_{15:0}$ (13 - 30%), and low amounts of unsaturated fatty acids (<3%). Fatty acid profiles of the dairy strains comply with this profile, and both species can be easily differentiated from one another based on different amounts of these major fatty acids, anteiso-$C_{15:0}$ and iso-$C_{15:0}$. *Bacillus thermoamylovorans* strains had major amounts of iso-$C_{15:0}$ (mean value 23.2%), anteiso-$C_{15:0}$ (mean value 23.4%) and $C_{16:0}$ (mean value 17.7%); iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, iso-$C_{14:0}$

	1	2	3	4	5	6	7
motility	-	v	+	w	+	+	+
spore position	ST	ST	T / ST	P / ST	T / ST	ST (P / T)	ST
sporangia swollen	-	v	+	-	+	slight	-
oxidase	+	+	-	-	-	v	w
starch hydrolysis	+ (w)	+	-	-	w	+	w
casein hydrolysis	+	-	+	-	w	-	-
ONPG production	-	+	NR	-	+	+	-
VP test	-	v (w)	NR	+	-	w	v
gelatin hydrolysis	+	+	-	+	v	+	-
nitrate reduction	+	+	-	-	v	v	-
acid from							
glycerol	-	w	+	v	+	+	+
L-arabinose	+	+	-	-	+	v	v
ribose	+	+	-	v	v	v	+
D-xylose	+	v	-	-	+	v	+
galactose	-	+	-	v	+	+	-
D-mannose	-	+	+	+	+	+	v
meso-inositol	-	-	-	-	+	v	-
mannitol	w	-	-	+	+	v	+
methyl-D-glucoside	-	+	-	v	+	v	v
N-acetylglucosamine	-	+	-	w	+	+	-
amygdalin	-	+	-	-	+	v	-
arbutin	-	+	-	-	+	v	-
salicin	-	+	-	-	+	v	-
D-cellobiose	-	+	-	-	+	v	-
lactose	-	+	-	v	+	v	-
D-melibiose	-	+	-	v	+	+	-
D-melezitose	-	v	-	v	+	-	-
D-raffinose	-	v	-	v	+	v	-
starch	w	+	-	-	+	+	-
glycogen	-	+	-	-	+	-	-
gentibiose	-	+	-	-	+	v	-
D-turanose	-	-	-	v	+	+	v
2-keto-D-gluconate	-	-	+	-	v	-	-
growth at							
30°C	-	-	-	+	+	+	+
60°C	+	-	+	-	-	+	+
growth in							
1% NaCl	-	+	+	+	+	+	+
2.5% NaCl	-	+	+	+	+	+	-
anaerobic growth	+	+	-	-	+	+	+

Table 6.11: Characters for distinguishing between *Bacillus thermolactis* sp. nov., *Bacillus thermoamylovorans* and other thermotolerant *Bacillus* species.

Taxa: 1, *Bacillus thermolactis* R-6488^T sp. nov.; 2, *Bacillus thermoamylovorans* LMG 18084^T; 3, *Bacillus alveayuensis* TM1^T; 4, *Bacillus fumarioli* LMG 17489^T; 5, *Bacillus circulans* LMG 13261^T; 6, *Bacillus coagulans* LMG 6326^T; 7, *Bacillus smithii* LMG 12526^T. Key: ST, subterminal; T, terminal; P, paracentral; +, > 85% positive; v, variable (26 – 74% positive); -, 0 – 15% positive; w, weak positive reaction; v (w), variable, and weak when positive; NR, not reported. Data for *B. alveayuensis* are from Bae *et al.* (2005) who obtained biochemical characters using similar methods to those used in the present work; all other data were obtained in the authors' laboratories using the same methods as those described here.

and $C_{14:0}$ were present in moderate amounts (mean values of 7.9%, 7.1%, 6.7%, 5.0% and 4.9%, respectively), and trace amounts could be observed of $C_{16:1}$ ω11c and $C_{18:0}$. *Bacillus thermolactis* strains had major amounts of $C_{16:0}$ (mean value 28.0%), iso-$C_{16:0}$ (mean value 12.1%) and iso-$C_{15:0}$ (mean value 12.0%); $C_{17:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$,iso-$C_{17:0}$, $C_{16:1}$ ω11c, $C_{14:0}$ and $C_{18:0}$ were present in moderate amounts (mean values of 10.0%, 7.6%, 5.7%, 5.4%, 4.9%, 4.9% and 4.6%, respectively), and trace amounts of iso-$C_{14:0}$ and $C_{16:1}$ ω7c were also detected.

Based on the cell wall composition, menaquinone analyses and major polar lipids, the dairy farm isolates could be allocated to the genus *Bacillus*. Furthermore, these data together with other phenotypic and genotypic data described above did indicate that strains R-33520, R-6488^T, R-6770, R-6491, R-7336, R-31288, R-7632, R-7165, R-28193, R-6492, R-6484, R-33367, R-26954, R-26950, R-26955, R-6486, R-26358 and R-28160 represented a novel species within the genus *Bacillus*, for which the name *Bacillus thermolactis* was proposed.

The four isolates R-7499, R-6546, R-7764 and R-7440 identified as *Bacillus thermoamylovorans* showed some discrepancies with the original species description (Combet-Blanc *et al*., 1995), therefore, an emended description of this species is given. In contrast to the original data, nitrate reduction was positive, and in the 50 CHB gallery positive results were obtained for acid production from D-melibiose and methyl-D-glucoside, a weak positive result for glycerol, and negative results for gluconate and rhamnose. Furthermore, endospore formation was observed, and this had not been reported in the original description of Combet-Blanc and co-workers (1995). In addition, fatty acid content has been determined, peptidoglycan, quinone and polar lipid analysis has also been performed.

6.2.3 Description of *Bacillus thermolactis* sp. nov.

Bacillus thermolactis [Gr. adj. thermos, hot; L. gen. n. lactis, from milk; N.L. gen. n. thermolactis, a thermotolerant bacterium isolated from milk].

Facultatively anaerobic, Gram-positive, rod-shaped cells (0.7 - 0.9 $\times$ 4 - 10 μm) that occur either singly or in short chains of two to four cells, and in filaments. Mainly non-motile but some strains are motile by means of peritrichous flagella. After 24 hours incubation at 50°C on TSA, colonies are circular in shape, cream-coloured, with irregular edges, slightly rough and matt surfaces with glossy centres, and diameters of approximately 1 - 4 mm. Endospores are formed within 24 hours of incubation at 50°C on TSA containing 5 mg L^{-1} $MnSO_4$; they are ellipsoidal, lie subterminally and do not swell the cells. Growth occurs at pH 7 but not at pH 6 or 8. Growth occurs between 40° and 60°C, optimally at 50°C, but does not occur at 30°C or 70°C. 1% NaCl (w/v) is not tolerated for growth. Casein is hydrolysed, starch is hydrolysed weakly, and aesculin hydrolysis is variable. Catalase and oxidase production are positive. In the API 20E strip, reactions for gelatin hydrolysis and nitrate reduction are positive. Arginine dihydrolase, citrate utilisation, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, ortho-nitrophenyl-ß-D-galactopyranosidase (ONPG), tryptophan deaminase, urease and the Voges-Proskauer (acetoin production) reactions are negative. Acid but no gas is produced from the following carbohydrates in the API 50 CHB Gallery: D-trehalose (weak), D-fructose, D-glucose, D-xylose, L-arabinose, mannitol (weak), ribose, starch (weak) and sucrose (weak). Acid production is variable between strains for maltose. No acid is produced from: 2-keto-D-gluconate, 5-keto-D-gluconate, adonitol, amygdalin, arbutin, D-raffinose, D-arabitol, D-cellobiose, D-fucose, D-melezitose, D-melibiose, D-turanose, D-arabinose, D-lyxose, D-mannose, D-tagatose, dulcitol, erythritol, galactose, gentibiose, gluconate, glycerol, glycogen, inulin, L-arabitol, L-fucose, lactose, L-sorbose, L-xylose,

meso-inositol, methyl-D-glucoside, methyl-D-mannoside, methyl-xyloside, N-acetylglucosamine, rhamnose, salicin, sorbitol and xylitol. In the variable reactions listed above, the type strain R-6488^T (LMG 25569^T, DSM 23332^T) is very weakly positive for acid production from maltose, and negative for aesculin hydrolysis. Major fatty acids are $C_{16:0}$ (28.0%), iso-$C_{16:0}$ (12.1%) and iso-$C_{15:0}$ (12.0%). Major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. MK-7 is the predominant menaquinone and the peptidoglycan type is A1γ. The mol% G+C of the DNA is 35.0 $\pm$ 0.2 mol%. Isolated from milk and dairy farm environments.

6.2.4 Emended description of *Bacillus thermoamylovorans*

Bacillus thermoamylovorans [Gr. adj. thermos, hot; Gr. n. amulon, starch, L. part. adj. vorans, devouring: N.L. part. adj. thermoamylovorans, utilizing starch at high temperature].

Facultatively anaerobic, Gram-positive, rod-shaped cells (0.45 - 0.5 $\times$ 3 - 4 μm) that occur either singly or in short chains of two to four cells. Usually non-motile but some strains are motile. After 24 hours incubation at 50°C on TSA, colonies are circular in shape, cream in colour, with smooth or irregular edges, and have diameters of 0.5 - 4 mm; colony surfaces range from smooth or glossy to slightly rough or mat. Endospores are formed within 24 hours of incubation at 50°C on TSA containing 5 mg L^{-1} $MnSO_4$; they are ellipsoidal, lie subterminally, and occasionally swell the cells. Growth does not occur at pH 5 but can occur between pH 6 and 9, with the optimum for growth being between pH 7 and 9. Growth occurs at 50°C, but does not occur at 40°C or 60°C. Tolerates 2.5% NaCl but does not tolerate 5% NaCl (w/v). Starch and aesculin are hydrolysed, but casein is not. Catalase and oxidase production are positive. In the API 20E strip, reactions for gelatin hydrolysis and nitrate reduction are positive. Reactions for ONPG production and the Voges-Proskauer (acetoin production) test are variable but the latter, when positive, is weak. Arginine dihydrolase, citrate utilisation, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease reactions are negative. Acid but no gas is produced from the following carbohydrates in the API 50 CHB Gallery: amygdalin, arbutin, D-trehalose, D-cellobiose, D-fructose, D-glucose, D-mannose, galactose, gentibiose, glycerol (weak), glycogen, lactose, L-arabinose, maltose, D-melibiose, methyl-D-glucoside, N-acetylglucosamine, ribose, salicin, starch and sucrose. Acid production is variable between strains for: D-raffinose, D-melezitose, D-xylose and methyl-D-mannoside. Acid is not produced from: 2-keto-D-gluconate, 5-keto-D-gluconate, adonitol, D-arabitol, D-fucose, D-turanose, D-arabinose, D-lyxose, D-tagatose, dulcitol, erythritol, gluconate, inulin, L-arabitol, L-fucose, L-sorbose, L-xylose, mannitol, meso-inositol, methyl-xyloside, rhamnose, sorbitol and xylitol. In the variable reactions listed above, the type strain LMG 18084^T has cream-coloured colonies with slightly rough, matt surfaces and irregular edges. It is positive for acid production from D-raffinose, D-melezitose, D-xylose and methyl-D-mannoside although the reactions are very weak; and negative for Voges-Proskauer and ONPG. Major fatty acids are iso-$C_{15:0}$ (23.2%), anteiso-$C_{15:0}$ (23.4%) and $C_{16:0}$ (17.7%). Major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. MK-7 is the predominant menaquinone and the peptidoglycan type is A1γ. The G+C content of the DNA of the type strain is 37.0 $\pm$ 0.2 mol%. Isolated from Senegalese palm wine, milking equipment, straw and grass.

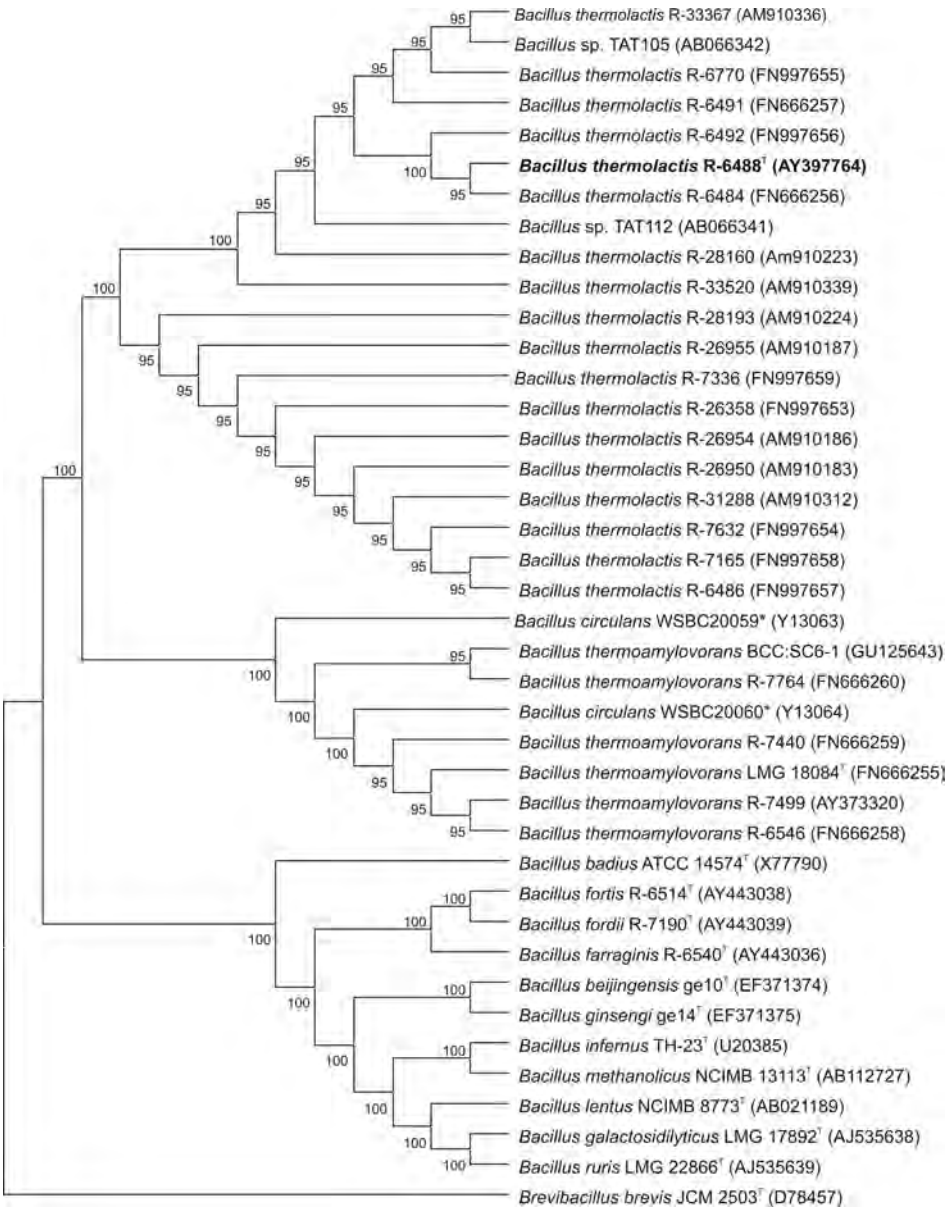


Figure 6.9: Consensus MP tree of the milk isolates with their closest relatives based on almost complete 16S rRNA gene sequences (1486 characters). Values at the branch points represent the percentage of trees showing that certain clade. Species are represented by species name, strain number and accession number of the 16S rRNA gene sequence between brackets. The 16S rRNA gene sequence of *Brevibacillus brevis* JCM 2503^T (D78457) was used as an outgroup to root the tree. The *Bacillus thermolactis* type strain R-6488^T is indicated in bold. *: these strains were probably misnamed and should be allocated to *Bacillus thermoamylovorans*.

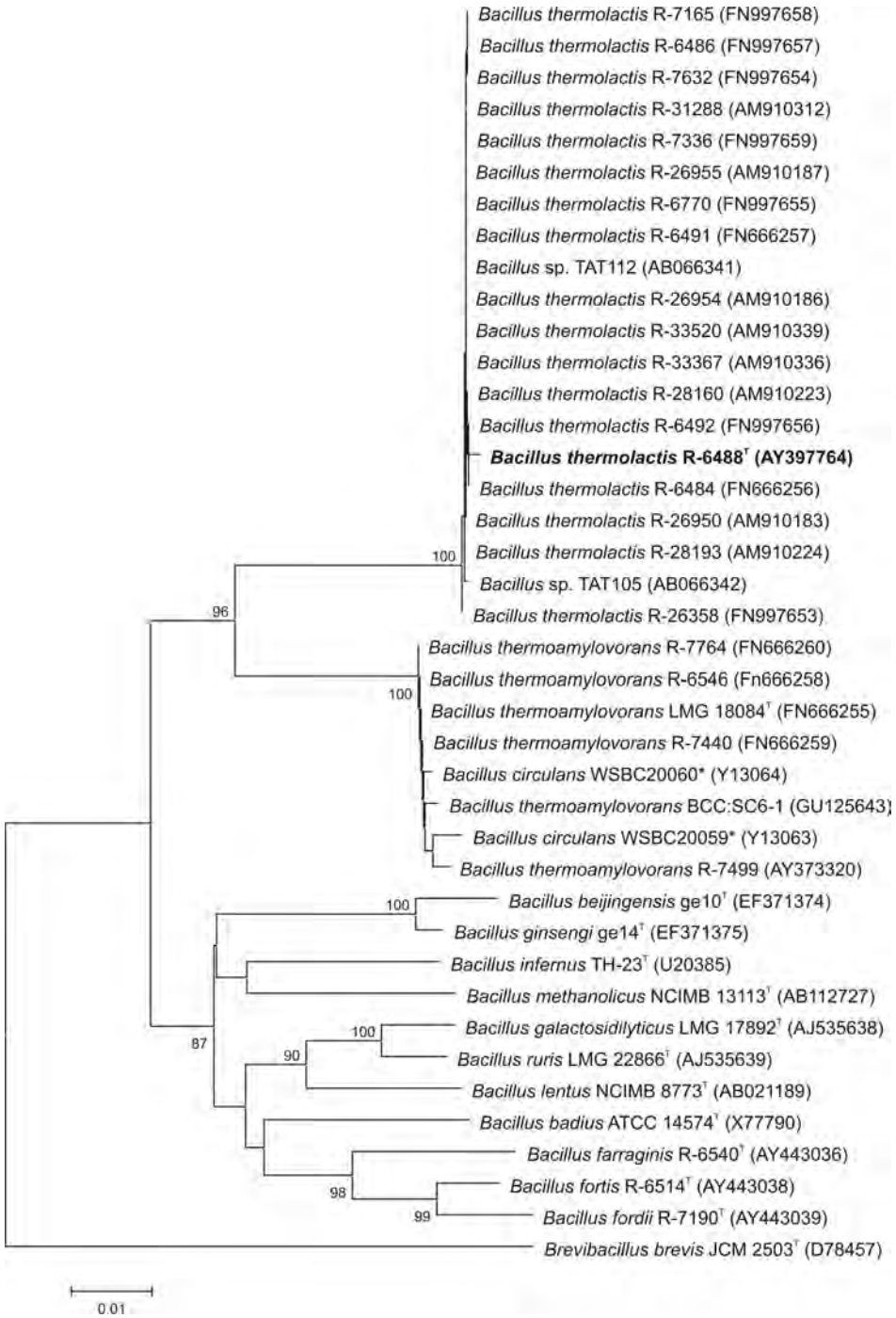


Figure 6.10: NJ phylogenetic tree of the milk isolates with their closest relatives based on almost complete 16S rRNA gene sequences (1486 characters). Bootstrap values above 85% are shown at the branch points. Species are represented by species name, strain number and accession number of the 16S rRNA gene sequence between brackets. The 16S rRNA gene sequence of *Brevibacillus brevis* JCM 2503^T (D78457) was used as an outgroup to root the tree. The *Bacillus thermolactis* type strain R-6488^T is indicated in bold. *: these strains were probably misnamed and should be allocated to *Bacillus thermoamylovorans*.

6.3 Toxinogenic and spoilage potential of the aerobic spore-formers

Representatives of the aerobic spore-forming microbiota of raw milk from the study described in §6.1 were tested for production of cytotoxic substances, spoilage enzymes and other deteriorative effects, giving a broad overview of possible harmful effects of aerobic spore-formers for the dairy industry. Unlike earlier studies, in which identification was usually performed according to biochemical properties and a limited database (Meer *et al.,* 1991), the investigated strains were taxonomically well-characterized using a polyphasic approach. This results in a better understanding of the effects caused by specific aerobic spore-forming bacteria in the dairy industry. Above this, extended knowledge on the microbiota of raw milk will allow the elaboration of preventive measurements for the dairy industry.

6.3.1 Screening for spoilage potential

Differential media were used as an initial screening method for spoilage potential on all isolates described in §6.1. Therefore a single colony was streaked out on SSM for detection of proteolysis, tributyrin agar (Oxoid) (TA) for lipolysis and SNA for lecithinase activity. The strains were also checked for β-galactosidase-activity as an indicator for lactose fermentation. Therefore, BHI plates (Oxoid) spread with 35 μL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (50 mg/mL dimethylformamide) and 20 μL filter-sterilized isopropyl-beta-D-thiogalactopyranoside (IPTG) (23.8 mg/mL) were used (X-GAL). All inoculated plates were incubated at 37°C for 72h and display of enzymatic activity was checked daily. This screening method revealed that isolates identified as *Paenibacillus polymyxa, Bacillus licheniformis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus clausii* and the *Bacillus cereus* group are capable of producing enzymes that adversely affect milk quality. Lecithinase activity seems to be a less abundant trait, as it was only found in isolates identified as the *Bacillus cereus* group and *P. polymyxa*. The results of this screening are summarized in Table 6.12, and the table in Appendix B.

A representative selection of isolates belonging to the most important species in raw milk, namely *B. licheniformis* (clusters D, F and L; n = 150), *B. cereus* group (cluster R, n = 44), *B. pumilus* (cluster G, n = 50), *B. amyloliquefaciens* (cluster H, n = 7) and *B. subtilis* (cluster F, n = 11), were subjected to $(GTG)_5$-fingerprinting to assess the intraspecific diversity, and to evaluate the genetic heterogeneity in the *Bacillus* microbiota from raw milk. Knowledge about intraspecies diversity can be useful to differentiate isolates of economical or clinical importance, to find contamination sources, track isolates along the food chain and to clarify the species distribution within highly related species clusters.

The results of these fingerprints are represented in Appendix B (different numbers represent different rep-types). Generally, different $(GTG)_5$-patterns observed within species could not be linked to any of the isolation parameters, such as isolation temperature, farm type or season. A link between psychrotrophic growth capacity and $(GTG)_5$-pattern was also not observed. Nonetheless, screening for the intraspecific diversity indicated a large difference in the population structure of important *Bacillus* species in raw milk, with the *B. cereus* group and *B. pumilus* showing the lowest and highest diversity respectively.

Indeed, for the *B. cereus* group one major type (R1 in Appendix B) could be traced back to three different (organic) farms. This result may point out a common contamination source, or this specific rep-type might be best capable of contaminating the raw milk. This finding is in

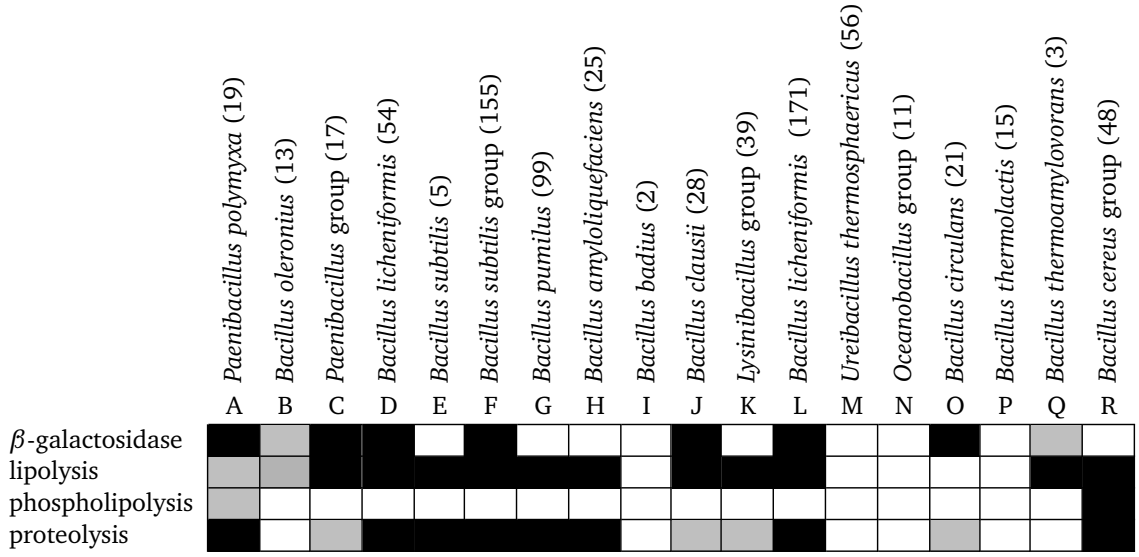


Table 6.12: Screening on differential media of aerobic spore-formers isolated from raw cow's milk. Clusters are named according to Figure 6.1. The number of isolates in each cluster is indicated between brackets. Black color of boxes indicates more than 70% of the isolates within that cluster screened positive, grey indicates between 30 and 70% of the isolates screened positive, and white coloured boxes indicate more than 70% of all isolates within that cluster scored negative.

agreement with the work of Svensson and co-workers (2004), who reported the presence of an in-house flora of mesophilic *B. cereus* strains in silo tanks at Scandinavian dairy plants that might have an ability to adhere to the surface of the tank. For one organic farm (farm 1), five different types of (R1 - R5 in Appendix B) of *B. cereus* group were found, in compliance with two Scandinavian studies that found different types of *B. cereus* contaminating the milk (Christiansson *et al.*, 1999; Svensson *et al.*, 2004). The different rep-types found in this study might represent different species within the *B. cereus* group or reflect the heterogeneity within a species or both. Further polyphasic research should clarify this issue.

Based on these rep-types, some representatives from proteolytic and lipolytic groups (revealed by the initial screening with differential media) were choosen to further investigate their proteolytic and lipolytic activity in a quantitative way, as described by De Jonghe and co-workers (2010). It was shown that strains belonging to the *B. cereus* group, *B. subtilis* and *P. polymyxa* were strongly proteolytic (++ in Appendix B), whereas strains belonging to *B. clausii*, *B. simplex* and *Lysinibacillus sphaericus* did not show any proteolytic activity (- in Appendix B), although they screened positive on SSM medium. The latter could be explained by the study of Jones and co-workers (2007), indicating the halo formed on skim milk medium might sometimes be caused by non-proteolytic activity as well. Strains identified as *B. amyloliquefaciens, B. licheniformis, B. pumilus* and *Lysinibacillus fusiformis* showed intermediate but significant proteolytic activity (+ in Appendix B). Furthermore, proteolytic activity showed some intraspecies variability as shown for *B. subtilis* and *B. licheniformis*. Surprisingly, lipolytic activity could only be demonstrated for three strains identified as *B. subtilis, B. pumilus* and *B. amyloliquefaciens* (++ and + in Appendix B; - if negative), although all selected strains scored positive on TA medium. These contradictory results could have several explanations. First, even though the quantitative method for determination of free fatty acids in milk is widely accepted for measurement of lipoly-

sis (IDF, 1989), the recovery of short chain fatty acids as a result of lipolysis is poor, due to the fact that they dissolve in the aqeous phase during the fat-extraction procedure (Duncan & Christen, 1991; IDF, 1991; Evers *et al.*, 2000). Secondly, the quantitative lipase-assay was performed in UHT-milk, in which the fat fraction is encapsulated in protein globules after homogenization and this probably makes the fat less accessible for lipolytic enzymes.

Some representatives (n = 7) that scored positive on X-GAL medium were further tested for their ability to ferment lactose as described by De Jonghe and co-workers (2010). This revealed four strains able to ferment lactose (+ Appendix B; - if negative), and one strain, R-31300, identified as *P. polymyxa* produced gas during fermentation (++ in Appendix B). This means that this strain has the potential to cause structural defects in cheese during fermentation. Indeed *P. polymyxa* has recently been implicated in blowing defects in Argentinean Cremoso and Mozarella cheeses (Quiberoni *et al.*, 2008).

Another important spoilage issue is the ability of spore-formers to reduce nitrate to nitrite, which may decrease the inhibitory effect of added nitrate to the germination of *Clostridium* spores, and thus promote 'late blowing' defects in Gouda type cheeses (Klijn *et al.*, 1995). Several representative strains were tested (n = 19) and nine scored positive (+ in Appendix B; - if negative), identified as *B. amyloliquefaciens, B. subtilis, L. sphaericus, P. polymyxa* and *B. clausii.*

6.3.2 Screening for toxinogenic potential

Main focus in this work is on spoilage of milk. However, for some *Bacillus* species it is known they do not only possess spoilage characteristics hampering milk quality but might also be imparted in food intoxications and thus hamper milk safety. Therefore, it was decided to screen some representative bacilli for their toxinogenic potential. This work was performed at the Institute for Agricultural and Fisheries Research, and results are summarized below (De Jonghe *et al.*, 2010).

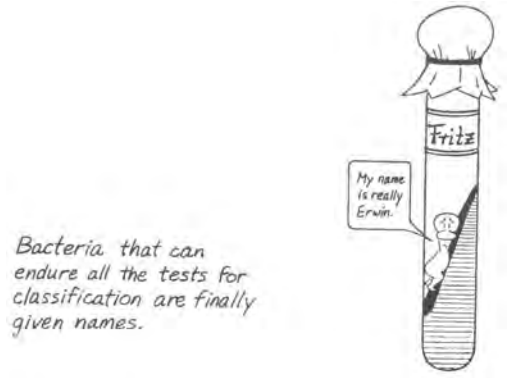
Representative strains belonging to different species and different rep-types were screened for toxin genes using PCR conditions and primers. The emetic toxin related gene *ces*, as well as genes encoding for enterotoxins HBL (*hblA*, *hblC* and *hblD*), NHE (*nheA*, *nheB* and *nheC*) and CytK (*cytK*) were targeted. Additionally, detection of emetic and other cytotoxic activity was performed. Complete operons for NHE and HBL enterotoxin were detected by PCR only in *B. cereus* group strains (NHE+, HBL+ in Appendix B). The CytK toxin gene was detected in *B. cereus* group strains and in *Paenibacillus cookii* strain R-26064 (CytK+ in Appendix B). None of the tested strains that were PCR-positive for the CytK toxin gene, showed the highly toxic *cytK-1* variant, they all contained the moderate *cytK-2* variant. A heat-stable cytotoxic component other than the emetic toxin was produced by strains belonging to *B. amyloliquefaciens* and *B. subtilis* (H-S+ in Appendix B). Heat-labile cytotoxic substances were produced by strains belonging to *B. amyloliquefaciens, B. subtilis, B. pumilus* and the *B. cereus* group (H-L+). Strain dependant variations could be noticed. Strains that were tested in the toxinogenic screening tests, but scored negative on all tests are indicated with - in Appendix B.

Acknowledgements

The author would like to thank Prof. Dr. Marc Heyndrickx and Dr. Valerie De Jonghe for the design and experimental setup of the isolation procedure, as well as for the assessment of the spoilage and toxinogenic potential of these bacilli. Prof. Dr. Niall Logan and Dr. Anna Dinsdale are acknowledged for their assistance in describing the novel species *Bacillus thermolactis*.

CHAPTER 7

Geobacillus taxonomy



At the very beginning of the diversity study on spore-forming bacilli in raw milk, it was assumed that a considerable part of the thermotolerant isolates would be attributed to the genus *Geobacillus*. Indeed, members of this genus are frequently associated with contamination of milk powders (Rueckert *et al.*, 2004; Scott *et al.*, 2007).

These expectations fitted in a joint cooperation research topic with Glasgow Caledonian University, studying a representative set of *Geobacillus* strains to adequately identify these strains and construct a reliable, satisfying taxonomy for members of this genus, as presented in this chapter. A good classification framework of a taxon results in reliable identification of its members, and it was thought resolving *Geobacillus* taxonomy would attribute in reliably identifying at least a part of the milk isolates.

Unfortunately, and unexpectedly, no *Geobacillus* isolates were retrieved from raw milk during the above described isolation campaign (§6.1). A possible explanation might be that major contamination source of milk powder with *Geobacillus* members is not primary introduction via raw milk but biofilms formed on milk powder production line equipment.

Despite the lack of isolates, it was decided to include this in-depth taxonomic study, as it nicely illustrates how the complex taxonomic situation encountered for the genera *Bacillus* and *Pseudomonas* could or should be resolved, in an ideal case scenario. Of course, due to the large amount of species harbored within both genera, the application of a similar approach is hardly feasible within a reasonable time-span. Nonetheless, one of the comments of the reviewers of the first paper dealing with *Geobacillus* taxonomy (§7.1) reflects his opinion on the taxonomic study presented in this chapter, thus justifying its inclusion in a taxonomically directed thesis: "This study is well overdue and represents a great step forward in sorting out the taxonomy of a group

of organisms that has been used and abused by various experts and opportunistic taxonomists over the years. The authors are experts in bacterial taxonomy and have brought together their collective knowledge and expertise to solve a bacterial taxonomy puzzle. I wish more groups would do the same and sort out some of the other puzzles that exist rather than just continuing to add species and subspecies to existing genera without complete studies of multiple strains. This paper sets the standard for future studies of strains of the genus *Geobacillus* and editors of the International Journal for Systematic and Evolutionary Microbiology should use it as an example of what the standard should be."

7.1 Emended descriptions of *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*

Nineteen thermophilic, aerobic, endospore-forming bacterial strains were subjected to 16S rRNA gene sequence analysis. Eight of these strains had been received as cultures of *Geobacillus kaustophilus, G. lituanicus, G. stearothermophilus, "G. thermoleovorans* subsp. *stromboliensis"*, *G. vulcani, "Bacillus caldolyticus"*, *"B. caldotenax"* and *"B. caldovelox"*, but they showed close relationships with the type strain of *G. thermoleovorans*, as did two other strains received as *G. thermoleovorans*. All strains underwent further taxonomic analysis by API, other phenotypic tests and FAME analysis, and selected strains were analysed for their polar lipids and for DNA relatedness. Eleven strains formed a *G. thermoleovorans* 16S rRNA cluster and also showed some phenotypic similarities; DNA relatedness data supported the reassignment of these strains - received as *G. kaustophilus, G. lituanicus, "G. thermoleovorans* subsp. *stromboliensis"*, *G. vulcani, "B. caldolyticus"*, *"B. caldotenax"* and *"B. caldovelox"* - and one of the *G. stearothermophilus* strains, as members of the species *G. thermoleovorans*. Four other strains received as *G. kaustophilus* were misnamed; two were identified as *G. stearothermophilus* and two appeared to be closely related to *Anoxybacillus rupiensis*. One strain received as *G. stearothermophilus* remained unidentified. On the basis of a single strain, *G. thermocatenulatus* was shown to represent a distinct species, but study of the type strain of *G. gargensis* showed this species to be a later heterotypic synonym of *G. thermocatenulatus*. Emended descriptions of *G. thermoleovorans* and *G. thermocatenulatus* are therefore presented.

7.1.1 Introduction

Following the discovery of novel thermophilic, aerobic endospore-formers in petroleum reservoirs, Nazina and co-workers (2001) proposed that the six species belonging to 16S rRNA group 5 (Ash *et al.*, 1991) should be accommodated in a new genus, *Geobacillus*, along with their two new species *G. subterraneus* and *G. uzenensis*. *Geobacillus* thus contained eight species: *G. stearothermophilus* (the type species), *G. kaustophilus, G. subterraneus, G. thermocatenulatus, G. thermodenitrificans, G. thermoglucosidasius, G. thermoleovorans* and *G. uzenensis*. For many years, the taxonomic positions of *G. kaustophilus* and *G. thermocatenulatus,* along with *"Bacillus caldolyticus", "B. caldotenax"* and *"B. caldovelox"* have been unclear. Metabolic studies and phage typing (Sharp *et al.*, 1980) revealed close relationships between these species. Also, White and co-workers (1993) considered whether *"B. caldolyticus", "B. caldotenax"* and *"B. caldovelox"* (Heinen & Heinen, 1972) were synonymous, and they recommended the revival of "*B. caldotenax*", but their DNA relatedness data were inconclusive. They also proposed an

emended description of *B. kaustophilus,* but did not validate this proposal. The 16S rRNA gene sequence studies of Rainey and co-workers (1994) showed that *B. kaustophilus, B. thermoleovorans, "B. caldolyticus", "B. caldotenax"* and *"B. caldovelox"* were closely related and that this group was related to *B. thermocatenulatus*. Sunna and co-workers (1997) proposed the merger of all these species on the basis of 72-88% DNA relatedness between representative strains, but their emended description of *B. thermoleovorans* was not validated. Nazina and co-workers (2004) did not support such a merger, however, as they found only 47-54% DNA relatedness between *G. kaustophilus, G. thermoleovorans,* and *G. thermocatenulatus*. The same authors (Nazina *et al*., 2004) also proposed the new species *G. gargensis*, and the transfer of *B. vulcani* (Caccamo *et al*., 2000) to *Geobacillus* on the basis of 99.4% 16S rRNA gene sequence similarity and 55% DNA relatedness with *G. kaustophilus.* Other taxa showing high 16S rRNA gene sequence similarities with *G. thermoleovorans* are *G. lituanicus* (Kuisiene *et al*., 2004), "*Geobacillus thermoleovorans* subsp. *stromboliensis*" (Romano *et al*., 2005b) and "*Geobacillus zalihae*" (Rahman *et al*., 2007). The present study subjected strains of *G. gargensis, G. kaustophilus, G. lituanicus, G. stearothermophilus*, *G. thermocatenulatus, G. thermoleovorans,* "*G. thermoleovorans* subsp. *stromboliensis*", *G. vulcani, "Bacillus caldolyticus", "B. caldotenax"* and *"B. caldovelox"* to a polyphasic taxonomic study, in order to resolve the taxonomic confusion.

7.1.2 Materials and methods

Bacterial strains. Strains were grown on TSA, unless otherwise stated, and incubated at 50°C or 60°C for 18 - 24 hours. An exception was *G. lituanicus*, which was grown on NA. Both media were supplemented with 5 mg L^{-1} $MnSO_4$ to encourage sporulation, and sporulated cultures were maintained at 3 - 5°C. The strains included in the study are listed in Table 7.1.

Phenotypic characterization. Colonial characters were recorded, and cellular and sporangial morphologies were observed by phase-contrast microscopy. Gram reaction, temperature and pH ranges, NaCl tolerance, production of catalase and oxidase, and starch and casein hydrolysis were tested by the methods of Logan and De Vos (2009). Characterization tests for each strain were performed in duplicate, and repeated if the results were ambiguous. Species represented by single strains were tested in triplicate. Biochemical characters were tested following the methods of Logan and Berkeley (1984) and Allan and co-workers (2005), and data were subjected to numerical taxonomic analysis using BioNumerics 5.1 software (Applied Maths, Belgium) using the general similarity coefficient of Gower (S_G; Gower, 1971) and UPGMA cluster analysis. Test error was calculated from mean similarities of several duplicated strains and the probability of error for an individual test was 0.25%.

FAME. For FAME-analysis, the method of Vauterin and co-workers (1991) was followed, and profiles were identified using the MIS software version 3.8. Cells were grown on TSBA at 52°C for 24 h as recommended in the MIDI-instructions (Microbiol ID, Newark, DE, USA).

Polar Lipids. Polar lipids of type and reference strains were extracted and separated by using two-dimensional thin-layer chromatography (Tindall, 1990a, b). The total lipid profiles were visualised by spraying with molybdatophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates) and α-naphthol (specific for sugars).

16S rRNA gene sequencing. Partial 16S rRNA gene sequences were generated as described by Heyrman and Swings (2001). Sequencing products were purified with the 'BigDye® XTermina-

name as received	present taxonomic status (if changed)	study number	other designations	source	16S rRNA acc. n°.
"*B. caldolyticus*"	*G. thermoleovorans*	R-32508	LMG 17975, DSM 405, B0211, Heinen YT-P	Superheated pool water USA; Atkinson	FN428646
"*B. caldotenax*"	*G. thermoleovorans*	R-32507	LMG 17974, DSM 406, B0212, Heinen YT-G	Superheated pool water USA; Atkinson	FN428645
"*B. caldovelox*"	*G. thermoleovorans*	R-32499	LMG 14463, DSM 411, B0213, Heinen YT-F	Superheated pool water USA; Atkinson	FN428638
G. gargensis	*G. thermocatenulatus*	R-35649^T	DSM 15378^T, LMG 23385^T, VKM B-2300^T, B3134^T	Garga hot spring, Eastern Siberia, Russia; DSMZ	FN428695
G. kaustophilus	*G. thermoleovorans*	R-35638^T	LMG 9819^T, DSM 7263^T, ATCC 8005^T B3061^T	Pasteurised milk; DSMZ	FN428686
G. kaustophilus	*G. stearothermophilus*	R-32635	B1552, Sharp 10007	Soil from Wales, UK; Sharp	FN428672
G. kaustophilus	*G. stearothermophilus*	R-32504	B1555, LUDA T28	Wolf	FN428642
G. kaustophilus	*Anoxybacillus rupiensis*	R-32636	B2598, Sharp 10332	Near Lake Taupo, New Zealand; Sharp	AM988775
G. kaustophilus	*Anoxybacillus rupiensis*	R-32637	B2599, Sharp 10315	Near Lake Taupo, New Zealand; Sharp	AM988776
G. lituanicus	*G. thermoleovorans*	R-35645^T	DSM 15325^T; LMG 23033^T, VKM B-2294^T, B3094^T	Lithuanian oilfield; Kuisiene	FN428693
G. stearothermophilus		R-35646^T	LMG 6939^T; DSM 22^T; ATCC 12980^T, B3117^T	Deteriorated canned food; DSMZ	FN428694
G. stearothermophilus	*G. thermoleovorans*	R-32642	DSM 3299, ATCC 15952, B3122, NCA 5520	DSMZ	FN428674
G. stearothermophilus	*Geobacillus* sp.	R-32643	DSM 13240, CIP 106956, B3124	Hot Springs, Yellowstone National Park, USA; DSMZ	FN428675
G. thermocatenulatus		R-35648^T	DSM 730^T; LMG 19007^T, VKM B-1259^T, B3129^T	Coating inside tube of hot-gas well, Southern Urals, USSR; DSMZ	FN538989
G. thermoleovorans		R-35636^T	LMG 9823^T, DSM 5366^T, ATCC 43513^T,B3059^T	Soil near hot water effluent, Bethlehem, USA; DSMZ	FN428684
G. thermoleovorans		R-32630	DSM 11667, B3131, Sunna HSR	Soil, Jordan; DSMZ	FN428671
G. thermoleovorans		R-32498	DSM 11668, B3132, Antranikian K-3d	Hot spring, Japan; DSMZ	FN428637
"*G. thermoleovorans* subsp. *stromboliensis*"	*G. thermoleovorans*	R-35650	DSM 15392, ATCC BAA 979, B3135, Pizzo	Soil close to crater of Stromboli, Italy; DSMZ	FN428696
G. vulcani	*G. thermoleovorans*	R-35635^T	DSM 13174^T, LMG 19852^T, CIP 106305^T, B3058^T, Caccamo 3S-1	Sediment, Vulcano-La Roya marine thermal vent, Eolian Islands, Italy; DSMZ	FN428683

Table 7.1: Overview of all strains in this study.

tor Purification Kit' (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, using sequential pipetting and a MixMate (Eppendorf, Hamburg, Germany) shaking device. Sequence analysis was performed using a *3100xl* DNA Sequencer (Applied Biosystems) according to the manufacturer's guidelines. Sequences were assembled and compared using BioNumerics 5.1 software. Phylogenetic trees were constructed using the ML, MP and NJ methods based on these partial 16S rRNA gene sequences, together with publicly available sequences of type strains within the genus *Geobacillus*. Alignment and clustering of sequences was performed using Mega4 (Tamura *et al.*, 2007) and PhyML (Guindon & Gascuel, 2003). The ML tree is represented in Figure 7.1, and is supported by the MP and NJ trees as represented in Figures 7.3 and 7.4, respectively.

DNA-DNA relatedness & G+C content. DNA was prepared and purified (Logan *et al.*, 2000) and hybridizations were performed using a modification (Willems *et al.*, 2001) of the microplate method described by Ezaki and co-workers (1989) with a reassociation temperature of 37°C. The G + C contents of the DNAs were determined by HPLC (Mesbah *et al.*, 1989; Logan *et al.*, 2000).

7.1.3 Results and Discussion

Our findings supported earlier proposals for merging the species *G. kaustophilus, G. thermoleovorans, "B. caldolyticus", "B. caldotenax"* and *"B. caldovelox"*. The 16S rRNA gene sequences of the type strains of *G. kaustophilus* R-35638^T, *G. lituanicus* R-35645^T and *G. thermoleovorans* R-35636^T, and of "*G. thermoleovorans* subsp. *stromboliensis*" R-35650 clustered at 99.9% S_P. "*B. caldolyticus*" R-32508, "*B. caldotenax*" R-32507 and "*B. caldovelox*" R-32499 clustered together at 99.8% S_P and joined the *G. thermoleovorans* cluster at 99.8% S_P (Figure 7.1), with *G. vulcani* 35635^T joining the cluster at 99.7% S_P, and R-32642 (received as *G. stearothermophilus*) joining at 99.6% S_P.

Reference strains for the species within this group showed over 71.5% DNA relatedness: values for the type strains of *G. kaustophilus, G. lituanicus,* and *G. vulcani,* and for strains of *"B. caldolyticus", "B. caldotenax", "B. caldovelox"* and "*G. thermoleovorans* subsp. *stromboliensis*", with *G. thermoleovorans* R-35636^T were 82.4%, 81%, 79.4%, 80.7%, 75.8%, 71.5% and 76.6%, respectively. These DNA relatedness data indicate that *G. kaustophilus* Priest *et al.,* 1989*, G. lituanicus* Kuisiene *et al.,* 2004 and *G. vulcani* Caccamo *et al.,* 2000 are later heterotypic synonyms of *G. thermoleovorans* Zarilla & Perry, 1988, and that *"B. caldolyticus", "B. caldotenax", "B. caldovelox"* and "*G. thermoleovorans* subsp. *stromboliensis*" are also members of *G. thermoleovorans*. These conclusions are consistent with the phylogenetic data of Nazina and co-workers (2005) and Zeigler (2005).

Only "*B. caldolyticus*" R-32508, "*B. caldotenax*" R-32507, and "*B. caldovelox*" R-32499 showed significant phenotypic similarities, clustering at 90% S_G (not shown), while joining with *G. thermoleovorans* R-35636^T at less than 85% S_G. *Geobacillus kaustophilus* R-35638^T and "*G. thermoleovorans* subsp. *stromboliensis*" R-35650 showed rather few phenotypic similarities with the *G. thermoleovorans* type strain. The considerable phenotypic diversity between these organisms makes a practically useful description difficult to write; indeed, Sunna and co-workers (1997) said that an emended *Bacillus thermoleovorans* would be "characterized by its phenotypic heterogeneity and its genotypic homogeneity". Further work, with more isolates, might discern biotypes within the species.

Polar lipid data were analysed for the type strains of *G. kaustophilus* R-35638^T, *G. lituanicus*

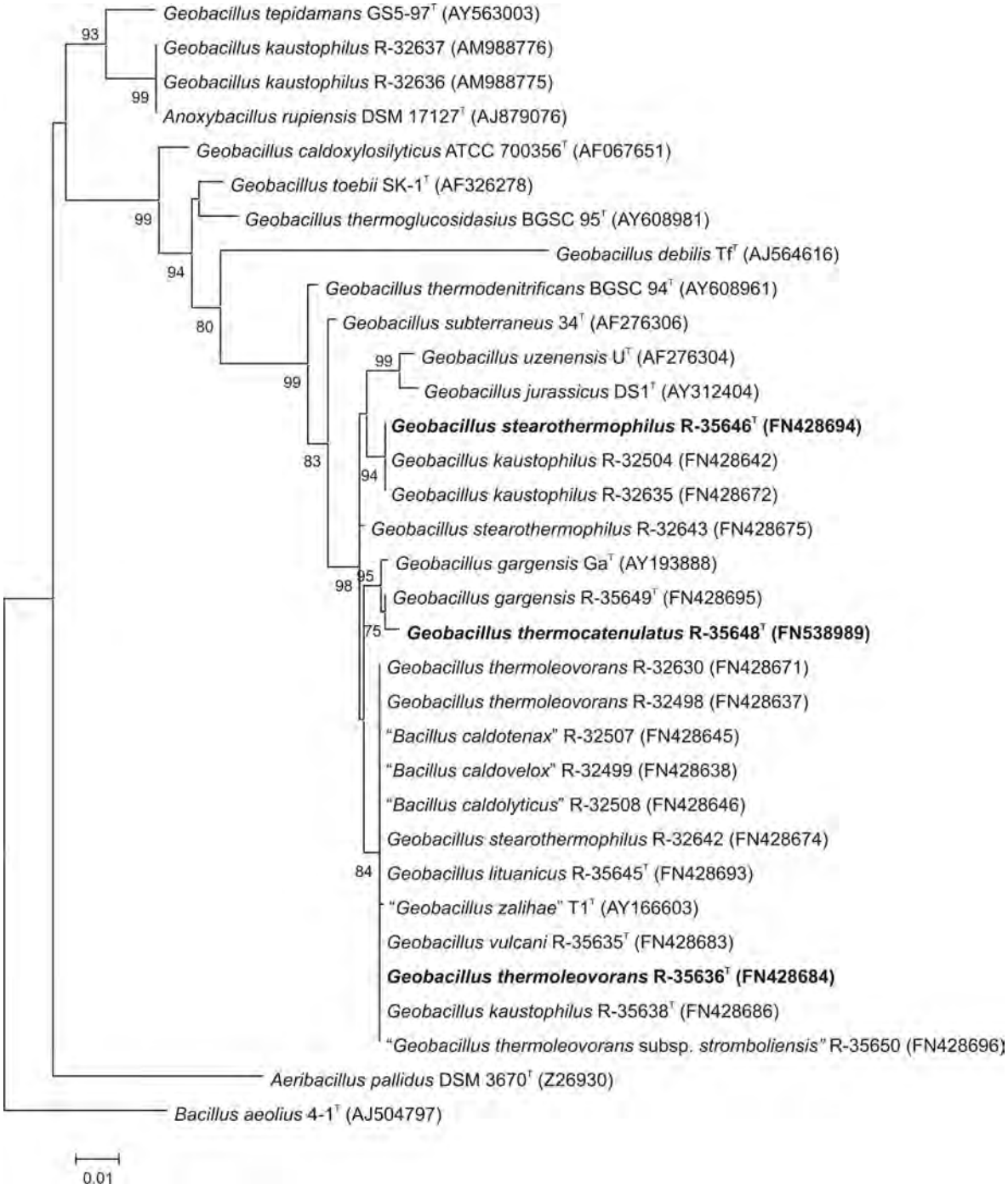


Figure 7.1: ML tree based on partial 16S rRNA gene sequences (1363 bp) of 19 *Bacillus* and *Geobacillus* strains. The 16S rRNA gene sequence of *Bacillus aeolius* (AJ504797) was selected as an outgroup to root the tree. aLRT values are shown at the branch points. Accession numbers for each strain are given in parentheses. All strains within the tree are represented by their names as received. The type strains, as studied, of the *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* clusters are indicated in bold. Bar, 0.01 substitutions per nucleotide position.

R-35645^T, *G. stearothermophilus* R-35646^T, *G. thermoleovorans* R-35636^T, *G. vulcani* R-35635^T, "*B. caldolyticus*" R-32508, "*B. caldotenax*" R-32507 and "*B. caldovelox*" R-32499. Polar lipid profiles were very similar for all strains, and exhibited diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unknown aminophosphoglycolipid (APGL) and some unknown aminolipids (ALs), a phosphoaminolipid (PAL), most probably phosphatidylserine (PS), and a lipid (L) in minor amounts. These polar lipid profiles for *Geobacillus* strains - including that of the type species *G. stearothermophilus* - showed PG, whereas Miñana-Galbis and co-workers (2010) reported its absence from the type strain of *G. stearothermophilus*. The polar lipid profile of the type strain of *G. thermoleovorans* is shown in Figure 7.2.

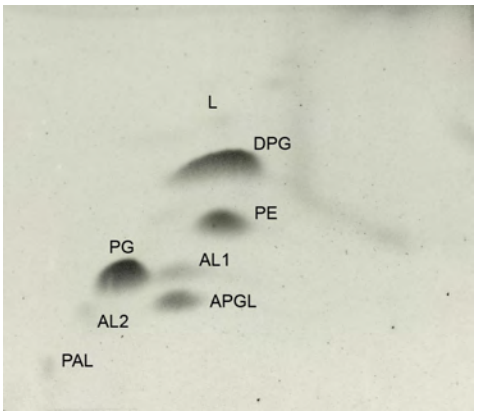


Figure 7.2: Polar lipid profile of *Geobacillus thermoleovorans* R-35636T after separation by two-dimensional thin layer chromatography. DPG: diphosphatidylglycerol, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, AL 1-2: aminolipid, PAL: phosphoaminolipid, APGL: aminophosphoglycolipid, L: lipid.

The polar lipid profiles of the species of *Geobacillus* here examined are clearly distinguishing from other endospore-forming taxa, showing *meso*-diaminopimelic acid in the peptidoglycan and a quinone system with the predominant compound menaquinone MK-7 such as *Bacillus, Paenibacillus, Cohnella* (Kämpfer *et al.,* 2006) and *Falsibacillus* (Zhou *et al.,* 2009). *Bacillus subtilis* and *Paenibacillus polymyxa*, the type species of the latter two genera, exhibit the presence of glycolipids not detectable in *Geobacillus* species but are lacking the presence of aminophosphoglycolipid of *Geobacillus*. *Cohnella* species can be distinguished from *Geobacillus* based on the absence of the aminophosphoglycoplipid and the presence of two aminophospholipids and a phospholipid (Kämpfer *et al*., 2006). *Falsibacillus* (Zhou *et al*., 2009), different from *Geobacillus*, exhibits two other aminophosphoglycolipids, two phosphoglycolipids and a glycolipid.

An emended description of *G. thermoleovorans* is given below, and characters to differentiate this species from other members of the genus are shown in Table 7.2.

In the study of Rahman and co-workers (2007), *"Geobacillus zalihae*" clustered with members of the *G. thermoleovorans* group and showed 68.2 and 73.6% DNA homology with the type strains of *G. thermoleovorans* and *G. kaustophilus* respectively, so this strain appears to be yet another representative of *G. thermoleovorans.* This was confirmed by a 16S rRNA gene sequence similarity percentage of 99.5% between "*G. zalihae"* and *G. thermoleovorans* (Figure 7.1). *Bacillus thermocatenulatus* was described by Golovacheva and co-workers (1991), but later was classed as *species incertae sedis* (Claus & Berkeley, 1986). It was later transferred to *Geobacillus* on the basis of 16S

	G. stearothermophilus	*G. caldoproteolyticus*	*G. caldoxylosilyticus*	*G. debilis*	*G. jurassicus*	*G. subterraneus*	*G. tepidamans*	*G. thermocatenulatus*	*G. thermodenitrificans*	*G. thermoglucosidasius*	*G. thermoleovorans*	*G. toebii*	*G. uzenesis*
n	15	1	1	1	2	1	1	2	9	8	11	6	2
sporangia													
cylindrical spores	-	+	v	+	-	-	-	d	-	d	-	-	-
sporangia swollen	v	-	+	+	v	-	+	-	-	d	-	+	d
spores subterminal	+	+	-	-	+	+	-	d	+	+	+	-	-
spores terminal	+	+	+	+	-	v	+	+	+	+	+	+	+
spores central/paracentral	-	-	+	-	-	-	-	d	-	-	-	-	-
hydrolysis of													
aesculin	+/w	+	+	+	+	+	+	+	+	+	+	-	+
casein	+/w	+	v	v	+	-	-	-	-	-	d	-	-
gelatin	+	+	+	+	+	+	-	+	d	+	+	+	+
ONPG	-	-	-	+	-	-	+	-	-	-	d	-	-
starch	+	+/w	+	-	+	+	+	-	+/w	d (w)	+	+/w	+
catalase	+	+	+	-	-	+	+	+	+	+	+	+	
oxidase	-	+	+	+	+	-	-	+	-	w	+	+	
nitrate reduction	d	-	+	-	+	+	-	d	d	d	+	+	+
VP											d		
Voges-Proskauer	+	+	-	-	-	-	-	+	d	d	d (w)	+	-
acid from													
N-Acetylglucosamine	-	+	-	+	-	-	+	d	-	+	d	-	-
amygdalin	-	-	+	+	-	-	+	+	-	d	-	-	-
L-Arabinose	-	-	+	-	+	-	+	-	d (w)	d	-	-	+
arbutin	-	v	+	w	-	-	+	+	-	d	-	-	w
D-Cellobiose	-	+	+	+	+	+	+	+	+	+	d	-	+
galactose	w	+	+	+	+	+	+	+	-	-	d	+	+
gentiobiose	-	-	+	+	-	-	+	-	-	d	-	-	-
glycerol	w	+	w	-	+	+	+	+	-	w	+	-	+
glycogen	+	+	+	-	-	+	-	-	-	-	d	-	+
lactose	-	-	+	+	-	-	+	-	-	w	d (w)	-	-
meso-inositol	-	-	-	-	-	+	+	-	-	-	d	-	-
mannitol	-	+	-	-	+	+	-	+	w	+	d	w	+
D-Melezitose	+	-	-	-	+	+	+	+	d (w)	-	d (w)	-	-
D-Melibiose	+	-	+	-	-	-	+	+	d	-	d	d	-
methyl-D-glucoside	+	-	w	-	+	-	+	d	-	+	d	-	+
D-Raffinose	+	-	+	-	-	-	+	+	-	-	d	-	-
ribose	-	+	+	-	+	-	w	+	w	+	+	d	-
salicin	-	+	+	+	-	+	+	+	d	+	w	-	+
sucrose	+	+	+	-	+	+	+	+	d	+	d	d	+
D-Trehalose	+	+	+		+	+	+	+	d	+	d	+	+
D-Turanose	+	w	+	w	+	-	+	-	-	+	-	-	-
D-Xylose	-	+	+	w	+	+	+	-	w	+	d	-	-
anaerobic growth	w/-	-	+/w	-	w	+	+	w	+	+	w	+	+
growth at pH 5	-	+	+	-	+	-	-	+	-	-	w	-	-
growth at pH 9	+	+	+	+	+	-	+	+	+	w	w	+	-
growth at 37°C	w	+	-	-	-	+	-	+	-	-	d	-	-
optimum temperature (°C)	40-60	60	50-65	>60	60	55-60	55	60	50	50	60	60	55-60
growth at 70°C	d	+	+	+	+	+	-	+	+	-	d	+	-
growth in 1% NaCl (w/v)	+	-	+	-	+	+	+	+	-	-	d	+	+
growth in 5% NaCl (w/v)	-	-	-	-	+	-	-	+	-	-	-	-	-

Table 7.2: Differentiation table. Data for *G. uzenensis* were taken from Logan *et al.* (2009); all other data were obtained in the course of the present study, and no entry indicates that data were not available. Symbols: +, >85% strains give positive reaction; -, 0-15% strains give positive reaction +/w, positive or weakly positive reaction; w, weak reaction; w/-, weak or negative reaction; d different strains give different reactions; d (w), different strains give different reactions, but positive reactions are weak; v, result varies within strains.

rRNA gene sequence and DNA relatedness data (Nazina *et al.*, 2001). Subsequently, a novel isolate from a hot spring was proposed as *G. gargensis* (Nazina *et al.*, 2004). They found 99.0% 16S rRNA sequence similarity between *G. thermocatenulatus* and *G. gargensis,* similar FAME profiles, and many shared phenotypic characteristics; however, a DNA relatedness value of 43% for the two type strains supported the proposal of a new species. In the present work *G. gargensis* R-35649^T and *G. thermocatenulatus* R-35648^T clustered at 94.5% S_G on the basis of phenotypic characters, at 98.9% S_P on the basis of 16S rRNA gene sequence analysis (Figure 7.1), and showed 85.6% DNA relatedness. The phylogenetic tree (Figure 7.1) shows slight differences between the 16S rRNA gene sequences of *G. gargensis* strains Ga^T (AY193888) and R-35649^T (FN428695). However, close examination of sequence AY193888 reveals a long stretch of undefined base pair positions (positions 874 to 907), whereas these positions have been determined in sequence FN428695. We therefore conclude that *G. gargensis* Nazina *et al.,* 2004 is a later heterotypic synonym of *G. thermocatenulatus* Golovacheva *et al*., 1991, and an emended description is given below; characters to differentiate the species from other members of the genus are shown in Table 7.2.

Strain R-32643, received as *G. stearothermophilus*, showed 98,9% and 99,2% S_P in 16S rRNA with the type strains of *G. stearothermophilus* and *G. thermoleovorans*. Despite these high similarity values, the strain could not be identified as its position in the phylogenetic tree indicates a clear separation between the *G. thermoleovorans* group and strain R-32643 (Figure 7.1). Four cultures received as *G. kaustophilus* were believed to be misnamed. Strains R-32635 and R-32504 showed 98.7 and 99.2% S_P in 16S rRNA with the type strain of *G. stearothermophilus* (Figure 7.1), respectively. DNA relatedness between R-32635 and R-32504 was 67.1%, and these strains' relatedness values with the type strain of *G. stearothermophilus* R-35646^T were 72.4% and 66.4%, respectively. Thus R-32504 is clearly a member of *G. stearothermophilus*, while R-32635 lies at the margin of this species. Strains R-32636 and R-32637, both isolated from the Lake Taupo volcanic region in New Zealand and received as *G. kaustophilus,* showed high phenotypic (96% S_G) and genotypic (99.9% S_P) similarities to each other, but were distinct from all the other taxa studied; however R-32637 showed 99.2% S_P with *Anoxybacillus rupiensis* (Derekova *et al.,* 2007) in 16S rRNA and so these Lake Taupo isolates most probably belong to *A. rupiensis*.

7.1.4 Emended description of *Geobacillus thermoleovorans* (Zarilla & Perry, 1988) Nazina et al., 2001 (*Bacillus thermoleovorans* Zarilla & Perry, 1988) Effective publication: Zarilla & Perry 1987.

ther.mo'le.o.vo'rans. Gr. n. *therme* heat; L. n. *oleum* oil; L. v. *vorare* to devour; N.L. pres. part. *thermoleovorans* indicating heat-requiring bacteria capable of utilizing oil (hydrocarbons).

Gram-positive, rod-shaped cells, 0.7 - 1.5 μm by 2 - 6 μm. Some strains are peritrichously motile. Ellipsoidal endospores are borne terminally and subterminally and do not swell the sporangia. Facultatively anaerobic, but growth is weak in the absence of oxygen. Colonies are circular in shape, creamy white in colour, with smooth and glossy or rough surfaces, entire or irregular margins, and diameters of 0.5 - 5 mm following incubation at 60°C for 18 - 24 h. Grows between 37 - 40°C and 65 - 70°C, with optimum at 60°C; no growth at 30 or 80°C. Grows sparsely at pH 5 or 9; optimum growth at pH 6 - 7. Tolerates 0.5 - 1% NaCl. Catalase and oxidase are positive. Aesculin and starch are hydrolysed. Casein hydrolysis is variable.

In the API 20E strip, gelatin is hydrolysed and nitrate is reduced; ONPG is variable; the Voges-Proskauer test is variable and weak when positive; and citrate utilization (Simmons'), arginine dihydrolase, hydrogen sulphide production, indole production, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, and urease production are negative. In the API 50 CHB gallery acid without gas is produced from D-fructose, D-glucose, glycerol, maltose, D-mannose, and ribose. Acid is produced weakly from salicin and production is variable from D-cellobiose, galactose, glycogen, mannitol, D-melezitose (weak when positive), D-melibiose, *meso*-inositol, lactose (weak when positive), methyl-D-glucoside, *N*-acetylglucosamine, D-raffinose, starch, sucrose, D-trehalose, D-xylose. No acid is produced from adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, inulin, 2- and 5-keto-D-gluconate, D-lyxose, methyl-D-mannoside, methyl-xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol and L-xylose. The following carbohydrates are assimilated: D-cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, lactose, maltitol, maltose, maltotriose, mannitol, D-mannose, D-melezitose, D-melibiose, 1-0-methyl-β-D-glucopyranoside, palatinose, D-ribose, sucrose, D-trehalose, and D-turanose; assimilation is variable from aesculin, glycerol, *meso*-inositol, lactulose, D-lyxose, 1-0- and 3-0-methyl-α-D-glucopyranoside, 1-0-methyl-α-galactopyranoside (weak when positive), 1-0-methyl-β-glucopyranoside, D-raffinose and D-xylose; and negative for adonitol, L-arabinose, D- and L-arabitol, dulcitol, erythritol, L-fucose, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose and xylitol. The following organic acids are assimilated: *N*-acetylglucosmine, fumarate, D-gluconate, DL-glycerate, DL-β-hydroxybutyrate (3-hydroxybutyrate), L-malate and succinate; assimilation is variable for 2-keto-D-gluconate (weak when positive), DL-lactate, D-malate (weak when positive), phenylacetate and quinate; and negative for *cis*- and *trans*-aconitate, 4-aminobutyrate, 4-aminovalerate, benzoate, caprate, caprylate, citrate, *m*-coumarate, D-galacturonate, gentisate, D-glucoronate, glutarate, 3- and 4-hydroxybenzoate, itaconate, α-ketoglutarate, 5-keto-D-gluconate, malonate, mucate, 3-phenylpropionate, propionate, protocatechuate, D-saccharate, D-, L- and meso-tartrate and tricarballylate. The following amino acids are assimilated: D-glucosamine, L-glutamate, and L-histidine; assimilation is variable for D- and L-alanine, histamine (weak when positive), L-proline (weak when positive) and L-serine; and negative for L-aspartate, betaine, ethanolamine, putrescine, trigonelline, tryptamine, L-tryptophan and L-tyrosine.

In the variable reactions listed above the type strain is positive for hydrolysis of casein; negative for ONPG and positive for Voges-Proskauer; acid is produced from galactose, glycogen, mannitol, D-melezitose (weakly), D-melibiose, *meso*-inositol, D-raffinose, starch, sucrose, D-trehalose, and D-xylose; acid is not produced from D-cellobiose, lactose, methyl-D-glucoside, and *N*-acetylglucosamine; positive for assimilation of aesculin, glycerol, *meso*-inositol, lactulose (weak), 1-0-methyl-β-glucopyranoside, D-raffinose, D-xylose, DL-lactate, phenylacetate, quinate, D- and L-alanine, L-proline and L-serine; and negative for D-lyxose, 1-0- and 3-0-methyl-α-D-glucopyranoside, 1-0-methyl-α-galactopyranoside, 2-keto-D-gluconate, D-malate and histamine. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0.}$

Isolated from pasteurized milk, geothermally-heated pools and springs and associated soil, marine sediment of a hydrothermal vent, and crude oil. The mol% G+C content of DNA ranges from 52 - 53%, and is 52.4% for the type strain. Type strain: LMG 9823^T (= ATCC 43513^T = DSM 5366^T). EMBL/GenBank accession number (16S rRNA) is Z26923 (DSM 5366^T).

7.1.5 Emended description of *Geobacillus thermocatenulatus* (Golovacheva *et al.*, 1991) Nazina *et al.*, 2001 (*Bacillus thermocatenulatus* Golovacheva *et al.*, 1991)

ther.mo'ca.ten'ul.at.us Gr. n. *therme* heat; N.L. adj. *catenulatus* chain-like; N.L. masc. adj. *thermocatenulatus*, thermophilic and chain-like, referring to two of the organism's features.

Gram-positive, peritrichously motile rods, 0.9 - 1.5 μm by 6 to 12 μm. Description is based upon two strains. Facultatively anaerobic, but growth is weak in the absence of oxygen. Occurs singly or in long chains. Ellipsoidal or cylindrical spores are located terminally, occasionally subterminally and paracentrally, and do not swell the sporangia. Colonies on trypticase soy agar are butyrous, cream or yellowish in colour, round and raised, with entire margins; may become partially heaped-up and viscid. Growth can occur at 37 and 80^{o}C, with optimum growth occurring at 60^{o}C. Tolerates 5% but not 7% NaCl. Grows at pH 5 and pH 9, and optimally at pH 7. Aesculin is hydrolysed; casein and starch are not hydrolysed. Catalase and oxidase are positive. In the API 20E strip gelatin is hydrolysed and the Voges-Proskauer test is positive; citrate utilization (Simmons') and nitrate reduction are variable, and arginine dihydrolase, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, ONPG, tryptophan deaminase and urease production are negative. In the API 50CHB gallery acid without gas is produced from amygdalin, arbutin, D-cellobiose, D-fructose, galactose, D-glucose, glycerol, maltose, mannitol, D-mannose, D-melezitose, D-melibiose, D-raffinose, ribose, salicin, starch, sucrose, and D-trehalose. Acid production is variable from methyl-D-glucoside and *N*-acetylglucosamine. Acid production is negative from adonitol, D- and L-arabinose, D-and L-arabitol, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycogen, inulin, 2- and 5-keto-D-gluconate, lactose, D-lyxose, *meso*-inositol, methyl-D-mannoside, methyl-xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol, D-xylose and L-xylose.

In the variable reactions listed above, the type strain produces acid from methyl-D-glucoside and *N*-acetylglucosamine, and does not utilize citrate or reduce nitrate. A number of hydrocarbons (C_{10}-C_{16}) may be utilized as carbon and energy sources. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0}$, making up more than 60% of the total fatty acids (Nazina *et al.*, 2001).

The type strain was isolated from a slime layer inside a hot-gas borehole pipe, another strain was isolated from the Garga hot spring in the Transbaikal region, Russia. The mol% G+C of the DNA is 51.2% for the type strain. Type strain*:* LMG 19007^{T} (= DSM 730^{T} = VKM B-1259^{T} = BGSC 93^{T} = strain 178^{T}). EMBL/GenBank accession number (16S rRNA) is AY608935 (BGSC 93^{T}).

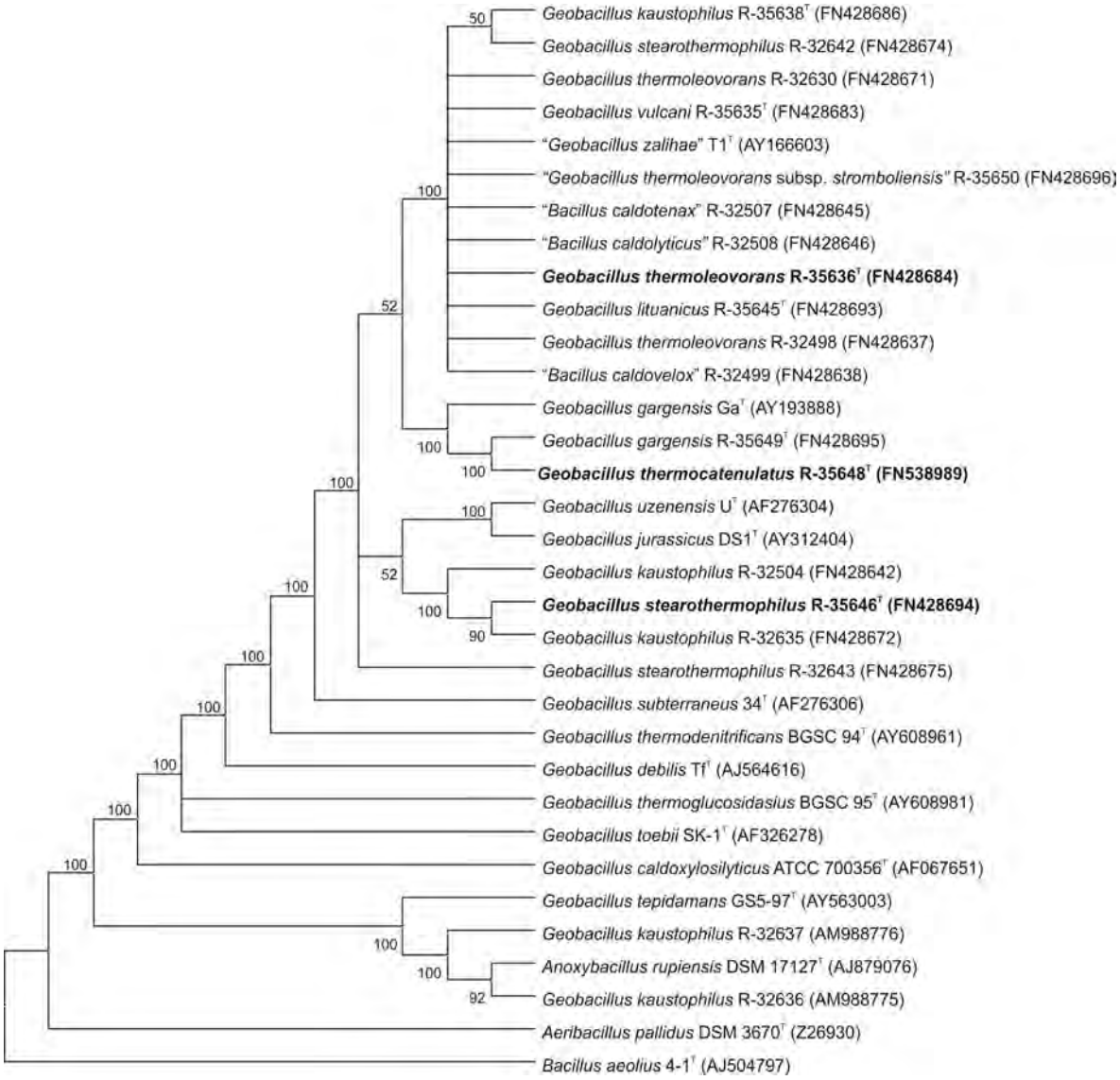


Figure 7.3: Consensus MP tree based on partial 16S rRNA gene sequences (1363 bp) of 19 *Bacillus* and *Geobacillus* strains. The 16S rRNA gene sequence of *Bacillus aeolius* (AJ504797) was selected as an outgroup to root the tree. Alignment and clustering of sequences was performed using MEGA4. Consensus percentages are given at the branch points. Accession numbers for each strain are given in parentheses. All strains within the tree are represented by their names as received. The type strains, as studied, of the *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* clusters are indicated in bold.

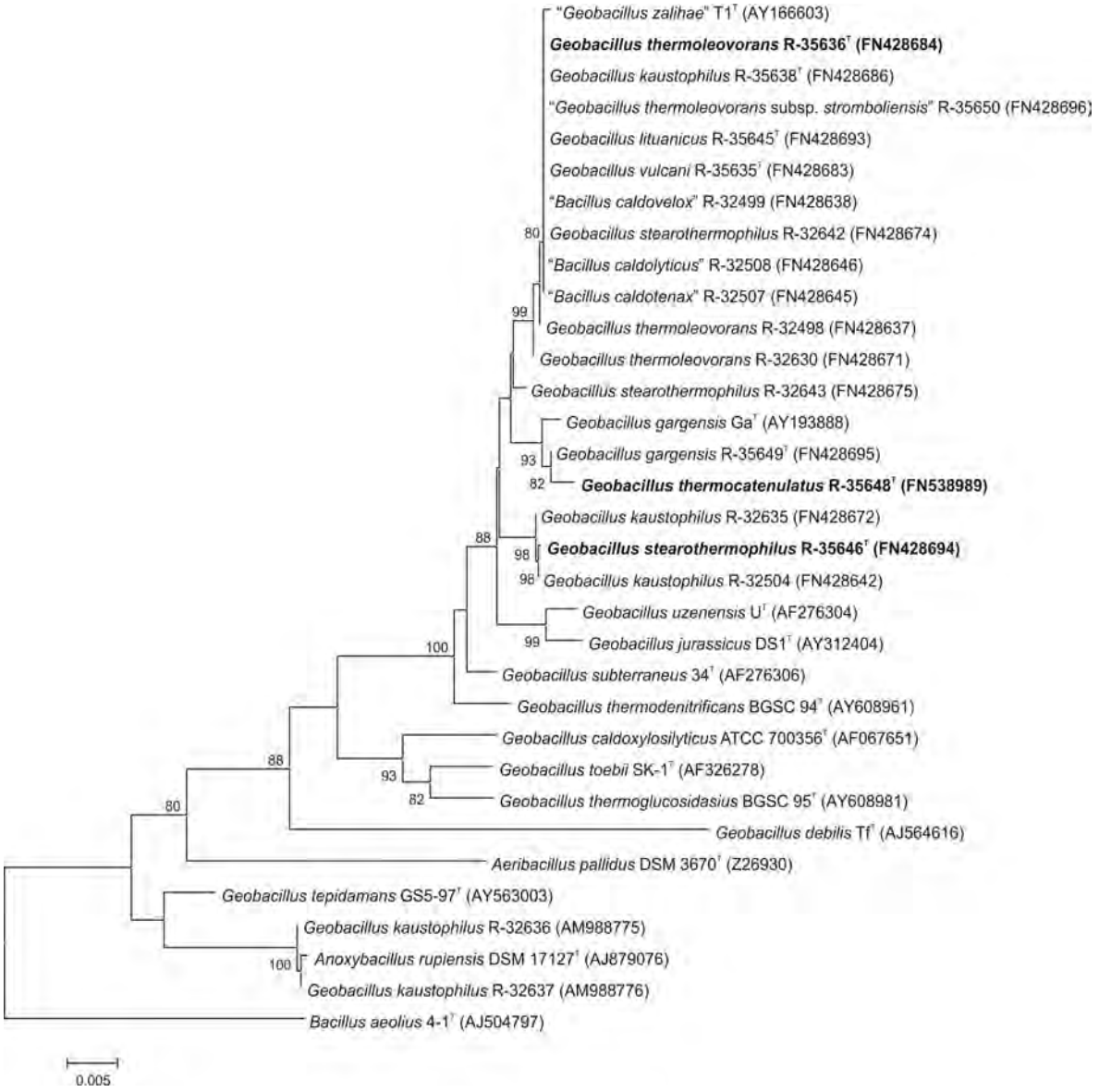


Figure 7.4: NJ tree based on partial 16S rRNA gene sequences (1363 bp) of 19 *Bacillus* and *Geobacillus* strains. The 16S rRNA gene sequence of *Bacillus aeolius* (AJ504797) was selected as an outgroup to root the tree. Alignment and clustering of sequences was performed using MEGA4. Bootstrap values above 70% are indicated at the branch points. Accession numbers for each strain are given in parentheses. All strains within the tree are represented by their names as received. The type strains, as studied, of the *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* clusters are indicated in bold.

7.2 Taxonomic revision of the genus *Geobacillus*: emendation of *Geobacillus*, *G. stearothermophilus*, *G. jurassicus, G. toebii*, *G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly "*thermoglucosidasius*"); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus*; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* and proposal of *Anoxybacillus caldiproteolyticus* sp. nov.

Sixty-two strains of thermophilic aerobic endospore-forming bacteria were subjected to polyphasic taxonomic study including 16S rRNA gene sequence analysis, polar lipid and fatty acid analysis, phenotypic characterization, and DNA-DNA hybridization experiments. Distinct clusters of the species *Geobacillus stearothermophilus, Geobacillus thermodenitrificans, Geobacillus toebii* and *Geobacillus thermoglucosidasius* were formed, allowing their descriptions to be emended, and the distinctnesses of the poorly represented species *Geobacillus jurassicus*, *Geobacillus subterraneus* and *Geobacillus caldoxylosilyticus* were confirmed. It is proposed that the name *Geobacillus thermoglucosidasius* be corrected to *G. thermoglucosidans*. *Bacillus thermantarcticus* clustered between *Geobacillus* species on the basis of 16S rRNA gene sequence analysis, and its transfer to *Geobacillus* is proposed. The above-mentioned species, together with *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus,* form a monophyletic cluster representing the genus *Geobacillus*. The distinctness of "*Geobacillus caldoxylosilyticus*" was confirmed and it is proposed that it be accommodated, along with *Geobacillus tepidamans*, in *Anoxybacillus*. The type strain of *Geobacillus debilis* was not closely related to any members of *Anoxybacillus* and *Geobacillus*, and it is proposed that this species be placed in the new genus *Caldibacillus*.

7.2.1 Introduction

For many years *Bacillus stearothermophilus* (Donk, 1920) was the only validly published, obligately thermophilic species of the genus *Bacillus*. After 1980, further thermophilic species were proposed on the basis of phenotypic analyses, and the subsequent application of 16S rRNA gene sequencing to members of *Bacillus* indicated that *B. stearothermophilus, B. kaustophilus* and *B. thermoglucosidasius* formed a generic lineage distinct from *Bacillus* s.s. (Ash *et al*., 1991b). Nazina and co-workers (2001) proposed that the six species of that lineage, namely *B. stearothermophilus, B. kaustophilus, B. thermoglucosidasius, B. thermocatenulatus, B. thermoleovorans* and *B. thermodenitrificans*, should be accommodated in a new genus, *Geobacillus*, along with the two new species, *G. subterraneus* and *G. uzenensis*. Subsequently, *Bacillus pallidus* (Scholz *et al.*, 1987), *Saccharococcus caldoxylosilyticus* (Ahmad *et al*., 2000) and *Bacillus vulcani* (Caccamo *et al.*, 2000) were transferred to *Geobacillus* (Fortina *et al*., 2001; Banat *et al*., 2004; Nazina *et al.*, 2004) and then the further transfer of *G. pallidus* to the new genus *Aeribacillus* was proposed (Minana-Galbis *et al.*, 2010). Also, six further species, *G. toebii* (Sung *et al*., 2002), *G. gargensis* (Nazina *et al*., 2004), *G. debilis* (Banat *et al.*, 2004), *G. lituanicus* (Kuisiene *et al*., 2004), *G. tepidamans* (Schäffer *et al*., 2004) and *G. jurassicus* (Nazina *et al*., 2005) have been proposed, while "*Geobacillus caldoproteolyticus*" (Chen *et al*., 2004) and the subspecies "*G. toebii* subsp. *decanicus*" (Poli *et al*., 2006b) await valid publication. The transfer of *Bacillus thermantarcticus* to

Geobacillus, recommended by Zeigler (2005), awaits formal proposal.

In contrast to this expansion of the number of species in *Geobacillus*, Dinsdale and co-workers (2011) showed that *G. kaustophilus*, *G. lituanicus*, *G. vulcani*, and several other strains with invalid names, were all synonyms of *G. thermoleovorans* and that *G. gargensis* was a synonym of *G. thermocatenulatus*. These mergers leave 12 validly published species in *Geobacillus*. The description of *G. stearothermophilus* - the type species of the genus - that was given by Nazina and co-workers (2001) was largely based on the one given by Claus and Berkeley (1986), who recognized the species to be heterogeneous because for many years most thermophilic *Bacillus* isolates were automatically assigned to it. *G. stearothermophilus* thus lacks a modern description based upon polyphasic taxonomic study. We therefore subjected representatives of members of *Geobacillus* and *Bacillus thermantarcticus* to a polyphasic study in order to resolve their taxonomy and allow their descriptions to be emended where necessary.

7.2.2 Materials and methods

Bacterial strains. Strains were grown on TSA, unless otherwise stated, and incubated at 50 or 60°C for 18 - 24 hours. Exceptions were *Bacillus thermantarcticus*, which was grown on $\frac{1}{2}$-strength BFA (Forsyth & Logan, 2000) or $\frac{1}{2}$-strength NA, and *G. tepidamans*, which was grown on NA. All solid growth media were supplemented with 5 mg L^{-1} $MnSO_4$ to encourage sporulation, and sporulated cultures were maintained at 3 - 5°C. All strains used in this study are listed in Table 7.3, and were assigned a Research (R)-number.

Phenotypic characterization. Colonial and microscopic morphologies were observed, and the Gram-reaction, temperature and pH ranges, NaCl tolerance, production of catalase and oxidase, and starch and casein hydrolysis were tested according to Logan and De Vos (2009). Characterization tests for each strain were performed in duplicate, and repeated if results were ambiguous. Species represented by single strains were tested in triplicate. Biochemical characters were tested following the methods of Logan and De Vos (2009) and Allan and co-workers (2005), and data were subjected to numerical taxonomic analysis using BioNumerics 4.61 software (Applied Maths, Belgium) using the general similarity coefficient of Gower (S_G, Gower, 1971) and UPGMA cluster analysis. Test error was calculated from mean similarities of several duplicated strains and the probability of error for an individual test was found to be 0.25%.

Chemotaxonomic characterization. For FAME analysis, the method of Vauterin and co-workers (1991) was followed, including standardization of incubation temperature (52°C), incubation time (24 hours) and physiological age (overlap area of the second and third quadrant from a quadrant streak); profiles were identified and clustered using the MIS software and MIDI TSBA database version 5.0. Polar lipids were extracted and separated after growth on TSA at 52°C for 24 hours (Dinsdale *et al.*, 2011).

Genotypic characterization. DNA-extraction and 16S rRNA gene sequencing was carried out as described by Coorevits and co-workers (2011c). All sequences were aligned using the integrated aligner of arb (Ludwig *et al.,* 2004) and manual refinement. Sequences were then exported to MEGA5 software (Tamura *et al.,* 2011) to construct a maximum likelihood (ML) tree. Reliability of clusters was assessed by calculating bootstrap values based on 1000 replications. DNA-DNA hybridization experiments and determination of DNA G +C content were performed at a hybridization temperature of 37°C (Dinsdale *et al.*, 2011).

Table 7.3: List of strains included in this study. * The type strain of *G. uzenensis* DSM 13551^T is actually a strain of *G. subterraneus*.

subculture used (R-number)	name as received	identity based on this study (if changed)	source / reference or comment	16S rRNA acc. n°
32606	*Geobacillus stearothermophilus* DSM 231		unknown	FN428658
32607	*Geobacillus stearothermophilus* DSM 2357		U.V. mutant from NCIMB 10278	FN428659
32765	*Geobacillus stearothermophilus* DSM 458		sugar beet juice	FN428681
32504	*Geobacillus stearothermophilus* LUDA T28		Wolf	FN428642
32635	*Geobacillus stearothermophilus* Sharp 10007		soil from Wales, U.K.	FN428672
32640	*Geobacillus stearothermophilus* DSM 457		sugar beet juice	FN428673
32496	*Geobacillus stearothermophilus* CECT 43		duplicate type strain	FN428633
32501	*Geobacillus stearothermophilus* ATCC 10149		unknown, Iowa	FN428640
32605	*Geobacillus stearothermophilus* Sharp 11182		unknown	FN428657
32604	*Geobacillus stearothermophilus* LMG 8193		steam sterilization control strain	FN428656
32513	*Geobacillus stearothermophilus* Sharp 12026		unknown	FN428649
32603	*Geobacillus stearothermophilus* Sharp 12001		sugar beet juice	FN428655
32602	*Geobacillus stearothermophilus* Sharp 12026		unknown	FN428654
32601	*Geobacillus stearothermophilus* Sharp 11200		unknown	FN428653
32600	*Geobacillus stearothermophilus* Sharp 12045		deteriorated canned food	FN428652
32502	*Geobacillus stearothermophilus* LMG 17899		flat sour spoilage of canned foods	FN428641
32512	*Geobacillus stearothermophilus* Wolf T210		duplicate type strain	FN428648
35646^T	*Geobacillus stearothermophilus* DSM 22^T		deteriorated canned food	FN428694
35648^T	*Geobacillus thermocatenulatus* DSM 730^T		coating inside tube of hot gas well, USSR	FN538989
35636^T	*Geobacillus thermoleovorans* DSM 5366^T		soil near hot water effluent, USA	FN428684
35651^T	*Geobacillus jurassicus* DSM 15726^T		formation water, Dagang oil field, China	FN428697
32597	*Geobacillus jurassicus* DSM 15727		formation water, Dagang oil field, China	FN428651
32641^T	*Geobacillus subterraneus* DSM 13552^T		formation water of the Liaohe oil field, China	FN428689
35640^T	*Geobacillus uzenensis** DSM 13551^T		formation water of the Uzen oil field, Kazakhstan	FN428688
32500	*Geobacillus thermodenitrificans* Sharp 12643		Ayutthaya, Thailand	FN428639
32506	*Geobacillus thermodenitrificans* Sharp 11033		atypical phenotype	FN428644
32616	*Geobacillus thermodenitrificans* Sharp 12823		soil from Iceland	FN428660
32617	*Geobacillus thermodenitrificans* Sharp 11024		atypical phenotype	FN428661
32618	*Geobacillus thermodenitrificans* Sharp 10097		river Cam, Cambridge, U.K.	FN428662
32511	*Geobacillus thermodenitrificans* Sharp 10078		soil, France	FN428647
32619	*Geobacillus thermodenitrificans* Sharp 11025		atypical phenotype	FN428663
32621	*Geobacillus thermodenitrificans* Sharp 12019		sugar beet juice, Austria	FN428664
32622	*Geobacillus thermodenitrificans* Sharp 11022		unknown	FN428665
32623	*Geobacillus thermodenitrificans* DSM 13147		soil, Italy	FN428666

continued on next page ...

subculture used (R-number)	name as received	identity based on this study (if changed)	source / reference or comment	16S rRNA acc. n°
32624	*Geobacillus thermodenitrificans* DSM 13148		soil, Indonesia	FN428667
32614	*Geobacillus thermodenitrificans* Sharp 12628		Deli peoples' commune, China	FN538994
32615	*Geobacillus thermodenitrificans* Sharp 11020		atypical phenotype	FN538995
32625	*Geobacillus thermodenitrificans* DSM 13149		soil, Saudi Arabia	FN538990
35647^T	*Geobacillus thermodenitrificans* DSM 465^T		sugar beet juice	FN538993
35644^T	*Bacillus thermantarcticus* DSM 9572^T	*Geobacillus thermantarcticus*	Mount Melbourne, Antarctica	FN428692
32639	*Geobacillus stearothermophilus* DSM 1550	*Geobacillus toebii*	evaporated milk	FN538992
32652	*Geobacillus toebii* Sharp 11056		unknown	FN538991
32651	*Geobacillus toebii* Sharp 12670		soil, Thailand	FN428679
32650	*Geobacillus toebii* Sharp 12633		soil, Thailand	FN538988
32653	*Geobacillus toebii* Sharp 12668		soil, Thailand	FN428680
35642^T	*Geobacillus toebii* DSM 14590^T		hay compost, Korea	FN428690
35637^T	*Geobacillus thermoglucosidasius* DSM2542^T		soil, Japan	FN428685
32515	*Geobacillus thermoglucosidasius* Sharp 12251		Yellowstone national park, USA	FN428650
32505	*Geobacillus thermoglucosidasius* Sharp 12641		Ayttahya, Thailand	FN428643
32627	*Geobacillus thermoglucosidasius* Sharp 12678		soil, China	FN428668
32646	*Geobacillus thermoglucosidasius* Sharp 12656		soil, China	FN428676
32628	*Geobacillus thermoglucosidasius* Sharp 12673		soil, Thailand	FN428669
32648	*Geobacillus thermoglucosidasius* Sharp 11101		unknown	FN428677
32649	*Geobacillus thermoglucosidasius* Sharp 12620		China	FN428678
32629	*Geobacillus thermoglucosidasius* DSM 2543		soil	FN428670
35639^T	*Geobacillus caldoxylosilyticus* DSM 12041^T		soil, Australia	FN428687
35653^T	*Geobacillus debilis* DSM 16016^T	*Caldibacillus debilis*	undisturbed subsurface soil, Ireland	FN428699
35652^T	"*Geobacillus caldoproteolyticus*" DSM 15730	*Anoxybacillus caldoproteolyticus*	sewage sludge, Singapore	FN428698
35643^T	*Geobacillus tepidamans* DSM 16325^T	*Anoxybacillus tepidamans*	suger beet extraction juice	FN428691
32637	"*Geobacillus kaustophilus*" Sharp 10332	*Anoxybacillus rupiensis*	near lake Taupo, New Zealand	AM988775
32636	"*Geobacillus kaustophilus*" Sharp 10315	*Anoxybacillus rupiensis*	near lake Taupo, New Zealand	AM988776
35634^T	*Aeribacillus pallidus* LMG 19006^T		yeast factory sewage	FN428682

7.2.3 Results and discussion

On the basis of 16S rRNA gene sequence analysis, the strains studied fell into four distinct clusters, corresponding with four different genera, namely *Geobacillus, Anoxybacillus, Aeribacillus* and the new genus *Caldibacillus* that is proposed herein (Figure 7.5). These clusters are discussed individually below, and some differentiating features for the four genera are listed in Table 7.4.

	Geobacillus	*Aeribacillus*	*Anoxybacillus*	*Caldibacillus*
polar lipids	DPG, PG, PE, AL1 PAL*, APGL*, AL2* GL1°, PL1-2°	DPG, PG PGL, GLs	DPG, PE, PG ALs, Ls	PE APGL, PGLs, GLs, PLs
DNA G + C (mol%)	42 - 55	39 - 41[1]	41 - 54[2]	50
fatty acids				
$C_{14:00}$	1,3 (0,7)	3,2	2,6 (1,2)	3,2
anteiso-$C_{15:0}$	2,9 (1,1)	3,4	5,0 (3,2)	3,8
iso-$C_{15:0}$	35,6 (11,9)	22,3	55,1 (7,3)	4,7
$C_{16:00}$	12,4 (7,8)	25	9,8 (4,5)	50,4
iso-$C_{16:0}$	10,3 (4,5)	12,4	3,8 (1,1)	12,4
$C_{16:1}$ ω11c		1,9		
$C_{17:00}$				3,4
anteiso-$C_{17:0}$	9,0 (4,1)	11,8	5,3 (2,1)	10,2
iso-$C_{17:0}$	22,3 (5,0)	17,9	15,0 (5,9)	8,9
$C_{18:00}$	1,3 (3,2)*			2,1
iso-$C_{17:1}$ ω5c	1,2 (2,2)*			
summed feature 3	1,7 (2,0)			

Table 7.4: Characters useful for differentiating between the genera *Geobacillus, Caldibacillus, Aeribacillus* and *Anoxybacillus*. *Only for MSC-members, ° only for non-MSC members. AL, aminolipid; APGL, aminophosphoglycolipid; DPG, diphosphatidylglycerol; GL, glycolipid; L, lipid; PAL, phosphoaminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGL, phosphoglycolipid; PL, phospholipid. [1]Data from Miñana-Galbis *et al.*, 2010. [2]Data from Pikuta (2009). All other data are from this study (strains in Table 7.3). For fatty acid analysis mean values of all profiles included are shown with their standard deviation values in parentheses. Summed feature 3 represents $C_{16:1}$ iso I and $C_{14:0}$ 3OH. Trace amounts (<1%) are not shown.

7.2.3.1 *Geobacillus*

16S rRNA gene sequence analysis revealed a monophyletic cluster of 56 strains assigned to the genus *Geobacillus*. Although interspecies 16S rRNA gene sequence similarities are high in *Geobacillus*, ranging from 96.0% to 99.4%, intraspecies similarities are generally higher, ranging from 99.3% to 100.0%, allowing reliable differentiation of species, supported by high bootstrap values. Nevertheless, the resolution of the 16S rRNA gene is often considered insufficient and

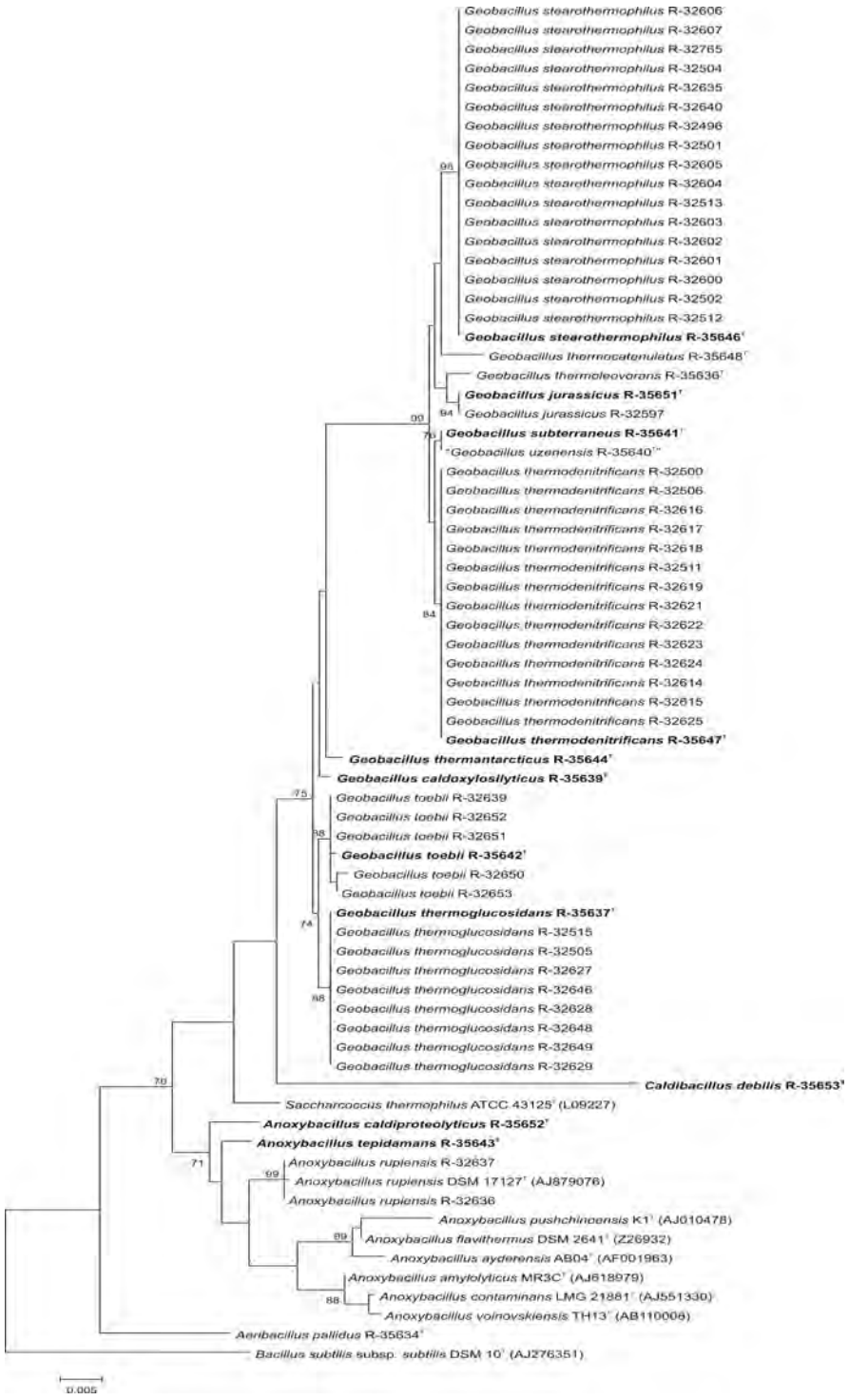


Figure 7.5: ML phylogenetic tree based on 16S rRNA gene sequences of all strains under study and some representatives of closest relatives. Bootstrap values above 69% are given at the branch points. All type strains that have been dealt with in this paper are indicated in bold. The 16S rRNA gene sequence of *Bacillus subtilis* subsp. *subtilis* DSM 10^{T} was choosen as an outgroup to root the tree. The strain of "*G. uzenensis*" DSM 13551^{T} is a strain of *G. subterraneus*.

several other genes were investigated for their applicability to *Geobacillus* taxonomy. The most promising approach so far appears to be *recN* gene sequencing (Zeigler, 2005), and this resulted in exactly the same grouping of *Geobacillus* as obtained with 16S rRNA gene sequencing, but with higher resolution. The reliability of species clusters in Figure 7.5 was therefore further confirmed by comparing all *Geobacillus* strains from Table 7.3 with *Geobacillus* strains identified by Zeigler (2005) based on 16S rRNA and *recN* gene sequencing (data not shown).

Other housekeeping genes investigated for their usability in *Geobacillus* taxonomy were *spo0A* (Kuisiene *et al*., 2009), *recA* (Weng *et al*., 2009), *rpoB* (Meintanis *et al.*, 2008; Weng *et al*., 2009), *gyrB* and *parE* (Tourova *et al*., 2010). Although resolution of these genes was often higher than for 16S rRNA, some of them (such as *spo0A* and *rpoB)* failed to differentiate *Geobacillus* species and others were not universally applicable (such as *gyrB*, *parE* and *recA*) so that only a few species have been analyzed. The resolutions and abilities to differentiate *Geobacillus* species of these genes is graphically presented in Fig 7.6. Although it has been criticized for poor resolution, it can be concluded that the 16S rRNA gene is a good taxonomic marker for *Geobacillus*, and our groupings and conclusion correlate well with other studies using the above-mentioned housekeeping genes.
One monophyletic subgeneric cluster (MSC), harboring the type species *G. stearothermophilus* together with *G. thermocatenulatus, G. thermoleovorans, G. jurassicus, G. subterraneus* and *G. thermodenitrificans* can be differentiated from other *Geobacillus* species and the former *B. thermantarcticus* based on 16S rRNA gene sequencing (Figure 7.5). This distinction was confirmed by some small chemotaxonomic differences in fatty acid and polar lipid composition, as shown in Table 7.4. In general, phenotypic profiles across the genus showed little diversity, so that there are few features characteristic of individual species; the groupings of White and co-workers (1993) also showed rather small margins of separation. FAME profiles were not found to be reliable for species differentiation, and these data are only used in species descriptions. However, FAME analysis does allow a clear distinction of *Geobacillus* members from the other three genera, *Anoxybacillus, Aeribacillus* and *Caldibacillus* (Table 7.4).

Major polar lipids detected within *Geobacillus* species were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). For the minor polar lipids, differences were observed between profiles of MSC members and non-MSC members, as represented by *G. stearothermophilus* R-35646^{T}, *G. thermoleovorans* R-35636^{T}, *G. thermoglucosidans* (formerly *G. thermoglucosidasius*) R-35637^{T} and *G. toebii* R-35642^{T} in Figure 7.7. Whereas MSC members contain an aminophosphoglycolipid (APGL) and a phosphoaminolipid (PAL), the non-MSC members contain a glycolipid (GL). *Geobacillus* species can be easily differentiated from members of *Caldibacillus* by the presence of DPG and PG, from members of *Aeribacillus* by the presence of PE, and from *Anoxybacillus* by the presence of GL (non-MSC members) or PAL and APGL (MSC-members) (Table 7.4. DNA G + C content within the genus varies from 42.4 mol% to 54.5 mol%. Other characteristics for each of the species within the genus are discussed individually below.

Geobacillus stearothermophilus. The original description for *G. stearothermophilus* (Donk, 1920) was based on an unstated number of strains for which few characters were given, and the methods used then are not comparable with current ones. Gordon and co-workers (1973) published a description of 31 strains that they believed to represent "*Bacillus stearothermophilus*", and they included "*B. calidolactis*", *B. kaustophilus* and "*B. thermoliquefaciens*" in the synonymy. Subsequently, the heterogeneity of *B. stearothermophilus* was indicated by wide ranges of DNA compositions and phenotypic properties among strains assigned to it (Walker & Wolf, 1971; Logan & Berkeley, 1984). In the present study, 19 strains were received as *G. stearothermophilus*, and

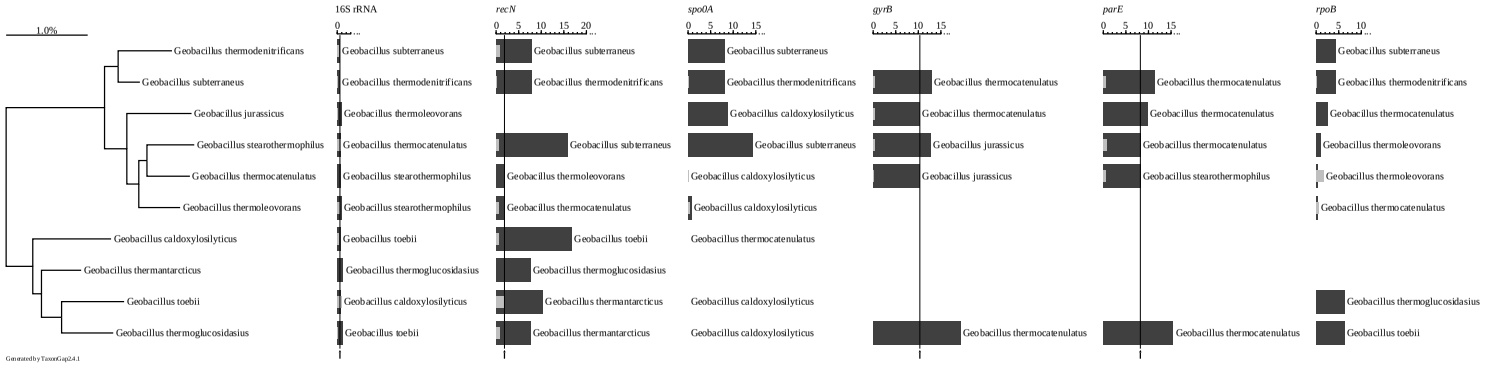


Figure 7.6: Taxonomic resolution of house-keeping genes used for *Geobacillus*, presented by Taxongap software v.4.2.1 (Slabbinck *et al.*, 2008). For each species (represented in the phylogenetic tree at the left), intra- and interspecies variability is represented by grey bars, for each gene separately. Heterogeneity within species is represented as light grey horizontal bars, while species separability is represented as dark grey horizontal bars. Next to the bars, the closest related species is given based on that particular sequence. If length of the light grey bar exceeds length of the dark grey bar, species differentiation is not possible. The greater the length of the dark grey bars, the better the resolution of the gene. All sequences used for this analysis are listed in Table 7.5.

Table 7.5: Overview of all gene sequences used for Taxongap analysis as represented in Figure 7.6.

species	16S rRNA	*recN*	*spo0A*	*gyrB*	*parE*	*rpoB*
Geobacillus thermodenitrificans	FN428639, FN428644, FN428660, FN428661, FN428662, FN428647, FN428663, FN428664, FN428665, FN428666, FN428667, FN538994, FN538995, FN538990, FN538993, AY608961, AY608963, AY608964, AY608977, AY608979, AY608965, AY608968, AY608970, AY608962, AY608967, AY608972, AY608974, AY608978, AY608973, AY608969, AY608976, AY608971, AY608966	AY609044, AY609046, AY609036, AY609043, AY609037, AY609038, AY609040, AY609030, AY609027, AY609031, AY609045, AY609028, AY609029, AY609032, AY609034, AY609033, AY609039, AY609035, AY609041	FJ226599			DQ642081
Geobacillus subterraneus	FN428689, AY608957, AY608958, FN428688	AY609023, AY609026, AY609024, AY609025	FJ226597, FJ226603	GU459230, GU459227, GU459231	GU459242, GU459241, GU459240	DQ642077, DQ642078
Geobacillus jurassicus	FN428697, FN 428651		FJ226593	GU459229, GU459228	GU459243	DQ642075
Geobacillus stearothermophilus	FN428672, FN428642, FN428673, AY608930, AY608933, AY608929, FN428641, FN428649, FN428653, FN428655, FN428656, FN428657, FN428640, FN428633, FN428658, FN428659, AY608927, FN428652, FN428694, AY608932, AY608931, FN428648, FN428654, FN428681	AY608994, AY608996, AY608997, AY608995, AY609000, AY608999, AY608998	FJ226596	GU459238, GU459239	GU994006, GU459249	DQ642073
Geobacillus thermocatenulatus	FN538989, FN428695		FJ226598, FJ226592	GU323952, GU459237	GU459247, GU323953	DQ642064, DQ642074
Geobacillus thermoleovorans	FN428646, FN428638, FN428645, FN428684, FN428686, FN428693, AY608941, AY608942, AY608940, AY608946, AY608939, AY608943	AY609003, AY609013, AY609006, AY609010, AY609001, AY609004, AY609009, AY609007, AY609008, AY609002 AY609005, AY609012	FJ226604, FJ226595, FJ226594, FJ226601,			DQ642068, DQ642065 DQ642067, DQ642063, DQ642069, DQ642071 DQ642062
Geobacillus caldoxylosilyticus	AY608949, AY608950, AY608954, FN428687, AY608952	AY609018, AY609017, AY609016, AY609021, AY609019	FJ226591			

continued on next page . . .

species	16S rRNA	*recN*	*spo0A*	*gyrB*	*parE*	*rpoB*
Geobacillus thermantarcticus	FN428692	AY609047				
Geobacillus toebii	FN 428690, FN428679, AY608987, FN538992, AY608992, FN538991, FN428680, FN538988, AY608983	AY609059, AY609049, AY609054, AY609050	FJ226602			DQ642061
Geobacillus thermoglucosidans	AY608989, AY608990, AY608991, FN428643, FN428676, FN428669, FN428677, AY608984, FN428678, FN428685, FN428670, AY608985, AY608986, AY608988, AY608993, FN428650, FN428668	AY609048, AY609051, AY609055, AY609056, AY609060, AY609052, AY609057, AY609058, AY609053	FJ226600	GU323951	GU459246	EU484373

18 of those were confirmed as belonging to that species on the basis of 16S rRNA gene sequence analysis (Table 7.3). The remaining strain, R-32639, was a member of *G. toebii* and showed high phenotypic similarities with *Geobacillus toebii* strains R-32650, R-32653 and R-32651 (Figure 7.8). Thirteen strains showed high phenotypic similarities (91% S_G) to the type strain R-35646^T, again supporting their allocation to *G. stearothermophilus,* yet in contrast to the original description of Donk (1920), they were found to hydrolyse gelatin but not produce acid from lactose. The remaining four strains (R-32640, R-32765, R-32635 and R-32504) were recovered in other parts of the phenogram (Figure 7.8), but 16S rRNA gene sequence similarities above 99.4% (Figure 7.5) and DNA relatedness values above 70% (Table 7.6) clearly showed these were all strains of *G. stearothermophilus*; nonetheless R-32504 lies at the margin of the species with a DNA relatedness value of only 66.4% with the type strain R-35646^T.

	R-32640	R-32765	R-32635	R-32504	R-35646^T	R-32649	R-35637^T	R-32653	R-35642^T	R-35652^T	DSM 17127^T	R-32636	R-32637	R-35643
R-32640	100													
R-32765	99.4	100												
R-32635	85.8	87.3	100											
R-32504	71.3	70.1	67.2	100										
R-35646^T	77.7	72.3	72.4	66.4	100									
R-32649						100								
R-35637^T						89.2	100							
R-32653								100						
R-35642^T								89.9	100					
R-35652^T										100				
DSM 17127^T										29.7	100			
R-32636										28.5	93.4	100		
R-32637										24.6	97.8	90.7	100	
R-35643										33.6	37.9	27.7	23.7	100

Geobacillus stearothermophilus

Geobacillus thermoglucosidasius

Geobacillus toebii

Anoxybacillus

Table 7.6: DNA relatedness values between representatives of strains under study. Values represented here (%) are mean values of two reciprocals. Values above 70%, and thus representing the same species, are indicated in grey.

Geobacillus thermodenitrificans. This species was described by Ambroz (1913) as *Denitrobacterium thermophilum,* was renamed *Bacillus thermodenitrificans* by Mishustin (1950), and included in the Approved List of Bacterial Names (Skerman *et al.*, 1980). Manachini and co-workers (2000) emended the species description based on 10 strains, and Nazina and co-workers (2001) transferred it to *Geobacillus* on the basis of 16S rRNA gene sequence comparisons and DNA relatedness. Although all 15 of our strains received as *G. thermodenitrificans* could be confirmed in their identities, clustering with the type strain R-35647^T at 99.7% S_P in the 16S rRNA gene sequence analysis, only seven clustered closely with the type strain in the phenotypic analysis of 14 strains (Figure 7.8). Of the remainder, two strains (R-32619 and R-32621) clustered at 87.5% S_G with the type strain, three (R-32506, R-32617 and R-32625) joined with *G. jurassicus* at 86% S_G and one (R-32615) remained unclustered. This implied that phenotypic description would be difficult, but careful examination of profiles showed variations in strength of reaction rather than substantial pattern differences. All strains were found to be negative for acid production from galactose and lactose, in contrast to the results of Manachini and co-workers (2000).

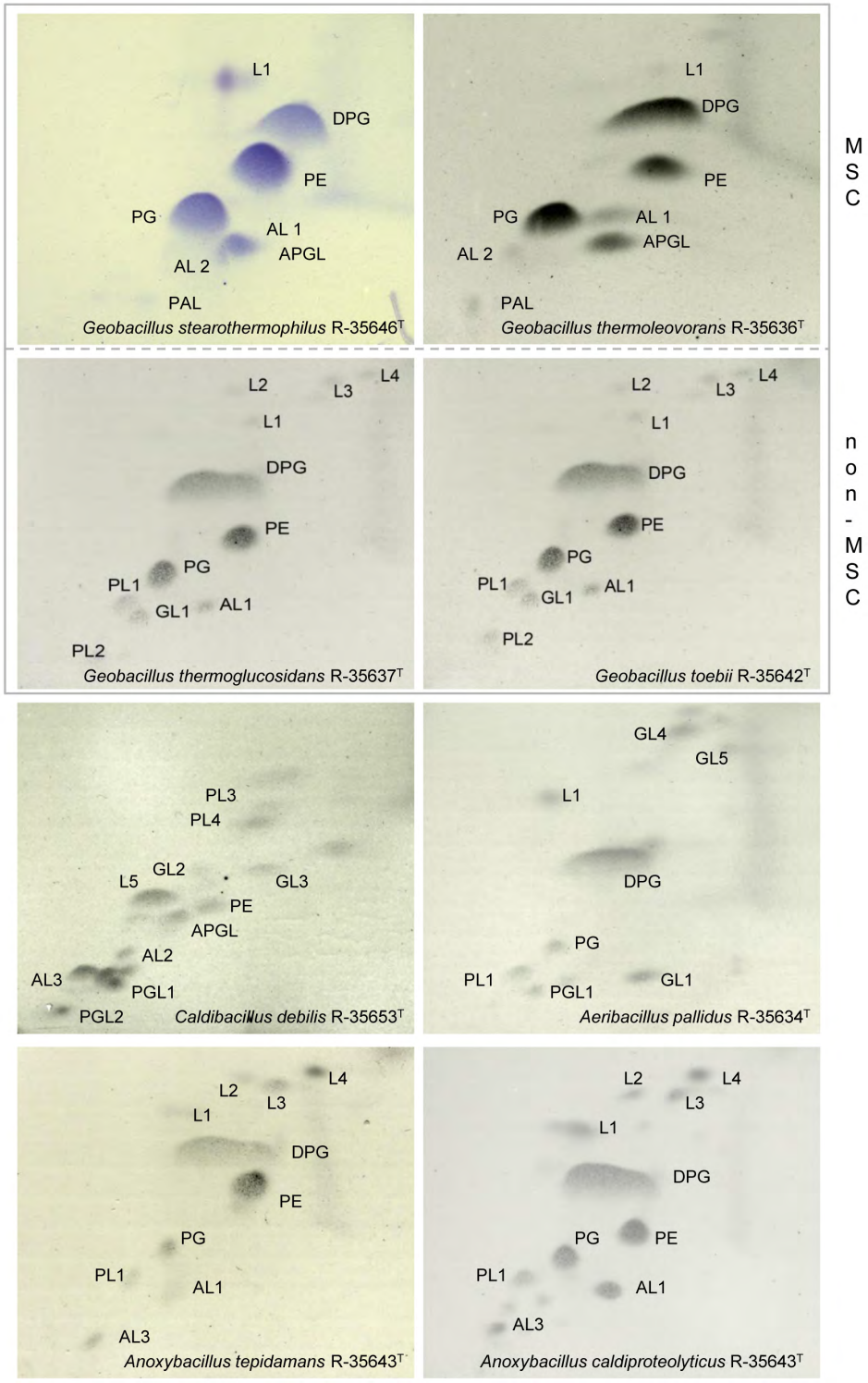


Figure 7.7: Polar lipid profiles of representative strains for the genera *Geobacillus, Anoxybacillus, Aeribacillus* and *Caldibacillus*. Lipids were visualized by staining with molybdatophosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, aminolipid; PAL, phosphoaminolipid; APGL, aminophosphoglycolipid; L: lipid; GL, glycolipid; PGL, phosphoglycolipids.

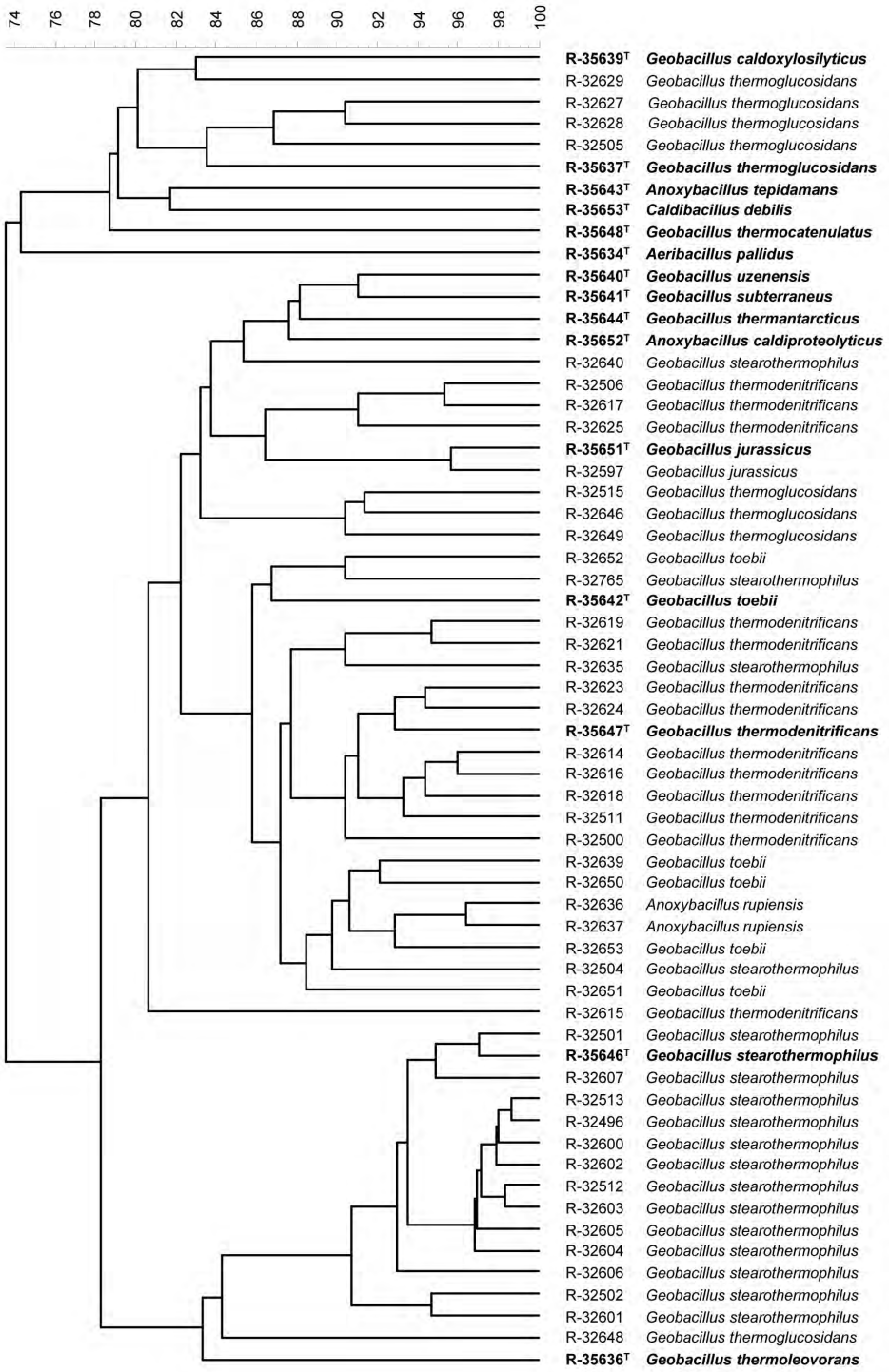


Figure 7.8: Phenogram based upon the UPGMA clustering of similarity coefficients (S_G) of 115 phenotypic characters of *Aeribacillus*, *Anoxybacillus, Caldibacillus,* and *Geobacillus* strains.

Geobacillus uzenensis. This species was proposed in 2001 (Nazina *et al.*, 2001) but it appears the strain deposited as the type of *G. uzenensis* in DSMZ (DSM 13551^T), and studied in the present work, is not the authentic strain U^T, but actually a strain of *G. subterraneus*. This was confirmed by 100% 16S rRNA gene pairwise sequence similarity and complete DNA relatedness between strain R-35640^T (DSM 13551^T) and strain R-32641^T (DSM 13552^T).

Geobacillus subterraneus. This species was also proposed by Nazina and co-workers (2001). We provide additional characters for the type strain of *G. subterraneus* in Table 7.7.

Geobacillus jurassicus. This species was proposed in 2005 (Nazina *et al.*, 2005) on the basis of two isolates, both of which (R-35651^T and R-32597) were included in our study. They showed similar and distinct phenotypic profiles (96% S_G, Figure 7.8), and the closest phenotypic profile was that of three *G. thermodenitrificans* strains, but a number of characteristics allow distinction of these species (Table 7.8).

Geobacillus thermoglucosidasius. This species was proposed on the basis of six strains (Suzuki *et al.*, 1983), and it was transferred to *Geobacillus* without its description being emended (Nazina *et al.*, 2001). Of the thirteen strains received as *G. thermoglucosidasius* only three (R-32627, R-32628 and R-32505) showed appreciable phenotypic similarity (84% S_G) with the type strain R-35637^T (Figure 7.8). These strains and R-32515, R32646, R-32649, R-32629 and R-32648 clustered with the type strain at above 99.9% S_P in 16S rRNA gene sequence comparisons (Figure 7.5). One representative, R-32649, showed 89.2% DNA relatedness to the type (Table 7.6), and so all eight strains may be identified as *G. thermoglucosidasius*. Strains R-32515, R-32649 and R-32646 differed from R-32505, R-32627, R-32628 and R-35637^T in giving negative results for aesculin hydrolysis and for acid production from amygdalin, arbutin, gentiobiose, salicin and sorbitol. In contrast with Suzuki and co-workers (1983), but in agreement with Nazina and co-workers (2001), all *G. thermoglucosidasius* strains in the present study hydrolysed gelatin. Four other strains received as *G. thermoglucosidasius*, R-32650, R-32651, R-32652 and R-32653, were identified as *G. toebii* (see below). We propose an emended description of *G. thermoglucosidasius* with correction of the name to *G. thermoglucosidans.*

Saccharococcus caldoxylosilyticus was described in 2000 (Ahmad *et al.*, 2000) and transferred to *Geobacillus* in 2001 (Fortina *et al.*, 2001). The type strain R-35639^T was the only strain available and had no near neighbours in the phenotypic analysis (Figure 7.8); its closest relative in 16S rRNA gene sequence analysis was *G. toebii,* ranging from 98.5 to 99.2% S_P. Our data confirm the distinction of the species, and additional characters for the type strain are provided in Table 7.7.

Geobacillus toebii. The type strain (R-35642^T) of this single-strain species (Sung *et al.*, 2002) clustered at over 99.4% S_P in 16S rRNA gene sequence comparisons with a strain (R-32639) received as *G. stearothermophilus*, and with four strains (R-32650, R-32651, R-32652, R-32653) received as *G. thermoglucosidasius* (Figure 7.5). DNA relatedness between R-35642^T and R-32653 was 89.9% (Table 7.6), confirming that they are members of the same species. In the phenotypic analysis R-35642^T only clustered loosely with these five other strains at about 86% S_G, and so the species does not have a distinct phenotypic profile (Figure 7.8). The description of *G. toebii* is now based upon six strains, and is emended accordingly. "*Geobacillus toebii* subsp. *decanicus*", isolated from compost (Poli *et al.*, 2006b) has not been validated, and was not included in our study.

Bacillus thermantarcticus was proposed (with the name "*Bacillus thermoantarcticus*") (Nicolaus *et al.*, 1996) on the basis of a single strain. Zeigler (2005) recommended, on the basis of *recN* and 16S rRNA gene sequences, that this species should be transferred to the genus *Geobacillus*,

character	*Aeribacillus pallidus* LMG 19006^T	*Anoxybacillus rupiensis* R-32636, R-32637	*Geobacillus subterraneus* LMG 23035^T	*Geobacillus caldoxylosilyticus R-35639*^{T}
acid from				
N-acetylglucosamine	-	-	-	-
adonitol	+	-		-
amygdalin	+	-	-	+
D-arabinose	-	-		-
L-arabinose	-	+		
arbutin	+	-	-	+
D-arabitol	+	-	-	-
L-arabitol	-	-	-	-
D-cellobiose	-	-		
dulcitol	-	-	-	-
erythritol	-	-	-	-
D-fructose	+	+		
D-fucose	-	-	-	-
L-fucose	w	-	-	-
galactose	-	-		
gentiobiose	-	-	-	+
D-glucose	+	+		
gluconate	-	-	-	-
glycerol	+	-		w
glycogen	-	w	+	
meso-inositol	+	-	-	-
inulin	-	-	-	-
2-keto-D-gluconate	-	-	-	-
5-keto-D-gluconate	-	-	-	-
lactose	-	-	-	
D-lyxose	-	-	-	-
maltose	+	+		
mannitol	-	-	+	-
D-mannose	+	+		+
D-melibiose	-	-	-	+
D-melezitose	-	-	+	-
methyl-D-glucoside	+	-	-	w
methyl-D-mannoside	-	-		-
methyl-xyloside	-	-	-	-
D-raffinose	-	-		
rhamnose	w	-		
ribose	+	+	-	
salicin	+	-	+	+
sorbitol	+	-		-
L-sorbose	w	-	-	-
starch	+	w	+	

character	*Aeribacillus pallidus* LMG 19006^T	*Anoxybacillus rupiensis* R-32636, R-32637	*Geobacillus subterraneus* LMG 23035^T	*Geobacillus caldoxylosilyticus R-35639*^{T}
sporangia				
ellipsoidal spores		+		
sporangia swollen		-		
spores subterminal		+		
spores terminal		+		
anaerobic growth			+	w
aesculin hydrolysis		-		+
starch hydrolysis		+		
oxidase		w		+
ONPG	-	-	-	
arginine dihydrolase	-	-		-
lysine decarboxylase	-	-	-	-
ornithine decarboxylase	-	-	-	-
citrate	-	-	-	
H_2S production	-	-	-	-
urease	-	-	-	
tryptophan deaminase	-	-	-	-
indole	-	-	-	
Voges-Proskauer	w	-/w		
gelatin hydrolysis	-	v		
growth at				
pH 6	-			
pH 7	+			
pH 8	+			
pH 9	+			
growth at				
30°C	-			
37°C	+			
65°C	+			
70°C	-			
growth in				
1% NaCl (w/v)	+	w		
5% NaCl (w/v)	-	-		
acid from				
sucrose	+	w		
D-tagatose	-	-	-	-
D-trehalose	+	+	+	
D-turanose	w	-	-	+
xylitol	+	-	-	-
D-xylose	-	+	+	
L-xylose	-	-	-	

Table 7.7: Additional characters for *Aeribacillus pallidus, Geobacillus subterraneus*, *Geobacillus caldoxylosilyticus* and *Anoxybacillus rupiensis*. The major fatty acids for *Anoxybacillus rupiensis* R-32636, R32637 measured at 55°C were iso-$C_{15:0}$ (58 - 64% of total), iso-$C_{17:0}$ (27 - 33%), with small proportions of $C_{16:0}$ (3%) and anteiso-$C_{17:0}$ (2 - 3%). Characters shown are additional to those already published in original descriptions of species. Symbols: +, > 85% strains give positive reaction; -, < 15% of the strains give positive reaction; w, weak reaction; -/w, negative or weakly positive reaction; v, results varies.

with further analysis being required to determine whether it be a novel species, or a subspecies of *G. thermoglucosidasius*. In our analysis, *B. thermantarcticus* R-35644^T did not show a close relationship with the type strain of *G. thermoglucosidasius* (now *G. thermoglucosidans*) in 16S rRNA gene sequence or phenotypic analyses, implying it should indeed be transferred to the genus *Geobacillus* as the separate species *Geobacillus thermantarcticus*.

7.2.3.2 *Caldibacillus debilis* gen. nov., comb. nov

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Only the type strain of *G. debilis* (R-35653^T) was available for study. It did not grow well below 60°C, and even at or above this temperature it often grew poorly, with cells appearing distorted and stressed. The phenotypic characters were therefore tested on several occasions, in order to be confident of consistent and accurate results. The phenotypic profile was distinct, and this strain did not show a significant similarity with any other species in the study (Table 7.8, Figure 7.8). The 16S rRNA gene sequence did not imply a strong relationship with other *Geobacillus* strains either, the closest being members of *G. thermoglucosidans* with similarities between 93.3 and 93.6%. The polar lipid profile of strain R-35653^T showed remarkable differences with the profiles of members of *Geobacillus* (Table 7.4, Figure 7.7), as the major polar lipids DPG and PG were absent. Instead, two phosphoglycolipids (PGL), two glycolipids (GL), and two phospholipids (PL) were detected. The fatty acid pattern was also quite different from those of *Geobacillus* species (Table 7.4) with $C_{16:00}$ as the major fatty acid (50.4%). We conclude that *Geobacillus debilis* represents a genus separate from *Geobacillus*, and we supplement the description given by Banat and co-workers (2004) in our proposal to transfer *G. debilis* to the novel, single-species genus *Caldibacillus*.

7.2.3.3 *Aeribacillus pallidus*

Our data support the proposal that *Geobacillus pallidus* be reclassified as *Aeribacillus pallidus* (Minana-Galbis *et al.*, 2010), and we provide additional characters based on extensive analysis of strain R-35634^T (Table 7.7). Polar lipid profile of strain R-35634^T is provided in Figure 7.7, and matches the profile described by Miñana-Galbis and co-workers (2010), with DPG, PG as major polar lipids. It can be easily differentiated from *Geobacillus, Caldibacillus* and *Anoxybacillus* by the absence of PE (Table 7.4). It should also be noted that the name *Bacillus pallidus* was recently proposed for a mesophilic soil organism (Zhou *et al.*, 2008), but as this is a homonym of the validly published *Bacillus pallidus* Scholz *et al.*, 1988, it was renamed *Falsibacillus pallidus* (Zhou *et al.*, 2009).

7.2.3.4 *Anoxybacillus tepidamans* comb. nov. and *Anoxybacillus caldiproteolyticus* sp. nov.

The type strain of *Geobacillus tepidamans* (R-35643^T) and the strain received as "*Geobacillus caldoproteolyticus*" (R-35652) clustered together in 16S rRNA gene sequence analysis, and were recovered in a group harboring *Anoxybacillus* species. Overall, they showed higher similarities with members of the genus *Anoxybacillus* than with any of the species in *Geobacillus*. Their respective G + C values of 43.2 and 40.2 mol% are consistent with the range of 41.6 - 44.4 mol% seen for most other species in *Anoxybacillus*. The first-reported DNA G + C values for *A. kestanbolensis, A. ayderensis* and *A. gonensis* appear to be rather high for this genus, at 50.0, 54.0 and 57.0 mol%

respectively, but Kevbrin and co-workers (2005) gave a redetermined value of 42.8 mol% for *A. gonensis*. However, later, a G + C value of 53.5 mol% was reported for *A. thermarum* (Poli *et al.*, 2009), and this species clustered closely with *A. kestanbolensis, A. ayderensis* and *A. gonensis* in their 16S rRNA gene sequence analysis. The polar lipid profiles for both strains, R-35643^T and R-35652, were very similar with DPG, PG and PE as the major polar lipids (Figure 7.7); two aminolipids, a phospholipid and three new lipids were also detected. These findings correlate well with the profile given for *Anoxybacillus pushchinoensis* (Miñana-Galbis *et al.*, 2010). Other polar lipid profiles for *Anoxybacillus* species also show DPG, PG and PE as the major polar lipids, although *Anoxybacillus thermarum* lacks PG (Poli *et al.,* 2006a; Gul-Guven *et al.*, 2008; Poli *et al.*, 2009). Fatty acid profiles of R-35643^T and R-35652 were consistent with the fatty acid composition of other *Anoxybacillus* species, with iso-$C_{15:00}$, iso-$C_{17:00}$ and $C_{16:00}$ as the major fatty acids (De Clerck *et al.*, 2004). The phenotypic profile of *G. tepidamans* R-35643^T was distinct (Table 7.8) and it did not cluster with any other of the type strains analysed in the phenotypic analysis (Figure 7.8). We supplement the description given by Schäffer and co-workers (2004) with further phenotypic data in our description of the new combination *Anoxybacillus tepidamans*. The name "*Geobacillus caldoproteolyticus*" was proposed for a single, sewage sludge isolate (Chen *et al.*, 2004), but was not validly published. The phenotypic profile (Table 7.8) of strain R-35652^T was distinct from that of *G. tepidamans* and other *Anoxybacillus* species and it showed low DNA relatedness with *A. rupiensis* (DSM 17127^T) (Table 7.6). These data demonstrate conclusively that this strain should be classified as a new species in the genus *Anoxybacillus,* for which the name *Anoxybacillus caldiproteolyticus* is proposed. It was earlier reported that strains R-32636 and R-32637, isolated from near Lake Taupo, New Zealand and received as *Geobacillus kaustophilus,* most probably belonged to *Anoxybacillus rupiensis* (Dinsdale *et al.*, 2011). DNA relatedness values confirmed this (Table 7.6), and phenotypic data for these two strains for characters that were not included in the original description of this species are given in Table 7.7.

7.2.4 Descriptions

Characters useful for differentiating the species are shown in Table 7.8.

7.2.4.1 Emended description of *Geobacillus* Nazina *et al.,* 2001

Geobacillus (Ge.o.ba.cil'lus. Gr. n. *Ge* the Earth; L. dim. n. *bacillus* small rod; N.L. masc. n. Geobacillus, earth or soil small rod).

Obligate thermophiles. Vegetative cells are rod-shaped and produce one endospore per cell. Cells occur either singly or in short chains and are motile by means of peritrichous flagella or they are non-motile. The cell wall structure is Gram-positive, but the Gram-stain reaction may vary between positive and negative. Ellipsoidal endospores are located subterminally or terminally in non-swollen or slightly swollen sporangia. Colony morphology and size are variable. Chemoorganotrophs. Aerobic or facultatively anaerobic, but anaerobic growth may be weak. Oxygen is the terminal electron acceptor, replaceable in some species by nitrate. The temperature range for growth is 30 to 80°C, with an optimum at 50 - 60°C. Neutrophilic. Growth occurs within a pH range of 5.0 to 9.0, with optima between pH 6.2 and 7.5. Growth factors, vitamins, NaCl and KCl are not required by most species. Many species can utilize *n*-alkanes as carbon and energy sources. Most species produce acid but no gas from fructose, glucose, maltose, mannose, sucrose and trehalose. Most species do not produce acid from lactose and gentiobiose. Produce

	G. stearothermophilus	*G. jurassicus*	*G. subterraneus*	*G. thermoleovorans*	*G. thermocatenulatus*	*G. thermodenitrificans*	*G. uzenesis*	*G. thermoglucosidans*	*G. caldoxylosilyticus*	*G. thermantarcticus*	*G. toebii*	*A. caldiproteolyticus*	*A. rupiensis*	*A. tepidamans*	*G. debilis*	*Aeribacillus pallidus*
n	15	2	1	11	2	9	2	8	1	1	6	1	2	1	1	1
sporangia																
cylindrical spores	-	-	-	-	d	-	-	d	v	-	-	+	-	-	+	v
sporangia swollen	v	v	-	-	-	-	d	d	+	+	+	-	-	+	+	+
spores subterminal	+	+	+	+	d	+	-	+	-	+	-	+	+	-	-	+
spores terminal	+	-	v	+	+	+	+	+	+	+	+	+	+	+	+	v
spores central/paracentral	-	-	-	-	d	-	-	-	+	-	-	-	-	-	-	+
Hydrolysis of																
aesculin	+/w	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+
casein	+/w	+	-	d	-	-	-	-	v	-	-	+	+	-	w	-
gelatin	+	+	w	+	+	d	+	+	+	+	+	+	d	-	+	-
ONPG	-	-	-	d	-	-	-	-	-	-	-	-	-	+	+	-
starch	+	+	+	+	-	+/w	+	d (w)	+	-	+/w	+/w	+	+	-	w
catalase	+	+	+	+	+	+		+	+	+	+	+	+	+	-	+
oxidase	-	+	-	+	+	-		w	+	+/w	+	+	w	-	+	+
nitrate reduction	d	+	+	+	d	+/d	+	d	+	-	+	-	-	-	-	-
Voges-Proskauer	+	-	+	d (w)	+	+/d	-	d	-	w	v	+	w/-	+	w/-	+/w
Acid from																
N-acetylglucosamine	-	-	-	d	d	-	-	+	-	-	-	+	-	+	+	-
amygdalin	-	-	-	-	+	-	-	d	+	-	-	-	-	+	+	+
L-Arabinose	-	+	-	-	-	d (w)	+	d	+	-	-	-	+	+	-	-
arbutin	-	-	-	-	+	-	w	d	+	-	-	v	-	+	w	+
D-Cellobiose	-	+	+	d	+	+	+	+	+	+	-	+	-	+	+	-
galactose	w	+	+	d	+	-	+	-	+	w	+	+	-	+	+	-
gentiobiose	-	-	-	-	-	-	-	d	+	-	-	-	-	+	+	-
glycerol	w	+	+	+	+	-	+	w	w	w	-	+	-	+	-	+
glycogen	+	-	+	d	-	-	+	-	+	-	-	+	w	-	-	-
lactose	-	-	-	d (w)	-	-	-	w	+	-	-	-	-	+	+	-
meso-inositol	-	-	+	d	-	-	-	-	-	-	-	-	-	+	-	+
mannitol	-	+	+	d	+	w	+	+	-	-	w	+	-	-	-	-
D-Melezitose	+	+	+	d (w)	+	d (w)	-	-	-	-	-	-	-	+	-	-
D-Melibiose	+	-	-	d	+	d	-	-	+	-	d	-	-	+	-	-
Methyl-D-glucoside	+	+	-	d	d	-	+	+	w	+	-	-	-	+	-	+
D-Raffinose	+	-	-	d	+	-	-	-	+	-	-	-	-	+	-	-
ribose	-	+	-	+	+	w	-	+	+	-	d	+	+	w	-	+
salicin	-	-	+	w	+	d	+	+	+	+	-	+	-	+	+	+
sucrose	+	+	+	d	+	d	+	+	+	+	d	+	w	+	-	+
D-Trehalose	+	+	+	d	+	d	+	+	+	+	+	+	+	+	+	+
D-Turanose	+	+	-	-	-	-	-	+	+	+	-	w	-	+	w	w
D-Xylose	-	+	+	d	-	w	-	+	+	+	-	+	+	+	w	-
anaerobic growth	w/-	w	+	w	w	+	+	+	+	-	+	-	-	+	-	+
growth at pH 5	-	+	-	w	+	-	-	-	-	-	-	+	-	-	-	-
growth at pH 9	+	+	-	w	+	+	-	w	+	+	+	+	-	+	+	+
minimum growth temperature °C	30-45	45	37	37-40	37	50	45	40	50	37	37	37	35	40	50	37
maximum growth temperature °C	60-70	65	60	70	80	70	65	<60	70	80	70	70	67	65	70	65
optimum temperature (°C)	40-60	60	55-60	60	60	50	55-60	50	50-65	60	60	60	55	55	65	50
growth in 1% NaCl (w/v)	+	+	+	d	+	-	+	-	-	+	+	-	+	+	+	+
growth in 5% NaCl (w/v)	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-

Table 7.8: Characters useful for differentiating between *Geobacillus* (*G.*) species, *Caldibacillus* (*C.*), *Aeribacillus* and some *Anoxybacillus* (*A.*) species. Data for *G. uzenensis* were taken from Logan *et al.* (2009); all other data were obtained in the course of the present study or were taken from Dinsdale *et al.* (2011). No entry indicates the data are not available. All strains studied were motile, formed ellipsoidal spores, and produced acid from fructose, glucose, maltose and mannose. Symbols: +, > 85% strains give positive reaction; -, < 15% of the strains give positive reaction; +/w, positive or weakly positive reaction; w, weak reaction; w/-, weak or negative reaction; +/d, usually positive, but different strains give different reactions; d, different strains give different reactions; d (w), different strains give different reactions, but positive reactions are weak; v, result varies.

catalase. Oxidase reaction varies. Phenylalanine is not deaminated, tyrosine is not degraded, indole is not produced, and the Voges-Proskauer reaction varies between species. Aesculin and gelatin are hydrolysed, starch is hydrolysed by most species, casein is hydrolysed by some species, and ONPG is rarely hydrolysed. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0}$, which make up more than 60% of the total. Major polar lipids for members of this genus are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminolipids and an aminophosphoglycolipid. The main menaquinone type is MK-7. The mol% G + C of the DNA of the species ranges from 48.4 to 54.5. The type species is *Geobacillus stearothermophilus* and the type strain is LMG 6939^T.

7.2.4.2 Emended description of *Geobacillus stearothermophilus* (Donk, 1920) Nazina *et al.*, 2001 (*Bacillus stearothermophilus* Donk, 1920)

Geobacillus stearothermophilus (ste.a.ro.ther.mo'phi.lus. Gr. n.. *stear* fat; Gr. n. *therme* heat; Gr. adj. *philos* loving. N.L. adj. heat and fat loving).

Gram-positive, -negative and -variable, motile rods, 2 to 3.5 by 0.6 to 1.0 μm, occurring singly or in short chains. Ellipsoidal spores are located subterminally and/or terminally within sporangia that may be swollen. Colonies on TSA are circular and 0.5 - 2 mm in diameter after 24 - 48 hours at 60°C, usually convex, and may be smooth-surfaced and with crenate edges. Grows weakly if at all in the absence of oxygen. Minimum growth temperature 30 to 45°C, maximum 60 to 70°C; most strains grow between 40 and 60°C. Growth occurs at pH 6 and pH 9. Tolerates 1% but not 5% NaCl. Aesculin, casein, gelatin and starch are hydrolysed, but aesculin and casein reactions may be weak. Oxidase negative. Gelatin is hydrolysed and the Voges-Proskauer test is positive; nitrate reduction is variable, and arginine dihydrolase, citrate utilization (Simmons'), hydrogen sulphide production, indole production, lysine decarboxylase, ornithine decarboxylase, ONPG (*ortho*-nitrophenyl-β-galactosidase), tryptophan deaminase, and urease production are negative. Acid without gas is produced from D-fructose, D-glucose, glycogen, maltose, D-mannose, D-melezitose, D-melibiose, methyl-D-glucoside, D-raffinose, starch, sucrose, D-trehalose, and D-turanose; and acid is produced weakly from galactose and glycerol. Acid production is variable between strains for inulin and methyl-D-mannoside, and positive reactions are often weak. No acid is produced from *N*-acetylglucosamine, adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, 2- and 5-keto-D-gluconate, lactose, D-lyxose, mannitol, *meso*-inositol, methyl-xyloside, rhamnose, ribose, salicin, sorbitol, L-sorbose, D-tagatose, D-xylose, L-xylose and xylitol. In the variable reactions listed above, the type strain does not have swollen sporangia, it reduces nitrate, produces acid from methyl-D-mannoside, and produces acid weakly from inulin. The polar lipid profile of the type strain consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-ethanolamine, aminolipids and an aminophosphoglycolipid. Hydrocarbons (C_{10}, C_{11}) may be used as carbon and energy sources (Nazina *et al.*, 2001). The major fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0}$, which comprise more than 60% of the total fatty acids. The DNA G+C content of the type strain LMG 6939^T (= DSM 22^T = ATCC 12980^T = R-35646^T) is 52.8 mol% (by HPLC). Originally isolated from deteriorated canned corn and beans; also isolated from evaporated milk, sugar beet juice from extraction installations, hot springs, hydrothermal vents, petroleum reservoirs and soil.

7.2.4.3 Emended description of *Geobacillus jurassicus* Nazina *et al.*, 2005

Geobacillus jurassicus (ju.ras.si'cus, N.L. masc. adj. *jurassicus,* of Jurassic, referring to the geological period of the oil-bearing formation from where the strains were isolated.

Gram-positive rods, motile by peritrichous flagella, and producing subterminally located ellipsoidal spores in slightly swollen sporangia. Description is based upon two isolates. On nutrient agar colonies are round, mucoid, colourless and glossy, and reach a diameter of about 2 mm after 24 - 48h of incubation. Aerobic and chemoorganotrophic; anaerobic growth is weak. Oxidase positive. Grows between 45 and 65°C, with optimum growth at 58 - 60°C. Grows at pH 5 and pH 9, with optimum growth at pH 7.0 - 7.2. Can tolerate 5 - 5.5% (w/v) NaCl; grows weakly in the presence of up to 7% NaCl (w/v). Can grow in nutrient broth and on potato agar. Produces NH_3 from peptone. Does not grow autotrophically on H_2+CO_2. Aesculin, casein, gelatin and starch are hydrolyzed. Reactions for ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulphide production, urease, tryptophan deaminase, indole, Voges–Proskauer, tyrosine degradation, dihydroxyacetone production, egg-yolk, and the methyl red test are negative. Phenylalanine is not deaminated. Fe^{2+} is not used as an electron acceptor. Nitrate is reduced to nitrite. Acid but no gas is produced from the following: L-arabinose, D-cellobiose, D-fructose, galactose, D-glucose, glycerol, maltose, mannitol, D-mannose, D-melezitose, methyl-D-glucoside, ribose, starch (weak), sucrose, D-trehalose, D-turanose, and D-xylose. No acid is produced from: *N*-acetylglucosamine, adonitol, amygdalin, D-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycogen, *meso*-inositol, inulin, 2- and 5-keto-D-gluconate, D-lyxose, D-melibiose, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, salicin, sorbitol, L-sorbose, D-tagatose, xylitol and L-xylose. The following are utilized as carbon and energy sources: hydrocarbons (C_6, C_{10}, C_{11}, C_{14} and C_{16}), methane-naphthenic oil, acetate, butyrate, pyruvate, lactate, benzoate, fumarate, succinate, malate, ethanol, peptone, tryptone, and yeast extract. Cannot grow on methanol, propanol, butanol, *iso*-butanol, phenol, phenylacetate, alanine, glutamate, serine, formate, propionate. Poor growth is observed on asparagine and glutamine. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. The G+C content of DNA is 53.8 - 54.5 mol% and 54.5 mol% for the type strain LMG 23069^T (= DSM 15726^T = VKM B-2301^T = R-35651^T). Isolated from the formation water of the high-temperature Dagang oilfield, China.

7.2.4.4 Emended description of *Geobacillus thermodenitrificans* (Manachini *et al.*, 2000) Nazina *et al.*, 2001 (*Bacillus thermodenitrificans* (*ex* Klaushofer & Hollaus 1970) Manachini *et al.* 2000, "*Denitrobacterium thermophilum*" Ambroz, 1913; "*Bacillus thermodenitrificans*" Mishustin, 1950; "*Bacillus thermodenitrificans*" Klaushofer & Hollaus, 1970)

Geobacillus thermodenitrificans (ther.mo.de.ni.tri'fi.cans. Gr. n. *therme* heat; N.L. part. adj. *denitrificans* denitrifying; N.L. adj. *thermodenitrificans* thermophilic denitrifying, referring to two of the organism's features).

Gram-positive, motile, straight rods 0.5 to 1 by 1.5 to 2.5 μm. Endospores are ellipsoidal, lie subterminally and/or terminally and do not distend the sporangium. Facultatively anaerobic. Colonies on trypticase soy agar are flat with irregular margins, off-white to beige in colour, with non-glossy surfaces; they show some motility and are 1 to 4 mm in diameter after incubation at 60°C for 24 h. Growth occurs between 50 and 70°C with optimum at 50°C. Grows at pH

6 and 9 but not at pH 5; optimum is pH 7. Tolerates 0.5% but not 1% NaCl. Oxidase negative. Aesculin and starch are hydrolysed, the latter sometimes only weakly; and casein is not hydrolysed. Nitrate reduction and the Voges-Proskauer test are variable, but usually positive, gelatin hydrolysis is variable, and arginine dihydrolase, citrate utilization, hydrogen sulphide production, indole production, lysine decarboxylase, ornithine decarboxylase, ONPG, tryptophan deaminase and urease production are negative. Acid without gas is produced from D-cellobiose, D-fructose, D-glucose, maltose, D-mannose; acid is produced weakly from mannitol, ribose, starch, and D-xylose. Acid production is variable between strains from D-melibiose, salicin, sucrose and D-trehalose, and variable, but weak when positive, from L-arabinose and D-melezitose. No acid is produced from *N*-acetylglucosamine, adonitol, amygdalin, arbutin, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, galactose, gentiobiose, gluconate, glycerol, glycogen, inulin, 2- and 5-keto-D-gluconate, lactose, D-lyxose, *meso*-inositol, methyl-D-glucoside, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, D-tagatose, sorbitol, L-sorbose, D-turanose, L-xylose, and xylitol. In the variable reactions listed above, the type strain gives positive reactions for gelatin hydrolysis, nitrate reduction and the Voges-Proskauer test; acid is produced from L-arabinose, D-melibiose, sucrose and D-trehalose; and produced weakly from salicin and D-melezitose. The main fatty acids of this species are iso-$C_{15:0}$and iso-$C_{17:0}$, which account for over 66 to 69% of the total; the minor acids are $C_{16:0}$, iso-$C_{16:0}$and anteiso-$C_{17:0}$. The DNA G + C content of the type strain LMG 17532^{T} (= ATCC 29492^{T}= DSM 465^{T} = R-35647^{T}) is 48.4 mol% (by HPLC). Originally isolated from soil; also isolated from sugar beet juice from extraction installations, hot compost and hydrothermal vents.

7.2.4.5 Emended description of *Geobacillus thermoglucosidans* nom. corrig.

Geobacillus thermoglucosidans (ther.mo.glu.co'si.dans Gr. n. *therme* heat; N.L. part. adj. *glucosidans* glucosidating, *i.e.* releasing glucoside units; N.L. part. adj. *thermoglucosidans* indicating the production of heat-stable glucosidase).

Basonym: *Bacillus thermoglucosidasius* Suzuki *et al.*, 1984

Gram-positive, motile rods 0.5 to 1.2 by 3.0 to 7.0 μm. Short ellipsoidal to cylindrical endospores are borne subterminally and terminally and occasionally swell the sporangia. Facultatively anaerobic. Colonies on trypticase soy agar are 0.5 to 5 mm in diameter, flat, smooth, translucent, glossy, circular and entire, and faintly brown in colour after 50^{o}C incubation for 24 - 48 h. Viscid pellicles are formed in broth. Optimum growth at 50°C; no growth at 37 or 60^{o}C. Optimum growth at pH 8, weak growth at pH 7 and 9, and no growth at pH 6. Tolerates up to 0.5% NaCl. Catalase positive, oxidase weakly positive. Aesculin is hydrolysed, casein is not. Starch hydrolysis is variable and weak when positive. Citrate is not utilized; arginine dihydrolase, hydrogen sulphide, indole, lysine decarboxylase, tryptophan deaminase and urease production are negative; and the Voges-Proskauer test and nitrate reduction are variable. Acid without gas is produced from D-cellobiose, D-glucose, D-fructose, maltose, mannitol, D-mannose, methyl-D-glucoside, *N*-acetylglucosamine, ribose, salicin, sucrose, D-trehalose, D-turanose, and D-xylose. Acid is produced weakly from glycerol and lactose. Acid production is variable from amygdalin, L-arabinose, arbutin, gentiobiose, rhamnose, sorbitol, L-sorbose (weak when positive) and starch. No acid is produced from adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, galactose, gluconate, glycogen, inulin, 2- and 5-keto-D-gluconate, D-lyxose, D-melezitose, D-melibiose, *meso*-inositol, methyl-D-mannoside, methyl-xyloside, D-raffinose, D-tagatose, xylitol and L-xylose. In the variable reactions listed above the type strain does not produce cylindri-

cal spores or swollen sporangia; it hydrolyses starch weakly; is negative for nitrate reduction and Voges-Proskauer test, and produces acid from L-arabinose, amygdalin, arbutin, gentiobiose, rhamnose, sorbitol, L-sorbose (weakly) and starch. Large amounts of exo-oligo-1,6-glucosidase are synthesized (Suzuki *et al.*, 1984). The main cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, iso-$C_{17:0}$and anteiso-$C_{17:0}$, which make up 90% of the total. The mol% G + C of the DNA is: 43.1 - 43.4, and 43.4 (by HPLC) for the type strain LMG 7137^{T} (= ATCC 43742^{T}= DSM 2542^{T}= NCIMB 11955^{T} = R-35637^{T}). Isolated from soil.

7.2.4.6 Description of *Geobacillus thermantarcticus* comb. nov.

Geobacillus thermantarcticus (therm.ant.arct'.ic.us. Gr. n. *therme* heat; L. masc. adj. *antarcticus* southern, belonging to Antarctica; N.L. masc. adj *thermantarcticus* a thermophile from Antarctica).

Basonym: *Bacillus thermantarcticus* Nicolaus *et al.* 2002 (*Bacillus thermoantarcticus* [*sic*] Nicolaus *et al.* 1996).

Description is based upon a single strain. Gram-positive, motile rods, 0.5 to 2.0 μm by 3.0 to 5.0 μm, with ellipsoidal endospores that are borne terminally or subterminally in swollen sporangia. Strictly aerobic. After 24 h incubation at 60°C colonies are whitish to cream in colour, opaque, flat and circular with entire margins, glossy surfaces and diameters of less than 0.5 mm. Grows at 37 and 80°C, and optimally at 60°C. Grows between pH 5.5 and 9; optimum is pH 6.0. Tolerates up to 3% NaCl and is inhibited by 5% NaCl. Oxidase positive, but may be weak. Aesculin is hydrolysed, casein and starch are not. Growth occurs on yeast extract; glucose, trehalose and xylose can be utilized as sole carbon sources; propionate is not utilized; hippurate and tyrosine are not degraded. Gelatin hydrolysis is positive, and Voges-Proskauer reaction is weak, but citrate utilization, nitrate reduction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulphide production, indole production, tryptophan deaminase, and urease production are negative. Acid without gas is produced from: D-cellobiose, D-fructose, galactose (weak), D-glucose, glycerol (weak), maltose, D-mannose, methyl-D-glucoside, salicin, L-sorbose (weak), starch, sucrose, D-trehalose, D-turanose, and D-xylose. No acid is produced from *N*-acetylglucosamine, adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycogen, inulin, 2- and 5-keto-D-gluconate, lactose, D-lyxose, mannitol, D-melezitose, D-melibiose, *meso*-inositol, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, ribose, sorbitol, D-tagatose, xylitol and L-xylose. In stationary phase of growth an exopolysaccharide, exo- and endocellular α-glucosidases, an intracellular alcohol dehydrogenase, and an exocellular xylanase are produced (Nicolaus *et al.,* 1996). The major fatty acids at 60°C are anteiso-$C_{17:0}$(36% of total), iso-$C_{17:0}$(27%), iso-$C_{15:0}$(15%) and iso-$C_{16:0}$(13%). The DNA G+C content is 53.7 mol% for the type strain LMG 23032^{T} (= DSM 9572^{T}= strain M1^{T} = R-35644^{T}). Isolated from Antarctic geothermal soil.

7.2.4.7 Emended description of *Geobacillus toebii* comb. nov., Sung *et al.,* 2002.

Geobacillus toebii (toe'bi.i. N.L. gen. n. *toebii* derived from toebi, a special, farmland compost in Korea, from which the organism was isolated).

Facultatively anaerobic, Gram-positive, motile rods, 0.5 to 0.9 by 2.0 to 3.5 μm. Ellipsoidal spores are located terminally in swollen sporangia. Cells are motile and form colonies that

are cream in colour, with smooth, glossy surfaces and diameters of 0.5 - 2 mm after 24 - 48 h at 60°C. Grows at 37°C (weakly) and 70°C; optimum growth at 60°C. No growth at 30 or 80°C. Grows at pH 6.0 and 9.0; optimum at pH 7.5. Tolerates up to 1% NaCl. Oxidase positive. Starch is hydrolysed (reaction may be weak), but aesculin and casein are not hydrolysed. Gelatin is hydrolysed and nitrate is reduced; the Voges-Proskauer test is variable; and citrate utilization, arginine dihydrolase, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, and urease production are negative. Acid without gas is produced from D-fructose, galactose, D-glucose, maltose, mannitol (weak), D-mannose, and D-trehalose. Acid production is variable from D-melibiose, ribose, starch and sucrose. Acid is not produced from *N*-acetylglucosamine, adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycerol, glycogen, inulin, 2- and 5-keto-D-gluconate, lactose, D-lyxose, D-melezitose, *meso*-inositol, methyl-D-glucoside, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, salicin, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol, D- and L-xylose. In the variable reactions listed above, the colonies of the type strain are motile, Voges-Proskauer test and acid production from D-melibiose are positive, and acid production from ribose, starch and sucrose are negative. Sung *et al*. (2002) reported that the type strain utilizes *n*-alkanes but not acetate, formate or lactate. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0}$, which comprise over 85% of the total. The type strain is reported to produce factors that stimulate the growth of *Symbiobacterium toebii*. The mol% G + C of the DNA (by HPLC) is 42.3 - 42.4 mol% and 42.4 for the type strain LMG 23037^{T} (= DSM 14590^{T}= KCTC 0306BP^{T}= strain SK-1^{T} = R-35642^{T}). Isolated from farmland hay compost in Kongju, Korea, from soil in Thailand, and evaporated milk.

7.2.4.8 Description of *Caldibacillus* gen. nov.

Caldibacillus (Cal.di.ba.cil'lus. L. adj. *caldus* warm, hot;L. masc. n. *bacillus* a small staff or rod; N.L. masc. n. *Caldibacillus*warm bacillus, referring to the organism's thermophily).

Gram-negative, motile rods 0.5 to 1.0 by 1.0 to 14.0 μm that produce one endospore per cell. Ellipsoidal or cylindrical endospores are located terminally in sporangia that may be swollen. Obligately thermophilic and strictly aerobic. Colonies on trypticase soy agar are flat and cream-coloured with smooth margins, and are approximately 0.5 mm in diameter after 24 h at 60°C. The temperature range for growth is 50 to 70°C, with optimum at 65°C. Growth occurs within a pH range of 7.0 to 9.5. Grows in presence of 0% to 2.5% NaCl. Catalase weakly positive and oxidase positive. Aesculin, arginine, casein, gelatin and ONPG are hydrolysed by some strains, but starch is not hydrolysed. The Voges-Proskauer reaction is negative or weak. The polar lipid profile consists of phosphatidylethanolamine, two phosphoglycolipids, two glycolipids, an aminophosphoglycolipid, two phospholipids and two aminolipids. The major cellular fatty acids, which comprise more than 60% of the total, are iso-$C_{15:0}$, iso-$C_{16:0}$and iso-$C_{17:0}$. The main menaquinone type is MK-7. The mol% G + C of the DNA of the type strain of the type species is 49.9 (by HPLC). The type species is *Caldibacillus debilis* and the type strain is LMG 23386^{T}.

Description of Caldibacillus debilis comb. nov.

Caldibacillus debilis (*de'bil.is.* L. masc. adj. *debilis* weak orfeeble, referring to the restricted substrate range for thisspecies).

Basonym: *Geobacillus debilis* Banat *et al.,* 2004.

The type strain exhibits the following properties in addition to those given in the genus description. Reactions for arginine dihydrolase, gelatin hydrolysis and ONPG are positive; those for hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease production, nitrate reduction and citrate utilization are negative. Acid but no gas is produced from the following: amygdalin, D-cellobiose, D-fructose, galactose, gentiobiose, D-glucose, lactose, maltose, D-mannose, *N*-acetylglucosamine, raffinose. salicin, and starch; and weakly produced from arbutin, D-tagatose, D-trehalose, D-turanose and D-xylose. Acid is not produced from: adonitol, D- and L-arabinose, D- and L-arabitol, dulcitol, erythritol, gluconate, glycerol, glycogen, inulin, D- and L-fucose, 2- and 5-keto-D-gluconate, D-lyxose, mannitol, D-melezitose, D-melibiose, *meso*-inositol, methyl-D-glucoside, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, ribose, sorbitol, L-sorbose, sucrose, xylitol and L-xylose. The mol% G+C of the DNA is 49.9 (by HPLC). The type strain, strain LMG 23386^{T} (= DSM 16016^{T} = NCIMB 13995^{T} = Tf^{T} = R-35653^{T}) was isolated from undisturbed subsurface soil in Northern Ireland.

7.2.4.9 Description of *Anoxybacillus caldiproteolyticus* sp. nov.

Anoxybacillus caldiproteolyticus (cal.di.pro.teo.ly'ti.cus. L. adj. *caldus* hot; N.L. masc. adj. *proteolyticus* proteolytic; N.L. masc. adj. *caldiproteolyticus* hot, and protein degrading).

Synonym: "*Geobacillus caldoproteolyticus*" Chen *et al.,* 2004.

Description is based upon study of a single isolate. Gram-positive, motile rods. Strictly aerobic. Endospores are formed within 24 h of incubation at 60°C on nutrient agar containing 5 mg L^{-1} $MnSO_4$; they are ellipsoidal and sometimes cylindrical, lie subterminally and/or terminally and do not swell the sporangia. After 24 h incubation at 60°C on nutrient agar, colonies are circular in shape with smooth edges, glossy surfaces and diameters of approximately 5 mm. Grows between 37 and 70°C (at 48 h), with optimum at 60°C. Grows at pH 5 and 9 with optimum growth at pH 6.5-7. Tolerates up to 0.5% NaCl. Hydrolyses aesculin, casein and (sometimes weakly) starch. Catalase and oxidase are positive. Positive for gelatin hydrolysis and the Voges-Proskauer test; negative for citrate utilization, arginine dihydrolase, hydrogen sulphide production, indole production, lysine decarboxylase production, nitrate reduction, ornithine decarboxylase, ONPG (*ortho*-nitrophenyl-β-galactosidase), tryptophan deaminase and urease production. Acid without gas is produced from D-cellobiose, D-fructose, galactose, D-glucose, glycerol, glycogen, maltose, mannitol, D-mannose, *N*-acetylglucosamine, ribose, salicin, starch, sucrose, D-trehalose, D-turanose (weak), and D-xylose. Acid production from arbutin is variable. No acid is produced from adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, inulin, 2-and 5-keto-D-gluconate, lactose, D-lyxose, D-melezitose, D-melibiose, *meso*-inositol, methyl-D-glucoside, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, sorbitol, L-sorbose, D-tagatose, xylitol and L-xylose. The DNA G + C of the type strain DSM 15730^{T} (= ATCC BAA-818^{T} = LMG 26209^{T} = R-35652^{T}) is 40.2 mol% (by HPLC). Isolated from sewage sludge from a water reclamation plant in Singapore.

7.2.4.10 Description of *Anoxybacillus tepidamans* comb. nov.

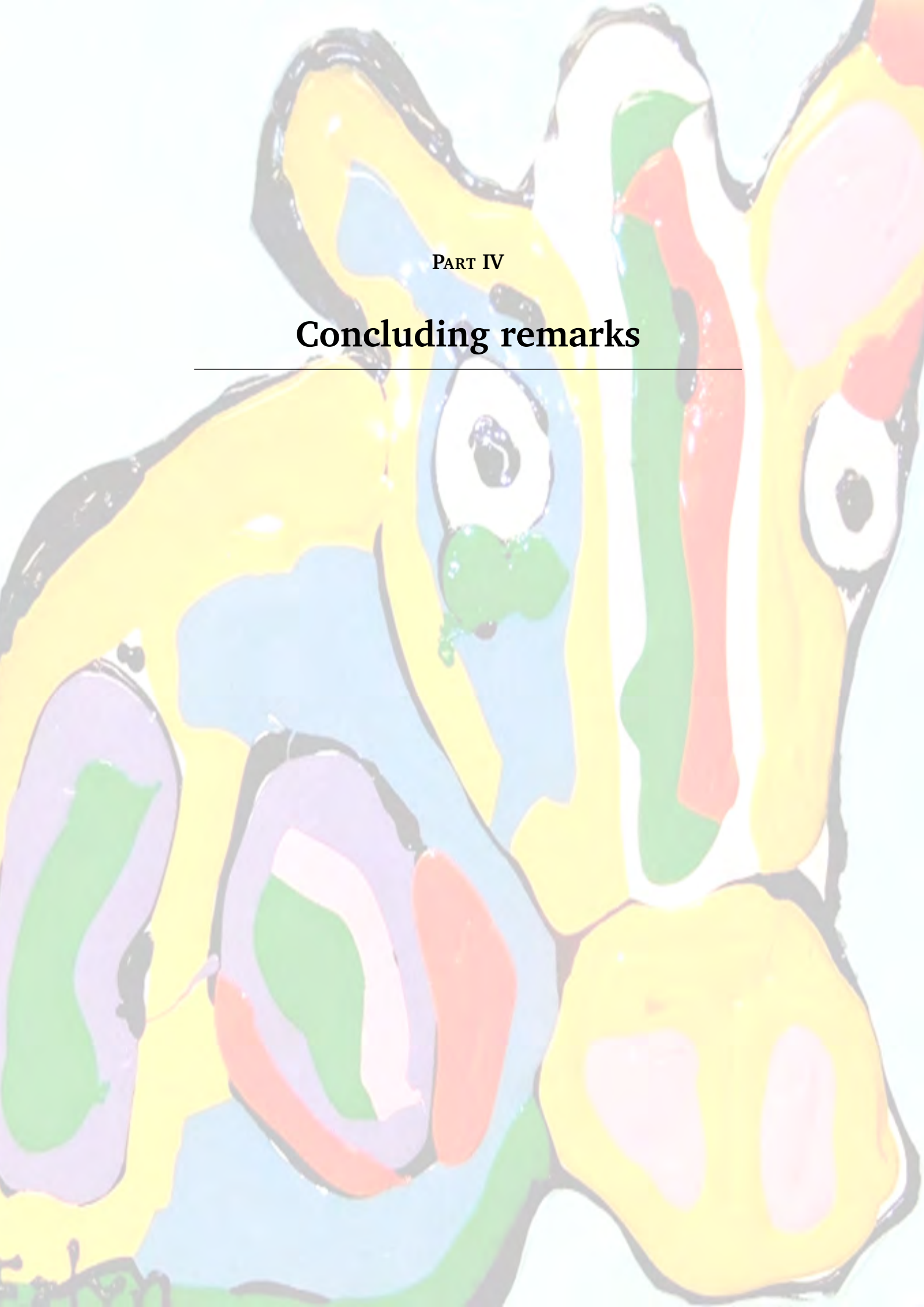
Anoxybacillus tepidamans (*te.pid.a'mans*. L. adj. *tepidus* (luke)warm; L. part adj. *amans* loving; N.L. part adj. *tepidamans* lovingwarm conditions).

Basonym: *Geobacillus tepidamans* Schäffer *et al*. 2004.

Data are from study of the type strain. Facultatively anaerobic, Gram-positive rods 3 to 5 by 0.9 to 1.2 μm, producing ellipsoidal endospores that lie terminally and swell the sporangia. After 24 h at 60°C on NA, colonies are opaque and white in colour, circular, with smooth edges and glossy surfaces, and diameters of approximately 0.5 mm. Grows at 40-65°C, with an optimum at 55°C. Grows between pH 6 and 9; no growth at pH 5. Tolerates up to 2% NaCl. Aesculin and starch are hydrolysed, but casein is not. Catalase positive and oxidase negative. ONPG and the Voges-Proskauer test (this test was recorded as negative by Schäffer *et al*. 2004) are positive; reactions for arginine dihydrolase production, citrate utilization, gelatin hydrolysis, hydrogen sulphide, indole, lysine decarboxylase, nitrate reduction (this test was recorded as positive by Schäffer *et al*. 2004), ornithine decarboxylase, tryptophan deaminase and urease production are negative. Acid without gas is produced from amygdalin, L-arabinose, arbutin, D-cellobiose, D-fructose, galactose, gentiobiose, D-glucose, glycerol, lactose, maltose, D-mannose, D-melezitose, D-melibiose, *meso*-inositol, methyl-D-glucoside, *N*-acetylglucosamine, D-raffinose, ribose (weak), salicin, starch, sucrose, D-trehalose, D-turanose, and D-xylose. No acid is produced from: adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gluconate, glycogen, inulin, 2- and 5-keto-D-gluconate, D-lyxose, mannitol, methyl-D-mannoside, methyl-xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, L-xylose, and xylitol. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine, together with two aminolipids, a phospholipid and some new lipids. The DNA G + C (by HPLC) is 42.4 - 43.2 mol%, and 43.2 for the type strain LMG 26208$^{\mathrm{T}}$ (= ATCC BAA-942^{T}= DSM 16325$^{\mathrm{T}}$ = R-35643$^{\mathrm{T}}$). The type strain was isolated from a beet sugar factory in Austria, and another strain was isolated from geothermally-heated soil, YellowstoneNational Park, USA.

Acknowledgements

The author would like to thank Prof. Dr. Niall Logan, Dr. Anna Dinsdale and Dr. Gillian Halket for the phenotypic characterization of *Geobacillus* strains at Glasgow Caledonian University.



PART IV

Concluding remarks

CHAPTER 8

Conclusions and future perspectives

8.1 General conclusion

The bacterial diversity of raw cow's milk, with a focus on aerobic spore-formers and psychrotrophic pseudomonads, was investigated and the spoilage potential of members of both groups was assessed.

It can be concluded for both studies that our knowledge on the diversity of milk spoilage bacteria is far from complete. Species frequently associated with milk spoilage were most abundantly isolated such as *Bacillus licheniformis*, *Bacillus subtilis, Bacillus pumilus* and *Bacillus cereus*, confirming earlier studies. Nonetheless, a considerable part of the isolates was identified as species that have not been associated with milk spoilage before, such as *Bacillus clausii*, and several possibly new species within the *Pseudomonas fluorescens* group (*Pseudomonas fluorescens* like), all showing considerable proteolytic and lipolytic potential. Novel species are also recovered, one of which is described as *Bacillus thermolactis.* As for the pseudomonads, spoilage was for long time mainly attributed to *Pseudomonas fluorescens*, however the study of Marchand *et al*. (2008), and the study presented here clearly indicate members of *Pseudomonas lundensis* and *Pseudomonas fragi* as the main spoilers, together with the so-called *Pseudomonas fluorescens* like strains.

The study on diversity of pseudomonads **(Chapter 5)** not only showed a considerable diversity of species recovered from raw cow's milk, but also indicates the importance of cold storage of

raw milk and derived products throughout the dairy chain. Even the smallest deviation of the optimal storage temperature has great impact on the outgrowth of spoilage bacteria, and thus the production of spoilage enzymes. Prolonged storage time of raw cow's milk before processing fortifies this effect.

Additionally to mapping the diversity, the study presented in **Chapter 6** on aerobic spore-formers also attempted to determine whether differences in operational management on the farm and seasonal variation can influence the bacterial flora in milk. Indeed, seasonal variations were observed, with *Ureibacillus thermosphaericus* and *Bacillus cereus* mostly isolated during late summer / autumn, while *Bacillus amyloliquefaciens* was almost exclusively isolated in winter. Members of the *Bacillus cereus* group are more frequently isolated at organic farms, while milk from cows accommodated at conventional farms contain more thermotolerant organisms, especially *Ureibacillus thermosphaericus* and *Bacillus licheniformis*. These differences might be correlated with differences in feeding and housing strategies between both types of farming.

The polyphasic identification approaches used for both diversity studies approves very useful. For aerobic spore-formers, a first grouping and tentative identification was obtained by analyzing the fatty acid methyl ester patterns of all isolates. Representatives of these groups were further identified with rep-fingerprinting and 16S rRNA gene sequencing. Some species groups within the genus *Bacillus* suffer from insufficient resolution of the 16S rRNA gene to differentiate between species. For instance, *gyrB* gene sequencing was proposed to identify strains within the *Bacillus subtilis* group (Wang *et al*., 2007), but the combination of fatty acids with 16S rRNA, sometimes aided with rep-fingerprints proved satisfactory in this study. A similar approach could not be used for the pseudomonads, as fatty acid methyl ester analysis does not allow a distinct grouping of isolates. Therefore, isolates were first grouped based on DNA-fingerprint patterns, and representative strains were further identified by FAME (to confirm or repudiate their allocation as pseudomonads), 16S rRNA gene sequencing and *rpoB* gene sequencing. Additionally, for some representatives a *rpoD* gene sequence was obtained. Isolates could be identified onto the species or species group level for both studies.

8.2 Future perspectives

Pseudomonads and bacilli are two very diverse, heterogeneous taxa, and taxonomic work in these groups seems like a hopeless task. Both genera, *Pseudomonas* and *Bacillus*, but especially the genus *Bacillus*, are actually melting pots of bacteria that probably could be attributed to various different genera. Finding one satisfying identification approach for a whole genus during the spam of one PhD project is thus not feasible. Therefore, focus for the bacilli was on the genus *Geobacillus*, originating from the genus *Bacillus*, and harboring thermophilic aerobic endospore-formers of mostly industrial importance. A thorough polyphasic approach on several members of this genus resulted in a reshuffling with some species being merged (e.g. *Geobacillus thermocatenulatus* and *Geobacillus gargensis*) and others being thoroughly emended. For the pseudomonads, focus was on the *Pseudomonas fluorescens* group, harboring more than 50 species, and including important milk spoilers. 16S rRNA gene sequence similarities between members of this group are high (more than 97.0%) and hamper reliable species allocation. Therefore, the applicability of the MLSA-scheme proposed by Mulet and co-workers (2010) was validated, but proved unsatisfactory on the species level. However, no alternatives were found yet, and the need for multiple laborious, time-consuming DNA-DNA hybridization experiments, seriously slow

down any progress in this field.

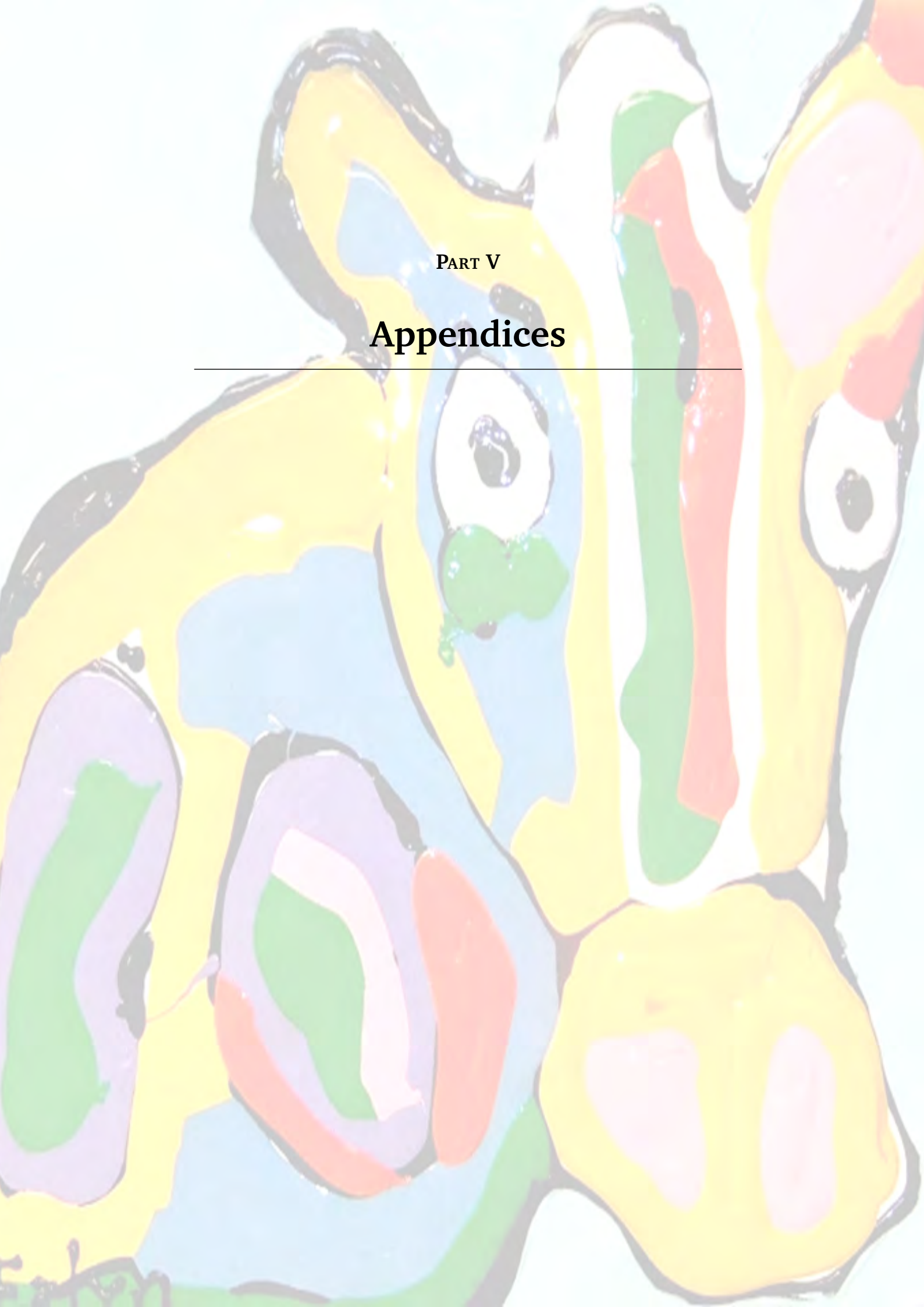
The dairy industry occasionally encounters economical losses due to spoilage of milk and derived products and any test quickly tracking the responsible bacteria would be welcomed.

Any such test however depends on reliable identification of these spoilage bacteria and this in turn depends on the taxonomic framework of the taxa these isolates fit in. Spoilage of milk has mainly been attributed to members of the genus *Bacillus* and *Pseudomonas*, both genera displaying an extremely complex taxonomic situation, thus hampering reliable identification. A major challenge for taxonomists seems to lie in resolving this complex situation. Using an approach similar to the one used for the genus *Geobacillus* is definitely the most accurate one, taking into account as many data as possible, phenotypic and genotypic, and supported by DNA-relatedness values. However, the genus *Bacillus* largely outnumbers the genus *Geobacillus*, and a similar approach would take many, many years, and the same could be said about the genus *Pseudomonas*. Due to the huge phenotypic and genotypic diversity within both genera, it is very likely that no uniform identification approach will be found. Therefore, a more feasible method for the genus *Bacillus* would probably be to further subdivide the genus in several species groups harboring phylogenetically related species (as already done for the genus *Pseudomonas*), and step by step investigate such species groups with an approach similar to the one used for the genus *Geobacillus*. This, in combination with whole genome sequence data, could help in allocating good taxonomic markers to differentiate *Bacillus* species. For the genus *Pseudomonas* species groups have already been assessed and are established in *Pseudomonas* taxonomy. However species differentiation within these groups is far from satisfying based on currently available methods, and here also step by step thorough analysis of members of the species groups could result in finding good taxonomic markers. As said, reliable identification is dependant of the classification system, and for now this is still based on phylogenetic relationships as represented by 16S rRNA gene sequencing. Future taxonomic reshuffling surely depends on the evolution of the species definition and on the development and use of genetic markers and fast whole-genome sequencing techniques.

As for the dairy industry, although suffering from these spoilers, there are no parameters to monitor their specific presence as routinely done for germ number, presence of antibiotics, etc. The dairy industry itself would benefit from a quick test immediately indicating if fresh incoming milk has a high *Bacillus* or *Pseudomonas* load. This would provide the necessary information to determine how to process the milk further downstream the dairy chain. For instance, milk subject to severe endospore-formers contamination could be further processed to UHT-products, rather than to pasteurized products, while milk subject to pseudomonad contamination could be processed to short shelf-life products, thus inhibiting the proteolytic and lipolytic activity. The main restriction for such a test however is time. Indeed, as soon as raw milk arrives at the dairy plant, it should be processed as quickly as possible, and there is no time to wait for the outcome of a 3-day incubation test, not even for a PCR-test of a few hours. Normal practice in the dairy industry is the 3-minutes test to decide whether or not a fresh load of milk is accepted or not, and any additional test should preferably fit in the same time schedule. The solution could be an immunological detection test on the protein level, but further research is needed to evaluate this.

Another important strategy for the dairy industry could be, rather than detecting whether these spoilers are present or not, to prevent their initial income in the raw milk. A first step then should be to determine the contamination source of these bacteria, through source tracking. The rep-fingerprinting patterns generated throughout the studies presented here could be used as reference material for that purpose. Some preliminary results on pseudomonads isolated from

biofilms on milking equipment already clearly indicated these biofilms could be a contamination source of raw milk for *Pseudomonas lundensis*.



PART V

Appendices

APPENDIX A

Overview of Pseudomonas isolates

Overview of all milk isolates obtained during the study of Chapter 5. The strain number is indicated, with identification based on fatty acid methyl ester analysis (FAME FATAL ID), 16S rRNA gene sequences (16S rRNA gene ID, with accession numbers), *rpoB* gene sequences (*rpoB* gene ID, with accession numbers), and the consensus identification (Final ID in study of De Jonghe *et al.*, 2011). The BOX-cluster the isolates were assigned to is indicated (cl.) as well as the isolation parameters, namely simulation (S.), cooling conditions (C., being optimal: green or suboptimal: red), isolation point (P., RM1 to RM14, see Table 5.1), and medium (M., CFC or PCMA). The enzymatic activity is represented per isolate with color boxes: white: no activity, grey: intermediate activity and black: strong activity for 1: protease and 2: lipase. Isolates in purple do not belong to the genus *Pseudomonas*.

R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37100						*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37262						*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37263						*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37265	*Pseudomonas tolaasii*	*P. fragi*	FN650724	*Pseudomonas* sp.	FN650766	*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37266						*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37267	*Pseudomonas tolaasii*	*P. fragi*	FN650725	*Pseudomonas* sp.	FN650767	*Pseudomonas* sp. nov. 1	A	1		RM14	CFC		
37242						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37245	*Pseudomonas tolaasii*			*Pseudomonas* sp.	FN650763	*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37248						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37251						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37252						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37253						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37254						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37255						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37256						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37257	*Pseudomonas tolaasii*	*P. fragi*	FN650722	*Pseudomonas* sp.	FN650764	*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37258						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37259						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37338						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37340						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37455						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37543						*Pseudomonas* sp. nov. 2	B	1		RM8	CFC		
37059						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37061						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37064						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37071						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37075						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37079						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37081						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37083						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37084						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37087	*Pseudomonas proteolytica*					*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37090						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37093	*Pseudomonas proteolytica*			*P. fluorescens* group	FN650752	*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37095	*Pseudomonas fluorescens*	*P. fluorescens* group	FN650713	*P. fluorescens* group	FN650753	*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37096	*Pseudomonas proteolytica*	*P. fluorescens* group	FN650714	*P. fluorescens* group	FN650754	*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37106						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37110						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37116	*Pseudomonas proteolytica*			*P. fluorescens* group	FN650756	*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37118						*Pseudomonas fluorescens* like	C	1		RM14	CFC		
37123	*Pseudomonas lurida*	*P. fluorescens* group	FN650715	*P. fluorescens* group	FN650757	*Pseudomonas fluorescens* like	C	1		RM1	CFC		
37147	*Pseudomonas brenneri*	*P. fluorescens* group	FN650716	*P. fluorescens* group	FN650758	*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37148						*Pseudomonas fluorescens* like	C	1		RM8	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37149						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37150						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37152						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37154	*Pseudomonas azotoformans*	*P. fluorescens* group	FN650718	*P. fluorescens* group	FN750759	*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37156						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37158						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37159						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37160						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37161						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37163						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37164						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37167						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37169						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37170						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37172						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37173						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37181						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37186						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37187						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37195						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37207						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37220						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37221						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37222						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37223						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37244						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37246						*Pseudomonas fluorescens* like	C	1		RM8	CFC		
37270						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37271						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37272						*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37323	*Pseudomonas proteolytica*	*P. fluorescens* group	FN650727	*P. fluorescens* group	FN650769	*Pseudomonas fluorescens* like	C	1		RM11	CFC		
37332						*Pseudomonas fluorescens* like	C	1		RM1	CFC		
37063						*Pseudomonas fluorescens* like	D	1		RM8	CFC		
37070						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37074						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37076						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37077						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37101						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37111						*Pseudomonas fluorescens* like	D	1		RM14	CFC		
37194	*Pseudomonas libanensis*	*P. fluorescens* group	FN650720	*P. fluorescens* group	FN650762	*Pseudomonas fluorescens* like	D	1		RM11	CFC		
37276						*Pseudomonas fluorescens* like	D	1		RM11	CFC		
37279						*Pseudomonas fluorescens* like	D	1		RM11	CFC		
38091						*Pseudomonas fluorescens* like	D	2		RM11	CFC		
38103						*Pseudomonas fluorescens* like	D	2		RM11	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38554						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38555						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38557						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38584						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38586						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38600						*Pseudomonas fluorescens* like	E	3		RM14	PCMA		
38617						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38619						*Pseudomonas fluorescens* like	E	3		RM11	PCMA		
38638						*Pseudomonas fluorescens* like	E	3		RM8	PCMA		
38641						*Pseudomonas fluorescens* like	E	3		RM8	PCMA		
38664						*Pseudomonas fluorescens* like	E	3		RM14	PCMA		
38675						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38677						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38679						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38683						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38684						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38686						*Pseudomonas fluorescens* like	E	3		RM1	CFC		
38689						*Pseudomonas fluorescens* like	E	3		RM1	CFC		
38746						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38749						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38750						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38751						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38752						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38753						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38756						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38757	*Pseudomonas azotoformans*	*P. fluorescens* group	FN650742	*P. fluorescens* BT A	FN650787	*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38760						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38763						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38765						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38767						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38771						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38773	*Pseudomonas brenneri*	*P. fluorescens* group	FN650743	*P. fluorescens* BT A	FN650788	*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38775						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38777						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38778						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38780						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38781						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38810						*Pseudomonas fluorescens* like	E	3		RM8	PCMA		
38818						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
38826						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38828						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
38830						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38831						*Pseudomonas fluorescens* like	E	3		RM8	CFC		
38837						*Pseudomonas fluorescens* like	E	3		RM11	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38842						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
38845						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
39375						*Pseudomonas fluorescens* like	E	3		RM14	CFC		
39376						*Pseudomonas fluorescens* like	E	3		RM14	CFC		
39381						*Pseudomonas fluorescens* like	E	3		RM14	CFC		
39388						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
39393						*Pseudomonas fluorescens* like	E	3		RM11	CFC		
39399	*Pseudomonas brenneri*					*Pseudomonas fluorescens* like	E	3		RM14	CFC		
39424						*Pseudomonas fluorescens* like	E	3		RM14	CFC		
39426						*Pseudomonas fluorescens* like	E	3		RM14	CFC		
37901						*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37904	*Pseudomonas tolaasii*	*P. fluorescens* group	FN650729	*P. fluorescens* BT A	FN650771	*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37905						*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37916						*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37918	*Pseudomonas proteolytica*					*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37923	*Pseudomonas libanensis*	*P. fluorescens* group	FN650923	*P. fluorescens* BT A	FN650774	*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37926						*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37927						*Pseudomonas fluorescens* like	F	2		RM8	CFC		
37965						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
37967						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
37970						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
37987	*Pseudomonas tolaasii*			*P. fluorescens* BT A	FN750776	*Pseudomonas fluorescens* like	F	2		RM11	CFC		
37992						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
37993						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38073	*Pseudomonas fulgida*					*Pseudomonas fluorescens* like	F	2		RM14	CFC		
38079						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38081						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38114						*Pseudomonas fluorescens* like	F	2		RM14	CFC		
38118						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38121						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38122						*Pseudomonas fluorescens* like	F	2		RM11	CFC		
38235	*Pseudomonas graminis*	*P. fluorescens* group	FN650735	*Pseudomonas* sp.	FN650779	*Pseudomonas fluorescens* like	F	2		RM14	CFC		
38434						*Pseudomonas fluorescens* like	F	2		RM14			
38440						*Pseudomonas fluorescens* like	F	2		RM14	CFC		
37085	*Pseudomonas extremorientalis*					*Pseudomonas fluorescens* like	G	1		RM14	CFC		
37895						*Pseudomonas fluorescens* like	G	2		RM0	CFC		
37897						*Pseudomonas fluorescens* like	G	2		RM0	CFC		
37909						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37913						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37915						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37917						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37934						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37935						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37937						*Pseudomonas fluorescens* like	G	2		RM8	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37939						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37941						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37944						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37958						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37959						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37968						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
37985						*Pseudomonas fluorescens* like	G	2		RM8	CFC		
37986						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
37988						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
37989						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
37990						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
37991						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38075						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38080						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38083	*Pseudomonas putida*	*P. fluorescens* group	FN650733	*P. fluorescens* group	FN650777	*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38088						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38089						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38092						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38099						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38100						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38102						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38107						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38108						*Pseudomonas fluorescens* like	G	2		RM11	CFC		
38113						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38239						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38441						*Pseudomonas fluorescens* like	G	2		RM14	CFC		
38626						*Pseudomonas fluorescens* like	G	3		RM14	PCMA		
38685						*Pseudomonas fluorescens* like	G	3		RM1	CFC		
38696						*Pseudomonas fluorescens* like	G	3		RM14	PCMA		
38811						*Pseudomonas fluorescens* like	G	3		RM1	CFC		
39368						*Pseudomonas fluorescens* like	G	3		RM11	CFC		
37060						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37067	*Pseudomonas putida*			*P. fluorescens* group	FN650748	*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37072						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37082	*Pseudomonas antarctica*	*P. fluorescens* group	FN650710	*P. fluorescens* group	FN650749	*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37086						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37091						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37092	*Pseudomonas putida*	*P. fluorescens* group	FN650712	*P. fluorescens* group	FN650751	*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37094						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37097						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37107						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37114						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37120						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37121						*Pseudomonas fluorescens* like	H	1		RM14	CFC		

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		16S rRNA		*rpoB*		final ID in study of		isol. param.				enz. act.	
R-number	FAME FATAL ID	ID	acc. n°	ID	acc. n°	De Jonghe *et al.* (2011)	cl.	S.	C.	P.	M.	1	2
37124						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37128						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37129						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37130						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37133						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37139						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37140						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37141						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37142						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37144						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37145						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37146						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37151						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37153	*Pseudomonas putida*	*P. fluorescens* group	FN650717			*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37155						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37157						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37166						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37168						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37174						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37175						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37176	*Pseudomonas antarctica*	*P. fluorescens* group	FN650719	*P. fluorescens* group	FN650760	*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37180						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37219						*Pseudomonas fluorescens* like	H	1		RM8	CFC		
37225						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37226						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37230						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37231						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37234						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37235						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37236						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37237						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37240	*Pseudomonas antarctica*	*P. fluorescens* group	FN650721			*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37264						*Pseudomonas fluorescens* like	H	1		RM14	CFC		
37274						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37275						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37277						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37278						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37280						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37282						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37283	*Pseudomonas antarctica*	*P. fluorescens* group	FN650726	*P. fluorescens* group	FN650768	*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37319						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37320						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37321						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37322						*Pseudomonas fluorescens* like	H	1		RM11	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37324						*Pseudomonas fluorescens* like	H	1		RM11	CFC		
37327						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37328						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37334						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
37542						*Pseudomonas fluorescens* like	H	1		RM1	CFC		
38768	*Pseudomonas salomonii*					*Pseudomonas fluorescens* like	H	3		RM8	CFC		
38783						*Pseudomonas fluorescens* like	H	3		RM8	CFC		
39382						*Pseudomonas fluorescens* like	H	3		RM14	CFC		
38558						*Pseudomonas* sp.	I	3		RM11	PCMA		
38615						*Pseudomonas* sp.	I	3		RM11	PCMA		
38624						*Pseudomonas* sp.	I	3		RM14	PCMA		
38668	*Pseudomonas rhodesiae*	*P. fluorescens* group	FN650741			*Pseudomonas* sp.	I	3		RM1	CFC		
38672						*Pseudomonas* sp.	I	3		RM1	CFC		
38695						*Pseudomonas* sp.	I	3		RM14	PCMA		
38748						*Pseudomonas* sp.	I	3		RM8	CFC		
38754						*Pseudomonas* sp.	I	3		RM8	CFC		
38825						*Pseudomonas* sp.	I	3		RM8	CFC		
38841						*Pseudomonas* sp.	I	3		RM11	CFC		
39428						*Pseudomonas* sp.	I	3		RM14	CFC		
39429						*Pseudomonas* sp.	I	3		RM14	CFC		
37900						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37906						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37907	*Pseudomonas mandelii*	*P. fluorescens* group	FN650730	*P. gessardii*	FN650772	*Pseudomonas gessardii* like	J	2		RM8	CFC		
37938						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37945						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37947						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37954						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37955						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37956						*Pseudomonas gessardii* like	J	2		RM8	CFC		
37979						*Pseudomonas gessardii* like	J	2		RM11	CFC		
38241						*Pseudomonas gessardii* like	J	2		RM14	CFC		
38242	*Pseudomonas mandelii*	*P. fluorescens* group		*P. gessardii*	FN650780	*Pseudomonas gessardii* like	J	2		RM14	CFC		
38243						*Pseudomonas gessardii* like	J	2		RM14	CFC		
38248						*Pseudomonas gessardii* like	J	2		RM14	CFC		
38251						*Pseudomonas gessardii* like	J	2		RM14	CFC		
38432						*Pseudomonas gessardii* like	J	2		RM14	CFC		
37936						*Pseudomonas* sp. nov. 5	K	2		RM8	CFC		
37940						*Pseudomonas* sp. nov. 5	K	2		RM8	CFC		
37951						*Pseudomonas* sp. nov. 5	K	2		RM8	CFC		
37952						*Pseudomonas* sp. nov. 5	K	2		RM8	CFC		
37977	*Pseudomonas graminis*					*Pseudomonas* sp. nov. 5	K	2		RM11	CFC		
37984	*Pseudomonas graminis*					*Pseudomonas* sp. nov. 5	K	2		RM8	CFC		
38093	*Pseudomonas fuscovaginae*					*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38104						*Pseudomonas* sp. nov. 5	K	2		RM11	CFC		

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		16S rRNA		*rpoB*		final ID in study of		isol. param.				enz. act.	
R-number	FAME FATAL ID	ID	acc. n°	ID	acc. n°	De Jonghe *et al.* (2011)	cl.	S.	C.	P.	M.	1	2
38124						*Pseudomonas* sp. nov. 5	K	2		RM11	CFC		
38234						*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38247	*Pseudomonas graminis*			*Pseudomonas* sp.	FN650781	*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38257						*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38258	*Pseudomonas graminis*					*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38259						*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38260						*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
38262						*Pseudomonas* sp. nov. 5	K	2		RM14	CFC		
37903						*Pseudomonas gessardii*	L	2		RM8	CFC		
37910						*Pseudomonas gessardii*	L	2		RM8	CFC		
37948	*Pseudomonas fuscovaginae*	*P. fluorescens* group	FN650948	*Pseudomonas* sp.	FN650775	*Pseudomonas gessardii*	L	2		RM8	CFC		
37949						*Pseudomonas gessardii*	L	2		RM8	CFC		
37962						*Pseudomonas gessardii*	L	2		RM11	CFC		
37964						*Pseudomonas gessardii*	L	2		RM11	CFC		
37969						*Pseudomonas gessardii*	L	2		RM11	CFC		
37971						*Pseudomonas gessardii*	L	2		RM11	CFC		
37978						*Pseudomonas gessardii*	L	2		RM11	CFC		
38076						*Pseudomonas gessardii*	L	2		RM14	CFC		
38077						*Pseudomonas gessardii*	L	2		RM11	CFC		
38085						*Pseudomonas gessardii*	L	2		RM11	CFC		
38106						*Pseudomonas gessardii*	L	2		RM11	CFC		
38237						*Pseudomonas gessardii*	L	2		RM14	CFC		
38238						*Pseudomonas gessardii*	L	2		RM14	CFC		
38249						*Pseudomonas gessardii*	L	2		RM14	CFC		
38263						*Pseudomonas gessardii*	L	2		RM14	CFC		
38603						*Pseudomonas gessardii*	M	3		RM11	PCMA		
38609						*Pseudomonas gessardii*	M	3		RM14	PCMA		
38612						*Pseudomonas gessardii*	M	3		RM8	PCMA		
38620						*Pseudomonas gessardii*	M	3		RM11	PCMA		
38697						*Pseudomonas gessardii*	M	3		RM1	CFC		
38819						*Pseudomonas gessardii*	M	3		RM11	CFC		
38833						*Pseudomonas gessardii*	M	3		RM8	CFC		
38836						*Pseudomonas gessardii*	M	3		RM11	CFC		
38838						*Pseudomonas gessardii*	M	3		RM11	CFC		
38839						*Pseudomonas gessardii*	M	3		RM11	CFC		
39396	*Pseudomonas libanensis*	*P. fluorescens* group	FN650745	*P. gessardii*	FN650790	*Pseudomonas gessardii*	M	3		RM11	CFC		
39461						*Pseudomonas gessardii*	M	3		RM14	CFC		
37902	*Pseudomonas brenneri*	*P. fluorescens* group	FN650728	*P. gessardii*	FN650770	*Pseudomonas gessardii* like	N	2		RM8	CFC		
37922						*Pseudomonas gessardii* like	N	2		RM8	CFC		
37928						*Pseudomonas gessardii* like	N	2		RM8	CFC		
37974						*Pseudomonas gessardii* like	N	2		RM11	CFC		
37976						*Pseudomonas gessardii* like	N	2		RM11	CFC		
38111	*Pseudomonas vancouverensis*					*Pseudomonas gessardii* like	N	2		RM14	CFC		
38431						*Pseudomonas gessardii* like	N	2		RM14	CFC		

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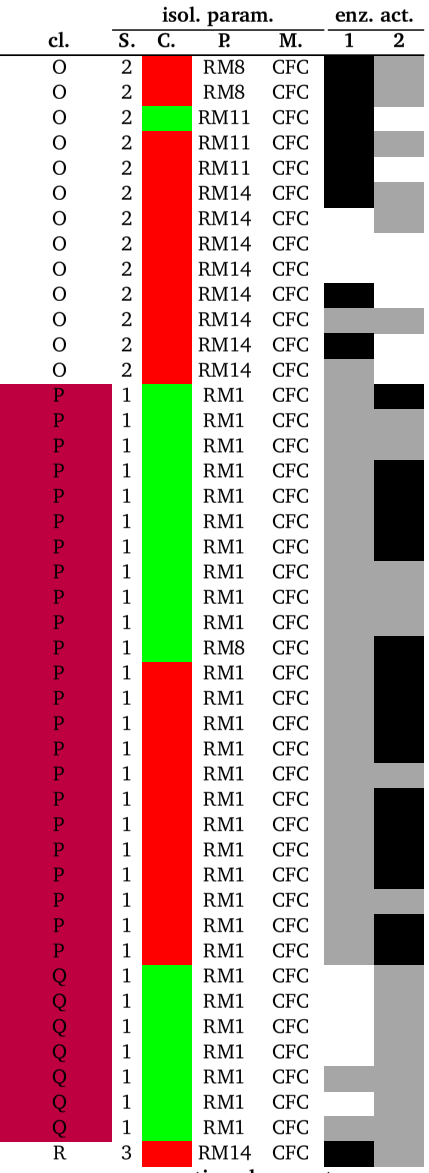
R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37942						*Pseudomonas gessardii* like	O	2		RM8	CFC		
37950						*Pseudomonas gessardii* like	O	2		RM8	CFC		
37966						*Pseudomonas gessardii* like	O	2		RM11	CFC		
38084						*Pseudomonas gessardii* like	O	2		RM11	CFC		
38087						*Pseudomonas gessardii* like	O	2		RM11	CFC		
38236						*Pseudomonas gessardii* like	O	2		RM14	CFC		
38255	*Pseudomonas brenneri*	*P. fluorescens* group	FN650737	*P. gessardii*	FN650783	*Pseudomonas gessardii* like	O	2		RM14	CFC		
38256						*Pseudomonas gessardii* like	O	2		RM14	CFC		
38261	*Pseudomonas proteolytica*					*Pseudomonas gessardii* like	O	2		RM14	CFC		
38433						*Pseudomonas gessardii* like	O	2		RM14	CFC		
38435						*Pseudomonas gessardii* like	O	2		RM14	CFC		
38437						*Pseudomonas gessardii* like	O	2		RM14	CFC		
38438						*Pseudomonas gessardii* like	O	2		RM14	CFC		
37122						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37125						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37126						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37132						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37134						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37135						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37136						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37137						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37138						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37143						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37162						*Stenotrophomonas* sp.	P	1		RM8	CFC		
37233						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37238						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37239						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37281	*Stenotrophomonas* sp.					*Stenotrophomonas* sp.	P	1		RM1	CFC		
37330						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37331						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37333						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37335						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37336						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37337						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37453						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37454						*Stenotrophomonas* sp.	P	1		RM1	CFC		
37127						*Delftia* sp.	Q	1		RM1	CFC		
37131						*Delftia* sp.	Q	1		RM1	CFC		
37212						*Delftia* sp.	Q	1		RM1	CFC		
37215						*Delftia* sp.	Q	1		RM1	CFC		
37216						*Delftia* sp.	Q	1		RM1	CFC		
37217						*Delftia* sp.	Q	1		RM1	CFC		
37218	*Delftia* sp.					*Delftia* sp.	Q	1		RM1	CFC		
38456						*P. fragi*	R	3		RM14	CFC		



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		16S rRNA		*rpoB*		final ID in study of		isol. param.				enz. act.	
R-number	FAME FATAL ID	ID	acc. n°	ID	acc. n°	De Jonghe *et al.* (2011)	cl.	S.	C.	P.	M.	1	2
38564						*P. fragi*	R	3		RM14	PCMA		
38565						*P. fragi*	R	3		RM14	PCMA		
38566						*P. fragi*	R	3		RM14	PCMA		
38567						*P. fragi*	R	3		RM14	PCMA		
38568						*P. fragi*	R	3		RM14	PCMA		
38569						*P. fragi*	R	3		RM8	PCMA		
38570						*P. fragi*	R	3		RM8	PCMA		
38571						*P. fragi*	R	3		RM8	PCMA		
38572						*P. fragi*	R	3		RM8	PCMA		
38573						*P. fragi*	R	3		RM8	PCMA		
38583						*P. fragi*	R	3		RM14	PCMA		
38587						*P. fragi*	R	3		RM8	PCMA		
38591						*P. fragi*	R	3		RM8	PCMA		
38592						*P. fragi*	R	3		RM14	PCMA		
38593						*P. fragi*	R	3		RM14	PCMA		
38594						*P. fragi*	R	3		RM14	PCMA		
38597						*P. fragi*	R	3		RM14	PCMA		
38598						*P. fragi*	R	3		RM14	PCMA		
38599						*P. fragi*	R	3		RM14	PCMA		
38601						*P. fragi*	R	3		RM14	PCMA		
38605						*P. fragi*	R	3		RM11	PCMA		
38608						*P. fragi*	R	3		RM14	PCMA		
38613						*P. fragi*	R	3		RM8	PCMA		
38614						*P. fragi*	R	3		RM8	PCMA		
38616						*P. fragi*	R	3		RM11	PCMA		
38618	*Pseudomonas proteolytica*	*P. fragi*	FN650738			*P. fragi*	R	3		RM11	PCMA		
38622						*P. fragi*	R	3		RM14	PCMA		
38627						*P. fragi*	R	3		RM14	PCMA		
38628						*P. fragi*	R	3		RM11	PCMA		
38629						*P. fragi*	R	3		RM8	PCMA		
38630						*P. fragi*	R	3		RM8	PCMA		
38631						*P. fragi*	R	3		RM8	PCMA		
38636						*P. fragi*	R	3		RM8	PCMA		
38640						*P. fragi*	R	3		RM8	PCMA		
38642						*P. fragi*	R	3		RM14	PCMA		
38644						*P. fragi*	R	3		RM14	PCMA		
38645	*Pseudomonas proteolytica*	*P. fragi*	FN650739	*P. fragi*	FN650784	*P. fragi*	R	3		RM14	PCMA		
38646						*P. fragi*	R	3		RM14	PCMA		
38648						*P. fragi*	R	3		RM14	PCMA		
38651						*P. fragi*	R	3		RM8	PCMA		
38654						*P. fragi*	R	3		RM1	CFC		
38655						*P. fragi*	R	3		RM1	CFC		
38657						*P. fragi*	R	3		RM1	CFC		
38662						*P. fragi*	R	3		RM14	PCMA		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38663						*P. fragi*	R	3		RM14	PCMA		
38669						*P. fragi*	R	3		RM1	CFC		
38670						*P. fragi*	R	3		RM1	CFC		
38671						*P. fragi*	R	3		RM1	CFC		
38673						*P. fragi*	R	3		RM1	CFC		
38687						*P. fragi*	R	3		RM1	CFC		
38694						*P. fragi*	R	3		RM14	PCMA		
38698						*P. fragi*	R	3		RM1	CFC		
38702						*P. fragi*	R	3		RM1	CFC		
38705						*P. fragi*	R	3		RM1	CFC		
38706						*P. fragi*	R	3		RM14	PCMA		
38708						*P. fragi*	R	3		RM8	PCMA		
38813						*P. fragi*	R	3		RM11	CFC		
38814						*P. fragi*	R	3		RM11	CFC		
38815						*P. fragi*	R	3		RM11	CFC		
38817						*P. fragi*	R	3		RM11	CFC		
38820						*P. fragi*	R	3		RM11	CFC		
38834						*P. fragi*	R	3		RM11	CFC		
38835						*P. fragi*	R	3		RM11	CFC		
38840						*P. fragi*	R	3		RM11	CFC		
38843						*P. fragi*	R	3		RM11	CFC		
39013						*P. fragi*	R	3		RM11	CFC		
39364						*P. fragi*	R	3		RM11	CFC		
39366						*P. fragi*	R	3		RM11	CFC		
39385						*P. fragi*	R	3		RM11	CFC		
39392						*P. fragi*	R	3		RM11	CFC		
39427						*P. fragi*	R	3		RM14	CFC		
39433						*P. fragi*	R	3		RM14	CFC		
39434						*P. fragi*	R	3		RM14	CFC		
39435						*P. fragi*	R	3		RM14	CFC		
39436						*P. fragi*	R	3		RM14	CFC		
39437						*P. fragi*	R	3		RM14	CFC		
39438						*P. fragi*	R	3		RM14	CFC		
39439						*P. fragi*	R	3		RM14	CFC		
39440						*P. fragi*	R	3		RM14	CFC		
39441						*P. fragi*	R	3		RM14	CFC		
39442						*P. fragi*	R	3		RM14	CFC		
39443	*Pseudomonas marginalis*	*P. fragi*	FN650746	*P. fragi*	FN650791	*P. fragi*	R	3		RM14	CFC		
39444						*P. fragi*	R	3		RM14	CFC		
39445						*P. fragi*	R	3		RM14	CFC		
39446						*P. fragi*	R	3		RM14	CFC		
39447						*P. fragi*	R	3		RM14	CFC		
39449						*P. fragi*	R	3		RM14	CFC		
39450						*P. fragi*	R	3		RM14	CFC		

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		16S rRNA		*rpoB*		final ID in study of		isol. param.				enz. act.	
R-number	FAME FATAL ID	ID	acc. n°	ID	acc. n°	De Jonghe *et al.* (2011)	cl.	S.	C.	P.	M.	1	2
39451						*P. fragi*	R	3		RM14	CFC		
39452						*P. fragi*	R	3		RM14	CFC		
39453						*P. fragi*	R	3		RM14	CFC		
39454						*P. fragi*	R	3		RM14	CFC		
39455						*P. fragi*	R	3		RM14	CFC		
39457						*P. fragi*	R	3		RM14	CFC		
39459						*P. fragi*	R	3		RM14	CFC		
39460						*P. fragi*	R	3		RM14	CFC		
39462						*P. fragi*	R	3		RM14	CFC		
38579						*P. fragi*	S	3		RM14	PCMA		
38580						*P. fragi*	S	3		RM14	PCMA		
38581						*P. fragi*	S	3		RM14	PCMA		
38582						*P. fragi*	S	3		RM14	PCMA		
38595						*P. fragi*	S	3		RM14	PCMA		
38596						*P. fragi*	S	3		RM14	PCMA		
38637						*P. fragi*	S	3		RM8	PCMA		
38639						*P. fragi*	S	3		RM8	PCMA		
38643						*P. fragi*	S	3		RM14	PCMA		
38647	*Pseudomonas proteolytica*	*P. fragi*	FN650740	*P. fragi*	FN650785	*P. fragi*	S	3		RM14	PCMA		
37065	*Pseudomonas libanensis*					*Pseudomonas lundensis*	T + U + V	1		RM8	CFC		
37073						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37078						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37088	*Pseudomonas tolaasii*	*P. lundensis*	FN650711	*P. lundensis*	FN650750	*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37089						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37099						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37102	*Pseudomonas tolaasii*			*P. lundensis*	FN650755	*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37103						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37104						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37105						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37119						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37178						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37182						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37183						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37184	*Pseudomonas chlororaphis*			*P. lundensis*	FN650761	*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37188						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37191						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37192						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37199						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37203						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37206						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37224						*Pseudomonas lundensis*	T + U + V	1		RM11	CFC		
37243						*Pseudomonas lundensis*	T + U + V	1		RM8	CFC		
37260	*Pseudomonas tolaasii*	*P. lundensis*	FN650723	*P. lundensis*	FN650765	*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		
37269						*Pseudomonas lundensis*	T + U + V	1		RM14	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
37339						*Pseudomonas lundensis*	T + U + V	1		RM8	CFC		
37911						*Pseudomonas lundensis*	T + U + V	2		RM8	CFC		
37924						*Pseudomonas lundensis*	T + U + V	2		RM8	CFC		
37925						*Pseudomonas lundensis*	T + U + V	2		RM8	CFC		
37946						*Pseudomonas lundensis*	T + U + V	2		RM8	CFC		
37961						*Pseudomonas lundensis*	T + U + V	2		RM8	CFC		
38000						*Pseudomonas lundensis*	T + U + V	2		RM11	CFC		
38001						*Pseudomonas lundensis*	T + U + V	2		RM11	CFC		
38003						*Pseudomonas lundensis*	T + U + V	2		RM11	CFC		
38097						*Pseudomonas lundensis*	T + U + V	2		RM14	CFC		
38098						*Pseudomonas lundensis*	T + U + V	2		RM14	CFC		
38110						*Pseudomonas lundensis*	T + U + V	2		RM14	CFC		
38123						*Pseudomonas lundensis*	T + U + V	2		RM11	CFC		
38240	*Pseudomonas vancouverensis*					*Pseudomonas lundensis*	T + U + V	2		RM14	CFC		
38245						*Pseudomonas lundensis*	T + U + V	2		RM14	CFC		
38666				*P. lundensis*	FN650786	*Pseudomonas lundensis*	T + U + V	3		RM1	CFC		
38692						*Pseudomonas lundensis*	T + U + V	3		RM14	PCMA		
38700						*Pseudomonas lundensis*	T + U + V	3		RM1	CFC		
38766						*Pseudomonas lundensis*	T + U + V	3		RM8	CFC		
38769						*Pseudomonas lundensis*	T + U + V	3		RM8	CFC		
38822						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
38824						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39023						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39027						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39187						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39188						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39189						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39190						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39192						*Pseudomonas lundensis*	T + U + V	3		RM11	CFC		
39380						*Pseudomonas lundensis*	T + U + V	3		RM14	CFC		
39430						*Pseudomonas lundensis*	T + U + V	3		RM14	CFC		
39458						*Pseudomonas lundensis*	T + U + V	3		RM14	CFC		
39464						*Pseudomonas lundensis*	T + U + V	3		RM8	CFC		
39468						*Pseudomonas lundensis*	T + U + V	3		RM8	CFC		
39469						*Pseudomonas lundensis*	T + U + V	3		RM8	CFC		
37908	*Pseudomonas tolaasii*			*P. fragi*	FN650773	*P. fragi* like	W	2		RM8	CFC		
37912						*P. fragi* like	W	2		RM8	CFC		
38244						*P. fragi* like	W	2		RM14	CFC		
38253	*Pseudomonas vancouverensis*	*P. fragi*	FN650736	*P. fragi*	FN650782	*P. fragi* like	W	2		RM14	CFC		
38436						*P. fragi* like	W	2		RM14	CFC		
38439						*P. fragi* like	W	2		RM14	CFC		
38606						*P. fragi* like	W	3		RM14	PCMA		
38621						*P. fragi* like	W	3		RM14	PCMA		
38632						*P. fragi* like	W	3		RM8	PCMA		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38812	*Pseudomonas chlororaphis*	*P. fragi*	FN650744	*P. fragi*	FN650789	*P. fragi* like	W	3		RM11	CFC		
39387						*P. fragi* like	W	3		RM11	CFC		
37062	*Pseudomonas libanensis*					*Pseudomonas* sp.		1		RM8	CFC		
37069	*Pseudomonas libanensis*					*Pseudomonas* sp.		1		RM8	CFC		
37098						*Pseudomonas* sp.		1		RM14	CFC		
37108	*Pseudomonas extremorientalis*					*Pseudomonas* sp.		1		RM14	CFC		
37109	*Pseudomonas salomonii*					*Pseudomonas* sp.		1		RM14	CFC		
37112	*Pseudomonas tolaasii*					*Pseudomonas* sp.		1		RM14	CFC		
37113	*Pseudomonas costantinii*					*Pseudomonas* sp.		1		RM14	CFC		
37115	*Pseudomonas antarctica*					*Pseudomonas* sp.		1		RM14	CFC		
37117	*Pseudomonas extremorientalis*					*Pseudomonas* sp.		1		RM14	CFC		
37171	*Pseudomonas extremorientalis*					*Pseudomonas* sp.		1		RM8	CFC		
37177	*Pseudomonas proteolytica*					*Pseudomonas* sp.		1		RM11	CFC		
37198						*Pseudomonas* sp.		1		RM11	CFC		
37201	*Pseudomonas abietaniphila*					*Pseudomonas* sp.		1		RM11	CFC		
37202	*Pseudomonas proteolytica*					*Pseudomonas* sp.		1		RM11	CFC		
37204						*Pseudomonas* sp.		1		RM11	CFC		
37232	*Pseudomonas agarici*					*Pseudomonas* sp.		1		RM1	CFC		
37241						*Pseudomonas* sp.		1		RM1	CFC		
37273	*Pseudomonas fluorescens*					*Pseudomonas* sp.		1		RM11	CFC		
37329	*Rhizobium* sp.					*Rhizobium* sp.		1		RM1	CFC		
37894	*Pseudomonas aeruginosa*					*Pseudomonas aeruginosa*		2		RM0	CFC		
37896						*Pseudomonas* sp.		2		RM0	CFC		
37898	*Stenotrophomonas* sp.					*Stenotrophomonas* sp.		2		RM1	CFC		
37899	*Pseudomonas lundensis*					*Pseudomonas* sp.		2		RM1	CFC		
37914						*Pseudomonas* sp.		2		RM8	CFC		
37920	*Pseudomonas brenneri*					*Pseudomonas* sp.		2		RM8	CFC		
37921	*Pseudomonas lurida*					*Pseudomonas* sp.		2		RM8	CFC		
37929						*Pseudomonas* sp.		2		RM8	CFC		
37932						*Pseudomonas* sp.		2		RM1	CFC		
37933						*Pseudomonas* sp.		2		RM1	CFC		
37943						*Pseudomonas* sp.		2		RM8	CFC		
37963						*Pseudomonas* sp.		2		RM11	CFC		
37973	*Pseudomonas brenneri*					*Pseudomonas* sp.		2		RM11	CFC		
37975	*Pseudomonas abietaniphila*					*Pseudomonas* sp.		2		RM11	CFC		
37980	*Pseudomonas tolaasii*					*Pseudomonas* sp.		2		RM11	CFC		
37982	*Stenotrophomonas* sp.					*Stenotrophomonas* sp.		2		RM1	CFC		
37983						*Pseudomonas* sp.		2		RM0	CFC		
38072	*Pseudomonas brenneri*					*Pseudomonas* sp.		2		RM14	CFC		
38074	*Pseudomonas brenneri*					*Pseudomonas* sp.		2		RM14	CFC		
38078	*Pseudomonas libanensis*					*Pseudomonas* sp.		2		RM11	CFC		
38082	*Pseudomonas proteolytica*					*Pseudomonas* sp.		2		RM11	CFC		
38086	*Pseudomonas agarici*					*Pseudomonas* sp.		2		RM11	CFC		
38090						*Pseudomonas* sp.		2		RM11	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38094						*Pseudomonas* sp.		2		RM14	CFC		
38095						*Pseudomonas* sp.		2		RM14	CFC		
38096	*Pseudomonas antarctica*					*Pseudomonas* sp.		2		RM14	CFC		
38101	*Pseudomonas putida*					*Pseudomonas* sp.		2		RM14			
38105						*Pseudomonas* sp.		2		RM11	CFC		
38109	*Pseudomonas putida*					*Pseudomonas* sp.		2		RM11	CFC		
38112						*Pseudomonas* sp.		2		RM14	CFC		
38119						*Pseudomonas* sp.		2		RM11	CFC		
38120	*Pseudomonas chlororaphis*					*Pseudomonas* sp.		2		RM14	CFC		
38246	*Pseudomonas brenneri*					*Pseudomonas* sp.		2		RM14	CFC		
38250	*Pseudomonas extremorientalis*					*Pseudomonas* sp.		2		RM14	CFC		
38252	*Pseudomonas lurida*					*Pseudomonas* sp.		2		RM14	CFC		
38254	*Pseudomonas proteolytica*					*Pseudomonas* sp.		2		RM14	CFC		
38556	*Rahnella aquatilis*							3		RM11	PCMA		
38559	*Rahnella aquatilis*							3		RM11	PCMA		
38560						*Pseudomonas* sp.		3		RM11	PCMA		
38561	*Rahnella aquatilis*							3		RM11	PCMA		
38562						*Pseudomonas* sp.		3		RM11	PCMA		
38563						*Pseudomonas* sp.		3		RM11	PCMA		
38574						*Pseudomonas* sp.		3		RM11	PCMA		
38575						*Pseudomonas* sp.		3		RM11	PCMA		
38576						*Pseudomonas* sp.		3		RM11	PCMA		
38577	*Pseudomonas putida*					*Pseudomonas* sp.		3		RM11	PCMA		
38578						*Pseudomonas* sp.		3		RM11	PCMA		
38585	*Rahnella aquatilis*							3		RM11	PCMA		
38588						*Pseudomonas* sp.		3		RM8	PCMA		
38589	*Pseudomonas marginalis*					*Pseudomonas* sp.		3		RM8	PCMA		
38590						*Pseudomonas* sp.		3		RM8	PCMA		
38604	*Pseudomonas brenneri*					*Pseudomonas* sp.		3		RM11	PCMA		
38607	*Pseudomonas tolaasii*					*Pseudomonas* sp.		3		RM14	PCMA		
38610	*Rahnella aquatilis*							3		RM14	PCMA		
38611						*Pseudomonas* sp.		3		RM14	PCMA		
38623	*Rahnella aquatilis*							3		RM14	PCMA		
38633	*Enterobacter intermedius*							3		RM1	PCMA		
38635	*Enterobacter intermedius*							3		RM1	PCMA		
38649	*Rahnella aquatilis*							3		RM1	PCMA		
38656						*Pseudomonas* sp.		3		RM1	CFC		
38658						*Pseudomonas* sp.		3		RM14	PCMA		
38659	*Rahnella* sp.							3		RM14	PCMA		
38660						*Pseudomonas* sp.		3		RM14	PCMA		
38665	*Stenotrophomonas* sp.							3		RM1	CFC		
38680	*Pseudomonas brenneri*					*Pseudomonas* sp.		3		RM8	CFC		
38681						*Pseudomonas* sp.		3		RM8	CFC		
38682						*Pseudomonas* sp.		3		RM8	CFC		

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R-number	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	*rpoB* ID	*rpoB* acc. n°	final ID in study of De Jonghe *et al.* (2011)	cl.	isol. param. S.	C.	P.	M.	enz. act. 1	2
38688	*Pseudomonas libanensis*					*Pseudomonas* sp.		3		RM1	CFC		
38691						*Pseudomonas* sp.		3		RM1	CFC		
38707						*Pseudomonas* sp.		3		RM1	CFC		
38755						*Pseudomonas* sp.		3		RM8	CFC		
38759						*Pseudomonas* sp.		3		RM8	CFC		
38762						*Pseudomonas* sp.		3		RM8	CFC		
38770						*Pseudomonas* sp.		3		RM8	CFC		
38816						*Pseudomonas* sp.		3		RM11	CFC		
38827	*Pseudomonas libanensis*					*Pseudomonas* sp.		3		RM8	CFC		
38844						*Pseudomonas* sp.		3		RM11	CFC		
39007	*Pseudomonas azotoformans*					*Pseudomonas* sp.		3		RM11	CFC		
39008						*Pseudomonas* sp.		3		RM11	CFC		
39014	*Pseudomonas tolaasii*					*Pseudomonas* sp.		3		RM11	CFC		
39367						*Pseudomonas* sp.		3		RM11	CFC		
39369						*Pseudomonas* sp.		3		RM11	CFC		
39370						*Pseudomonas* sp.		3		RM14	CFC		
39372	*Pseudomonas lundensis*					*Pseudomonas* sp.		3		RM14	CFC		
39374	*Pseudomonas fuscovaginae*					*Pseudomonas* sp.		3		RM14	CFC		
39378	*Pseudomonas lurida*					*Pseudomonas* sp.		3		RM14	CFC		
39386	*Pseudomonas brenneri*					*Pseudomonas* sp.		3		RM11	CFC		
39390	*Pseudomonas lundensis*					*Pseudomonas* sp.		3		RM11	CFC		
39397	*Pseudomonas chlororaphis*					*Pseudomonas* sp.		3		RM14	CFC		
39400						*Pseudomonas* sp.		3		RM14	CFC		
39431						*Pseudomonas* sp.		3		RM14	CFC		
40253	*Acidovorax* sp.					*Acidovorax* sp.		3		RM1	CFC		

APPENDIX B

Overview of Bacillus isolates

Overview of all strains under study in Chapter6 about aerobic spore-formers in milk. The strain number (R-number) is given with an identification based on FAME (GC subgroup between brackets), on 16S rRNA gene sequencing (with the accession n°) and the final identification based on these two techniques and rep-fingerprinting. Clusters based on FAME are indicated (Cl.), as well as the isolation paramaters (Isol. param.): farm (F; O: 1 - 5; C: 6 - 10), isolation medium (SM, BHI or NAE), season (S / W; summer / winter) and isolation temperature (T.). Psychrotrophic growth capacity was evaluated after 7 days of incubation at 7°C and scored + or -. Enzymatic activity is represented based on screening on elective media. Bars are indicative for the whole cluster, with black bars: > 70% of the strains scored positive, grey bars: 30 - 70% of the strains scored positive, and white bars: more than 70% scored negative; 1: X-GAL medium (indicative for lactose fermentation), 2: TA-medium (indicative for lipolytic activity), 3: NAE-medium (indicative for phospholipase activity), 4: SM-medium (indicative for protease activity). If (GTG)5 fingerprinting was performed, the rep-type is indicated in 'REP'. Quantitative assays for proteolytic activity (P) and lipolytic activity (L) are indicated as well as the ability to ferment lactose (F) and to reduce nitrate to nitrite (N). Toxins represent the PCR-based assay as well as the screening for heat-stable and heat-labile cytotoxic compounds.
Used abbreviations: B., Bacillus; B. thermoamylo., Bacillus thermoamylovorans; B. sporothermo., Bacillus sporothermodurans; G., Geobacillus; U., Ureibacillus.

R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
31311	*Paenibacillus polymyxa*	99.1% *P. polymyxa*	AM910321	*P. polymyxa*	A	9	SM	W	20°C	++							
30959	*Paenibacillus polymyxa*	97.9% *P. polymyxa*	AM910284	*P. polymyxa*	A	10	BHI	W	37°C								
30834	*Paenibacillus polymyxa*	99.6% *P. polymyxa*	AM910260	*P. polymyxa*	A	7	SM	W	37°C								
31179	*Paenibacillus polymyxa*	98.9% *P. polymyxa*	AM910301	*P. polymyxa*	A	1	SM	W	20°C	+++							
30602	*Paenibacillus polymyxa*	99.1% *P. polymyxa*	AM910237	*P. polymyxa*	A	3	SM	W	37°C								
31029	*Paenibacillus polymyxa*	99.1% *P. polymyxa*	AM910292	*P. polymyxa*	A	9	SM	W	37°C								
31300	*Paenibacillus polymyxa*	99.1% *P. polymyxa*	AM910314	*P. polymyxa*	A	10	SM	W	20°C	++			++	-	++	+	-
30957	*Paenibacillus polymyxa*	98.9% *P. polymyxa*	AM910283	*P. polymyxa*	A	10	BHI	W	37°C								
27609	*Paenibacillus polymyxa*	99.1% *P. polymyxa*	AM910220	*P. polymyxa*	A	7	BHI	S	37°C								
31308	*Paenibacillus polymyxa*	99.0% *P. polymyxa*	AM910320	*P. polymyxa*	A	7	SM	W	20°C	++							
27993	*Paenibacillus polymyxa*	99.1% *P. lactis*	AM910222	*P. lactis*	A	10	BHI	S	37°C								-
30642	*Paenibacillus polymyxa*	98.3% *P. terrae*	AM910249	*P. terrae*	A	1	SM	W	37°C								-
27624	*Paenibacillus polymyxa*	99.5% *P. polymyxa*	AM910213	*P. polymyxa*	A	8	SM	S	37°C								
30627	*Paenibacillus polymyxa*	98.6% *P. polymyxa*	AM910243	*P. polymyxa*	A	1	SM	W	37°C								-
31326	*Paenibacillus polymyxa*	98.8% *P. lautus*	AM910327	*P. polymyxa*	A	1	BHI	W	20°C	++							
30846	*Paenibacillus polymyxa*	98.8% *P. polymyxa*	AM910265	*P. polymyxa*	A	7	SM	W	37°C								
26064	*Nesterenkonia halobia*	99.6% *P. cookii*	HE586332	*P. cookii*	A	1	NAE	S	37°C								cytK+
30653	*Brevibacterium liquefaciens*	*Paenibacillus* sp. 3	AM910250	*Paenibacillus* sp. 3	A	2	BHI	W	37°C								
27413	*Paenibacillus polymyxa*	*Paenibacillus* sp. 2	HE586333	*Paenibacillus* sp. 2	A	5	BHI	S	37°C								
31021	*Bacillus oleronius*	99.8% *B. oleronius*	AM910290	*B. oleronius*	B	9	BHI	W	37°C								
30728	*Bacillus oleronius*			*B. oleronius*	B	4	BHI	W	37°C								
31022	*Bacillus oleronius*			*B. oleronius*	B	9	BHI	W	37°C								
31028	*Bacillus oleronius*			*B. oleronius*	B	9	BHI	W	37°C								
27302	*Bacillus oleronius*			*B. oleronius*	B	4	BHI	S	37°C								
30630	*Bacillus atrophaeus*			*B. oleronius*	B	1	BHI	W	37°C								
32842	*Bacillus* (22)	100% *B. oleronius*	AM910330	*B. oleronius*	B	9	BHI	W	37°C								
27269	*Bacillus oleronius*	99.8% *B. oleronius*	HE586334	*B. oleronius*	B	3	BHI	S	37°C								
30607	*Bacillus oleronius*			*B. oleronius*	B	2	BHI	S	37°C								
30633	*Bacillus oleronius*			*B. oleronius*	B	1	BHI	W	37°C								
26050	*Bacillus oleronius*			*B. oleronius*	B	1	BHI	S	37°C								
27330	*Bacillus oleronius*	99.9% *B. oleronius*	AM910195	*B. oleronius*	B	7	BHI	S	37°C								-
30629	*Bacillus* (22)	99.5% *B. oleronius*	AM910244	*B. oleronius*	B	1	BHI	W	37°C								
27600	*Paenibacillus polymyxa*	98.3% *P. terrae*	AM910215	*P. terrae*		7	SM	S	37°C		– – – –						
31009	*Paenibacillus polymyxa*	*Paenibacillus* sp.	AM910287	*Paenibacillus* sp. 4		10	SM	W	20°C	++	– – – –						
26866	*Brevibacillus choshinensis*	99.9% *B. simplex*	AM910174	*B. simplex*		7	BHI	S	20°C	++++	– – – –						
31314	*Bacillus megaterium* (B)	99.6% *B. licheniformis*	AM910322	*B. licheniformis*		9	BHI	W	20°C	+++	– – – –						
30935	*Paenibacillus validus*	98.4% *P. lactis*	AM910281	*P. lactis*		8	BHI	W	37°C		– – – –						
27333	*Paenibacillus lautus*	98.8% *P. lactis*	AM910196	*P. lactis*	C	8	BHI	S	37°C								
30939	*Paenibacillus popilliae*	98.8% *P. lactis*	AM910282	*P. lactis*	C	8	BHI	W	37°C								
27359	*Paenibacillus lautus*	98.4% *P. lactis*	AM910202	*P. lactis*	C	9	BHI	S	37°C								
31035	*Bacillus lentus*	99% *P. lactis*	AM910294	*P. lactis*	C	9	BHI	W	37°C								
30637	*Bacillus lentus*	98.7% *P. odorifer*	AM910248	*P. odorifer*	C	1	BHI	W	37°C								
27028	*Bacillus clausii*	*Oceanobacillus* sp.	AM910192	*Oceanobacillus* sp. 1	C	6	BHI	S	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	isol. param. M.	isol. param. S/W	isol. param. T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
31297	*Bacillus clausii*	99.2% *B. thermoamylovorans*	AM910313	*B. thermoamylovorans*	C	10	BHI	W	55°C								
26208	*Bacillus circulans*	*Bacillus* sp.	HE586335	*Bacillus* sp. 1	C	1	BHI	S	37°C								
26217	*Bacillus lentus*	99.9% *B. galactosidilyticus*	HE586336	*B. galactosidilyticus*	C	1	BHI	S	37°C								
30910	*Paenibacillus polymyxa*	99.6% *P. macerans*	AM910269	*P. macerans*	C	10	BHI	W	37°C								
26267	*Bacillus alcalophilus*	99.1% *B. clausii*	HE586337	*B. clausii*	C	2	BHI	S	37°C								
27422	*Paenibacillus pabuli*	*Paenibacillus* sp.	HE586338	*Paenibacillus* sp. 1	C	5	BHI	S	37°C								
28338	*Paenibacillus pabuli*	*Paenibacillus* sp.	AM910228	*Paenibacillus* sp. 1	C	9	BHI	S	37°C								
27420	*Paenibacillus pabuli*			*Paenibacillus* sp. 1	C	5	BHI	S	37°C								
27029	*Paenibacillus alvei* (A)	98.5% *P. lautus*	AM910193	*P. lautus*	C	7	BHI	S	37°C								
30631	*Paenibacillus alvei* (A)	*Paenibacillus* sp.	AM910245	*Paenibacillus* sp. 1	C	1	BHI	W	37°C								
27370	*NO MATCH*	98.8% *B. thermoamylovorans*	AM910205	*B. thermoamylo.*	C	9	BHI	S	37°C								
30608	*Cellulomonas fimi* (A)	99.5% *B. licheniformis*	AM910238	*B. licheniformis*	D	2	SM	W	37°C			L2					-
30928	*Cellulomonas fimi* (A)			*B. licheniformis*	D	6	BHI	W	37°C								
30941	*Micrococcus luteus* (B)			*B. licheniformis*	D	8	BHI	W	37°C								
30650	*Virgibacillus pantothenticus*			*B. licheniformis*	D	2	BHI	W	37°C								
30902	*Virgibacillus pantothenticus*			*B. licheniformis*	D	10	SM	W	37°C			L1					-
30609	*Bacillus atrophaeus*			*B. licheniformis*	D	2	SM	W	37°C								
30924	*Bacillus atrophaeus*	98.1% *B. licheniformis*	AM910275	*B. licheniformis*	D	6	BHI	W	55°C			L8					-
27306	*Bacillus megaterium* (A)			*B. licheniformis*	D	4	BHI	S	37°C								
27307	*Bacillus pumilus* (A)			*B. licheniformis*	D	4	BHI	S	37°C			L1					
31043	*Bacillus subtilis*			*B. licheniformis*	D	8	SM	W	37°C								
27026	*Bacillus subtilis*			*B. licheniformis*	D	6	BHI	S	37°C			F4					
30624	*Bacillus atrophaeus*			*B. licheniformis*	D	1	BHI	W	37°C								
27020	*Staphylococcus schleiferi*			*B. licheniformis*	D	6	BHI	S	37°C								
31197	*Bacillus laevolacticus*			*B. licheniformis*	D	7	BHI	W	20°C	-		L1					
27036	*Bacillus licheniformis*			*B. licheniformis*	D	6	BHI	S	37°C			L1					
27631	*Bacillus licheniformis*	99.9% *B. licheniformis*	AM910214	*B. licheniformis*	D	8	BHI	S	37°C			L1					
30718	*Bacillus licheniformis*			*B. licheniformis*	D	4	SM	W	37°C			L1					
26235	*Bacillus laevolacticus*			*B. licheniformis*	D	1	NAE	S	37°C			L1					
30832	*Bacillus licheniformis*			*B. licheniformis*	D	7	BHI	W	37°C								
30929	*Bacillus subtilis*			*B. licheniformis*	D	6	BHI	W	37°C								
30628	*Bacillus licheniformis*			*B. licheniformis*	D	1	BHI	W	37°C			L2					
27332	*Bacillus atrophaeus*			*B. licheniformis*	D	8	BHI	S	37°C								
27364	*Bacillus atrophaeus*			*B. licheniformis*	D	9	SM	S	37°C			L3					
27611	*Virgibacillus pantothenticus*			*B. licheniformis*	D	7	BHI	S	37°C								
30649	*Virgibacillus pantothenticus*			*B. licheniformis*	D	2	BHI	W	37°C								
27383	*Bacillus subtilis*	99.7% *B. licheniformis*	HE586339	*B. licheniformis*	D	4	NAE	S	37°C			L1					
30603	*Bacillus licheniformis*			*B. licheniformis*	D	3	SM	W	37°C								
31327	*Bacillus licheniformis*			*B. licheniformis*	D	1	BHI	W	20°C	-		L2					
31034	*Bacillus subtilis*			*B. licheniformis*	D	9	BHI	W	37°C								
30623	*Bacillus coagulans*			*B. licheniformis*	D	1	BHI	W	37°C								
30644	*Bacillus atrophaeus*			*B. licheniformis*	D	1	SM	W	37°C			L1					
30839	*Bacillus coagulans*			*B. licheniformis*	D	7	SM	W	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
26300	*Bacillus pumilus* (B)			*B. licheniformis*	D	2	BHI	S	37°C		■ ■ ■	L1					
30940	*Bacillus subtilis*	99.5% *B. licheniformis*	AM910280	*B. licheniformis*	D	8	BHI	W	37°C		■ ■ ■						
30835	*Virgibacillus pantothenticus*	99.1% *B. licheniformis*	AM910261	*B. licheniformis*	D	7	SM	W	37°C		■ ■ ■	L1	+	-	+	-	-
30907	*Virgibacillus pantothenticus*			*B. licheniformis*	D	10	SM	W	37°C		■ ■ ■						
30920	*Bacillus atrophaeus*			*B. licheniformis*	D	10	BHI	W	37°C		■ ■ ■						
30645	*Virgibacillus pantothenticus*			*B. licheniformis*	D	1	SM	W	37°C		■ ■ ■	L1					
30938	*Bacillus atrophaeus*			*B. licheniformis*	D	8	BHI	W	37°C		■ ■ ■						
30840	*Virgibacillus pantothenticus*			*B. licheniformis*	D	7	SM	W	37°C		■ ■ ■						
31214	*Paenibacillus macerans*	98.8% *B. subtilis*	AM910311	*B. subtilis*	D	2	BHI	W	20°C	-	■ ■ ■						H-S+, H-L+
30908	*Virgibacillus pantothenticus*			*B. licheniformis*	D	10	SM	W	37°C		■ ■ ■						
30926	*Bacillus atrophaeus*			*B. licheniformis*	D	6	SM	W	37°C		■ ■ ■						
30823	*Bacillus coagulans*			*B. licheniformis*	D	7	SM	W	37°C		■ ■ ■						
28349	*Virgibacillus pantothenticus*		AM910231	*B. licheniformis*	D	8	SM	S	37°C		■ ■ ■	L2					
30611	*Virgibacillus pantothenticus*			*B. licheniformis*	D	2	SM	W	37°C		■ ■ ■	L1					
30838	*Bacillus atrophaeus*			*B. licheniformis*	D	7	SM	W	37°C		■ ■ ■						
30999	*Bacillus licheniformis*			*B. licheniformis*	D	10	BHI	W	20°C	-	■ ■ ■	L1					
27416	*Bacillus atrophaeus*			*B. licheniformis*	D	5	BHI	S	37°C		■ ■ ■	L1					
30905	*Bacillus licheniformis*	99.9% *B. licheniformis*	AM910267	*B. licheniformis*	D	10	SM	W	37°C		■ ■ ■						
30620	*Bacillus atrophaeus*	99.8% *B. licheniformis*	AM910240	*B. licheniformis*	D	3	BHI	W	37°C		■ ■ ■						
30933	*Bacillus coagulans*	99.7% *B. licheniformis*	AM910278	*B. licheniformis*	D	8	SM	W	37°C		■ ■ ■						
30656	*Bacillus laevolacticus*	99.8% *B. licheniformis*	AM910251	*B. licheniformis*	D	2	BHI	W	37°C		■ ■ ■						
30923	*Staphylococcus aureus* (G)	97.7% *B. licheniformis*	AM910274	*B. licheniformis*	D	6	BHI	W	55°C		■ ■ ■						
27304	*Paenibacillus alvei* (A)			*B. subtilis*	E	4	BHI	S	37°C		■						
30845	*Paenibacillus alvei* (A)		AM910264	*B. subtilis*	E	7	SM	W	37°C		■						
30707	*Paenibacillus macerans*	99.1% *B. subtilis*	AM910252	*B. subtilis*	E	4	BHI	W	37°C		■						
27355	*Paenibacillus alvei* (A)		AM910200	*B. subtilis*	E	9	BHI	S	37°C		■						
30621	*Paenibacillus lentimorbus*	99.5% *B. amyloliquefaciens*	AM910241	*B. subtilis*	E	3	BHI	W	37°C		■						
30837	*Bacillus subtilis*	99.8% *B. subtilis*	AM910262	*B. subtilis*	F	7	SM	W	37°C		■ ■ ■	F4	+			+	H-L+
30932	*Bacillus subtilis*			*B. subtilis*	F	8	SM	W	37°C		■ ■ ■						
31040	*Bacillus laevolacticus*			*B. licheniformis*	F	8	SM	W	37°C		■ ■ ■						
27406	*Bacillus oleronius*			*B. licheniformis*	F	5	BHI	S	37°C		■ ■ ■						
27380	*Bacillus subtilis*			*B. subtilis*	F	4	SM	S	37°C		■ ■ ■	L4					
27616	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C		■ ■ ■						
33525	*Virgibacillus pantothenticus*	100% *B. licheniformis*	AM910341	*B. licheniformis*	F	4	BHI	W	20°C	-	■ ■ ■	L2					
27592	*Bacillus licheniformis*	99.8% *B. licheniformis*	HE586340	*B. licheniformis*	F	5	SM	S	37°C		■ ■ ■						-
30652	*Bacillus licheniformis*			*B. licheniformis*	F	2	BHI	W	37°C		■ ■ ■						
30704	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C		■ ■ ■	L1					
30724	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C		■ ■ ■	L1					
31036	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	37°C		■ ■ ■						
28006	*Bacillus licheniformis*			*B. licheniformis*	F	10	BHI	S	37°C		■ ■ ■						
27423	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	S	37°C		■ ■ ■	L1					
31303	*Bacillus subtilis*	99.9% *B. subtilis*	AM910316	*B. subtilis*	F	7	SM	W	20°C	-	■ ■ ■	F1					
33577	*Bacillus subtilis*			*B. subtilis*	F	7	SM	W	37°C		■ ■ ■						
27630	*Bacillus licheniformis*	98.4% *B. licheniformis*	AM910210	*B. licheniformis*	F	8	BHI	S	37°C		■ ■ ■						

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
31194	*Bacillus licheniformis*	99.6% *B. licheniformis*	AM910306	*B. licheniformis*	F	7	BHI	W	20°C	-		L2					
27997	*Bacillus subtilis*			*B. subtilis*	F	10	SM	S	37°C								
26221	*Bacillus laevolacticus*			*B. licheniformis*	F	1	SM	S	37°C								
27350	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	S	37°C								-
30836	*Bacillus subtilis*			*B. subtilis*	F	7	SM	W	37°C			F4					
27390	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	S	37°C			L1					
27994	*Bacillus licheniformis*			*B. licheniformis*	F	10	BHI	S	37°C								
27358	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	37°C			L1					
30727	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C			L1					
31031	*Bacillus licheniformis*			*B. licheniformis*	F	9	SM	W	37°C								
27301	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	S	37°C			L2					
27356	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	37°C			L2	+	-		-	-
27588	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	S	37°C								
28340	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	37°C			L1					
31027	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	37°C								
30825	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	W	37°C								
31122	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	20°C	-		L2					
30712	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C								
31020	*Bacillus licheniformis*	99.8% *B. licheniformis*	AM910289	*B. licheniformis*	F	9	NAE	W	37°C			L1					
30709	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C								
31023	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	37°C								
27267	*Bacillus licheniformis*			*B. licheniformis*	F	3	BHI	S	37°C			L1					
27365	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	37°C			L2					
27391	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	S	37°C			L1					
30831	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C			L1					
27270	*Bacillus licheniformis*			*B. licheniformis*	F	3	BHI	S	37°C								
27264	*Bacillus licheniformis*	98.5% *B. licheniformis*	HE586341	*B. licheniformis*	F	3	BHI	S	37°C			L2					
28334	*Bacillus licheniformis*			*B. licheniformis*	F	8	SM	S	37°C			L1					
30725	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C								
27421	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C			L2					
30723	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C			L1					
30705	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C								
26272	*Bacillus licheniformis*	99.8% *B. licheniformis*	HE586342	*B. licheniformis*	F	2	BHI	S	37°C								
26281	*Bacillus licheniformis*			*B. licheniformis*	F	2	SM	S	37°C								
27262	*Bacillus licheniformis*			*B. licheniformis*	F	3	BHI	S	37°C			L1					
30816	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C			L1					
27407	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C			L1					-
27352	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	S	37°C			L1					
31016	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	W	37°C								
30922	*Bacillus licheniformis*			*B. licheniformis*	F	6	BHI	W	55°C			L1					
27331	*Bacillus licheniformis*			*B. licheniformis*	F	8	BHI	S	37°C								
27265	*Bacillus licheniformis*			*B. licheniformis*	F	3	SM	S	37°C								
31019	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	W	37°C								
31171	*Bacillus licheniformis*			*B. licheniformis*	F	2	BHI	W	20°C	-							

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
26299	*Bacillus licheniformis*	99.6% *B. licheniformis*	HE586343	*B. licheniformis*	F	2	BHI	S	37°C			L2					-
31091	*Bacillus subtilis*	99.7% *B. licheniformis*	AM910296	*B. subtilis*	F	4	SM	W	20°C	-		F3					
27268	*Bacillus licheniformis*			*B. licheniformis*	F	3	BHI	S	37°C								
27032	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C			L2					
30720	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C								
31196	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	20°C	-		L1					
31193	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	20°C	-		L2					
31209	*Bacillus licheniformis*			*B. licheniformis*	F	1	BHI	W	20°C	-							
26283	*Bacillus licheniformis*			*B. licheniformis*	F	2	SM	S	37°C			L1					
31181	*Bacillus amyloliquefaciens*			*B. licheniformis*	F	1	BHI	W	20°C	-		F1					
31186	*Bacillus subtilis*			*B. subtilis*	F	7	BHI	W	20°C	++		F3					
31026	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	37°C			L4					
31184	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	20°C	+		L2					
27031	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C			L2					
31212	*Bacillus licheniformis*			*B. licheniformis*	F	1	BHI	W	20°C	-							
31188	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	20°C	-							
26273	*Bacillus licheniformis*			*B. licheniformis*	F	2	BHI	S	37°C			L2					
26279	*Bacillus licheniformis*			*B. licheniformis*	F	2	SM	S	37°C								
31025	*Bacillus licheniformis*	99.6% *B. licheniformis*	AM910291	*B. licheniformis*	F	9	BHI	W	37°C								
30903	*Bacillus licheniformis*	99.9% *B. licheniformis*	AM910266	*B. licheniformis*	F	10	SM	W	37°C			L2					
26210	*Bacillus subtilis*	99.9% *B. subtilis*	HE586344	*B. subtilis*	F	1	BHI	S	37°C								
30719	*Bacillus subtilis*			*B. subtilis*	F	4	SM	W	37°C			F3					
30619	*Bacillus subtilis*			*B. subtilis*	F	3	BHI	W	37°C								
33576	*Bacillus subtilis*			*B. subtilis*	F	7	SM	W	37°C								
27601	*Bacillus subtilis*	99.5% *B. subtilis*	HE586345	*B. subtilis*	F	7	SM	S	37°C								
26051	*Bacillus amyloliquefaciens*			*B. licheniformis*	F	1	BHI	S	37°C								
27386	*Bacillus subtilis*			*B. subtilis*	F	4	BHI	S	37°C								
31306	*Bacillus subtilis*	99.8% *B. subtilis*	AM910318	*B. subtilis*	F	7	SM	W	20°C	-		F1	++			+	H-S+, H-L+
26284	*Bacillus subtilis*			*B. subtilis*	F	2	SM	S	37°C								
30614	*Bacillus subtilis*	99.8% *B. subtilis*	AM910239	*B. subtilis*	F	3	BHI	W	37°C			F3	++	+		+	H-L+
27379	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	S	37°C								
31032	*Bacillus licheniformis*			*B. licheniformis*	F	9	SM	W	37°C			L1					
27337	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C			L1					
30600	*Bacillus licheniformis*			*B. licheniformis*	F	3	SM	W	37°C								
31861	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	37°C								
30829	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C			L2					
27414	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C								
28000	*Bacillus licheniformis*			*B. licheniformis*	F	10	SM	S	37°C								
27989	*Bacillus licheniformis*			*B. licheniformis*	F	10	BHI	S	37°C								
27613	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	S	37°C			L1					
30952	*Bacillus licheniformis*			*B. licheniformis*	F	6	SM	W	37°C								
28005	*Bacillus subtilis*			*B. subtilis*	F	10	BHI	S	37°C								
27599	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	S	37°C								
27604	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
27351	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	S	37°C			L1					
30703	*Bacillus laevolacticus*			*B. licheniformis*	F	4	BHI	W	37°C			L2					
31123	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	20°C	-							
31121	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	20°C	-							
26220	*Bacillus licheniformis*	99.5% *B. licheniformis*	HE586346	*B. licheniformis*	F	1	SM	S	37°C			L4	+	-	+	-	-
28332	*Bacillus licheniformis*			*B. licheniformis*	F	8	SM	S	37°C			L2					
31014	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	W	37°C								
27375	*Bacillus licheniformis*	99.7% *B. licheniformis*	HE586347	*B. licheniformis*	F	4	BHI	S	37°C			L1					
27378	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	S	37°C			L1					
26218	*Bacillus licheniformis*			*B. licheniformis*	F	1	SM	S	37°C								
26219	*Bacillus laevolacticus*			*B. licheniformis*	F	1	SM	S	37°C								
31316	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	W	20°C	-		L1					
30956	*Bacillus licheniformis*			*B. licheniformis*	F	6	BHI	W	55°C			L2					
27405	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C			L2					
30811	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C			L1					
27999	*Bacillus licheniformis*			*B. licheniformis*	F	10	SM	S	37°C								
30819	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C			L2					
30827	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	37°C								
28344	*Bacillus licheniformis*			*B. licheniformis*	F	9	SM	S	37°C			L2					
27410	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	S	37°C			L4					
27415	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C			L1					
27623	*Bacillus licheniformis*			*B. licheniformis*	F	8	SM	S	37°C			L2					
27627	*Bacillus licheniformis*			*B. licheniformis*	F	8	BHI	S	37°C			L2					
27603	*Bacillus licheniformis*	99.7% *B. licheniformis*	AM910209	*B. licheniformis*	F	7	BHI	S	37°C			L1					-
28333	*Bacillus laevolacticus*			*B. licheniformis*	F	8	SM	S	37°C								
27417	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C			L2					
27027	*Bacillus licheniformis*			*B. licheniformis*	F	6	BHI	S	37°C								
26855	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	20°C	-							
27589	*Bacillus licheniformis*			*B. licheniformis*	F	5	SM	S	37°C								
27387	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	S	37°C								
27371	*Bacillus licheniformis*			*B. licheniformis*	F	9	BHI	S	37°C								
33620	*Bacillus licheniformis*			*B. licheniformis*	F	2	SM	W	37°C								
30822	*Bacillus licheniformis*			*B. licheniformis*	F	7	SM	W	37°C			L1					
31307	*Bacillus laevolacticus*	99.3% *B. licheniformis*	AM910319	*B. licheniformis*	F	7	SM	W	20°C	-		L1					
27392	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	S	37°C			L2					
33285	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	S	37°C								
31343	*Bacillus licheniformis*			*B. licheniformis*	F	10	BHI	W	55°C			L10					
27591	*Bacillus licheniformis*			*B. licheniformis*	F	5	BHI	S	37°C								
31187	*Bacillus licheniformis*			*B. licheniformis*	F	7	BHI	W	20°C	-							
31192	*Bacillus licheniformis*	99.5% *B. licheniformis*	AM910305	*B. licheniformis*	F	7	BHI	W	20°C	-		L9					
31183	*Bacillus licheniformis*			*B. licheniformis*	F	1	BHI	W	20°C	+		L2					
31317	*Bacillus licheniformis*			*B. licheniformis*	F	8	SM	W	20°C	-							
27636	*Bacillus licheniformis*			*B. licheniformis*	F	8	BHI	S	37°C			L1					
33619	*Bacillus licheniformis*			*B. licheniformis*	F	2	SM	W	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
30717	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C		■ ■ ■						
30721	*Bacillus licheniformis*			*B. licheniformis*	F	4	SM	W	37°C		■ ■ ■						
30706	*Bacillus licheniformis*			*B. licheniformis*	F	4	BHI	W	37°C		■ ■ ■						
26034	*Bacillus licheniformis*			*B. licheniformis*	F	1	BHI	S	37°C		■ ■ ■						
30913	*Bacillus atrophaeus*	99.6% *B. licheniformis*	AM910271	*B. licheniformis*	F	10	BHI	W	37°C		■ ■ ■	L2					
27015	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	HE586348	*B. licheniformis*	F	2	BHI	S	37°C		■ ■ ■						
26216	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	S	37°C		■ ■ ■	G13					
26388	*Bacillus pumilus* (B)	99.2% *B. pumilus*	HE586349	*B. pumilus*	G	2	BHI	S	20°C	++++	■ ■ ■						
26387	*Bacillus pumilus* (B)			*B. pumilus*	G	2	BHI	S	20°C	+++	■ ■ ■	G8					
31085	*Bacillus pumilus* (B)			*B. pumilus*	G	5	SM	W	20°C	++++	■ ■ ■						
31082	*Bacillus pumilus* (B)			*B. pumilus*	G	4	SM	W	20°C	+++	■ ■ ■						
31114	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	20°C	+++	■ ■ ■	G13					
27389	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	S	37°C		■ ■ ■	G16					
27434	*Bacillus pumilus* (B)			*B. pumilus*	G	9	BHI	S	37°C		■ ■ ■						
26056	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	S	37°C		■ ■ ■	G7					
27403	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	S	37°C		■ ■ ■	G14					
27419	*Bacillus pumilus* (B)			*B. pumilus*	G	5	BHI	S	37°C		■ ■ ■	G11					
28343	*Bacillus megaterium* (A)			*B. pumilus*	G	9	BHI	S	37°C		■ ■ ■						
28517	*Bacillus megaterium* (A)			*B. pumilus*	G	8	SM	S	37°C		■ ■ ■						
27339	*Bacillus megaterium* (A)			*B. pumilus*	G	8	SM	S	37°C		■ ■ ■	G10					
26271	*Bacillus megaterium* (A)			*B. pumilus*	G	2	BHI	S	37°C		■ ■ ■						
27620	*Bacillus pumilus* (B)			*B. pumilus*	G	8	SM	S	37°C		■ ■ ■	G1					
27621	*Bacillus pumilus* (B)			*B. pumilus*	G	8	SM	S	37°C		■ ■ ■	G1					
27418	*Bacillus pumilus* (B)	99% *B. pumilus*	HE586350	*B. pumilus*	G	5	BHI	S	37°C		■ ■ ■	G1					
31086	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	20°C	+++	■ ■ ■	G1					
31312	*Bacillus pumilus* (B)			*B. pumilus*	G	9	SM	W	20°C	+++	■ ■ ■	G1					
31202	*Bacillus pumilus* (B)			*B. pumilus*	G	3	BHI	W	20°C	++	■ ■ ■						
30824	*Bacillus pumilus* (B)			*B. pumilus*	G	7	SM	W	37°C		■ ■ ■						
27305	*Bacillus pumilus* (B)	98.6% *B. pumilus*	HE586351	*B. pumilus*	G	4	BHI	S	37°C		■ ■ ■	G1					-
30821	*Bacillus pumilus* (B)	98.5% *B. pumilus*	AM910257	*B. pumilus*	G	7	BHI	W	37°C		■ ■ ■						
31090	*Bacillus pumilus* (B)			*B. pumilus*	G	4	SM	W	20°C	+++	■ ■ ■						
27992	*Bacillus pumilus* (B)			*B. pumilus*	G	10	BHI	S	37°C		■ ■ ■						
31118	*Bacillus pumilus* (B)			*B. pumilus*	G	4	SM	W	20°C	+++	■ ■ ■						
32494	*Bacillus pumilus* (B)			*B. pumilus*	G	7	BHI	W	20°C	++	■ ■ ■						
31004	*Bacillus pumilus* (B)			*B. pumilus*	G	10	BHI	W	20°C	++	■ ■ ■						
31006	*Bacillus pumilus* (B)			*B. pumilus*	G	10	BHI	W	20°C	+++	■ ■ ■						
31002	*Bacillus pumilus* (B)	98.9% *B. pumilus*	AM910286	*B. pumilus*	G	10	BHI	W	20°C	+++	■ ■ ■	G1					
31198	*Bacillus pumilus* (B)	99.2% *B. pumilus*	AM910307	*B. pumilus*	G	7	BHI	W	20°C	++++	■ ■ ■	G9					
30615	*Bacillus pumilus* (B)			*B. pumilus*	G	3	BHI	W	37°C		■ ■ ■						
30909	*Bacillus megaterium* (A)	99.3% *B. pumilus*	AM910268	*B. pumilus*	G	10	SM	W	37°C		■ ■ ■	G9					
27308	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	S	37°C		■ ■ ■	G13					
30844	*Bacillus pumilus* (B)			*B. pumilus*	G	7	BHI	W	37°C		■ ■ ■						
31322	*Bacillus pumilus* (B)			*B. pumilus*	G	8	BHI	W	20°C	+++	■ ■ ■	G14					
30729	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	37°C		■ ■ ■						

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1	2	3	4	rep	P	L	F	N	toxins
30953	*Bacillus megaterium* (A)			*B. pumilus*	G	6	SM	W	37°C											
27634	*Bacillus pumilus* (B)			*B. pumilus*	G	8	BHI	S	37°C						G14					
31003	*Bacillus megaterium* (A)			*B. pumilus*	G	10	BHI	W	20°C	+++										
31041	*Bacillus megaterium* (A)			*B. pumilus*	G	8	SM	W	37°C											
31088	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	20°C	++++					G9					
31211	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	++										
31173	*Bacillus pumilus* (B)			*B. pumilus*	G	2	SM	W	20°C	+++										
31007	*Bacillus pumilus* (B)			*B. pumilus*	G	10	SM	W	20°C	+++					G17					
31318	*Bacillus pumilus* (B)			*B. pumilus*	G	8	SM	W	20°C	+++										
33193	*Bacillus pumilus* (B)			*B. pumilus*	G	10	BHI	S	37°C						G9					
31208	*Bacillus pumilus* (B)	99.8% *B. pumilus*	AM910309	*B. pumilus*	G	1	BHI	W	20°C	+++										
31015	*Bacillus megaterium* (A)			*B. pumilus*	G	5	SM	W	37°C											
31176	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	20°C	+++										
30612	*Bacillus megaterium* (A)			*B. pumilus*	G	2	SM	W	37°C											
30625	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C						G2					
30643	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C						G3					
31203	*Bacillus pumilus* (B)			*B. pumilus*	G	3	BHI	W	20°C	+++					G1					
30654	*Bacillus pumilus* (B)			*B. pumilus*	G	2	BHI	W	37°C											
27628	*Bacillus pumilus* (B)			*B. pumilus*	G	8	BHI	S	37°C						G3					
31084	*Bacillus pumilus* (B)			*B. pumilus*	G	5	SM	W	20°C	+++										
30708	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	37°C						G8					
31309	*Bacillus pumilus* (B)			*B. pumilus*	G	7	SM	W	20°C	++					G6					
26033	*Bacillus pumilus* (B)	99% *B. pumilus*	HE586352	*B. pumilus*	G	1	BHI	S	37°C						G3					H-L+
31010	*Bacillus pumilus* (B)			*B. pumilus*	G	7	SM	W	20°C	+++					G1					
31210	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	+++					G1					
31216	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	+++										
31178	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	20°C	++++					G12					
31218	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	++++										
27361	*Bacillus pumilus* (B)	99% *B. pumilus*	AM910203	*B. pumilus*	G	9	SM	S	37°C						G9					-
31217	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	++++										
27622	*Bacillus pumilus* (B)			*B. pumilus*	G	8	SM	S	37°C						G9					
30601	*Bacillus pumilus* (B)			*B. pumilus*	G	3	SM	W	37°C						G9					
30646	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C											
30843	*Bacillus pumilus* (B)			*B. pumilus*	G	7	BHI	W	37°C											
31321	*Bacillus pumilus* (B)	99.1% *B. pumilus*	AM910325	*B. pumilus*	G	8	BHI	W	20°C	+++					G13					H-L+
30647	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C											
26206	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	S	37°C						G9					
26223	*Bacillus pumilus* (B)	99.7% *B. pumilus*	HE586353	*B. pumilus*	G	1	SM	S	37°C						G9	+	-		-	H-L+
31087	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	20°C	++++										
31115	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	W	20°C	+++					G13					
30638	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C											
31215	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	20°C	++++										
27598	*Bacillus pumilus* (B)	99.4% *B. pumilus*	AM910207	*B. pumilus*	G	7	SM	S	37°C											
28341	*Bacillus pumilus* (B)			*B. pumilus*	G	9	BHI	S	37°C											

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
27377	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	S	37°C			G15					-
31299	*Bacillus pumilus* (B)			*B. pumilus*	G	6	BHI	W	20°C	++		G7	+	++		-	H-L+
26269	*Bacillus pumilus* (B)			*B. pumilus*	G	2	BHI	S	37°C			G12					
26278	*Bacillus pumilus* (B)			*B. pumilus*	G	2	SM	S	37°C			G9					
26268	*Bacillus pumilus* (B)			*B. pumilus*	G	2	BHI	S	37°C			G1					
30954	*Bacillus pumilus* (B)			*B. pumilus*	G	8	BHI	W	37°C			G4					
27998	*Bacillus pumilus* (B)			*B. pumilus*	G	10	SM	S	37°C								
27991	*Bacillus megaterium* (A)			*B. pumilus*	G	10	BHI	S	37°C								
30616	*Bacillus pumilus* (B)			*B. pumilus*	G	3	BHI	W	37°C			G13					
30604	*Bacillus pumilus* (B)			*B. pumilus*	G	2	BHI	W	37°C								
30626	*Bacillus pumilus* (B)			*B. pumilus*	G	1	SM	W	37°C								
30634	*Bacillus pumilus* (B)			*B. pumilus*	G	1	BHI	W	37°C								
30925	*Kurthia sibirica*	99% *B. pumilus*	AM910276	*B. pumilus*	G	6	SM	W	37°C								
27388	*Bacillus pumilus* (B)			*B. pumilus*	G	4	BHI	S	37°C			G5					
31117	*Bacillus pumilus* (B)	99.6% *B. pumilus*	AM910298	*B. pumilus*	G	4	BHI	W	20°C	+++		G5					
31018	*Bacillus pumilus* (B)	98.6% *B. pumilus*	AM910288	*B. pumilus*	G	5	BHI	W	37°C								
30930	*Bacillus megaterium* (A)	99.3% *B. pumilus*	AM910277	*B. pumilus*	G	6	BHI	W	37°C			G10					
30820	*Paenibacillus macerans*	99.6% *B. amyloliquefaciens*	AM910256	*B. amyloliquefaciens*	H	7	BHI	W	37°C								
33514	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	7	BHI	W	20°C	-		H2					
31042	*Bacillus subtilis*			*B. amyloliquefaciens*	H	8	SM	W	37°C								
30713	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	4	BHI	W	37°C								
31089	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	4	SM	W	20°C	-							
31315	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	7	BHI	W	20°C	-							
31304	*Paenibacillus macerans*	99.5% *B. amyloliquefaciens*	AM910317	*B. amyloliquefaciens*	H	7	SM	W	20°C	-							
31119	*Paenibacillus macerans*	99.2% *B. amyloliquefaciens*	AM910299	*B. amyloliquefaciens*	H	4	SM	W	20°C	+							
30722	*Bacillus subtilis*			*B. amyloliquefaciens*	H	4	SM	W	37°C								
30833	*Bacillus subtilis*	99.9% *B. subtilis*	AM910259	*B. amyloliquefaciens*	H	7	SM	W	37°C								
30812	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	7	BHI	W	37°C								
30605	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	2	BHI	W	37°C								
27625	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	8	BHI	S	37°C			H2					
31116	*Paenibacillus macerans*	99.2% *B. amyloliquefaciens*	AM910297	*B. amyloliquefaciens*	H	4	SM	W	20°C	+		H1					
31125	*Bacillus amyloliquefaciens*			*B. amyloliquefaciens*	H	4	BHI	W	20°C	-							
30622	*Bacillus subtilis*	99.3% *B. amyloliquefaciens*	AM910242	*B. amyloliquefaciens*	H	3	BHI	W	37°C								
31008	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	10	SM	W	20°C	+		H1					
30813	*Paenibacillus macerans*	99.2% *B. amyloliquefaciens*	AM910255	*B. amyloliquefaciens*	H	7	BHI	W	37°C			H2	+			+	H-L+, H-S+
30810	*Paenibacillus lentimorbus*			*B. amyloliquefaciens*	H	7	BHI	W	37°C								
30830	*Paenibacillus macerans*			*B. amyloliquefaciens*	H	7	BHI	W	37°C			H2					
33515	*Paenibacillus lentimorbus*			*B. amyloliquefaciens*	H	7	BHI	W	20°C	+							
31200	*Paenibacillus macerans*	99.5% *B. amyloliquefaciens*	AM910308	*B. amyloliquefaciens*	H	3	BHI	W	20°C	+		H1	+	+		+	H-L+, H-S+
30809	*Paenibacillus lentimorbus*			*B. amyloliquefaciens*	H	7	BHI	W	37°C								
31305	*Bacillus agaradhaerens*			*B. amyloliquefaciens*	H	7	SM	W	20°C	+							
31199	*Bacillus amyloliquefaciens*			*B. amyloliquefaciens*	H	7	BHI	W	20°C	+							
26049	*Kurthia sibirica*	98.9% *B. badius*	HE586354	*B. badius*	I	1	BHI	S	37°C								
28336	*Kurthia sibirica*	98.9% *B. badius*	AM910226	*B. badius*	I	9	BHI	S	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1	2	3	4	rep	P	L	F	N	toxins
26048	*Kurthia sibirica*	*Bacillus* sp.	HE586355	*Bacillus* sp. 2		1	BHI	S	37°C		–	–	–	–						
26211	*Bacillus laevolacticus*	99.5% *B. licheniformis*	HE586356	*B. licheniformis*		1	BHI	S	37°C		–	–	–	–						
31126	*Bacillus clausii*	99.4% *B. clausii*	AM910300	*B. clausii*	J	4	BHI	W	20°C	-										
31174	*Bacillus clausii*			*B. clausii*	J	2	BHI	W	20°C	-										
28002	*Bacillus clausii*			*B. clausii*	J	10	BHI	S	37°C											
31313	*Bacillus clausii*			*B. clausii*	J	9	BHI	W	20°C	-										
27367	*Bacillus clausii*			*B. clausii*	J	9	BHI	S	37°C											
31120	*Bacillus clausii*			*B. clausii*	J	4	BHI	W	20°C	-										
26270	*Bacillus clausii*	99.3% *B. clausii*	HE586357	*B. clausii*	J	2	BHI	S	37°C											
30918	*Bacillus clausii*			*B. clausii*	J	10	BHI	W	37°C											
30934	*Paenibacillus validus*	98% *P. lactis*	AM910279	*B. clausii*	J	8	BHI	W	37°C											
28339	*Hyphomonas hirschiana*	99.2% *P. lactis*	AM910229	*B. clausii*	J	9	BHI	S	37°C									+	+	-
31124	*Bacillus clausii*			*B. clausii*	J	4	BHI	W	20°C	-										
26046	*Bacillus clausii*			*B. clausii*	J	1	BHI	S	37°C											
33528	*Bacillus clausii*			*B. clausii*	J	8	BHI	W	20°C	+										
30711	*Bacillus clausii*			*B. clausii*	J	4	BHI	W	37°C											
30815	*Bacillus clausii*			*B. clausii*	J	7	BHI	W	37°C											
30818	*Bacillus alcalophilus*			*B. clausii*	J	7	BHI	W	37°C											
26265	*Bacillus clausii*	99.7% *B. clausii*	HE586358	*B. clausii*	J	2	BHI	S	37°C									-	-	-
31000	*Bacillus clausii*	98.5% *B. clausii*	AM910285	*B. clausii*	J	10	BHI	W	20°C	-						-	-	-	+	-
30817	*Hyphomonas hirschiana*			*B. clausii*	J	7	BHI	W	37°C											
27303	*Bacillus clausii*			*B. clausii*	J	4	BHI	S	37°C											
31039	*Bacillus clausii*			*B. clausii*	J	9	BHI	W	37°C											
31182	*Bacillus clausii*			*B. clausii*	J	1	BHI	W	20°C	+										
27404	*Bacillus clausii*			*B. clausii*	J	4	BHI	S	37°C											
27400	*Bacillus clausii*	97.2% *B. clausii*	HE586359	*B. clausii*	J	4	BHI	S	37°C											
31185	*Bacillus clausii*			*B. clausii*	J	7	BHI	W	20°C	++										
30606	*Bacillus clausii*			*B. clausii*	J	2	BHI	W	37°C											
30842	*Bacillus clausii*	99.1% *B. clausii*	AM910263	*B. clausii*	J	7	BHI	W	37°C											
26266	*Bacillus clausii*			*B. clausii*	J	2	BHI	S	37°C											
26349	*Kytococcus sedentarius*	99.9% *U. thermosphaericus*	HE586360	*U. thermosphaericus*		2	BHI	S	55°C		–	–	–	–						
27341	*Bacillus* (22)	*Bacillus* sp.	AM910198	*Bacillus* sp. 6	K	8	BHI	S	37°C											
31037	*Bacillus* (22)	*Bacillus* sp.	AM910295	*Bacillus* sp. 6	K	9	BHI	W	37°C											
31190	*Bacillus* (22)	*Bacillus* sp.	AM910303	*Bacillus* sp. 6	K	7	BHI	W	20°C	+++										
31189	*Bacillus circulans*	*Bacillus* sp.	AM910302	*Bacillus* sp. 6	K	1	BHI	W	20°C	++										
31320	*Bacillus* (22)	*Bacillus* sp.	AM910324	*Bacillus* sp. 6	K	8	BHI	W	20°C	++										
27335	*Bacillus* (22)	99.3% *B. sporothermodurans*	AM910197	*B. sporothermo.*	K	8	BHI	S	37°C											
26276	*Bacillus megaterium* (A)	*Bacillus* sp.	HE586361	*Bacillus* sp. 3	K	2	BHI	S	37°C											
26275	*Brevibacillus reuszeri*	*Bacillus* sp.	HE586362	*Bacillus* sp. 3	K	2	BHI	S	37°C											
31323	*Bacillus circulans*	*Sporosarcina* sp.	AM910326	*Sporosarcina* sp.	K	8	BHI	W	20°C	+++										
31301	*Arthrobacter viscosus*	99% *Brevibacillus invocatus*	AM910315	*Br. invocatus*	K	10	BHI	W	20°C											
27357	*Bacillus sphaericus* (D)	*Bacillus* sp.	AM910201	*Bacillus* sp. 5	K	9	BHI	S	37°C											
28348	*Bacillus circulans*	*Bacillus* sp.	AM910230	*Bacillus* sp. 5	K	9	BHI	S	37°C											
27368	*Bacillus sphaericus* (D)	*Bacillus* sp.	AM910204	*Bacillus* sp. 5	K	9	BHI	S	37°C											

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1	2	3	4	rep	P	L	F	N	toxins
26869	*Bacillus sphaericus* (E)	98.4% *L. fusiformis*	AM910176	*L. fusiformis*	K	8	BHI	S	20°C							+	-		-	-
30912	*Bacillus sphaericus* (E)	99.9% *L. fusiformis*	AM910270	*L. fusiformis*	K	10	BHI	W	37°C											
27607	*Bacillus sphaericus* (E)	99.8% *L. fusiformis*	AM910221	*L. fusiformis*	K	7	BHI	S	37°C											
28515	*Bacillus sphaericus* (E)	99% *L. fusiformis*	AM910232	*L. fusiformis*	K	8	BHI	S	20°C	++++										
31869	*Bacillus sphaericus* E	99.9% *L. fusiformis*	AM910329	*L. fusiformis*	K	8	SM	W	20°C	+++										
26047	*Bacillus sphaericus* (D)	99% *L. fusiformis*	HE586363	*L. fusiformis*	K	1	BHI	S	37°C											
34297	*Bacillus sphaericus* (D)	98.7% *L. fusiformis*	AM910348	*L. fusiformis*	K	1	BHI	W	37°C											
26228	*Bacillus* (22)	98.2% *S. silvestris*	HE586364	*S. silvestris*	K	1	BHI	S	37°C											
26865	*Bacillus sphaericus* (A)	99.5% *S. silvestris*	AM910173	*S. silvestris*	K	7	BHI	S	20°C	+++										
26205	*Bacillus sphaericus* (A)	99.1% *L. sphaericus*	HE586365	*L. sphaericus*	K	1	BHI	S	37°C											
31030	*Bacillus sphaericus* (E)	98.8% *L. sphaericus*	AM910293	*L. sphaericus*	K	9	SM	W	37°C							-		-	+	-
31319	*Bacillus sphaericus* (D)	*Bacillus* sp.	AM910323	*Bacillus* sp. 5	K	8	BHI	W	20°C											
30915	*Bacillus sphaericus* (D)	*Bacillus* sp.	AM910273	*Bacillus* sp. 5	K	10	BHI	W	37°C											
27016	*Bacillus sphaericus* (C)	99.7% *L. fusiformis*	HE586366	*L. fusiformis*	K	2	SM	S	37°C											
27024	*Bacillus sphaericus* (D)	98.7% *L. fusiformis*	AM910191	*L. fusiformis*	K	6	BHI	S	37°C											
27019	*Bacillus sphaericus* (C)	99.8% *L. fusiformis*	AM910188	*L. fusiformis*	K	6	BHI	S	37°C							+	-		-	-
30635	*Bacillus sphaericus* (D)	99.1% *L. fusiformis*	AM910247	*L. fusiformis*	K	1	BHI	W	37°C											
27408	*Bacillus sphaericus* (D)	99.8% *B. massiliensis*	HE586367	*L. fusiformis*	K	5	BHI	S	37°C											
31191	*Bacillus sphaericus* (B)	99.9% *L. fusiformis*	AM910304	*L. fusiformis*	K	7	BHI	W	20°C	++++										
26864	*Bacillus sphaericus* (A)	99.5% *S. silvestris*	AM910172	*S. silvestris*	K	7	BHI	S	20°C	+++										
28337	*Bacillus sphaericus* (A)	99.6% *S. silvestris*	AM910227	*S. silvestris*	K	9	BHI	S	37°C											
26868	*Bacillus sphaericus* (A)	99.4% *S. silvestris*	AM910175	*S. silvestris*	K	8	BHI	S	20°C	+++										
26045	*Bacillus sphaericus* (A)	99.5% *S. silvestris*	HE586368	*S. silvestris*	K	1	BHI	S	37°C											
30914	*Brevibacillus agri/borstelensis*	*Bacillus* sp.	AM910272	*Bacillus* sp. 7	K	10	BHI	W	37°C											
26373	*Bacillus insolitus*	99.2% *B. simplex*	HE586369	*B. simplex*	K	1	BHI	S	20°C	++++						-			-	-
26859	*Bacillus* (22)	98.8% *B. simplex*	AM910170	*B. simplex*	K	9	BHI	S	20°C	+++										
33518	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	NAE	W	55°C											
33554	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	AM910344	*B. licheniformis*	L	10	NAE	W	55°C						L2					
26881	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	BHI	S	55°C											
27927	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	S	55°C						L4					
28175	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	S	55°C											
27928	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	S	55°C											
26329	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	NAE	S	55°C											
28176	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	S	55°C						L2					
27940	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	NAE	S	55°C											
27343	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	NAE	S	55°C											
33517	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	SM	W	55°C						L1					
28170	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	S	55°C											
27429	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	BHI	S	55°C											
28174	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	S	55°C											
27925	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	SM	S	55°C											
28156	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C						L1					
33553	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	NAE	W	55°C						L2					

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
27346	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C			L1					
28513	*Staphylococcus sciuri*			*B. licheniformis*	L	4	BHI	S	55°C			L1					
27941	*Staphylococcus sciuri*			*B. licheniformis*	L	10	NAE	S	55°C								
27349	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C			L1					
33549	*Staphylococcus sciuri*	99.7% *B. licheniformis*	AM910342	*B. licheniformis*	L	5	BHI	W	55°C			L2					
33373	*Staphylococcus sciuri*			*B. licheniformis*	L	1	BHI	W	55°C								
33454	*Staphylococcus sciuri*			*B. licheniformis*	L	7	BHI	W	55°C								
26937	*Staphylococcus schleiferi*	99.7% *B. licheniformis*	AM910180	*B. licheniformis*	L	7	BHI	S	55°C								
26952	*Staphylococcus schleiferi*			*B. licheniformis*	L	7	BHI	S	55°C								
27431	*Thermus aquaticus* (B)	99.7% *B. licheniformis*	AM910206	*B. licheniformis*	L	9	BHI	S	55°C			L1					
26352	*Staphylococcus schleiferi*			*B. licheniformis*	L	2	BHI	S	55°C								
26355	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C								
26915	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
33449	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
26351	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C								
26912	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
27348	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C								
26344	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C								
33448	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	W	55°C			L11					
26923	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
28173	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	S	55°C								
33575	*Thermus aquaticus* (B)	100% *B. licheniformis*	AM910346	*B. licheniformis*	L	4	BHI	W	55°C			L1					
26924	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
28516	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
33456	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
28172	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	HE586370	*B. licheniformis*	L	4	BHI	S	55°C			L2					
28514	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
33375	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	BHI	W	55°C			L2					
27426	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C								
33455	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C								
26956	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C			L2					
33287	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	W	55°C			L1					
33567	*Staphylococcus sciuri*			*B. licheniformis*	L	4	BHI	W	55°C								
28157	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C								
33450	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C								
26333	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	BHI	S	55°C			L1					
33371	*Staphylococcus sciuri*			*B. licheniformis*	L	1	BHI	W	55°C			L1					
27329	*Staphylococcus sciuri*			*B. licheniformis*	L	8	BHI	S	55°C								
26916	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
27432	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	BHI	S	55°C			L6					
26940	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C			L1					
33364	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	W	55°C								
33444	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	W	55°C								
26316	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	SM	S	55°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
33184	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	S	55°C								
28181	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	BHI	S	55°C			L2					
28182	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	BHI	S	55°C								
33286	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
33366	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	W	55°C			L2					
33618	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	BHI	W	55°C								
33519	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	W	55°C								
26315	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	SM	S	55°C			L2					
26326	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	NAE	S	55°C								
26930	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	SM	S	55°C								
27923	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	SM	S	55°C								
33560	*Thermus aquaticus* (B)			*B. licheniformis*	L	5	BHI	W	55°C								
26908	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
26353	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C			L1					
26350	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C								
26917	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
33451	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
33568	*Staphylococcus sciuri*			*B. licheniformis*	L	1	BHI	W	55°C								
33572	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	W	55°C			L1					
28195	*Staphylococcus sciuri*			*B. licheniformis*	L	10	NAE	S	55°C								
26946	*Staphylococcus sciuri*			*B. licheniformis*	L	7	BHI	S	55°C								
28177	*Staphylococcus sciuri*			*B. licheniformis*	L	5	BHI	S	55°C								
28180	*Staphylococcus sciuri*			*B. licheniformis*	L	5	SM	S	55°C			L1					
33372	*Thermus aquaticus* (B)			*B. licheniformis*	L	1	BHI	W	55°C			L1					
33555	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	W	55°C								
27922	*Staphylococcus sciuri*			*B. licheniformis*	L	10	SM	S	55°C								
27939	*Staphylococcus sciuri*			*B. licheniformis*	L	10	NAE	S	55°C								
27430	*Thermus aquaticus* (B)			*B. subtilis*	L	9	BHI	S	55°C			F2					-
33176	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	SM	S	55°C								
28185	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	SM	S	55°C								
27425	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C								
33377	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	W	55°C			L1					
28164	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	S	55°C			L2					
33183	*Staphylococcus sciuri*			*B. licheniformis*	L	10	BHI	S	55°C								
27427	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C			L1					
26919	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
26356	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	S	55°C			L1					-
28163	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	S	55°C			L4					
27038	*Staphylococcus schleiferi*			*B. licheniformis*	L	6	SM	S	55°C			L2					
33563	*Staphylococcus schleiferi*			*B. licheniformis*	L	4	BHI	W	55°C			L2					
26934	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C								
28522	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
28169	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	SM	S	55°C								
33175	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	SM	S	55°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
33551	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
26951	*Thermus aquaticus* (B)	99.4% *B. licheniformis*	AM910184	*B. licheniformis*	L	7	BHI	S	55°C			L1					
26910	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C			L1					
26927	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	SM	S	55°C								
33384	*Thermus aquaticus* (B)			*B. licheniformis*	L	3	BHI	W	55°C			L2					
28319	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	SM	S	55°C								
33185	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	S	55°C								
27424	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C								
28322	*Thermus aquaticus* (B)			*B. licheniformis*	L	3	BHI	S	55°C								
27325	*Staphylococcus schleiferi*			*B. licheniformis*	L	8	BHI	S	55°C								
27924	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	SM	S	55°C								
28165	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	S	55°C			L2					
28168	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	SM	S	55°C								
33376	*Thermus aquaticus* (B)			*B. licheniformis*	L	4	BHI	W	55°C								
33362	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	W	55°C								
33558	*Staphylococcus sciuri*			*B. licheniformis*	L	5	BHI	W	55°C								
28188	*Staphylococcus sciuri*			*B. licheniformis*	L	4	BHI	S	55°C								
33387	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	W	55°C								
33182	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	AM910333	*B. licheniformis*	L	10	BHI	S	55°C			L1					
33363	*Thermus aquaticus* (B)			*B. licheniformis*	L	2	BHI	W	55°C			L1					
26938	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C			L1					
28158	*Thermus aquaticus* (B)	99.9% *B. licheniformis*	AM910212	*B. licheniformis*	L	8	BHI	S	55°C								
33552	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C								
26914	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	BHI	S	55°C								
27014	*Bacillus pumilus* (B)			*B. licheniformis*	L	2	BHI	S	37°C								
26997	*Staphylococcus sciuri*			*B. licheniformis*	L	1	BHI	S	37°C								
27021	*Bacillus pumilus* (B)	99.6% *B. pumilus*	AM910189	*B. pumilus*	L	6	BHI	S	37°C			L5					
28542	*Thermus aquaticus* (B)	*Brevibacillus* sp.	AM910236	*Brevibacillus* sp.	L	7	SM	S	55°C								
26942	*Staphylococcus sciuri*			*B. licheniformis*	L	7	BHI	S	55°C								
33288	*Thermus aquaticus* (B)	99.6% *B. licheniformis*	AM910334	*B. licheniformis*	L	8	BHI	W	55°C								
33573	*Staphylococcus schleiferi*			*B. licheniformis*	L	4	NAE	W	55°C								
27022	*Bacillus laevolacticus*			*B. licheniformis*	L	6	SM	S	37°C								
27037	*Staphylococcus schleiferi*			*B. licheniformis*	L	6	BHI	S	37°C								
27023	*Staphylococcus schleiferi*	99.8% *B. licheniformis*	AM910190	*B. licheniformis*	L	6	SM	S	37°C								
26933	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C								
33571	*Bacillus clausii*			*B. licheniformis*	L	4	BHI	W	55°C			L2					
33562	*Staphylococcus schleiferi*			*B. licheniformis*	L	7	BHI	W	55°C								
33452	*Geobacillus stearothermophilus*			*B. licheniformis*	L	7	BHI	W	55°C								
26936	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	S	55°C								
28183	*Thermus aquaticus* (B)			*B. licheniformis*	L	9	SM	S	55°C			L1					
33379	*G. stearothermophilus*			*B. licheniformis*	L	2	BHI	W	55°C								
33442	*G. stearothermophilus*			*B. licheniformis*	L	8	BHI	W	55°C								
33374	*G. stearothermophilus*			*B. licheniformis*	L	1	BHI	W	55°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
28330	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	SM	S	55°C								
33561	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	BHI	W	55°C			L1					
27345	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	AM910199	*B. licheniformis*	L	8	BHI	S	55°C			L1					
33556	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	W	55°C								
27926	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	SM	S	55°C			L1					
27328	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	S	55°C			L1					
30828	*Bacillus laevolacticus*	99.6% *B. licheniformis*	AM910258	*B. licheniformis*	L	7	BHI	W	37°C			L1					
26925	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	SM	S	55°C			L1					
28523	*Thermus aquaticus* (B)	99.6% *B. licheniformis*	HE586371	*B. licheniformis*	L	3	BHI	S	37°C			L2					
33181	*Bacillus clausii*			*B. licheniformis*	L	10	BHI	S	55°C								
33177	*Bacillus clausii*			*B. licheniformis*	L	7	SM	S	55°C								
30775	*Bacillus laevolacticus*			*B. licheniformis*	L	4	SM	W	37°C			L7					
28146	*Thermus aquaticus* (B)			*B. licheniformis*	L	7	SM	S	55°C								
26357	*G. stearothermophilus*			*B. licheniformis*	L	2	BHI	S	55°C								
28162	*Bacillus clausii*			*B. licheniformis*	L	4	BHI	S	55°C								
31292	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	W	55°C								
31290	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	W	55°C			L1					
33443	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	BHI	W	55°C			L1					
31289	*Thermus aquaticus* (B)			*B. licheniformis*	L	10	BHI	W	55°C								
26929	*Thermus aquaticus* (B)			*B. licheniformis*	L	6	SM	S	55°C			L1					-
27428	*Thermus aquaticus* (B)			*B. licheniformis*	L	8	SM	S	55°C								
33570	*Thermus aquaticus* (B)	99.8% *B. licheniformis*	AM910345	*B. licheniformis*	L	4	SM	W	55°C			L1					
33574	*G. stearothermophilus*			*B. licheniformis*	L	4	SM	W	55°C								
28329	*Microbispora rosea*			*U. thermosphaericus*	M	8	BHI	S	55°C								
33535	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	W	55°C								
26920	*Microbispora rosea*	99.8% *U. thermosphaericus*	AM910178	*U. thermosphaericus*	M	6	BHI	S	55°C								
33530	*Lechevalieria flava*			*U. thermosphaericus*	M	4	BHI	W	55°C								
26943	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
28524	*U. thermosphaericus*			*U. thermosphaericus*	M	7	BHI	S	55°C								
28532	*Microbispora rosea*	99.5% *U. thermosphaericus*	AM910235	*U. thermosphaericus*	M	8	BHI	S	55°C								
33531	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	55°C								
33559	*Lechevalieria flava*			*U. thermosphaericus*	M	1	BHI	W	55°C								
27327	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
33532	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	55°C								
31867	*U. thermosphaericus*			*U. thermosphaericus*	M	9	BHI	W	37°C								
33527	*U. thermosphaericus*			*U. thermosphaericus*	M	9	BHI	W	55°C								
31864	*U. thermosphaericus*	99% *U. thermosphaericus*	AM910328	*U. thermosphaericus*	M	9	BHI	W	37°C								
27326	*Microbispora rosea*			*U. thermosphaericus*	M	8	BHI	S	55°C								
31866	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	37°C								
33614	*Lechevalieria flava*			*U. thermosphaericus*	M	4	BHI	W	55°C								
33533	*Lechevalieria flava*			*U. thermosphaericus*	M	4	BHI	W	55°C								
31862	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	37°C								
33178	*Lechevalieria flava*	99.7% *U. thermosphaericus*	AM910331	*U. thermosphaericus*	M	8	BHI	S	55°C								
27324	*Lechevalieria flava*	99.9% *U. thermosphaericus*	AM910194	*U. thermosphaericus*	M	8	BHI	S	55°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	isol. param. cl.	F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
28147	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
33772	*U. thermosphaericus*			*U. thermosphaericus*	M	9	BHI	W	37°C								
33617	*Lechevalieria flava*			*U. thermosphaericus*	M	1	BHI	W	55°C								
27323	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
28531	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
33521	*Lechevalieria flava*	100% *U. thermosphaericus*	AM910340	*U. thermosphaericus*	M	9	BHI	W	55°C								
28194	*U. thermosphaericus*			*U. thermosphaericus*	M	9	BHI	S	55°C								
28323	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
26907	*Lechevalieria flava*	99.8% *U. thermosphaericus*	AM910177	*U. thermosphaericus*	M	6	BHI	S	55°C								
26909	*U. thermosphaericus*			*U. thermosphaericus*	M	6	BHI	S	55°C								
26945	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
26935	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
26939	*U. thermosphaericus*			*U. thermosphaericus*	M	7	BHI	S	55°C								
26918	*U. thermosphaericus*			*U. thermosphaericus*	M	6	BHI	S	55°C								
26941	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
26947	*U. thermosphaericus*	99.7% *U. thermosphaericus*	AM910181	*U. thermosphaericus*	M	7	BHI	S	55°C								
26932	*U. thermosphaericus*			*U. thermosphaericus*	M	7	BHI	S	55°C								
30710	*U. thermosphaericus*			*U. thermosphaericus*	M	4	BHI	W	37°C								
26944	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
28153	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
28161	*Lechevalieria flava*			*U. thermosphaericus*	M	8	BHI	S	55°C								
28148	*Lechevalieria flava*			*U. thermosphaericus*	M	7	BHI	S	55°C								
28327	*Microbispora rosea*			*U. thermosphaericus*	M	8	BHI	S	55°C								
26911	*Lechevalieria flava*			*U. thermosphaericus*	M	6	BHI	S	55°C								
28184	*Lechevalieria flava*			*U. thermosphaericus*	M	9	SM	S	55°C								
31863	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	37°C								
27344	*Microbispora rosea*			*U. thermosphaericus*	M	8	BHI	S	55°C								
28328	*Thermus aquaticus* (B)			*U. thermosphaericus*	M	8	BHI	S	55°C								
26948	*Thermus aquaticus* (B)			*U. thermosphaericus*	M	7	BHI	S	55°C								
26921	*Thermus aquaticus* (B)			*U. thermosphaericus*	M	6	BHI	S	55°C								
28545	*Thermus aquaticus* (B)			*U. thermosphaericus*	M	3	BHI	S	55°C								
27433	*Microbispora rosea*			*U. thermosphaericus*	M	9	BHI	S	55°C								
31868	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	37°C								
31865	*Lechevalieria flava*			*U. thermosphaericus*	M	9	BHI	W	37°C								
26922	*Thermus aquaticus* (B)	99.8% *U. thermosphaericus*	AM910179	*U. thermosphaericus*	M	6	BHI	S	55°C								
27633	*Bacillus circulans*	*Oceanobacillus*	AM910211	*Oceanobacillus* sp. 3	N	8	BHI	S	37°C								
27626	*Bacillus lentus*	*Oceanobacillus*	AM910216	*Oceanobacillus* sp. 3	N	8	BHI	S	37°C								
27606	*Bacillus circulans*	*Oceanobacillus*	AM910217	*Oceanobacillus* sp. 3	N	7	BHI	S	37°C								
34296	*Bacillus circulans*	*Oceanobacillus*	AM910349	*Oceanobacillus* sp. 3	N	8	BHI	S	37°C								
31213	*Bacillus lentus*	*Oceanobacillus*	AM910310	*Oceanobacillus* sp. 3	N	2	BHI	W	20°C	++++							
27610	*Bacillus lentus*	*Oceanobacillus*	AM910219	*Oceanobacillus* sp. 3	N	7	BHI	S	37°C								
27612	*Bacillus lentus*	*Oceanobacillus*	AM910218	*Oceanobacillus* sp. 3	N	7	BHI	S	37°C								
28520	*Virgibacillus pantothenticus*	*Oceanobacillus*	AM910234	*Oceanobacillus* sp. 3	N	7	BHI	S	37°C								
28519	*Virgibacillus pantothenticus*	*Oceanobacillus*	AM910233	*Oceanobacillus* sp. 3	N	7	BHI	S	37°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	isol. param. M.	isol. param. S/W	isol. param. T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
27401	*Arthrobacter viscosus*	*Oceanobacillus*	HE586372	*Oceanobacillus* sp. 2	N	4	BHI	S	37°C								
27402	*Arthrobacter viscosus*	*Oceanobacillus*		*Oceanobacillus* sp. 2	N	4	BHI	S	37°C								
26384	*Listeria monocytogenes/innocua*			*B. circulans*	O	2	BHI	S	20°C	+							
31005	*Listeria monocytogenes/innocua*			*B. circulans*	O	10	BHI	W	20°C	+++							
30632	*Bacillus circulans*	99.4% *B. circulans*	AM910246	*B. circulans*	O	1	BHI	W	37°C								
27336	*Bacillus circulans*			*B. circulans*	O	7	BHI	S	37°C								
30732	*Bacillus circulans*			*B. circulans*	O	2	BHI	W	37°C								
31206	*Bacillus circulans*			*B. circulans*	O	1	BHI	W	20°C								
27369	*Bacillus circulans*			*B. circulans*	O	9	BHI	S	37°C								
30731	*Bacillus circulans*			*B. circulans*	O	1	BHI	W	37°C								
31001	*Bacillus circulans*			*B. circulans*	O	10	BHI	W	20°C	+							
27608	*Bacillus circulans*			*B. circulans*	O	7	BHI	S	37°C								
31324	*Bacillus circulans*			*B. circulans*	O	8	BHI	W	20°C								
26213	*Bacillus circulans*			*B. circulans*	O	1	BHI	S	37°C								
27605	*Bacillus circulans*	100% *B. circulans*	AM910208	*B. circulans*	O	7	BHI	S	37°C								
30734	*Bacillus circulans*			*B. circulans*	O	1	BHI	W	37°C								
30733	*Bacillus circulans*	99.5% *B. circulans*	AM910254	*B. circulans*	O	1	BHI	W	37°C								
30916	*Bacillus circulans*			*B. circulans*	O	10	BHI	W	37°C								
31177	*Bacillus circulans*			*B. circulans*	O	1	SM	W	20°C	+							
27637	*Bacillus circulans*			*B. circulans*	O	8	BHI	S	37°C								
31205	*Bacillus circulans*			*B. circulans*	O	1	BHI	W	20°C	+							
30730	*Bacillus circulans*	99% *B. circulans*	AM910253	*B. circulans*	O	1	BHI	W	37°C								
33179	*Bacillus circulans*	99.4% *B. circulans*	AM910332	*B. circulans*	O	9	BHI	S	37°C								-
26277	*Kurthia sibirica*	*Oceanobacillus*	HE586373	*Oceanobacillus* sp. 1		2	BHI	S	37°C		– – – –						
33052	*Arthrobacter mysorens*	100% *Oceanobacillus profundus*	HE586374	*O. profundus*		4	BHI	S	37°C		– – – –						
33520	*NO MATCH*	*Bacillus* sp.	AM910339	*Bacillus* sp. 4	P	4	BHI	W	55°C								
33550	*NO MATCH*	*Bacillus* sp.	AM910343	*Bacillus* sp. 4	P	9	BHI	W	55°C								
28193	*NO MATCH*	*Bacillus* sp.	AM910224	*Bacillus* sp. 4	P	9	BHI	S	55°C								
31288	*NO MATCH*	*Bacillus* sp.	AM910312	*Bacillus* sp. 4	P	10	BHI	W	55°C								
26955	*Amycolatopsis orientalis orientalis*	*Bacillus* sp.	AM910187	*Bacillus* sp. 4	P	7	BHI	S	55°C								
33378	*NO MATCH*	*Bacillus* sp.	AM910337	*Bacillus* sp. 4	P	4	BHI	W	55°C								
26358	*NO MATCH*	*Bacillus* sp.	FN997653	*Bacillus* sp. 4	P	2	BHI	S	55°C								
26949	*NO MATCH*	*Bacillus* sp.	AM910182	*Bacillus* sp. 4	P	7	BHI	S	55°C								
33858	*Cellulomonas flavigena*	*Bacillus* sp.	AM910350	*Bacillus* sp. 4	P	10	BHI	W	55°C								
33367	*G. stearothermophilus*	*Bacillus* sp.	AM910336	*Bacillus* sp. 4	P	2	BHI	W	55°C								
26954	*NO MATCH*	*Bacillus* sp.	AM910186	*Bacillus* sp. 4	P	7	BHI	S	55°C								
26950	*Streptococcus salivarius salivarius*	*Bacillus* sp.	AM910183	*Bacillus* sp. 4	P	7	BHI	S	55°C								
28160	*Streptococcus salivarius salivarius*	*Bacillus* sp.	AM910223	*Bacillus* sp. 4	P	8	BHI	S	55°C								

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	M.	S/W	T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
26953	*Amycolatopsis orientalis orientalis*	*Bacillus* sp.	AM910185	*Bacillus* sp. 4	P	7	BHI	S	55°C								
33857	*Lechevalieria flava*	*Bacillus* sp.	AM910347	*Bacillus* sp. 4	P	7	BHI	S	55°C								
33365	*Bacillus alcalophilus*	99.1% *B. thermoamylo.*	AM910335	*B. thermoamylovorans*	Q	2	BHI	W	55°C								
33380	*Bacillus alcalophilus*			*B. thermoamylo.*	Q	2	BHI	W	55°C								
33453	*G. stearothermophilus*	98.9% *B. thermoamylo.*	AM910338	*B. thermoamylovorans*	Q	7	BHI	W	55°C								
28324	*Staphylococcus sciuri*	*Brevibacillus* sp.	AM910225	*Brevibacillus* sp.		8	SM	S	55°C		– – – –						
26882	*Thermus aquaticus* (B)	99.1% *B. licheniformis*	HE586375	*B. licheniformis*		1	BHI	S	55°C		– – – –						
25364	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	20°C	++++		R5					
26209	*Bacillus cereus* (A)	99.8% *B. cereus group*	HE586376	*B. cereus group*	R	1	BHI	S	37°C			R1					
25362	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	20°C	++++		R1					
27409	*Bacillus cereus* (A)			*B. cereus group*	R	5	BHI	S	37°C			R1	++	-		-	NHE+, cytK+, H-L+
25388	*Bacillus cereus* (A)			*B. cereus group*	R	2	SM	S	20°C	++++		R1					
26237	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	37°C			R1					
25361	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	20°C	++++		R1					
25387	*Bacillus cereus* (A)			*B. cereus group*	R	2	NAE	S	20°C	++++		R1					
27595	*Bacillus cereus* (A)			*B. cereus group*	R	5	SM	S	37°C			R1					
26225	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	37°C			R1					
26229	*Bacillus cereus* (A)	99.3% *B. cereus group*	HE586377	*B. cereus group*	R	1	BHI	S	37°C			R1					
26226	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	37°C			R1					NHE+, cytK+
25369	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	20°C	++++		R1					
25363	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	20°C	++++		R1					
28004	*Bacillus cereus* (A)			*B. cereus group*	R	10	SM	S	37°C								
26290	*Bacillus cereus* (A)			*B. cereus group*	R	2	NAE	S	37°C			R1					
26293	*Bacillus cereus* (A)	99% *B. cereus group*	HE586378	*B. cereus group*	R	2	NAE	S	37°C			R1					
26287	*Bacillus cereus* (A)			*B. cereus group*	R	2	SM	S	37°C			R1					
26296	*Bacillus cereus* (A)			*B. cereus group*	R	2	SM	S	37°C			R1					
26288	*Bacillus cereus* (A)			*B. cereus group*	R	2	BHI	S	37°C			R1					
25384	*Bacillus cereus* (A)			*B. cereus group*	R	2	NAE	S	20°C	++++		R1					
25385	*Bacillus cereus* (A)	99.8% *B. cereus group*	HE586379	*B. cereus group*	R	2	BHI	S	20°C	+++		R1					
26061	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	37°C			R1					
26392	*Bacillus cereus* (A)			*B. cereus group*	R	2	NAE	S	20°C	++++		R1					NHE+, cytK+
25386	*Bacillus cereus* (A)			*B. cereus group*	R	2	SM	S	20°C	++++		R1					NHE+, cytK+
25389	*Bacillus cereus* (A)			*B. cereus group*	R	2	BHI	S	20°C	++++		R1					
26214	*Bacillus cereus* (A)			*B. cereus group*	R	1	BHI	S	37°C			R1					
26060	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	37°C			R1					
26372	*Bacillus cereus* (A)			*B. cereus group*	R	1	BHI	S	20°C	++++		R1					
26222	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	37°C			R1					
26298	*Bacillus cereus* (A)	99.7% *B. cereus group*	HE586380	*B. cereus group*	R	2	SM	S	37°C			R1					
26057	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	37°C			R1					
26063	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	37°C			R1					
26055	*Bacillus cereus* (A)	99.6% *B. cereus group*	HE586381	*B. cereus group*	R	1	SM	S	37°C			R1					

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R-n°	FAME FATAL ID	16S rRNA ID	16S rRNA acc. n°	final ID in this study	cl.	isol. param. F.	isol. param. M.	isol. param. S/W	isol. param. T.	psy. (7 d)	enz. act. 1 2 3 4	rep	P	L	F	N	toxins
26302	*Bacillus cereus* (A)			*B. cereus group*	R	2	SM	S	37°C			R1					
27002	*Bacillus cereus* (A)	99.7% *B. cereus group*	HE586382	*B. cereus group*	R	1	BHI	S	37°C			R1					NHE+, cytK+, H-L+
27385	*Bacillus cereus* (A)			*B. cereus group*	R	4	NAE	S	37°C								
26861	*Bacillus cereus* (A)			*B. cereus group*	R	7	BHI	S	20°C	++++							
26862	*Bacillus cereus* (A)	99.4% *B. cereus group*	AM910171	*B. cereus group*	R	7	BHI	S	20°C	+++							
31310	*Bacillus cereus* (A)			*B. cereus group*	R	9	NAE	W	20°C			R8					
25365	*Bacillus cereus* (A)			*B. cereus group*	R	1	NAE	S	20°C	++++		R2					
27334	*Bacillus cereus* (A)			*B. cereus group*	R	8	BHI	S	37°C			R6					
25368	*Bacillus cereus* (A)			*B. cereus group*	R	1	BHI	S	20°C	++++		R4					
25370	*Bacillus cereus* (A)	100% *B. cereus group*	HE586383	*B. cereus group*	R	1	SM	S	20°C	++++		R2					HBL+, NHE+
25366	*Bacillus cereus* (A)			*B. cereus group*	R	1	BHI	S	20°C	++++		R2					
26054	*Bacillus cereus* (A)			*B. cereus group*	R	1	SM	S	37°C			R3					HBL+
31325	*Bacillus cereus* (A)			*B. cereus group*	R	5	BHI	W	20°C	++++		R2					
30651	*Bacillus cereus* (A)			*B. cereus group*	R	2	BHI	W	37°C			R7					

Bibliography

[1] Abd El-Rahman, H.A., Fritze, D., Spröer, C. & Claus, D. (2002). Two novel psychrotolerant species, *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov., which contain ornithine in their cell walls. *Int J Syst Evol Microbiol* **52**, 2127-2133

[2] Achouak, W., Sutra, L., Heulin, T., Meyer, J.M., Fromin, N., Degraeve, S., Christen, R. & Gardan, L. (2000). *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov., two root-associated bacteria isolated from *Brassica napus* and *Arabidopsis thaliana*. *Int J Syst Evol Microbiol* **50**, 9-18

[3] Adams, D.M., Barach, J.T. & Speck, M.L. (1975). Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. *J Dairy Sci* **58**, 828-834

[4] Agnew, M.D., Koval, S.F. & Jarrell, K.F. (1995). Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. *Syst Appl Microbiol* **18**, 221-230

[5] Ahmad, S., Scopes, R.K., Rees, G.N. & Patel, B.K.C. (2000). *Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore-forming bacterium. *Int J Syst Evol Microbiol* **50**, 517-523

[6] Ahmed, I., Yokota, A. & Fujiwara, T. (2007a). A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. *Extremophiles* **11**, 217-224

[7] Ahmed, I., Yokota, A., Yamazoe, A. & Fujiwara, T. (2007b). Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *Int J Syst Evol Microbiol* **57**, 1117–1125

[8] Aino, K., Hirota, K., Matsuno, T., Morita, N., Nodasaka, Y., Fujiwara, T., Matsuyama, H., Yoshimune, K. & Yumoto, I. (2008). *Bacillus polygoni* sp. nov., a moderately halophilic, non-motile obligate alkaliphile isolated from indigo balls. *Int J Syst Evol Microbiol* **58**, 120-124

[9] Aizawa, T., Urai, M., Iwabuchi, N., Nakajima, M. & Sunaira, M. (2010). *Bacillus trypoxylicola* sp. nov., xylanase-producing alkaliphilic bacteria isolated from the guts of Japanese horned beetle larvae (*Trypoxylus dichotomus septentrionalis*). *Int J Syst Evol Microbiol* **60**, 61-66

[10] Ajithkumar, V.P., Ajithkumar, B., Iriye, R. & Sakai, T. (2002). *Bacillus funiculus* sp. nov., novel filamentous isolates from activated sludge. *Int J Syst Evol Microbiol* **52**, 1141-1144

[11] Akineden, O., Annemüller, C., Hassan, A.A., Lämmler, C., Wolter, W. & Zschock, M. (2001). Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clin Diagn Lab Immun* **8**, 959-964

[12] Albert, R.A., Archambault, J., Lempa, M., Hurst, B., Richardson, C., Gruenloh, S., Duran, M., Worliczek, H.L., Huber, B.E., Rossello-Mora, R., Schumann, P. & Busse, H.-J. (2007). Proposal of *Viridibacillus* gen. nov. and reclassification of *Bacillus arvi*, *Bacillus arenosi* and *Bacillus neidei* as *Viridibacillus arvi* gen. nov., comb. nov., *Viridibacillus arenosi* comb. nov. and *Viridibacillus neidei* comb. nov. *Int J Syst Evol Microbiol* **57**, 2729-2737

[13] Albert, R.A., Archambault, J., Rossello-Mora, R., Tindall, B.J. & Matheny, M. (2005). *Bacillus acidicola* sp. nov., a novel mesophilic, acidophilic species isolated from acidic *Sphagnum* peat bogs in Wisconsin. *Int J Syst Evol Microbiol* **55**, 2125-2130

[14] Albuquerque, L., Tiago, I., Taborda, M., Nobre, M.F., Verissimo, A. & Da Costa, M.S. (2008). *Bacillus isabeliae* sp. nov., a halophilic bacterium isolated from a sea salt evaporation pond. *Int J Syst Evol Microbiol* **58**, 226-230

[15] Alexander, B. & Priest, F.G. (1989). *Bacillus glucanolyticus*, a new species that degrades a variety of β-glucans. *Int J Syst Bacteriol* **39**, 112-115

[16] Alichanidis, E., Wrathall, J.H.M. & Andrews, A.T. (1986). Heat stability of plasmin (milk proteinase) and plasminogen. *J Dairy Res* **53**, 259-269

[17] Allan, R.N., Lebbe, L., Heyrman, J., De Vos, P., Buchanan, C.J. & Logan, N.A. (2005). *Brevibacillus levickii* sp. nov. and *Aneurinibacillus terranovensis* sp. nov., two new thermoacidophiles isolated from geothermal soils of northern Victoria Land, Antarctica. *Int J Syst Evol Microbiol* **55**, 1039-1050

[18] Ambroz, A. (1913). *Denitrobacterium thermophilum* spec. nova, ein Beitrag zur Biologie der thermophilen Bakterien. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg* **37**, 3-16

[19] Amoozegar, M.A., Sanchez-Porro, C., Rohban, R., Hajighasemi, M. & Ventosa, A. (2009). *Bacillus persepolensis* sp. nov., a moderately halophilic bacterium from a hypersaline lake. *Int J Syst Evol Microbiol* **59**, 2352-2358

[20] Andersch, I., Pianka, S., Fritze, D. & Claus, D. (1994). Description of *Bacillus laevolacticus* (*ex* Nakayama and Yanoshi 1967) sp. nov., nom. rev. *Int J Syst Bacteriol* **44**, 659-664

[21] Andersen, S.M., Johnsen, K., Sørensen, J., Nielsen, P. & Jacobsen, C.S. (2000). *Pseudomonas frederiksbergensis* sp. nov., isolated from soil at a coal gasification site. *Int J Syst Evol Microbiol* **50**, 1957-1964

[22] Andersson, A., Ronner, U. & Granum, P.E. (1995b). What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int J Food Microbiol* **28**, 145-155

[23] Andersson, M., Laukkanen, M., Nurmiaho-Lassila, E.L., Rainey, F.A., Niemelä, S.I. & Salkinoja-Salonen, M. (1995a). *Bacillus thermosphaericus* sp. nov. a new thermophilic ureolytic bacillus isolated from air. *Syst Appl Microbiol* **18**, 203-220

[24] Anisimova, M. & Gascuel, O. (2006). Approximate likelihood-ratio test for branches: a fast, accurate and powerful alternative. *Syst Biol* **55**, 539-552

[25] Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H. & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* **50**, 1563-1589

[26] Arahal, D.R., Castillo, A.M., Ludwig, W., Schleifer, K.-H. & Ventosa, A. (2002). Proposal of *Cobetia marina* gen. nov., comb. nov., within the family *Halomonadaceae*, to include the species *Halomonas marina*. *Syst Appl Microbiol* **25**, 207-211

[27] Arahal, D.R., Marquez, M.C., Volcani, B.E., Schleifer, K.-H. & Ventosa, A. (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. *Int J Syst Bacteriol* **49**, 521-530

[28] Arahal, D.R., Marquez, M.C., Volcani, B.E., Schleifer, K.-H. & Ventosa, A. (2000). Reclassification of *Bacillus marismortui* as *Salibacillus marismortui* comb. nov. *Int J Syst Evol Microbiol* **50**, 1501-1503

[29] Arfman, N., Dijkhuizen, L., Kirchhof, G., Ludwig, W., Schleifer, K.-H., Bulygina, E.S., Chumakov, K.M., Govorukhina, N.I., Trotsenko, Y.A., White, D. & Sharp, R.J. (1992). *Bacillus methanolicus* sp. nov., a new species of thermotolerant, methanol-utilizing, endospore-forming bacteria. *Int J Syst Bacteriol* **42**, 439-445

[30] Ash, C., Farrow, J.A.E., Wallbanks, S. & Collins, M.D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202-206

[31] Ash, C., Priest, F.G. & Collins, M.D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Ant Leeuw* **64**, 253-260

[32] Attafuah, A. & Bradbury, J.F. (1989). *Pseudomonas antimicrobica*, a new species strongly antagonistic to plant pathogens. *J Appl Bacteriol* **67**, 567-573

[33] Auling, G., Busse, H.-J., Pilz, F., Webb, L., Kneifel, H. & Claus, D. (1991). Rapid differentiation, by polyamine analysis, of *Xanthomonas* strains from phytopathogenic pseudomonads and other members of the class *Proteobacteria* interacting with plants. *Int J Syst Bacteriol* **41**, 223-228

[34] Auling, G., Reh, M., Lee, C.M. & Schlegel, H.G. (1978). Pseudomonas pseudoflava, a new species of hydrogen-oxidizing bacteria: its differentiation from Pseudomonas flava and other yellow-pigmented, Gram-negative, hydrogen oxidizing species. Int J Syst Bact 28, 82-95

[35] Austin, B. & Goodfellow, M. (1979). Pseudomonas mesophilica, a new species of pink bacteria isolated from leaf surfaces. Int J Syst Bact 29, 373-378

[36] Avakyan, Z.A., Pivovarova, T.A. & Karavaiko, G.I. (1986). Properties of a new species, *Bacillus mucilaginosus*. *Mikrobiologiya* **55**, 477-482

[37] Azegami, K., Nishiyama, K., Watanabe, Y., Kadota, I., Ohuchi, A. & Fukazawa, C. (1987). *Pseudomonas plantarii* sp. nov., the causal agent of rice seedling blight. *Int J Syst Bacteriol* **37**, 144-152

[38] Bae, S.S., Lee, J.H. & Kim, S.J. (2005). *Bacillus alveayuensis* sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu Trough. *Int J Syst Evol Microbiol* **55**, 1211-1215

[39] Baesman, S.M., Stolz, J.F., Kulp, T.R. & Oremland, R.S. (2009). Enrichment and isolation of *Bacillus beveridgei* sp. nov., a facultative anaerobic haloalkaliphile from Mono Lake, California, that respires oxyanions of tellurium, selenium and arsenic. *Extremophiles* **13**, 695-705

[40] Baïda, N., Yazourh, A., Singer, E. & Izard, D. (2001). *Pseudomonas brenneri* sp. nov., a new species isolated from natural mineral waters. *Res Microbiol* **152**, 493-502

[41] Baïda, N., Yazourh, A., Singer, E. & Izard, D. (2002). *Pseudomonas grimontii* sp. nov. *Int J Syst Evol Microbiol* **52**, 1497-1503

[42] Baik, K.S., Lim, C.H., Park, S.C., Kim, E.M., Rhee, M. & Seong, C.N. (2010). *Bacillus rigui* sp. nov., isolated from wetland fresh water. *Int J Syst Evol Microbiol* **60**, 2204-2209

[43] Balcazar, J.L., Pintado, J. & Planas, M. (2010). *Bacillus galliciensis* sp. nov., isolated from faeces of wild seahorses (*Hippocampus guttulatus*). *Int J Syst Evol Microbiol* **60**, 892-895

[44] Baldani, J.I., Pot, B., Kirchhof, G., Falsen, E., Baldani, V.L.D., Olivares, F.L., Hoste, B., Kersters, K., Hartmann, A., Gillis, M. & Döbereiner, J. (1996). Emended description of *Herbaspirillum*; inclusion of *Pseudomonas rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *Int J Syst Bacteriol* **46**, 802-810

[45] Banat, I.M., Marchant, R. & Rahman, T.J. (2004). *Geobacillus debilis* sp. nov., a novel obligately thermophilic bacterium isolated from a cool soil environment, and reassignment of *Bacillus pallidus* to *Geobacillus pallidus* comb. nov. *Int J Syst Evol Microbiol* **54**, 2197-2201

[46] Banks, W., Dalgleish, D.G. & Rook, J.A.F. (1981). Milk and milk processing. In '*Dairy Microbiology*'. Ed. Robinson, R.K. Applied Science, U.K.

[47] Bansal, B. & Dong Chen, X. (2006). A critical review of milk fouling in heat exchangers. *Comprehensive Reviews in Food Science and Food Safety* **5**, 27-33

[48] Barkley, M.B. & Delaney, P. (1980). An occurrence of bitty cream in Australian pasteurized milk. *Neth Milk Dairy J* **34**, 191-198

[49] Barkworth, H. (1958). Bitty cream. *J Soc Dairy Tech* **11**, 181-185

[50] Batchelor, M.D. (1919). Aerobic spore-bearing bacteria in the intestinal tract of children. J Bact 4, 23-34

[51] Baumann, L., Baumann, P., Mandel, M. & Allen, R.D. (1972). Taxonomy of aerobic marine eubacteria. J Bact 110, 402-429

[52] Baumann, L., Bowditch, R.D. & Baumann, P. (1983a). Description of *Deleya* gen. nov. created to accommodate the marine species *Alcaligenes aestus*, *A. pacificus*, *A. cupidus*, *A. venustus*, and *Pseudomonas marina*. *Int J Syst Bacteriol* **33**, 793-802

[53] Baumann, P., Bowditch, R.D., Baumann, L. & Beaman, B. (1983b). Taxonomy of marine *Pseudomonas* species: *P. stanieri* sp. nov.; *P. perfectomarina* sp. nov., nom. rev.; *P. nautica*; and *P. doudoroffii*. *Int J Syst Bacteriol* **33**, 857-865

[54] Beattie, S.H. & Williams, A.G. (1999). Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. *Lett Appl Microbiol* **28**, 221-225

[55] Behrendt, U., Schumann, P., Meyer, J.M. & Ulrich, A. (2009). *Pseudomonas cedrina* subsp. *fulgida* subsp. nov., a fluorescent bacterium isolated from the phyllosphere of grasses; emended description of *Pseudomonas cedrina* and description of *Pseudomonas cedrina* subsp. *cedrina* subsp. nov. *Int J Syst Evol Microbiol* **59**, 1331-1335

[56] Behrendt, U., Ulrich, A. & Schumann, P. (2003). Fluorescent pseudomonads associated with the phyllosphere of grasses; *Pseudomonas trivialis* sp. nov., *Pseudomonas poae* sp. nov. and *Pseudomonas congelans* sp. nov. *Int J Syst Evol Microbiol* **53**, 1461-1469

[57] Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. & Seyfarth, W. (1999). A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov. *Int J Syst Bacteriol* **49**, 297-308

[58] Behrendt, U., Ulrich, A., Schumann, P., Meyer, J.M. & Spröer, C. (2007). *Pseudomonas lurida* sp. nov., a fluorescent species associated with the phyllosphere of grasses. *Int J Syst Evol Microbiol* **57**, 979-985

[59] Bell, S.C. & Turner, J.M. (1973). Iodinin biosynthesis by a pseudomonad. *Biochem Soc Trans* **1**, 751-753

[60] Bennasar, A., Mulet, M., Lalucat, J. & Garcia-Valdes, E. (2010). PseudoMLSA: a database for multigenic sequence analysis of *Pseudomonas* species. *BMC Microbiol* **10**, 118-124

[61] Bennasar, A., Rossello-Mora, R., Lalucat, J. & Moore, E.R.B. (1996). 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and proposal of *Pseudomonas balearica* sp. nov. *Int J Syst Bacteriol* **46**, 200-205

[62] Bergey's Manual Trust (2011). Website: http://www.bergeys.org. Consulted April 2011.

[63] Bergey, D.H., Harrison, F.C., Breed, R.S., Hammer, B.W. & Huntoon, F.M. (1930). *Bergey's Manual of Determinative Bacteriology*. 3^{rd} ed. The Williams & Wilkins Co, Baltimore, pp. 1-589

[64] Berkeley, R.C.W. (2002). Whither *Bacillus*? In *'Applications and Systematics of Bacillus and relatives'*. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0.

[65] Berliner, E. (1915). Über die Schlaffsucht der Mehlmottenraupe (*Ephestia kühniella* Zell) und ihren Erreger *Bacillus thuringiensis* n. sp. *Zeitschrift fur angewandte Entomologie Berlin* **2**, 29-56

[66] Bernal, A., Ear, U. & Kyrpides, N. (2001). Genomes OnLine Database (GOLD): a monitor of genome projects world-wide. *Nuc Ac Res* **29**, 126-127

[67] Billing, E. & Cuthbert, W.A. (1958). "Bitty cream": The occurrence and significance of *Bacillus cereus* in raw milk supplies. *J Appl Bact* **21**, 65-78

[68] Bishop, J.R. & White, C.H. (1986). Assessment of dairy product quality and potential shelf-life - a review. *J Food Protect* **49**, 739-748

[69] Bligh & Dyer (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917

[70] Bodilis, J., Calbrix, R., Guérillon, J., Mérieau A., Pawlak, B., Orange, N. & Barray, S. (2003). Phylogenetic relationships between environmental and clinical isolates of *Pseudomonas fluorescens* and related species deduced from 16S rRNA gene and OprF protein sequences. *System Appl Microbiol* **27**, 93-108

[71] Bonjour, F. & Aragno, M. (1984). *Bacillus tusciae*, a new species of thermoacidophilic, facultatively chemolithoautotrophic, hydrogen oxidizing sporeformer from a geothermal area. *Arch Microbiol* **139**, 397-401

[72] Boone, D.R., Liu, Y., Zhao, Z.-J., Balkwill, D.L., Drake, G.R., Stevens, T.O. & Aldrich, H.C. (1995). *Bacillus infernus* sp. nov., an Fe(III) and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int J Syst Bacteriol* **45**, 441-448

[73] Borchert, M.S., Nielsen, P., Graeber, I. Kaesler, I., Szewzyk, U., Pape, T., Antranikian, G. & Schäfer, T. (2007). *Bacillus plakortidis* sp. nov. and *Bacillus murimartini* sp. nov., novel alkalitolerant members of rRNA group 6. *Int J Syst Evol Microbiol* **57**, 2888-2893

[74] Borsodi, A.K., Marialigetti, K., Szabo, G., Palatinszky, M., Pollak, B., Keki, Z., Kovacs, A.L., Schumann, P. & Toth, E.M. (2008). *Bacillus aurantiacus* sp. nov., an alkaliphilic and moderately halophilic bacterium isolated from Hungarian soda lakes. *Int J Syst Evol Microbiol* **58**, 845-851

[75] Bowman, J.P., Sly, L.I. & Hayward, A.C. (1988). *Pseudomonas mixta* sp. nov., a bacterium from soil with degradative activity on a variety of complex polysaccharides. *Syst Appl Microbiol* **11**, 53-69

[76] Bowman, J.P., Sly, L.I., Hayward, A.C., Spiegel, Y. & Stackebrandt, E. (1993). *Telluria mixta* (*Pseudomonas mixta* Bowman, Sly, and Hayward 1988) gen. nov., comb. nov., and *Telluria chitinolytica* sp. nov., soil-dwelling organisms which actively degrade polysaccharides. *Int J Syst Bacteriol* **43**, 120-124

[77] Bozal, N., Montes, M.J. & Mercadé, E. (2007). *Pseudomonas guineae* sp. nov., a novel psychrotolerant bacterium from an Antarctic environment. *Int J Syst Evol Microbiol* **57**, 2609-2612

[78] Bredemann, G. & Werner, W. (1933). Botanische beschreibung häufinger am buttersäureabbau beteiligter sporenbildender bakterienspezies. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **87**, 446-475

[79] Breed, R.S., Murray, E.G.D. & Hitchens, A.P. (1948). *Bergey's Manual of Determinative Bacteriology*. 6^{th} ed. The Williams & Wilkinson Co., Baltimore.

[80] Brodbeck, U., Denton, W.L., Tanahashi, N. & Ebner, K.E. (1967). The isolation and identification of the B protein of lactose-synthetase as α-lactalbumin. *J Biol Chem* **248**, 1391-1397

[81] Brown, K.L. (2000). Control of bacterial spores. *Br Med Bull* **56**, 158-171

[82] Brown, G.R., Sutcliffe, I.C. & Cummings, S.P. (2001). Reclassification of *Pseudomonas doudoroffii* (Baumann *et al*. 1983) into the genus *Oceanomonas* gen. nov. as *Oceanomonas doudoroffii* comb. nov., and description of a phenol-degrading bacterium from estuarine water as *Oceanomonas baumannii* sp. nov. *Int J Syst Evol Microbiol* **51**, 67-72

[83] Bull, A.T., Goodfellow, M. & Slater, J.H. (1992). Biodiversity as a source of innovation in biotechnology. *Ann Rev Microbiol* **46**, 219-252

[84] Burkholder, W.H. (1948). Genus I. *Pseudomonas* Migula. Bacterial plant pathogens. In '*Bergey's Manual of Determinative Bacteriology*'. Eds. Breed, R.S., Murray, E.G.D. & Hitchens, A.P. 6^{th} ed. The Williams & Wilkins Co, Baltimore, pp. 82-150

[85] Burr, S.E., Gobeli, S., Kuhnert, P., Goldsmith-Clermont, E. & Frey, J. (2010). *Pseudomonas chlororaphis* subsp. *piscium* subsp. nov., isolated from freshwater fish. *Int J Syst Evol Microbiol* **60**, 2753-2757

[86] Bylund, G. (1995). Dairy Processing Handbook. Ed. Teknotext AB, Tetra Pak Processing Systems AB, Lund, Sweden

[87] Caccamo, D., Gugliandolo, C., Stackebrandt, E. & Maugeri, T.L. (2000). *Bacillus vulcani* sp. nov., a novel thermophilic species isolated from a shallow marine hydrothermal vent. *Int J Syst Evol Microbiol* **50**, 2009-2012

[88] Callon, C., Duthoit, F., Delbès, C., Ferrand, M., Le Frileux, Y., De Crémoux, R. & Montel M.C. (2007). Stability of microbial communities in goat milk during a lactation year: molecular approaches. *Syst Appl Microbiol* **30**, 547-560

[89] Camara, B., Strömpl, C., Verbarg, S., Spröer C., Pieper, D.H. & Tindall, B.J. (2007). *Pseudomonas reinekei* sp. nov., *Pseudomonas moorei* sp. nov. and *Pseudomonas mohnii* sp. nov., novel species capable of degrading chlorosalicylates or isoprimaric acid. *Int J Syst Evol Microbiol* **57**, 923-931

[90] Campos, V.L., Valenzuela, C., Yarza, P. Kämpfer, P., Vidal, R., Zaror, C., Mondaca, M.A., Lopez-Lopez, A. & Rossello-Mora, R. (2010). *Pseudomonas arsenicoxydans* sp. nov., an arsenite-oxidizing strain isolated from the Atacama desert. *Syst Appl Microbiol* **33**, 193-197

[91] Carrasco, I.J., Marquez, M.C., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D. & Ventosa, A. (2007). *Bacillus chagannorensis* sp. nov., a moderate halophile from a soda lake in Inner Mongolia, China. *Int J Syst Evol Microbiol* **57**, 2084-2088

[92] Catara, V., Sutra, L., Morineau, A., Achouak, W., Christen, R. & Gardan, L. (2002). Phenotypic and genomic evidence for the revision of *Pseudomonas corrugata* and proposal of *Pseudomonas mediterranea* sp. nov. *Int J Syst Evol Microbiol* **52**, 1749-1758

[93] Cempirkova, R. (2002). Psychrotrophic vs. total bacterial counts in bulk milk samples. *Vet Med Czech* **47**, 227-233

[94]

[95] Cerritos, R., Vinuesa, P., Eguiarte, L.E., Herrer-Estrella, L., Alcaraz-Peraza, L.D., Arvizu-Gomez, J.L., Olmedo, G., Ramirez, E. & Souza, V. (2008). *Bacillus coahuilensis* sp. nov., a moderately halophilic species from a desiccation lagoon in the Cuatro Ciénegas Valley in Coahuila, Mexico. *Int J Syst Evol Microbiol* **58**, 919-923

[96] Chabeaud, P., De Groot, A., Bitter, W., Tommassen, J., Heulin, T. & Achouak, W. (2001). Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. *J Bact* **183**, 2117-2120

[97] Champagne, C.P., Laing, R.R., Roy, D., Mafu, A.A. & Griffiths, M.W. (1994). Psychrotrophs in dairy products: their effects and their control. *Crit Rev Food Sci Nutr* **34**, 1-30

[98] Chandler, R.E. & McMeekin, T.A. (1985). Temperature function integration and its relationship to the spoilage of pasteurized, homogenized milk. *Aust J Dairy Technol* **40**, 37-41

[99] Chanway, C.P. (2002). Plant growth promotion by *Bacillus* and relatives. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0

[100] Chen, L., Coolbear, T. & Daniel, R.M. (2003a). Characteristics of proteinases and lipases produced by seven *Bacillus* spp. isolated from milk powder production lines. *Int Dairy J* **14**, 495-504

[101] Chen L., Daniel, R.M. & Coolbear T. (2003b). Detection and impact of protease and lipase activities in milk and milk powders. *Int Dairy J* **13**, 255-275

[102] Chen, X.G., Stabnikova, O., Tay, J.H., Wang, J.Y. & Tay, S.T. (2004). Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus*, sp. nov., from sewage sludge. *Extremophiles* **8**, 489-498

[103] Chen, Y.G., Zhang, Y.Q., Wang, Y.X., Liu, Z.X., Klenk, H.P., Xiao, H.D., Tang, S.K., Cui, X.L. & Li, W.J. (2009). *Bacillus neizhouensis* sp. nov., a halophilic marine bacterium isolated from a sea anemone. *Int J Syst Evol Microbiol* **59**, 3035-3039

[104] Chen, Y.G., Zhang, L., Zhang, Y.Q., He, J.W., Klenk, H.P., Tang, S.K., Zhang, Y.X. & Li, W.J. (2011). *Bacillus nanhaiensis* sp. nov., isolated from an oyster. *Int J Syst Evol Microbiol* **61**, 888-893

[105] Cheshire, F.R. & Cheyne, W.W. (1885). The pathogenic history and history under cultivation of a new *Bacillus* (*B. alvei*), the cause of a disease of the hive bee hitherto known as foul brood. *Journal of the Royal Microscopic Society* **5**, 581-601

[106] Chester, F.D. (1898). Report of the mycologist: bacteriological work. *Del Agric Exp Stn Bull* **10**, 47-137

[107] Chester, F.D. (1901). A Manual of Determinative Bacteriology. The Macmillan Co., New York, pp. 1-401

[108] Cho, S.L., Jung, M.Y., Park, M.H. & Kim, W. (2010). *Bacillus chungangensis* sp. nov., a halophilic species isolated from sea sand. *Int J Syst Evol Microbiol* **60**, 1349-1352

[109] Christiansson, A., Bertilsson, J. & Svensson, B. (1999). *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. *J Dairy Sci* **82**, 305-314

[110] Cladera, A.M., Garcia-Valdés, E. & Lalucat, J. (2006). Genotype versus phenotype in the circumscription of bacterial species: the case of *Pseudomonas stutzeri* and *Pseudomonas chloritidismutans*. *Arch Microbiol* **184**, 353-361

[111] Clark, F.E. (1937). The relationship of *Bacillus siamensis* and similar pathogenic spore-forming bacteria to *Bacillus subtilis*. *J Bact* **33**, 435-443

[112] Clark, L.L., Dajcs, J.J., McLean, C.H., Bartell, J.G. & Stroman, D.W. (2006). *Pseudomonas otitidis* sp. nov., isolated from patients with otic infections. *Int J Syst Evol Microbiol* **56**, 709-714

[113] Claus, D. & Berkeley, R.C.W. (1986). Genus *Bacillus* (Cohn 1872). In *'Bergey's Manual of Systematic Bacteriology'*. Eds. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. The Williams and Wilkins Co., Baltimore

[114] Claydon, T.J. & Hammer, B.W. (1939). A skunk-like odor of bacterial origin in butter. J Bact 37, 251-258

[115] Clontech, 2011. Website: http://www.clontech.com/. Consulted June 2011

[116] Coenye, T., Gevers, D., Van De Peer, Y., Vandamme, P., Swings, J. (2005). Towards a prokaryotic genomic taxonomy. *FEMS Microbiol Rev* **29**, 147-167

[117] Coenye, T., Gillis, M. & Vandamme, P. (2000). *Pseudomonas antimicrobica* Attafuah and Bradbury 1990 is a junior synonym of *Burkholderia gladioli* (Severini 1913) Yabuuchi *et al*. 1993. *Int J Syst Evol Microbiol* **50**, 2135-2139

[118] Coenye, T., Laevens, S., Gillis, M. & Vandamme, P. (2001). Genotypic and chemotaxonomic evidence for the reclassification of *Pseudomonas woodsii* (Smith 1911) Stevens 1925 as *Burkholderia andropogonis* (Smith 1911) Gillis *et al*. 1995. *Int J Syst Evol Microbiol* **51**, 183-185

[119] Coenye, T., Spilker, T., Martin, A. & LiPuma, J. (2002). Comparative Assessment of Genotyping Methods for Epidemiologic Study of Burkholderia cepacia Genomovar III. J Clin Microbiol 40, 3300-3307

[120] Cohan, F.M. (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457-487

[121] Cohn, F. (1872). Untersuchungen über Bakterien. *Beitrage zur Biologie der Pflanzen* **1**, 127-224

[122] Coles, H. (2007). The lactase gene in Africa: Do you take milk? The Human Genome, Wellcome Trust. Website: http://genome.wellcome.ac.uk/doc_WTX038968.html. Consulted February 2011.

[123] Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J.A.E. (1994). The phylogeny of the genus *Clostridium:* proposal of five new genera and eleven new species combinations. *Int J Syst Bact* **44**, 812-826

[124]

[125] Collins, M.D. & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol Rev* **45**, 316-354

[126] Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B.K.C., Dwivedi, P.P., Pot, B., Prensier, G. & Garcia, J.L. (1995). *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *Int J Syst Bacteriol* **45**, 9-16

[127] Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126-140

[128] Coorevits, A., De Jonghe, V., Vandroemme, J., Van Landschoot, A., Heyndrickx, M. & De Vos, P. (2010). How can the type of dairy farming influence the bacterial flora in milk? In '*Organic Farming and Peanut Crops*'. Eds. Grossman, D.C. & Barrios, T.L. Nova Science Publishers, Inc., New York. ISBN: 978-1-60876-187-6

[129] Coorevits, A., Dinsdale, A.E., Heyrman, J., Schumann, P., Van Landschoot, A., Logan, N.A. & De Vos, P. (2011a). *Lysinibacillus macroides* sp. nov., nom. rev. *Int J Syst Evol Microbiol*, in press (doi 10.1099/ijs.0.027995-0)

[130] Coorevits, A., Dinsdale, A.E., Halket, G., Lebbe, L., De Vos, P., Van Landschoot, A. & Logan, N.A. (2011b). Taxonomic revision of the genus *Geobacillus*: emendation of *Geobacillus*, *G. stearothermophilus*, *G. jurassicus*, *G. toebii*, *G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly "*thermoglucosidasius*"); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus*; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. *Int J Syst Evol Microbiol*, in press (doi 10.1099/ijs.0.030346-0)

[131] Coorevits, A., Logan, N.A., Dinsdale, A.E., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos, P. (2011c). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans. Int J Syst Evol Microbiol* **61**, 1954-1961

[132] Coroler, L., Elomari, M., Hoste, B., Gillis, M., Izard, D. & Leclerc, H. (1996). *Pseudomonas rhodesiae* sp. nov., a new species isolated from natural mineral waters. *Syst Appl Microbiol* **19**, 600-607

[133] Cosentino, S., Mulargia, A.F., Pisano, F., Truveri, P., & Palmas, F. (1997). Incidence and biochemical characteristics of *Bacillus* flora in Sardinian dairy products. *Int J Food Microbiol* **38**, 235-238

[134] Cousin, M.A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *J Food Prot* **45**, 172-207

[135] Cousin, M. A. (1989). Physical and biochemical effects on milk components. In *'Enzymes of Psychrotrophs in Raw Food'.* Ed. McKellar, R.C. Boca Raton, Florida, CRC Press Inc

[136] Cousins, C.M. & Bramley, A.J. (1981). The microbiology of raw milk. In *'Dairy Microbiology'.* Ed. Robinson, R.K. London, UK, Applied Science

[137] Crielly, E.M., Logan, N.A. & Anderton, A. (1994). Studies on the *Bacillus* flora of milk and milk products. *J Appl Bacteriol* **77**, 256-263

[138] Curran, B., Jonas, D., Grundmann, H., Pitt, T. & Dowson, C.G. (2004). Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J Clin Microbiol* **42**, 5644-5649

[139] Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard D. & Leclerc, H. (1999a). *Pseudomonas libanensis*, sp. nov., a new species isolated from Lebanese spring waters. *Int J Syst Bacteriol* **49**, 1091-1101

[140] Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D. & Leclerc, H. (1999b). Taxonomic study of bacteria isolated from Lebanese spring waters: proposal of *Pseudomonas cedrella* sp. nov. and *P. orientalis* sp. nov. *Res Microbiol* **150**, 303-316

[141] Dabboussi, F., Hamze, M., Singer, E., Geoffroy, V., Meyer, M. & Izard, D. (2002). *Pseudomonas mosselii* sp. nov., a novel species isolated from clinical specimens. *Int J Syst Evol Microbiol* **52**, 363-376

[142] Darland, G. & Brock, T.D. (1971). Bacillus acidocaldarius sp. nov., an acidophilic thermophilic spore-forming bacterium. J Gen Bact 67, 9-15

[143] Datta, N. & Deeth, H.C. (2001). Age gelation of UHT-milk - a review. *Food Bioprod Process* **79**, 197-210

[144] Davis, D.H., Stanier, R.Y., Doudoroff, M. & Mandel, M. (1970). Taxonomic studies on some Gram-negative polarly flagellated "hydrogen bacteria" and related species. *Archiv Mikrobiol* **70**, 1-13

[145] Davis, D.H., Doudoroff, M., Stanier, R.Y. & Mandel, M. (1969). Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. *Int J Syst Bact* **19**, 375-390

[146] Dawyndt, P., Vancanneyt, M., Snauwaert, C., De Baets, B., De Meyer, H. & Swings, J. (2006). Mining fatty acid databases for detection of novel compounds in aerobic bacteria. *J Microbiol Meth* **66**, 410-433

[147] de Bary, A. (1884). Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bacterien. Wilhelm Engelmann, Leipzig

[148] De Clerck, E., Rodriguez-Diaz, M., Vanhoutte, T., Heyrman, J., Logan, N.A. & De Vos, P. (2004). *Anoxybacillus contaminans* sp. nov. and *Bacillus gelatini* sp. nov., isolated from contaminated gelatin batches. *Int J Syst Evol Microbiol* **54**, 941-946

[149] Deeth, H.C. & Fitz-Gerald, C.H. (1983). Lipolytic enzymes and hydrolytic rancidity in milk and milk products. In *'Developments in dairy chemistry'.* Ed. Fox, P.F. London, UK, Applied Science

[150] Deeth, H.C. & Fitz-Gerald, C.H. (1994). Lipolytic enzymes and hydrolytic rancidity in milk and milk products. In *'Advanced dairy chemistry'.* Ed. Fox, P.F. London, UK, Chapman & Hall

[151] Deinhard, G., Saar, J., Krischke, W. & Poralla, K. (1987). *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing ω-cycloheptane fatty acids. *Syst Appl Microbiol* **10**, 68-73

[152] de Jong, P. (1997). Impact and control of fouling in milk processes. *Trends Food Sci Tech* **8**, 401-405

[153] De Jonghe, V., Coorevits, A., De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., De Vos, P. & Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *Int J Food Microbiol* **136**, 318-325

[154] De Jonghe V., Coorevits A., Marchand S., Van Landschoot A., De Block J., De Vos P. & Heyndrickx M. (2011a). Microbial contamination and spoilage of consumer milk - facts and fiction. In *'Raw milk: production, consumption and health effects'*. Ed. Momani, J. & Natsheh, A. Nova Science Publishers, Inc., New York, ISBN 978-1-61470-751-6

[155] De Jonghe, V., Coorevits, A., Vandroemme, J., Heyrman, J., Herman, L., De Vos, P. & Heyndrickx, M. (2008). Intraspecific genotypic diversity of *Bacillus* species from raw milk. *Int Dairy J* **18**, 496-505

[156] De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2011b). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl Environ Microbiol* **77**, 460-470

[157] Delafield, F.P., Doudoroff, M., Palleroni, N.J., Lusty, C.J. & Contopoulou, R. (1965). Decomposition of poly-β-hydroxybutyrate by pseudomonads. *J Bact* **90**, 1455

[158] Delaporte, B., Raymond, M. & Daste, P. (1961). Une bactérie du sol capable d'utiliser, comme source de carbone, la fraction fixe de certaines oléorésines, *Pseudomonas resinovorans* n. sp. *Compte Rendu des Séances de l'Académie des Sciences* **252**, 1073-1075

[159] Delorme, S., Lemanceau, P., Christen, R., Corberand, T., Meyer, J.M. & Gardan, L. (2002). *Pseudomonas lini* sp. nov., a novel species from bulk and rhizospheric soils. *Int J Syst Evol Microbiol* **52**, 513-523

[160] De Ley, J., Segers, P. & Gillis, M. (1978). Intra- and intergeneric similarities of Chromobacterium and Janthinobacterium ribosomal ribonucleic acid cistrons. Int J Syst Bact 28, 154-168

[161] Demharter, W. & Hensel, R. (1989). *Bacillus thermocloaceae* sp. nov., a new thermophilic species from sewage sludge. *Syst Appl Microbiol* **11**, 272-276

[162] Denariaz, G., Payne, W.J. & Le Gall, J. (1989). A halophilic denitrifier, *Bacillus halodenitrificans* sp. nov. *Int J Syst Bacteriol* **39**, 145-151

[163] den Dooren De Jong, L.E. (1926). Bijdrage tot de kennis van het Mineralisatieproces. Thesis. Technische Hogeschool, Delft, pp. 1-199

[164] den Dooren De Jong, L.E. (1929). Über *Bacillus fastidiosus*. *Zentralblatt für Bakteriologie*, *Parasitenkunde*, *Infektionskrankheiten und Hygiene* **79**, 344-353

[165] Denizci, A.A., Kazan, D. & Erarslan, A. (2010). *Bacillus marmarensis* sp. nov., an alkaliphilic, protease-producing bacterium isolated from mushroom compost. *Int J Syst Evol Microbiol* **60**, 1590-1594

[166] Denner, E.B.M., Kämpfer, P., Busse, H.-J. & Moore, E.R.B. (1999). Reclassification of *Pseudomonas echinoides* Heumann 1962, 343, in the genus *Sphingomonas* as *Sphingomonas echinoides* comb. nov. *Int J Syst Bacteriol* **49**, 1103-1109

[167] Derekova, A., Sjøholm, C., Mandeva, R. & Kambourova, M. (2007). *Anoxybacillus rupiensis* sp. nov., a novel thermophilic bacterium isolated from Rupi basin (Bulgaria). Extremophiles **11**, 577-83

[168] De Vos, P. & De Ley, J. (1983). Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *Int J Syst Bacteriol* **33**, 487-509

[169] De Vos, P., Kersters, K., Falsen, E., Pot, B., Gillis, M., Segers, P. & De Ley, J. (1985). *Comamonas* Dacis and Park 1962 gen. nov., nom. rev. emend., and *Comamonas terrigena* Hugh 1962 sp. nov. nom. rev. *Int J Syst Bacteriol* **35**, 443-453

[170] Ding, L. & Yokota, A. (2004). Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for bacterial strains isolated from well water and reclassification of *Pseudomonas huttiensis*, *Pseudomonas lanceolata*, *Aquaspirillum delicatum* and *Aquaspirillum autotrophicum* as *Herbaspirillum huttiense* comb. nov., *Curvibacter lanceolatus* comb. nov., *Curvibacter delicatus* comb. nov. and *Herbaspirillum autotrophicum* comb. nov. *Int J Syst Evol Microbiol* **54**, 2223-2230

[171] Dinsdale, A.E., Halket, G., Coorevits, A., Van Landschoot, A., Busse, H.-J., De Vos, P. & Logan, N.A. (2011). Emended descriptions of *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus. Int J Syst Evol Microbiol* **61**, 1802-1810

[172] Dobson, S.J. & Franzmann, P.D. (1996). Unification of the genera *Deleya* (Baumann *et al*. 1983), *Halomonas* (Vreeland *et al*. 1980), and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *Int J Syst Bacteriol* **46**, 550-558

[173] Dogan, B. & Boor, K.J. (2003). Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. *App Environ Microbiol* **69**, 130-138

[174] Döhler, K., Huss, V.A.R. & Zumft, W.G. (1987). Transfer of *Pseudomonas perfectomarina* Baumann, Bowditch, Baumann, and Beaman 1983 to *Pseudomonas stutzeri* (Lehmann and Neumann 1896) Sidjerius 1946. *Int J Syst Bacteriol* **37**, 1-3

[175] Donk, P.J. (1920). A highly resistant thermophilic organism. J Bact 5, 373-374

[176] Doolittle, W.F. & Papke, R.T. (2006). Genomics and the bacterial species problem. *Genome Biol* **7**, 116.1-116.7

[177] Doolittle, W.F. (1999). Lateral genomics. *Trends Genet* **15**, M5-M8

[178] Doudoroff, M. (1940). The oxidative assimilation of sugars and related substances by *Pseudomonas saccharophila* with a contribution to the problem of the direct respiration of di- and polysaccharides. *Enzymologia* **9**, 59-72

[179] Dowson, W.J. (1939). On the systematic position and generic names of the Gram negative bacterial plant pathogens. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **100**, 177-193

[180] Drobniewski, F.A. (1993). *Bacillus cereus* and related species. *Clin Microbiol Rev* **6**, 324-338

[181] Duncan, S.E. & Christen, G.L. (1991). Sensory detection and recovery by acid degree value of fatty acids added to milk. *J Dairy Sc* **74**, 2855-2859

[182] Dutky, S.R. (1940). Two new spore-forming bacteria causing milky diseases of Japanese beetle larvae. *J Agri Res* **61**, 57-68

[183] Ellis, K.A., Innocent, G.T., Mihm, M., Cripps, P., McLean, W.G., Howard, C.V. & Grove-White, D. (2007). Dairy cow cleanliness and milk quality on organic and conventional farms in the UK. *J Dairy Res* **74**, 302-310

[184] Elomari, M., Coroler, L, Hoste, B., Gillis, M., Izard, D. & Leclerc, H. (1996). DNA relatedness among *Pseudomonas* strains isolated from natural mineral waters and proposal of *Pseudomonas veronii* sp. nov. *Int J Syst Bacteriol* **46**, 1138-1144

[185] Elomari, M., Coroler, L., Verhille, S., Izard, D. & Leclerc, H. (1997). *Pseudomonas monteilii* sp. nov., isolated from clinical specimens. *Int J Syst Bacteriol* **47**, 846-852

[186] Eneroth, E., Christiansson, A., Brendehaug, J. & Molin, G. (1998). Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. *Int Dairy J* **8**, 829-834

[187] Escalante, A.E., Caballero-Mellado, J., Martinez-Aguilar, L., Rodriguez-Verdugo, A., Gonzalez-Gonzalez, A., Toribio-Jimenez, J. & Souza, V. (2009). *Pseudomonas cuatrocienegasensis* sp. nov., isolated from an evaporating lagoon in the Cuatro Cienegas valley in Coahuila, Mexico. *Int J Syst Evol Microbiol* **59**, 1416-1420

[188] Eurostat (2011). Statistical Office of the European Union. http://epp.eurostat.ec.europa.eu/ portal/page/portal/eurostat/home. Consulted January 2011.

[189] Evers, J.M., Luckman, M.S. & Palfreyman, K.R. (2000). The BDI method – part I: determination of free fatty acids in cream and whole milk powder. *Aust J Dairy Tech* **55**, 33-36

[190] Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224-229

[191] Fagerlund, A., Ween, O., Lund, T., Hardy, S.P. & Granum, P.E. (2004). Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiol* **150**, 2689-2697

[192] Farhadian, M., Vachelard, C., Duchez, D. & Larroche, C. (2008). In situ bioremediation of monoaromatic pollutants in groundwater: a review. *Biores Tech* **99**, 5296-5308

[193] Farkye, N.Y., Imafidon, G.I. & Fox, P.F. (1995). Thermal denaturation of indigenous milk enzymes. In *'Heat induced changes in milk'*. Ed. Fox, P.F. Brussels, Belgium

[194] Fendrich, C. (1988). *Halovibrio variabilis* gen. nov. sp. nov., *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid *Eubacterium* from Great Salt Lake, Utah, USA. *Syst Appl Microbiol* **11**, 36-43

[195] Fleet, G.H. (1990). Yeasts in dairy products. *J Appl Bacteriol* **68**, 199-211

[196] Flint, S. & Hartley, N. (1996). A modified selective medium for the detection of *Pseudomonas* species that cause spoilage of milk and dairy plants. *Int Dairy J* **6**, 223-230

[197] Flügge, C. (1886). Die Mikroorganismen. Ed. Vogel, F.C.W. Leipzig

[198] Food-info.net (2011). Food technology Department of Wageningen University. http://www.food-info.net/uk/index.htm. Consulted March 2011

[199] Forsyth, G. & Logan, N.A. (2000). Isolation of *Bacillus thuringiensis* from Northern Victoria Land, Antarctica. *Lett Appl Microbiol* **30**, 263-266

[200] Fortina, M.G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P.L. & Stackebrandt, E. (2001). *Ureibacillus* gen. nov., a new genus to accomodate *Bacillus thermosphaericus* (Andersson *et al.* 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447-455

[201] Foschino, R., Galli, A. & Ottogalli, G. (1990). Research on the microflora of UHT milk. *Ann Microbiol* **40**, 47-59

[202] Fox, P.F. (1981). Proteinases in dairy technology. *Neth Milk Dairy J* **35**, 233-253

[203] Frankland, G.C. & Frankland, P.F. (1887). Studies on some new microorganisms obtained from air. *Royal Society London*, *Philosophical Transactions Biological Sciences* **178**, 257-287

[204] Fritze, D. (1996). *Bacillus haloalkaliphilus* sp. nov. *Int J Syst Bacteriol* **46**, 98-101

[205] Fritze, D. (2002). *Bacillus* identification – traditional approaches. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0

[206] From, C., Hormazabal, V. & Granum, P.E. (2007a). Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. *Int J Food Microbiol* **115**, 319-324

[207] From, C., Hormazabal, V., Hardy, S.P. & Granum, P.E. (2007b). Cytotoxicity in *Bacillus mojavensis* is abolished following loss of surfactin synthesis: implications for assessment of toxicity and food poisoning potential. *Int J Food Microbiol* **117**, 43-49

[208] From, C., Pukall, R., Schumann, P., Hormazabal, V. & Granum, P.E. (2005). Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. *Appl Environ Microbiol* **71**, 1178-1183

[209] Fujita, T., Shida, O., Takagi, H., Kunugita, K., Pankrushina, A.N. & Matsuhashi, M. (1996). Description of *Bacillus carboniphilus* sp. nov. *Int J Syst Bacteriol* **46**, 116-118

[210] Galarneault, T.P. & Leifson, E. (1964). Pseudomonas vesiculare (Büsing et al.) nov. comb. International Bulletin of Bacteriological Nomenclature and Taxonomy 14, 165-168

[211] Garabito, M.J., Arahal, D.R., Mellado, E., Marquez, M.C. & Ventosa, A. (1997). *Bacillus salexigens* sp. nov., a new moderately halophilic *Bacillus* species. *Int J Syst Bacteriol* **47**, 735-741

[212] Garcia, M.L., Sanz, B., Garcia-Collia, P. & Ordonez, J.A. (1989). Activity and thermostability of the extracellular lipases and proteinases from pseudomonads isolated from raw milk. *Milchwissenschaft* **44**, 547-560

[213] Gardan, L., Bella, P., Meyer, J.M., Christen, R., Rott, P., Achouak, W. & Samson, R. (2002). *Pseudomonas salomonii* sp. nov., pathogenic on garlic, and *Pseudomonas palleroniana* sp. nov., isolated from rice. *Int J Syst Evol Microbiol* **52**, 2065-2074

[214] Gardan, L., Bollet, C., Abu Ghorrah, M., Grimont, F. & Grimont, P.A.D. (1992). DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. *Int J Syst Bacteriol* **42**, 606-612

[215] Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F. & Grimont, P.A.D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (*ex* Sutic and Dowson 1959). *Int J Syst Bacteriol* **49**, 469-478

[216] Gatson, J.W., Benz, B.F., Chandrasekaran, C., Satomi, M., Venkateswaran, K. & Hart, M.E. (2006). *Bacillus tequilensis* sp. nov., isolated from a 2000-year-old Mexican shaft-tomb, is closely related to *Bacillus subtilis*. *Int J Syst Evol Microbiol* **56**, 1475-1484

[217] Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J. & Fries, I. (2006). Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *Int J Syst Evol Microbiol* **56**, 501-511

[218] Gershman, M.D., Kennedy, D.J., Noble-Wang, J., Kim, C., Gullion, J., Kacica, M., Jensen, B., Pascoe, N., Saiman, L., McHale, J., Wilkins, M., Schoonmaker-Bopp, D., Clayton, J., Arduino, M., Srinivasan, A. & *Pseudomonas fluorescens* investigation team (2008). Multistate outbreak of *Pseudomonas fluorescens* bloodstream infection after exposure to contaminated heparinized saline flush prepared by a compounding pharmacy. *Clin Infect Dis* **47**, 1372-1379

[219] Gest, H. (1999). Bacterial classification and taxonomy: a 'primer' for the new millennium. *Microbiol Today* **26**, 70-72

[220] Gest, H. (2003). Names of bacteria and their evolutionary relationships. *Microbiol* **149**, 1956-1958

[221] Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F.L. & Swings, J. (2005). Re-evaluating prokaryotic species. *Nature Rev* **3**, 733-739

[222] Ghosh, A., Bhardwaj, M., Satyanarayana, T., Khurana, M., Mayilraj, S. & Jain, R.K. (2007). *Bacillus lehensis* sp. nov., an alkalitolerant bacterium isolated from soil. *Int J Syst Evol Microbiol* **57**, 238-242

[223] Gibb, A.P., Martin, K.M., Davidson, G.A., Walker, B. & Murphy, W.G. (1995). Rate of growth of *Pseudomonas fluorescens* in donated blood. *J Clin Path* **48**, 717-718

[224] Gibson, T. & Gordon, R.E. (1974). *Bacillus*. In '*Bergey's Manual of Determinative Bacteriology*'. Eds. Buchanan, R.E. & Gibbons, N.E. 8^{th} ed. Williams & Wilkins, Baltimore, MD

[225] Gibson, T. (1935). The urea-decomposing microflora of soils. Description and classification of the organisms. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **92**, 364-380

[226] Gillis, M., Tran Van, V., Bardin, R., Goor, M., Hebbar, P., Willems, A., Segers, P., Kersters, K., Heulin, T. & Fernandez, M.P. (1995). Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* **45**, 274-289

[227] Glazunova, O.O., Raoult, D. & Roux, V. (2006). *Bacillus massiliensis* sp. nov., isolated from cerebrospinal fluid. *Int J Syst Evol Microbiol* **56**, 1485-1488

[228] GOLD database (2011). http://www.genomesonline.org/. Consulted May 2011

[229] Golovacheva, R.S., Loginova, L.G., Salikhov, T.A., Kolesnikov, A.A. & Zaitzeva, G.N. (1975). A new thermophilic species *Bacillus thermocatenulatus* nov. sp. *Mikrobiologiya* **44**, 265-268

[230] Gonzalez, J.M., Mayer, F., Moran, M.A., Hodson, R.E. & Whitman, W.B. (1997). *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* **47**, 369-376

[231] Gordon, R.E. (1973). The genus *Bacillus*. In '*Handbook of Microbiology*'. Eds. Laskin, A.I. & Lechevalier, H.A. Cleveland, the Chemical Rubber Co. Press

[232] Goris, J., Konstantinidis, K., Klappenbach, J.A., Coenye, T., Vandamme, P. & Tiedje, J.M. (2007). DNA-DNA hybridisation values and their relationship to whole-genome sequence similarity. *Int J Syst Evol Microbiol* **57**, 81-91

[233] Goto, M. (1983). *Pseudomonas ficuserectae* sp. nov., the causal agent of bacterial leaf spot of *Ficus erecta* Thunb. *Int J Syst Bacteriol* **33**, 546-550

[234] Goto, K., Omura, T., Hara, Y. & Sadaie, Y. (2000). Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol* **46**, 1-8

[235] Gouy, M., Guindon, S. & Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**, 221-224

[236] Gower, J.C. (1971). A general coefficient of similarity and some of its properties. *Biometrics* **27**, 857-874

[237] Granum, P.E. (2002). *Bacillus cereus* and food poisoning. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R.C.W., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell Science, Oxford, UK.

[238] Gray, P.H.H. & Thornton, H.G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **73**, 74-96

[239] Green, P.N. & Bousfield, I.J. (1983). Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov., corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. *Int J Syst Bacteriol* **33**, 875-877

[240] Griffiths, M.W. (1983). Synergistic effects of various lipases and phospholipase C on milk fat. *J Food Tech* **18**, 495-505

[241] Griffiths, M.W. (1990). Toxin production by psychrotrophic *Bacillus* spp. present in milk. *J Food Prot* **53**, 790-792

[242] Griffiths, M.W., Phillips, J.D. & Muir, D.D. (1981). Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. *J Appl Bact* **50**, 289-303

[243] Grimes, D.J., Woese, C.R., MacDonelle, M.T. & Colwell, R.R. (1997). Systematic study of the genus *Vogesella* gen. nov. and its type species *Vogesella indigofera* comb. nov. *Int J Syst Bacteriol* **47**, 19-27

[244] Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234-239

[245] Grotiuz, G., Sirok, A., Gadea, P., Varela, G. & Schelotto, F. (2006). Shiga toxin 2-producing *Acinetobacter haemolyticus* associated with a case of bloody diarrhea. *J Clin Microbiol* **44**, 3838-3841

[246] Gruber, T. (1905). Ein weiterer Beiträg zür Aromabildung, speziell zür Bildung des Erdbeergeruches in der Gruppe "*Pseudomonas*", *Pseudomonas fragariae* II. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **14**, 122-123

[247] Gugliandolo, C., Maugeri, T.L., Caccamo, D. & Stackebrandt, E. (2003). *Bacillus aeolius* sp. nov. a novel thermophilic, halophilic marine *Bacillus* species from Eolian Islands (Italy). *Syst Appl Microbiol* **26**, 172-176

[248] Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696-704

[249] Gul-Guven, R., Guven, K., Poli, A. & Nicolaus, B. (2008). *Anoxybacillus kamchatkensis* subsp. *assacharedens* subsp. nov., a thermophilic bacterium isolated from a hot spring in Batman. *J Gen Appl Microbiol* **54**, 327-334

[250] Gupta, R.S. (2000). Evolutionary relationships among bacteria: does 16S rRNA provide all the answers? *ASM News* **66**, 189-190

[251] Gupta, S.K., Kumari, R., Prakash, O. & Lal, R. (2008). *Pseudomonas panipatensis* sp. nov., isolated from an oil-contaminated site. *Int J Syst Evol Microbiol* **58**, 1339-1345

[252] Ha, E. & Zemel, M.B. (2003). Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). *J Nutr Biochem* **14**, 251-258

[253] Haas, D. & Defago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* **3**, 307-319

[254] Hamdy, M.E.R. & Amin, A.S. (2002). Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet J* **163**, 299-305

[255] Hammer, B.W. (1915). Bacteriological studies on the coagulation of evaporated milk. *Iowa Agricultural Experimental Station Research Bulletin* **19**, 119-131

[256] Hammer, P., Lembke, F., Suhren, G. & Heeschen, W. (1995). Characterization of a heat resistant mesophilic *Bacillus* species affecting the quality of UHT-milk - a preliminary report. *Kieler Milchwirtschaftliche Forschungberichte* **47**, 303-311

[257] Hammer, B.W. & Trout, M.G. (1924). A study of the yellow cocci which survive pasteurization. *J Dairy Sci* **11**, 18-23

[258] Hammer, P. & Walte, H.G. (1996). Zur pathogenität hitzeresistenter mesophiler sporenbilder aus UHT-milch. *Kieler Milchwirtschaftliche Forschungberichte* **48**, 151-161

[259] Hanage, W.P., Fraser, C. & Spratt, B.G. (2006). Sequences, sequence clusters and bacterial species. *Phil Trans R Soc* **361**, 1917-1927.

[260] Hansen, D., Hilgenhöner, M. & Popp, W. (2008). ATP bioluminescence – for kitchen hygiene and cleaning control of surgical instruments. *Int J Infect Contr*, available online (doi:10.3396/ijic.V4i1.010.08)

[261] Hantsis-Zacharov, E. & Halpern, M. (2007). Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Appl Environ Microbiol* **73**, 7162-7168

[262] Hardham, J.F. (1998). Effect of protein standardization of milk by addition of UF milk permeate on the composition and storage stability of UHT processed milk. *Aust J Dairy Tech* **53**, 22-27

[263] Harrington, C.S. & On, S.L.W. (1999). Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *Int J Syst Bacteriol* **49**, 1171-1175.

[264] Harwalker, V.R. (1982). Age gelation of sterilized milks. In *'Development in Dairy Chemistry'*. Ed. Fox, P.F. London, UK, Elsevier Science Publisher Ltd

[265] Hatayama, K., Kawai, S., Shoun, H., Ueda, Y. & Nakamura, A. (2005). *Pseudomonas azotifigens* sp. nov., a novel nitrogen-fixing bacterium isolated from a compost pile. *Int J Syst Evol Microbiol* **55**, 1539-1544

[266] Hatayama, K., Shoun, H., Ueda, Y. & Nakamura, A. (2006). *Tuberibacillus calidus* gen. nov., sp. nov., isolated from a compost pile and reclassification of *Bacillus naganoensis* Tomimura *et al.* 1990 as *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *Bacillus laevolacticus* Andersch *et al.* 1994 as *Sporolactobacillus laevolacticus* comb. nov. *Int J Syst Evol Microbiol* **56**, 2545-2551

[267] Hauser, E., Kämpfer, P. & Busse, H.-J. (2004). *Pseudomonas psychrotolerans* sp. nov. *Int J Syst Evol Microbiol* **54**, 1633-1637

[268] Haynes, W.C. (1957). Genus I. *Pseudomonas* Migula 1894. In *'Bergey's Manual of Determinative Bacteriology'.* Eds. Breed, R.S., Murray, E.G.D. & Smith, N.R. 7^{th}edition. The Williams & Wilkins Co, Baltimore

[269] Heinen, U.J., & Heinen, W. (1972). Characteristics and properties of a caldoactive bacterium producing extracellular enzymes and two related strains. *Arch Mikrobiol* **82***,* 1-23

[270] Henz, S.R., Huson, D.H., Auch, A.F., Nieselt-Struwe, K. & Schuster, S.C. (2005). Whole-genome prokaryotic phylogeny. *Struct Bioinfo* **21**, 2329-2335

[271] Herman, L.M.F., Vaerewijck, M.J.M., Moermans, R.J.B., Waes, G.M.A.V.J. (1997). Identification and detection of *Bacillus sporothermodurans* spores in 1, 10 and 100 milliliters of raw milk by PCR. *Appl Environ Microbiol* **63**, 3139-3143

[272] Heumann, W. (1962). Die Methodik der Kreuzung sternbildender Bakterien. *Biolgisches Zentralblatt* **81**, 341-354

[273] Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J.J., Bos, M.H., Heilmann, F.G., Hofhuis, A., Hoekstra, T. & de Boer, E. (2009). Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. *Int J Food Microbiol* **134**, 70-74

[274] Heyman, M.B. (2006). Lactose-intolerance in children, infants and adolescents. *Pediatrics* **118**, 1279-1286

[275] Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N.A. (1998). *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. *Int J Syst Bacteriol* **48**, 99-106

[276] Heyndrickx, M., Lebbe, L., Vancanneyt, M., Kersters, K., De Vos, P., Logan, N.A., Forsyth, G., Nazli, S., Ali, N. & Berkeley, R.C.W. (1997). A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer *et al*. 1996) as *Aneurinibacillus thermoaerophilus* comb. nov., and emended descriptions of *A. aneurinilyticus* corrig., and *A. migulanus*, and *A. thermoaerophilus*. *Int J Syst Bacteriol* **47**, 808-817

[277] Heyndrickx, M., Logan, N.A., Lebbe, L., Rodriguez-Diaz, M., Forsyth, G., Goris, J., Scheldeman, P. & De Vos, P. (2004). *Bacillus galactosidilyticus* sp. nov., an alkali-tolerant β-galactosidase producer. *Int J Syst Evol Microbiol* **54**, 617-621

[278] Heyndrickx, M. & Scheldeman, P. (2002). Bacilli associated with spoilage in dairy and other food products. In *'Applications and Systematics of Bacillus and relatives'.* Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell Science, Oxford, UK

[279] Heyndrickx, M., Scheldeman, P., Forsyth, G., Lebbe, L., Rodriguez-Diaz, M., Logan, N.A. & De Vos, P. (2005). *Bacillus ruris* sp. nov., from dairy farms. *Int J Syst Evol Microbiol* **55**, 2551-2554

[280] Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., Logan, N.A., Ali, N. & Berkeley, R.C.W. (1996a). Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura 1984) Ash *et al*. 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) *larvae* (White 1906) Ash *et al*. 1994, as a subspecies of *P. larvae*, with emended descriptions of *P. larvae* as *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens*. *Int J Syst Bacteriol* **46**, 270-279

[281] Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Hoste, B., Kersters, K., De Vos, P., Logan, N.A., Aziz, A.M., Ali, N. & Berkeley, R.C.W. (1995). *Paenibacillus* (formerly *Bacillus*) *gordonae* (Pichinoty *et al*. 1986) Ash *et al*. 1994 is a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) *validus* (Nakamura 1984) Ash *et al*. 1994: emended description of *P. validus*. *Int J Syst Bacteriol* **45**, 661-669

[282] Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Kersters, K., De Vos, P., Logan, N.A., Aziz, A.M., Ali, N. & Berkeley, R.C.W. (1996b). A polyphasic reassessment of the genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and of *Bacillus peoriae* (Montefusco *et al*. 1993) as *Paenibacillus peoriae* comb. nov., and emended descriptions of *P. lautus* and of *P. peoriae*. *Int J Syst Bacteriol* **46**, 988-1003

[283] Heyrman, J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Logan, N., Swings, J. & De Vos, P. (2003a). *Virgibacillus carmomensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus pictorum* sp. nov., three new species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus* as *Virgibacillus marismortui* comb. nov., and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. *Int J Syst Evol Microbiol* **53**, 501-511

[284] Heyrman, J., Balcaen, A., Rodriguez-Diaz, M., Logan, N.A., Swings, J. & De Vos, P. (2003b). *Bacillus decolorationis* sp. nov., isolated from biodeteriorated parts of the mural paintings at the Servilia tomb (Roman necropolis of Carmona, Spain) and the Saint-Catherine chapel (Castle Herberstein, Austria). *Int J Syst Evol Microbiol* **53**, 459-463

[285] Heyrman, J., Logan, N.A., Rodriguez-Diaz, M., Scheldeman, P., Lebbe, L., Swings, J., Heyndrickx, M. & De Vos, P. (2005a). Study of mural painting isolates, leading to the transfer of *'Bacillus maroccanus'* and *'Bacillus carotarum'* to *Bacillus simplex*, emended description of *Bacillus simplex*, re-examination of the strains previously attributed to *'Bacillus macroides'* and description of *Bacillus muralis* sp. nov. *Int J Syst Evol Microbiol* **55**, 119-131

[286] Heyrman, J., Rodriguez-Diaz, M., Devos, J., Felske, A., Logan, N.A. & De Vos, P. (2005b). *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **55**, 111-117

[287] Heyrman, J. & Swings, J. (2001). 16S rDNA sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia tomb (necropolis of Carmona, Spain). *Syst Appl Microbiol* **46**, 417-422

[288] Heyrman, J., Vanparys, B., Logan, N.A., Balcaen, A., Rodriguez-Diaz, M., Felske, A. & De Vos, P. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. *Int J Syst Evol Microbiol* **54**, 47-57

[289] Heyrman, J., Verbeeren, J., Schumann, P., Swings, J. & De Vos, P. (2005c). Six novel *Arthrobacter* species isolated from deteriorated mural paintings. *Int J Syst Evol Microbiol* **55**, 1457-1464

[290] Hilario, E., Buckley, T.R. & Young, J. (2004). Improved resolution of the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD, carA, recA* and 16S rDNA. *Ant Leeuw* **86**, 51-64

[291] Hildebrand, D.C., Palleroni, N.J., Hendson, M., Toth, J. & Johnson, J.L. (1994). *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers. *Int J Syst Bacteriol* **44**, 410-415

[292] Hof, T. (1935). An investigation of the microorganisms commonly present in salted beans. *Recueil des Travaux Botaniques Néerlandais* **32**, 151-173

[293] Holland, D.F. (1920). Generic index of the commoner forms of bacteria. In 'The families and genera of the bacteria.' Eds. Winslow, C.E.A., Broadhurst, J., Buchanan, R.E., Krumwiede, C. Jr., Rogers, L.A. & Smith, G.H.

[294] Holmes, B., Owen, R.J., Evans, A., Malnick, H. & Willcox, W.R. (1977). Pseudomonas paucimobilis, a new species isolated from human clinical specimens, the hospital environment, and other sources. Int J Syst Bact 27, 133-146

[295] Holt, C. (1992). Structure and stability of bovine casein micelles. *Adv Protein Chem* **43**, 63-151

[296] Horne, D.S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. *Int Dairy J* **8**, 171-177

[297] Hu, X.F., Li, S.X., Wu, J.G., Wang, J.F., Fang, Q.L. & Chen, J.S. (2010). Transfer of *Bacillus mucilaginosus* and *Bacillus edaphicus* to the genus *Paenibacillus* as *Paenibacillus mucilaginosus* comb. nov. and *Paenibacillus edaphicus* comb. nov. *Int J Syst Evol Microbiol* **60**, 8-14

[298] Hu, F.P., Young, J.M. & Triggs, C.M. (1991). Numerical analysis and determinative tests for nonfluorescent plant-pathogenic *Pseudomonas* spp. and genomic analysis and reclassification of species related to *Pseudomonas avenae* Manns 1909. *Int J Syst Bacteriol* **41**, 516-525

[299] Hugh, R. (1981). *Pseudomonas maltophilia* sp. nov., nom. rev. *Int J Syst Bacteriol* **31**, 195

[300] Humm, H.J. (1946). Marine agar-digesting bacteria of the South Atlantic coast. *Bulletin of Duke University Marine Station* **3**, 45-75

[301] Hurley, W.L. (2010). Lactation Biology Website. http://classes.ansci.illinois.edu/ansc438/. Consulted March 2011.

[302] Hwang, C.Y., Zhang, G.I., Kang, S.H., Kim, H.J. & Cho, B.C. (2009). *Pseudomonas pelagia* sp. nov., isolated from a culture of the Antarctic green alga *Pyramimonas gelidicola*. *Int J Syst Evol Microbiol* **59**, 3019-3024

[303] IDF, 1989. Milkfat products and butter. Determination of fat acidity: standard 6B. *International Dairy Federation*, Brussels, Belgium

[304] IDF, 1991. Determination of free fatty acids in milk and milk products. Bulletin N°265. *International Dairy Federation*, Brussels, Belgium

[305] Iizuka, H. & Komagata, K. (1963). New species of *Pseudomonas* belonged to fluorescent group (studies on the microorganisms of cereal grains). *Journal of the Agricultural Chemical Society of Japan* **37**, 137-141

[306] Iizuka, H. & Komagata, K. (1964). Microbiological studies on petroleum and natural gas. Determination of pseudomonads isolated from oil-brines and related materials. J Gen Appl Microbiol 10, 223-231

[307] IKM (2005). Kwaliteitsbewaking in de zuivelketen. Press conference 08-12-2005, by the Integrale Kwaliteitszorg Melk (http://www.ikm.be/news/index_nl.phtml)

[308] Imanaka, H., Kousaka, M., Tamura, G. & Arima, K. (1965). Studies on pyrrolnitrin, a new antibiotic. Taxonomic studies on pyrrolnitrin-producing strain. *J Antibiotics* **18**, 205-206

[309] Ito, H. & Iizuka, H. (1971). Taxonomic studies on radio-resistant *Pseudomonas*. Studies on the microorganisms of cereal grains. *Agr Biol Chem* **35**, 1566-1571

[310] Ivanova, E.P., Alexeeva, Y.A., Zhukova, N.V., Gorshkova, N.M., Buljan, V., Nicolau, D.V., Mikhailov, V.V. & Christen, R. (2004). *Bacillus algicola* sp. nov., a novel filamentous organism isolated from brown alga *Fucus evanescens*. *Syst Appl Microbiol* **27**, 301-307

[311] Ivanova, E.P., Christen, R., Bizet, C., Clermont, D., Motreff, L., Bouchier, C., Zhukova, N.V., Crawford, R.J. & Kiprianova, E.A. (2009). *Pseudomonas brassicacearum* subsp. *neoaurantiaca* subsp. nov., orange-pigmented bacteria isolated from soil and the rhizosphere of agricultural plants. *Int J Syst Evol Microbiol* **59**, 2476-2481

[312] Ivanova, E.P., Gorshkova, N.M., Sawabe, T., Hayashi, K., Kalinovskaya, N.I., Lysenko, A.M., Zhukova, N.V., Nicolau, D.V., Kuznetsova, T.A., Mikhailov, V.V. & Christen, R. (2002). *Pseudomonas extremorientalis* sp. nov., isolated from a drinking water reservoir. *Int J Syst Evol Microbiol* **52**, 2113-2120

[313] Jaeger, K.E. & Eggert, T. (2002). Lipases for biotechnology. *Curr Opinion Microbiol* **13**, 390-397

[314] Janse, J.D., Rossi, P., Angelucci, L., Scortichini, M., Derks, J.H.J., Akkermans, A.D.L. De Vrijer, R. & Psallidas, P.G. (1996). Reclassification of *Pseudomonas syringae* pv. *avellanae* as *Pseudomonas avellanae* (spec. nov.), the bacterium causing canker of hazelnut (*Corylus avellana* L.). *Syst Appl Microbiol* **19**, 589-595

[315] Jayarao, B.M. & Wang, L. (1999). A study on the prevalence of gram-negative bacteria in bulk tank milk. *J Dairy Sci* **82**, 2620-2624

[316] Jendrossek, D. (2001). Transfer of *Pseudomonas lemoignei*, a Gram-negative rod with restricted catabolic capacity, to *Paucimonas* gen. nov. with one species, *Paucimonas lemoignei* comb. nov. *Int J Syst Evol Microbiol* **51**, 905-908

[317] Jensen, R.G. (1995). Handbook of Milk Composition. Academic Press, San Diego, USA

[318] Jeon, C.O., Lim, J.M., Lee, J.M., Xu, L.H., Jiang, C.L. & Kim, C.J. (2005). Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **55**, 1891-1896

[319] Jeyalakshmi, D. & Kanmani, S. (2008). Bioremediation of chromium contaminated soil by *Pseudomonas fluorescens* and indigenous micro-organisms. *J Environ Sci* **50**, 1-6

[320] Johnson, J.C. (1956). Pod twist: A previously unrecorded bacterial disease of French bean (*Phaseolus vulgaris* L.). *Queensland Journal of Agricultural Science* **13**, 127-158

[321] Jones, B.V., Sun, F. & Marchesi, J.R. (2007). Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. *Lett Appl Microbiol* **45**, 418-420

[322] Jordan, E.O. (1890). A report on certain species of bacteria observed in sewage. In '*A Report of the Biological Work of the Lawrence Experiment Station, Including an Account of Methods Employed and Results Obtained in the Microscopical and Bacteriological Investigation of Sewage and Water. Report on Water Supply and Sewerage. The Report of the Massachusetts Board of Health*'. Ed. Sedgewick, W.T.

[323] Jung, M.Y., Kim, J.S. & Chang, Y.H. (2009). *Bacillus acidiproducens* sp. nov., vineyard soil isolates that produce lactic acid. *Int J Syst Evol Microbiol* **59**, 2226-2231

[324] Kadota, H. (1951). Studies on the biochemical activities of marine bacteria. On the agar-decomposing bacteria in the sea. *Memoirs of the College of Science Kyoto University* **59**, 54-67

[325] Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86-98

[326] Kämpfer, P. (2002). Whole-cell fatty acid analysis in the systematic of *Bacillus* and related genera. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0

[327] Kämpfer, P., Rossello-Mora, R., Falsen, E., Busse, H.-J. & Tindall, B.J. (2006). *Cohnella thermotolerans* gen. nov., sp. nov., and classification of '*Paenibacillus hongkongensis*' as *Cohnella hongkongensis* sp. nov. *Int J Syst Microbiol* **56**, 781-786

[328] Kaneda, T. (1977). Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev* **41**, 391-418

[329] Kanso, S., Greene, A.C. & Patel, B.K.C. (2002). *Bacillus subterraneus* sp. nov., an iron- and manganese-reducing bacterium from a deep subsurface Australian thermal aquifer. *Int J Syst Evol Microbiol* **52**, 869-874

[330] Kanzawa, Y., Harada, A., Takeuchi, M., Yokota, A. & Harada, T. (1995). *Bacillus curdlanolyticus* sp. nov. and *Bacillus kobensis* sp. nov., which hydrolyze resistant curdlan. *Int J Syst Bacteriol* **45**, 515-521

[331] Kathariou, S. & Pine, L. (1991). The type strain(s) of *Listeria monocytogenes*: a source of continuing difficulties. *Int J Syst Bact* **41**, 328-330

[332] Kawai, Y. & Yabuuchi, E. (1975). Pseudomonas pertucinogena sp. n., an organism previously misidentified as Bordetella pertussis. Int J Syst Bact 25, 317-323

[333] Kelly, A.L. & Foley, J. (1997). Proteolysis and storage stability of UHT milk as influenced by milk plasmin activity, plasmin-β-lactoglobulin complexation, plasminogen activation and somatic cell count. *Int Dairy J* **7**, 411-420

[334] Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. & Schleifer, K.-H. (1996). Recent changes in the classification of the pseudomonads: an overview. *Syst Appl Microbiol* **19**, 465-477

[335] Kevbrin, V.V., Zengler, K., Lysenko, A.M. & Wiegel, J. (2005). *Anoxybacillus kamchatkensis* sp. nov., a novel thermophilic facultative aerobic bacterium with a broad pH optimum from the Geyser valley, Kamchatka. Extremophiles **9**, 391-398

[336] Kim, K.H., Roh, S.W., Chang, H.W., Nam, Y.D., Yoon, J.H., Jeon, C.O., Oh, H.M. & Bae, J.W. (2009). *Pseudomonas sabulinigri* sp. nov., isolated from black beach sand. *Int J Syst Evol Microbiol* **59**, 38-41

[337] Kitahara, K. & Suzuki, J. (1963). Sporolactobacillus nov. subgen. J Gen Appl Microbiol 9, 59-71

[338] Klijn, N., Herman, L., Langeveld, L., Vaerewijck, M., Waegendorp, A.A., Huemer, I. & Weerkamp, A.H. (1997). Genotypical and phenotypical characterization of *Bacillus* strains, surviving UHT sterilisation. *Int Dairy J* **7**, 421-428

[339] Klijn, N., Nieuwenhof, F.F.J., Hoolwerf, J.D., Van Der Waals, C.B. & Weerkamp, A. (1995). Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Appl Environ Microbiol* **61**, 2919-2924

[340] Kluyver, A.J. (1956). Pseudomonas aureofaciens nov. spec. and its pigments. J Bact 72, 406-411

[341] Kluyver, A.J. & Van Niel, C.B. (1936). Prospects for a natural system of classification of bacteria. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **94**, 369-403

[342] Ko, K.S., Oh, W.S., Lee, M.Y., Lee, J.H., Peck, K.R., Lee, N.Y. & Song, J.H. (2006). *Bacillus infantis* sp. nov. and *Bacillus idriensis* sp. nov., isolated from a patient with neonatal sepsis. *Int J Syst Evol Microbiol* **56**, 2541-2544

[343] Kodama, K., Kimura, N. & Komagata, K. (1985). Two new species of *Pseudomonas*: *P. oryzihabitans* isolated from rice paddy and clinical specimens and *P. luteola* isolated from clinical specimens. *Int J Syst Bacteriol* **35**, 467-474

[344] Kohlmann, K.L., Nielsen, S.S., Steenson, L.R. & Ladisch, M.R. (1991). Production of proteases by psychrotrophic microorganisms. *J Dairy Sci* **74**, 3275-3283

[345] Koka, R.A. & Weimer, B.C. (2001). Influence of growth conditions on heat-stable phospholipase activity in *Pseudomonas*. *J Dairy Res* **68**, 109-116

[346] Konstantinidis, K.T., Ramette, A. & Tiedje, J.M. (2006). The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* **361**, 1929-1940

[347] Konstantinidis, K.T. & Tiedje, J.M. (2004). Genomic insights that advance the species definition for prokaryotes. *PNAS Microbiol* **102**, 2567-2572

[348] Konstantinidis, K.T. & Tiedje, J.M. (2005). Towards a genome-based taxonomy for prokaryotes. *J Bacteriol* **187**, 6258-6264

[349] Konstantinidis, K.T. & Tiedje, J.M. (2007). Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Curr Opinion Microbiol* **10**, 504-509

[350] Krasilnikov, N.A. (1949). Guide to the bacteria and actinomycetes. Moskva-Leningrad, Izdatel'stvo Akademii Nauk SSSR

[351] Krishnamurthi, S., Chakrabarti, T. & Stackebrandt, E. (2009). Re-examination of the taxonomic position of *Bacillus silvestris* Rheims *et al.* 1999 and proposal to transfer it to *Solibacillus* gen. nov. as *Solibacillus silvestris* comb. nov. *Int J Syst Evol Microbiol* **59**, 1054-1058

[352] Krishnamurthi, S., Ruckmani, A., Pukall, R. & Chakrabarti, T. (2010). *Psychrobacillus* gen. nov. and proposal for reclassification of *Bacillus insolitus* Larkin & Stokes, 1967, *B. psychrotolerans* Abd-El Rahman *et al.*, 2002 and *B. psychrodurans* Abd-El Rahman *et al.*, 2002 as *Psychrobacillus insolitus* comb. nov., *Psychrobacillus psychrotolerans* comb. nov. and *Psychrobacillus psychrodurans* comb. nov. *Syst Appl Microbiol* **33**, 367-373

[353] Kuhnert, P. & Korczak, B.M. (2006). Prediction of whole genome DNA-DNA similarity, determination of G + C content and phylogenetic analysis within the family Pasteurellaceae by multilocus sequence analysis (MLSA). *Microbiol* **152**, 2537-2548

[354] Kuhnigk, T., Borst, E.-M., Breunig, A., König, H., Collins, M.D., Hutson, R.H. & Kämpfer, P. (1995). *Bacillus oleronius* sp. nov., a member of hindgut flora of the termite *Reticulitermes santonensis* (Feytand). *Can J Microbiol* **41**, 699-706

[355] Kuisiene, N., Raugalas, J. & Chitavichius, D. (2004). *Geobacillus lituanicus* sp. nov. *Int J Syst Evol Microbiol* **54**, 1991-1995

[356] Kuisiene, N., Raugalas, J. & Chitavichius, D. (2009). Phylogenetic, inter, and intraspecific sequence analysis of *spo0A* gene of the genus *Geobacillus*. *Curr Microbiol* **58**, 547-553

[357] Kuisiene, N., Raugalas, J., Spröer, C., Kroppenstedt, R.M. & Chitavichius, D. (2008). *Bacillus butanolivorans* sp. nov., a species with industrial application for the remediation of n-butanol. *Int J Syst Evol Microbiol* **58**, 505-509

[358] Kumar, S., Kundu, S., Pakshirajan, K. & Venkata Dasu, V. (2008). Cephalosporins determination with a novel microbial biosensor based on permeabilized *Pseudomonas aeruginosa* whole cells. *Appl Biochem Biotech* **151**, 653-664

[359] Kumar, Y., Westram, R., Kipfer, P., Meier, H. & Ludwig, W. (2006). Evaluation of sequence alignments and oligonucleotide probes with respect to three-dimensional structure of ribosomal RNA using ARB software package. *BMC BioInformatics* **7**, 240 (1-11)

[360] Kumeta, H., Hoshino, T., Goda, T., Okayama, T., Shimada, T., Ohgiya, S., Matsuyama, H. & Ishizaki, K. (1999). Identification of a member of the serralysin family isolated from a psychrotrophic bacterium, *Pseudomonas fluorescens* 114. *Biosci Biotech Biochem* **63**, 1165-1170

[361] Kung, V.L., Ozer, E.A. & Hauser, A.R. (2010). The accessory genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol R* **74**, 621-641

[362] Kurita, T. & Tabei, H. (1967). On the pathogenic bacterium of bacterial grain rot of rice. *Annals of the Phytopathological Society* **33**, 111

[363] Kuroshima, K.I., Sakane, T., Takata, R. & Yokota, A. (1996). *Bacillus ehimensis* sp. nov. and *Bacillus chitinolyticus* sp. nov., new chitinolytic members of the genus *Bacillus*. *Int J Syst Bacteriol* **46**, 76-80

[364] Kwon, S.W., Kim, J.S., Park, I.C., Yoon, S.H., Park, D.H., Lim, C.K. & Go, S.J. (2003). *Pseudomonas koreensis* sp. nov., *Pseudomonas umsongensis* sp. nov. and *Pseudomonas jinjuensis* sp. nov., novel species from farm soils in Korea. *Int J Syst Evol Microbiol* **53**, 21-27

[365] Kwon, S.W., Lee, S.Y., Kim, B.Y., Weon, H.Y., Kim, J.B., Go, S.J. & Lee, G.B. (2007). *Bacillus niabensis* sp. nov., isolated from cotton-waste composts for mushroom cultivation. *Int J Syst Evol Microbiol* **57**, 1909-1913

[366] La Duc, M.T., Satomi, M. & Venkateswaran, K. (2004). *Bacillus odysseyi* sp. nov., a round-spore-forming bacillus isolated from the Mars Odyssey spacecraft. *Int J Syst Evol Microbiol* **54**, 195-201

[367] Lafarge, V., Ogier, J.C., Girard, V., Maladen, V., Leveau, J.Y., Gruss, A. & Delacroix-Buchet, A. (2004). Raw cow milk bacterial population shifts attributable to refrigeration. *Appl Environ Microbiol* **70**, 5644-5650

[368] Lai, Q. & Shao, Z. (2008). *Pseudomonas xiamenensis* sp. nov., a denitrifying bacterium isolated from activated sludge. *Int J Syst Evol Microbiol* **58**, 1911-1915

[369] Lalucat, J., Pares, R. & Schlegel, H.G. (1982). *Pseudomonas taeniospiralis* sp. nov., an R-body-containing hydrogen bacterium. *Int J Syst Bacteriol* **32**, 332-338

[370] Lang, E., Griese, B., Spröer, C., Schumann, P., Steffen, M. & Verbarg, S. (2007). Characterization of '*Pseudomonas azelaica*' DSM 9128, leading to emended descriptions of *Pseudomonas citronellolis* Seubert 1960 (Approved Lists 1980) and *Pseudomonas nitroreducens* Iizuka and Komagata 1964 (Approved Lists 1980), including *Pseudomonas multiresinivorans* as its later heterotypic synonym. *Int J Syst Evol Microbiol* **57**, 878-882

[371] Lapidus, A., Goltsman, E., Auger, S., Galleron, N., Segurens, B., Dossat, C., Land, M.L., Broussolle, V., Brillard, J., Guinebretiere, M.H., Sanchis, V., Nguen-The, C., Lereclus, D., Richardson, P., Wincker, P., Weissenbach, J., Ehrlich, S.D. & Sorokin, A. (2008). Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. *Chem-Biol Interact* **171**, 236-249

[372] Larkin, J.M. & Stokes, J.L. (1967). Taxonomy of psychrophilic strains of *Bacillus*. *J Bacteriol* **94**, 889-895

[373] Laubach, C.A. (1916). Studies on aerobic spore-bearing non-pathogenic bacteria. Spore-bearing organisms in water. J Bact 1, 505-512

[374] Law, B.A. (1979). Reviews in the progress of dairy science: enzymes of psychrotrophic bacteria and their effects on milk and milk products. *J Dairy Res* **46**, 573-588

[375] Law, B.A., Andrews, A.T. & Sharpe, M.E. (1977). Gelation of UHT-sterilized milk by proteases from a strain of *Pseudomonas fluorescens*, isolated from raw milk. *J Dairy Res* **44**, 145-148

[376] Lawson, P.A., Deutsch, C.E. & Collins, M.D. (1996). Phylogenetic characterization of a novel salt-tolerant *Bacillus* species: description of *Bacillus dipsosauri* sp. nov. *J Appl Bacteriol* **81**, 109-112

[377] Lechner, S., Mayr, R., Francis, K.P., Prüβ, B.M., Kaplan, T., Wieβner-Gunkel, E., Stewart, G.S.A.B. & Scherer, S. (1998). *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *Int J Syst Bacteriol* **48**, 1373-1382

[378] Lee, M. & Chandler, A.C. (1941). A study of the nature, growth and control of bacteria in cutting compounds. J Bact 41, 373-386

[379] Lee, J.C., Lee, G.S., Park, D.J. & Kim, C.J. (2008). *Bacillus alkalitelluris* sp. nov., an alkaliphilic bacterium isolated from sandy soil. *Int J Syst Evol Microbiol* **58**, 2629-2634

[380] Lee, J.C., Lim, J.M., Park, D.J., Jeon, C.O., Li, W.J. & Kim, C.J. (2006). *Bacillus seohaeanensis* sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall. *Int J Syst Evol Microbiol* **56**, 1893-1898

[381] Lee, D.H., Mokn, S.R., Park, Y.H., Kim, J.H., Kim, H., Parales, R.E. & Kahng, H.Y. (2010). *Pseudomonas taeanensis* sp. nov., isolated from a crude oil-contaminated seashore. *Int J Syst Evol Microbiol* **60**, 2719-2723

[382] Lee, J.S., Pyun, Y.R. & Bae, K.S. (2004). Transfer of *Bacillus ehimensis* and *Bacillus chitinolyticus* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov. *Int J Syst Evol Microbiol* **54**, 929-933

[383] Leifson, E. (1962a). The bacterial flora of distilled and stored water. New species of the genera Corynebacterium, Flavobacterium, Spirillum and Pseudomonas. International Bulletin of Bacteriological Nomenclature and Taxonomy 12, 161-170

[384] Leifson, E. (1962b). Pseudomonas spinosa n. sp. International Bulletin of Bacteriological Nomenclature and Taxonomy 12, 89-92

[385] Leifson, E. & Hugh, R. (1954). A new type of polar monotrichous flagellation. J Gen Microbiol 10, 68-70

[386] Lembke, F. (1995). Highly heat resistant spores in UHT-milk. Bulletin no. 302. International Dairy Federation

[387] Levine, M. & Anderson, D.Q. (1932). Two new species of bacteria causing mustiness in eggs. J Bact 23, 337-347

[388] Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T. & Ezaki, T. (2002). *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. *Int J Syst Evol Microbiol* **52**, 1205-1209

[389] Liao, C.H. & McCallus, D.E. (1998). Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. *Appl Environ Microbiol* **64**, 914-921

[390] Lim, J.M., Jeon, C.O. & Kim, C.J. (2006a). *Bacillus taeanensis* sp. nov., a halophilic Gram-positive bacterium from a solar saltern in Korea. *Int J Syst Evol Microbiol* **56**, 2903-2908

[391] Lim, J.M., Jeon, C.O., Lee, J.C., Ju, Y.J., Park, D.J. & Kim, C.J. (2006b). *Bacillus koreensis* sp. nov., a spore-forming bacterium, isolated from the rhizosphere of willow roots in Korea. *Int J Syst Evol Microbiol* **56**, 59-63

[392] Lim, J.M., Jeon, C.O., Lee, S.M., Lee, J.C., Xu, L.H., Jiang, C.L. & Kim, C.J. (2006c). *Bacillus salarius* sp. nov., a halophilic, spore-forming bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **56**, 373-377

[393] Lim, J.M., Jeon, C.O., Lee, J.R., Park, D.J. & Kim, C.J. (2007). *Bacillus kribbensis* sp. nov., isolated from a soil sample in Jeju, Korea. *Int J Syst Evol Microbiol* **57**, 2912-2916

[394] Lindsay, D., Mosupye, F.M., Brozel, V.S. & Von Holy, A (2000). Cytotoxicity of alkaline-tolerant dairy-associated *Bacillus* spp. *Lett Appl Microbiol* **30**, 364-369

[395] Liu, R., Liu, H., Feng, H., Wang, X., Zhang, C.X., Zhang, K.Y. & Lai, R. (2008). *Pseudomonas duriflava* sp. nov., isolated from a desert soil. *Int J Syst Evol Microbiol* **58**, 1404-1408

[396] Liu, M., Luo, X., Zhang, L., Dai, J., Wang, Y., Tang, Y., Li, J., Sun, T. & Fang, C. (2009a). *Pseudomonas xinjiangensis* sp. nov., a moderately thermotolerant bacterium isolated from desert sand. *Int J Syst Evol Microbiol* **59**, 1286-1289

[397] Liu, H., Zhou, Y., Liu, R., Zhang, K.Y. & Lai, R. (2009b). *Bacillus solisalsi* sp. nov., a halotolerant, alkaliphilic bacterium isolated from soil around a salt lake. *Int J Syst Evol Microbiol* **59**, 1460-1464

[398] Logan, N.A., Berge, O., Bishop, A.H., Busse, H.-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L., Salkinoja-Salkonen, M.S., Seldin, L., Ventosa, A. (2009). Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *Int J Syst Evol Microbiol* **59**, 2114-2121

[399] Logan, N.A. & Berkeley, R.C.W. (1984). Identification of *Bacillus* strains using the API system. *J Gen Microbiol* **130**, 1871-1882

[400] Logan, N.A. & De Vos P. (2009). Genus *Bacillus* Cohn 1872. In '*Bergey's Manual of Systematic Bacteriology*'. 2^{nd} ed. Eds. De Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. & Whitman, W.B. Springer-Verlag, New York.

[401] Logan, N.A. & Halket G. (2011). Developments in the taxonomy of aerobic, endospore-forming bacteria. In '*Endospore-forming soil bacteria*'. Eds. Logan, N.A. & De Vos, P. Soil Biology 27, Springer-Verlag Berlin Heidelberg, ISBN 978-3-642-19576-1

[402] Logan, N.A., Lebbe, L., Hoste, B., Goris, J., Forsyth, G., Heyndrickx, M., Murray, B.L., Syme, N., Wynn-Williams, D.D. & De Vos, P. (2000). Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of *Bacillus fumarioli* sp. nov. *Int J Syst Evol Microbiol* **50**, 1741-1753

[403] Logan, N.A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodriguez-Diaz, M., Heyndrickx, M. & De Vos, P. (2002). *Bacillus luciferensis* sp. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *Int J Syst Evol Microbiol* **52**, 1985-1989

[404] Logan, N.A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodriguez-Diaz, M., Heyndrickx, M. & De Vos, P. (2004). *Bacillus shackletonii* sp. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *Int J Syst Evol Microbiol* **54**, 373-376

[405] Lopez, N.I., Pettinari, M.J., Stackebrandt, E., Tribelli, P.M., Potter, M., Steinbüchel, A. & Mendez, B.S. (2009). *Pseudomonas extremaustralis* sp. nov., a poly(3-hydroxybutyrate) producer isolated from an Antarctic environment. *Curr Microbiol* **59**, 514-519

[406] Lovett, J., Francis, D.W. & Hunt, J.M. (1983). Isolation of *Campylobacter jejuni* from raw milk. *Appl Environ Microbiol* **46**, 459-462

[407] Lucey, J.A. (2002). Formation and Physical Properties of Milk Protein Gels. *J Dairy Sci* **85**, 281-294

[408] Ludwig, W. (2008). Reprint of 'Nucleic acid techniques in bacterial systematics and identification'. *Int J Food Microbiol* **125**, 1-12

[409] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. & Schleifer, K.-H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363-1371

[410] Lukasova J., Vyhnalkova, J. & Pacova, Z. (2001). *Bacillus* species in raw milk and in the farm environment. *Milchwissenschaft* **56**, 609-611

[411] Mabbit, L.A. (1981). The significant metabolic activity of bacteria in raw milk. *Kieler Milchwirtschaftliche Forschungberichte* **33**, 273-280

[412] Macé, E. (1889). Traité Pratique de Bactériologie. 1^{st} ed. J.B. Ballière & Sons, Paris

[413] Madhaiyan, M., Poonguzhali, S., Kwon, S.W. & Sa, T.M. (2010). *Bacillus methylotrophicus* sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *Int J Syst Evol Microbiol* **60**, 2490-2495

[414] Manachini, P.L., Fortina, M.G., Parini, C. & Craveri, R. (1985). *Bacillus thermoruber* sp. nov., nom. rev., a red-pigmented thermophilic bacterium. *Int J Syst Bacteriol* **35**, 493-496

[415] Manachini, P.L., Mora, D., Nicastro, G., Parini, C., Stackebrandt, E., Pukall, R. & Fortina, M.G. (2000). *Bacillus thermodenitrificans* sp. nov., nom. rev. *Int J Syst Evol Microbiol* **50**, 1331-1337

[416] Manaia, C.M. & Moore, E.R.B. (2002). *Pseudomonas thermotolerans* sp. nov., a thermotolerant species of the genus *Pseudomonas sensu stricto*. *Int J Syst Evol Microbiol* **52**, 2203-2209

[417] Manns, T.F. (1909). The blade blight of oats, a bacterial disease. *Bulletin of the Ohio Agriculture Experiment Station* **210**, 91-167

[418] Marchand, S., Coudijzer, K., Heyndrickx, M., Dewettinck, K. & De Block, J. (2008). Selective determination of the heat resistant proteolytic activity of bacterial origin in raw milk. *Int Dairy J* **18**, 514-519

[419] Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Herman, L., De Block, J. & Heyndrickx, M. (2009a). Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. *Environ Microbiol* **11**, 467-482

[420] Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M. & De Block,J. (2009b). Heterogeneity of heat resistant proteases from milk *Pseudomonas* species. *Int J Food Microbiol* **133**, 68-77

[421] Marcus, P.I. & Talalay, P. (1956). Induction and purification of α- and β-hydroxysteroid dehydrogenases. *J Biol Chem* **218**, 661-674

[422] Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109-118

[423] Marschall, B.J. & Ohye, D.F. (1966). Bacillus macquariensis n. sp., a psychrotrophic bacterium from sub-Antarctic soil. J Gen Microbiol 44, 41-46

[424] Matarante, A., Baruzzi, F., Cocconcelli, P.S. & Morea, M. (2004). Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages, *Appl Environ Microbiol* **70**, 5168-5176

[425] Mayr, R., Busse, H.-J., Worliczek, H.L., Ehling-Schulz, M. & Scherer, S. (2006). *Ornithinibacillus* gen. nov., with the species *Ornithinibacillus bavariensis* sp. nov. and *Ornithinibacillus californiensis* sp. nov. *Int J Syst Evol Microbiol* **56**, 1383-1389

[426] MCC Vlaanderen (2010). http://www.mcc-vlaanderen.be/content/. Consulted February 2011

[427] McGuiggan, J.T.M., McCleery, D.R., Hannan, A. & Gilmour, A. (2002). Aerobic spore-forming bacteria in bulk raw milk: factors influencing the number of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. *Int J Dairy Tech* **55**, 100-107

[428] McKellar, R.C. (1982). Factors influencing the production of extracellular proteinase by *Pseudomonas fluorescens*. *J Appl Bact* **53**, 305-316

[429] McKellar, R.C., Froehlich, D.A., Butler, G., Cholette, H. & Campbell, C. (1984). The effect of uncooled storage on proteolysis, bitterness and apparent viscosity in UHT milk. *Can I Food Sc Tech J* **17**, 14-17

[430] McTaggart, L.R., Richardson, S.E., Witkowska, M. & Zhang, S.X. (2010). Phylogeny and identification of *Nocardia* species based on MultiLocus Sequence Analysis. *J Clin Microbiol* **48**, 4525-4533

[431] Meer, R.R., Baker, J., Bodyfelt, F.W. & Griffiths, M.W. (1991). Psychotrophic *Bacillus* spp. in fluid milk products: a review. *J Food Prot* **54**, 969-979

[432] Meier-Stauffer, K., Busse, H.-J., Rainey, F.A., Burghardt, J., Scheberl, A., Hollaus, F., Kuen, B., Makristathis, A., Sleytr, U.B. & Messner, P. (1996). Description of *Bacillus thermoaerophilus* sp. nov., to include sugar beet isolates and *Bacillus brevis* ATCC 12990. *Int J Syst Bacteriol* **46**, 532-541

[433] Meintanis, C., Chalku, K.I., Kormas, K. A., Lymperopoulou, D.S., Katsifas, E.A., Hatzinikolaou, D.G. & Karagouni, A.D. (2008). Application of *rpoB* sequence similarity analysis, REP-PCR and BOX-PCR for the differentiation of species within the genus *Geobacillus*. *Lett Appl Microbiol* **46**, 395-401

[434] Mesbah, M., Premachandran, U. & Whitman, W. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159-167

[435] Metwalli, A.A.M., de Hongh, H.H.J. & van Boekel, M.A.J.S. (1998). Heat inactivation of bovine plasmin. *Int Dairy J* **8**, 47-56

[436] Meyer, J.-M., Geoffroy, V.A., Baida, N., Gardan, L., Izard, D., Lemanceau P., Achouak W. & Palleroni, N.J. (2002). Siderophore typing, a powerful tool for the identification of fluorescent and nonfluorescent *Pseudomonas* species. *Appl Environ Microbiol* **68**, 2745-2753

[437] Meyer, A. & Gottheil, O. (1901). In *'Botanische beschreibung einiger bodenbakterien*. *Zentralblatt für Bakteriologie*, *Parasitenkunde*, *Infektionskrankheiten und Hygiene'* **7**, 680-691

[438] Meyer, O., Lalucat, J. & Schlegel, H.G. (1980). *Pseudomonas carboxydohydrogena* (Sanjieva and Zavarzin) comb. nov., a monotrichous, nonbudding, strictly aerobic, carbon monoxide-utilizing hydrogen bacterium previously assigned to *Seliberia*. *Int J Syst Bacteriol* **30**, 189-195

[439] Meyer, A. & Neide, E. (1904). In: *Botanische beschreibung einiger sporenbildenden bakterien. Zentralblatt für Bakteriologie*, *Parasitenkunde*, *Infektionskrankheiten und Hygiene* **12**, 337-352

[440] Migula, W. (1900). System der Bakterien, Vol. 2, Gustav Fischer, Jena

[441] Migula, W. (1895). *Bacteriaceae* (Stabchenbacterien). In *'Die Naturlichen Pfanzenfamilien'*. Eds. Engler, A., Prantl, K. & Engelmann, W. Leipzig, Teil

[442] Mikkola, R., Kolari, M., Andersson, M.A., Helin, J. & Salkinoja-Salonen, M.S. (2000). Toxic lactonic lipopeptide from food poisoning isolates of *Bacillus licheniformis*. *Eur J Biochem* **267**, 4068-4074

[443] Minana-Galbis, D., Pinzon, D.L., Loren, J.G., Manresa, A. & Oliart-Ros, R.M. (2010). Reclassification of *Geobacillus pallidus* (Scholz *et al*. 1988) Banat *et al*. 2004 as *Aeribacillus pallidus* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **60**, 1600-1604

[444] Mishustin, E.N. (1950). Termofilnie mikroorganiszmi w prirode I praktike. Akademi Nauk SSSR, Moskwa

[445] Miyajima, K., Tani, A. & Akita, T. (1983). *Pseudomonas fuscovaginae* sp. nov., nom. rev. *Int J Syst Bacteriol* **33**, 656-657

[446] Mohn, W.W., Wilson, A.E., Bicho, P. & Moore, E.R.B. (1999). Physiological and phylogenetic diversity of bacteria growing on resin acids. *Syst Appl Microbiol* **22**, 68-78

[447] Molin, G., Ternström, A. & Ursing, J. (1986). *Pseudomonas lundensis*, a new bacterial species isolated from meat. *Int J Syst Bacteriol* **36**, 339-342

[448] Molin, G. & Ternström, A. (1986). Phenotypically based taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water, and soil. *Int J Syst Bact* **36**, 257-274

[449] Monias, B.L. (1928). Classification of *Bacterium alcaligenes pyocyaneum* and *fluorescens*. *J Infect Dis* **43**, 330-334

[450] Moniz, L. (1963). Leaf spot of apple blossom. Curr Sci 32, 177

[451] Montefusco, A., Nakamura, L.K. & Labeda, D.P. (1993). *Bacillus peoriae* sp. nov. *Int J Syst Bacteriol* **43**, 388-390

[452] Moore, E.R.B., Mau, M., Arnscheidt, A., Böttger, E.C., Hutson, R.A., Collins, M.D., Van De Peer, Y., De Wachter, R. & Timmis, K.N. (1996). The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intrageneric relationships. *Syst Appl Microbiol* **19**, 478-492

[453] Moore, E.R.B., Mihaylova, S.A., Vandamme, P., Krichevsky, M.I. & Dijkshoorn, L. (2010). Microbial systematics and taxonomy: relevance for microbial commons. *Res Microbiol* **161**, 430-438

[454] Morse, P.M., Jackson, H., McNaughton, C.H., Leggat, A.G., Landerkin, G.B. & Johns, C.K. (1968). Investigation of factors contributing to the bacteria count of bulk tank milk, bacteria in milk from individual cows. *J Dairy Sci* **51**, 1188-1191

[455] Mottar, J. (1989). Effect on the quality of dairy products. Ed. McKellar, R.C. Boca Raton, Florida: CRC Press Inc

[456] Mottar, J., Waes, G., Moermans, R. & Naudts, M. (1979). Sensoric changes in UHT milk during uncooled storage. *Milchwissenschaft* **34**, 257-262

[457] Mulet, M., Lalucat, J. & Garcia-Valdes, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* **12**, 1513-1530

[458] Munsch-Alatossova, P. & Alatossova, T. (2006). Phenotypic characterization of raw milk associated psychrotrophic bacteria. *Microbiol Res* **161**, 334-346

[459] Munsch, P., Alatossava, T., Marttinen, N., Meyer, J.M., Christen, R. & Gardan, L. (2002). *Pseudomonas costantinii* sp. nov., another causal agent of brown blotch disease, isolated from cultivated mushroom sporophores in Finland. *Int J Syst Evol Microbiol* **52**, 1973-1983

[460] Nagel, M. & Andreesen, J.R. (1991). *Bacillus niacini* sp. nov., a nicotinate-metabolizing mesophile isolated from soil. *Int J Syst Bacteriol* **41**, 134-139

[461] Nakamura, L.K. (1984a). *Bacillus psychrophilus* sp. nov., nom. rev. *Int J Syst Bacteriol* **34**, 121-123

[462] Nakamura, L.K. (1993). DNA relatedness of *Bacillus brevis* Migula 1900 strains and proposal of *Bacillus agri* sp. nov., nom. rev., and *Bacillus centrosporus* sp. nov., nom. rev. *Int J Syst Bacteriol* **43**, 20-25

[463] Nakamura, L.K. (1984c). *Bacillus amylolyticus* sp. nov., nom. rev., *Bacillus lautus* sp. nov., nom. rev., *Bacillus pabuli* sp. nov., nom. rev., and *Bacillus validus* sp. nov., nom. rev. *Int J Syst Bacteriol* **34**, 224-226

[464] Nakamura, L.K. (1987). *Bacillus alginolyticus* sp. nov. and *Bacillus chondroitinus* sp. nov., two alginate-degrading species. *Int J Syst Bacteriol* **37**, 284-286

[465] Nakamura, L.K. (1989). Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. *Int J Syst Bacteriol* **39**, 295-300

[466] Nakamura, L.K. (1998). *Bacillus pseudomycoides* sp. nov. *Int J Syst Bacteriol* **48**, 1031-1035

[467] Nakamura, L.K. (1984b). *Bacillus pulvifaciens* sp. nov., nom. rev. *Int J Syst Bacteriol* **34**, 410-413

[468] Nakamura, L.K. (1990). *Bacillus thiaminolyticus* sp. nov., nom. rev. *Int J Syst Bacteriol* **40**, 242-246

[469] Nakamura, L.K., Blumenstock, I. & Claus, D. (1988). Taxonomic study of *Bacillus coagulans* Hammer 1915 with a proposal for *Bacillus smithii* sp. nov. *Int J Syst Bacteriol* **38**, 63-73

[470] Nakamura, L.K., Roberts, M.S. & Cohan, F.M. (1999). Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. *Int J Syst Bacteriol* **49**, 1211-1215

[471] Nakamura, L.K., Shida, O., Takagi, H. & Komagata, K. (2002). *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., round-spored bacteria from soil. *Int J Syst Evol Microbiol* **52**, 501-505

[472] Nakamura, M., Schulz, J.A. & Cross, W.R. (1969). Factors affecting lecithinase activity and production in *Clostridium welchii*. *J Hyg* **67**, 153-162

[473] Nakhimovskaya, M.I. (1948). *Pseudomonas aurantiaca* n. sp. *Mikrobiologiya* **17**, 58-65

[474] Nazina, T.N., Lebedeva, E.V., Poltaraus, A.B., Tourova, T.P., Grigoryan, A.A., Sokolova, D.S.H., Lysenko, A.M. & Osipov, G.A. (2004). *Geobacillus gargensis* sp. nov., a novel thermophile from a hot spring, and the reclassification of *Bacillus vulcani* as *Geobacillus vulcani* comb. nov. *Int J Syst Evol Microbiol* **54**, 2019-2024

[475] Nazina, T.N., Sokolova, D.S., Grigoryan, A.A., Shestakova, N.M., Mikhailova, E.M., Poltaraus, A.B., Tourova, T.P., Lysenko, A. M., Osipov, G.A. & Belyaev, S.S. (2005). *Geobacillus jurassicus* sp. nov., a new thermophilic bacterium isolated from a high-temperature petroleum reservoir, and the validation of the *Geobacillus* species. *Syst Appl Microbiol* **28**, 43-53

[476] Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, G.A., Belyaev, S.S. & Ivanov, M.V. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of Bacillus stearothermophilus, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, Bacillus kaustophilus, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433-446

[477] Newcombe, D., Dekas, A., Mayilraj, S. & Venkateswaran, K. (2009). *Bacillus canaveralius* sp. nov., an alkali-tolerant bacterium isolated from a spacecraft assembly facility. *Int J Syst Evol Microbiol* **59**, 2015-2019

[478] Ney, K.H. (1979). Bitterness of peptides: amino acid composition and chain length. In '*Food Taste Chemistry*'. Ed. Boudreau, J.C. Washington: American Chemical Society

[479] Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J. & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* **64**, 548-572

[480] Nicolaus, B., Lama, L., Esposito, E., Manca, M.C., Di Prisco, G. & Gambacorta, A. (1996). "*Bacillus thermoantarcticus*" sp. nov., from Mount Melbourne, Antarctica: a novel thermophilic species. *Polar Biol* **16**, 101-104

[481] Nielsen, P., Fritze, D. & Priest, F.G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745-1761

[482] Nielsen, S.S. (2002). Plasmin system and microbial proteases in milk: characteristics, roles, and relationship. *J Agr Food Chem* **50**, 6628-6634

[483] Nishimori, E., Kita-Tsukamoto, K. & Wakabayashi, H. (2000). *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. *Int J Syst Evol Microbiol* **50**, 83-89

[484] Nogi, Y., Takami; H. & Horikoshi, K. (2005). Characterization of alkaliphilic *Bacillus* strains used in industry: proposal of five novel species. *Int J Syst Evol Microbiol* **55**, 2309-2315

[485] Noguchi, H., Uchino, M., Shida, O., Takano, K., Nakamura, L.K. & Komagata, K. (2004). *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. *Int J Syst Evol Microbiol* **54**, 2117-2120

[486] Nowlan, B., Dodia, M.S., Singh, S.P. & Patel, B.K.C. (2006). *Bacillus okhensis* sp. nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan. *Int J Syst Evol Microbiol* **56**, 1073-1077

[487] Ochman, H., Lawrence, J.G. & Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299-305

[488] O'Donnovan, K.O. (1959). The occurrence of *Bacillus cereus* in milk and on dairy equipment. *J Appl Bact* **22**, 131-137

[489] Ogimi, C. (1977). Studies on bacterial gall of chinaberry (*Melia Azedarach* Lin.), caused by *Pseudomonas meliae* n. sp. *Bull Coll. Agr., Univ of the Ryukyus* **24**, 497-556

[490] Olivecrona, T., Vilaro, S. & Olivecrona, G. (2003). Lipases in milk. In '*Advanced dairy chemistry*'. Eds. Fox, P.F. & McSweeney, P.L.H. New York, USA, Kluwer Academic Publishers/Plenum Press

[491] Olivera, N., Sineriz, F. & Breccia, J.D. (2005). *Bacillus patagoniensis* sp. nov., a novel alkalitolerant bacterium from the rhizosphere of *Atriplex lampa* in Patagonia, Argentina. *Int J Syst Evol Microbiol* **55**, 443-447

[492] Olsen, S.J., Ying, M., Davis, M.F., Deasy, M., Holland, B., Iampietro, L., Baysinger, C.M., Sassano, F., Polk, L.D., Gormley, B., Hung, M.J., Pilot, K., Orsini, M., Van Duyne, S., Rankin, S., Genese, C., Bresnitz, E.A., Smucker, J., Moll, M. & Sobel, J. (2004). Multidrug-resistant *Salmonella typhimurium* infection from milk contaminated after pasteurization. *Emerg Infect Dis* **10**, 932-935

[493] Outtrup, H. & Jørgensen, S.T. (2002). The importance of *Bacillus* species in the production of industrial enzymes. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0

[494] Pacova, Z., Svec, P., Stenfors, L.P., Vyletelova, M. & Sedlacek, I. (2003). Isolation of the psychrotolerant species *Bacillus weihenstephanensis* from raw cow's milk. *Czech J Anim Sci* **48**, 93-96

[495] Paine, S.G. (1919). Studies in bacteriosis. A brown blotch disease of cultivated mushrooms. *Ann Appl Biolog* **5**, 206-219

[496] Palleroni, N.J. (1975). General properties and taxonomy of the genus *Pseudomonas*. In '*Genetics and Biochemistry of Pseudomonas*'. Eds. Clarke, P.H. & Richmond, M.H. Wiley-Interscience, ISBN 0 471 15896 8, 1975, Great Britain

[497] Palleroni, N.J. (2003). Prokaryote taxonomy of the 20th century and the impact of studies on the genus *Pseudomonas*: a personal view. *Microbiol* **149**, 1-7

[498] Palleroni, N.J. (2005). Genus I. *Pseudomonas*. In '*Bergey's Manual of Systematic Bacteriology*'. Eds. Brenner, D.J., Krieg, N.R. & Staley, J.T. Springer, New York

[499] Palleroni, N.J. (2010). The *Pseudomonas* story. *Environ Microbiol* **12**, 1377-1383

[500] Palleroni, N.J., Ballard, R.W., Ralston, E. & Doudoroff, M. (1972). Deoxyribonucleic acid homologies among some *Pseudomonas* species. *J Bact* **110**, 1-11

[501] Palleroni, N.J. & Bradbury, J.F. (1993). *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (*Hugh* 1980) Swings *et al.*, 1983. *Int J Syst Bacteriol* **43**, 606-609

[502] Palleroni, N.J. & Holmes, B. (1981). *Pseudomonas cepacia* sp. nov., nom. rev. *Int J Syst Bacteriol* **31**, 479-481

[503] Palleroni, N.J., Doudoroff, M., Stanier, R.Y., Solanes, R.E. & Mandel, M. (1970). Taxonomy of the aerobic pseudomonads, the properties of the *Pseudomonas stutzeri* group. J Gen Microbiol 60, 215-231

[504] Palleroni, N.J., Kunisawa, R., Contopoulou, R. & Doudoroff, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *Int J Syst Bacteriol* **23**, 333-339

[505] Palmisano, M.M., Nakamura, L.K., Duncan, K.E., Istock, C.A. & Cohan, F.M. (2001). *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. *Int J Syst Evol Microbiol* **51**, 1671-1679

[506] Pappa, A., Sanchez-Porro, C., Lazoura, P., Kallimanis, A., Perisynakis, A., Ventosa, A., Drainas, C. & Koukkou, A.I. (2010). *Bacillus halochares* sp. nov., a halophilic bacterium isolated from a solar saltern. *Int J Syst Evol Microbiol* **60**, 1432-1436

[507] Park, Y.D., Lee, H.B., Yi, H., Kim, Y., Bae, K.S., Choi, J.E., Jung, H.S. & Chun, J. (2005). *Pseudomonas panacis* sp. nov., isolated from the surface of rusty roots of Korean ginseng. *Int J Syst Evol Microbiol* **55**, 1721-1724

[508] Park, Y.D., Yi, H., Baik, K.S., Seong, C.N., Bae, K.S., Moon, E.Y. & Chun, J. (2006). *Pseudomonas segetis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **56**, 2593-2595

[509] Pandey, K.K., Mayilraj, S. & Chakrabarti, T. (2002). *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int J Syst Evol Microbiol* **52**, 1559-1567

[510] Peak, K.K., Duncan, K.E., Veguilla, W., Luna, V.A., King, D.S., Heller, L., Heberlein-Larson, L., Reeves, F., Cannons, A.C., Amuso, P. & Cattani, J. (2007). *Bacillus acidiceler* sp. nov., isolated from a forensic specimen, containing *Bacillus anthracis* pX02 genes. *Int J Syst Evol Microbiol* **57**, 2031-2036

[511] Peconek, J.J., Gruber, C., Gallego, V., Ventosa, A., Busse, H.-J., Kämpfer, P., Radax, C. & Stan-Lotter, H. (2006). Reclassification of *Pseudomonas beijerinckii* Hof 1935 as *Chromohalobacter beijerinckii* comb. nov., and emended description of the species. *Int J Syst Evol Microbiol* **56**, 1953-1957

[512] Peix, A., Berge, O., Rivas, R., Abril, A. & Velazquez, E. (2005). *Pseudomonas argentinensis* sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Córdoba, Argentina. *Int J Syst Evol Microbiol* **55**, 1107-1112

[513] Peix, A., Ramirez-Bahena, M.-H. & Velazquez, E. (2009). Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infect Genet Evol* **9**, 1132-1147

[514] Peix, A., Rivas, R., Mateos, P.F., Martinez-Molina, E., Rodriguez-Barrueco, C. & Velazquez, E. (2003). *Pseudomonas rhizosphaerae* sp. nov., a novel species that actively solubilizes phosphate *in vitro*. *Int J Syst Evol Microbiol* **53**, 2067-2072

[515] Peix, A., Rivas, R., Santa-Regina, I., Mateos, P.F., Martinez-Molina, E., Rodriguez-Barrueco, C. & Velazquez, E. (2004). *Pseudomonas lutea* sp. nov., a novel phosphate-solubilizing bacterium isolated from the rhizosphere of grasses. *Int J Syst Evol Microbiol* **54**, 847-850

[516] Peix A., Valverde, A., Rivas, R., Igual, J.M., Ramírez-Bahena, M.H., Mateos, P.F., Santa-Regina, I., Rodríguez-Barrueco, C., Martínez-Molina, E. & Velázquez, E. (2007).Reclassification of Pseudomonas aurantiaca as a synonym of Pseudomonas chlororaphis and proposal of three subspecies, P. chlororaphis subsp. chlororaphis subsp. nov., P. chlororaphis subsp. aureofaciens subsp. nov., comb. nov. and P. chlororaphis subsp. aurantiaca subsp. nov., comb. nov. Int J Syst Evol Microbiol 57, 1286-1290

[517] Perez-Ibarra, B.M., Flores, M.E. & Garcia-Varela, M. (2007). Isolation and characterization of Bacillus thioparus sp. nov., chemolithoautotrophic, thiosulfate-oxidizing bacterium. FEMS Microbiol Lett 271, 289-296

[518] Peterson, M.C. (2003). Campylobacter jejuni enteritis associated with consumption of raw milk. J Environ Health 65, 20-21

[519] Pettersson, B., de Silva, S.K., Uhlen, M. & Priest, F.G. (2000). Bacillus siralis sp. nov., a novel species from silage with a higher order structural attribute in the 16S rRNA genes. Int J Syst Evol Microbiol 50, 2181-2187

[520] Pettersson, B., Lembke, F., Hammer, P., Stackebrandt, E. & Priest, F.G. (1996). Bacillus sporothermodurans, a new species producing highly heat-resistant endospores. Int J Syst Bacteriol 46, 759-764

[521] Pettersson, B., Ripperke, K.E., Yousten, A.A. & Priest, F.G. (1999). Transfer of Bacillus lentimorbus and Bacillus popilliae to the genus Paenibacillus with emended descriptions of Paenibacillus lentimorbus comb. nov. and Paenibacillus popilliae comb. nov. Int J Syst Bacteriol 49, 531-540

[522] Phelps, R.J. & McKillip, J.L. (2002). Enterotoxin production in natural isolates of Bacillaceae outside the Bacillus cereus group. Appl Environ Microbiol 68, 3147-3151

[523] Philips, J.D. & Griffiths, M.W. (1986). Factors contributing to the seasonal variation of Bacillus spp. in pasteurized dairy products. J Appl Bacteriol 61, 275-285

[524] Pichinoty, F., Asselineau, J. & Mandel, M. (1984). Charactérisation biochimique de *Bacillus benzoevorans* sp. nov., une nouvelle espèce filamenteuse, engainée et mésophile, dégradant divers acides aromatiques et phénols. *Ann Microbiol* **135**, 209-217

[525] Pichinoty, F., de Barjac, H., Mandel, M. & Asselineau, J. (1983). Description of *Bacillus azotoformans* sp. nov. *Int J Syst Bacteriol* **33**, 660-662

[526] Pichinoty, F., Waterbury, J.B., Mandel, M. & Asselineau, J. (1986). *Bacillus gordonae* sp. nov., une nouvelle espèce appartenant au second groupe morphologique, dégradant divers composés aromatiques. *Ann Inst Pasteur* **137**, 65-78

[527] Pikuta, E.V. (2009). Genus *Anoxybacillus* Pikuta *et al.*, 2000. In *'Bergey's Manual of Systematic Bacteriology'*. 2nd ed. Eds. De Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. & Whitman, W.B. Springer-Verlag, New York

[528] Pitcher, D.G., Saunders, N.A. & Owen, R.J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Bact* **8**, 151-156

[529] Poli, A., Esposito, E., Lama, L., Orlando, P., Nicolaus, G., de Appolonia, F., Gambacorta, A. & Nicolaus, B. (2006a). *Anoxybacillus amylolyticus* sp. nov., a thermophilic amylase producing bacterium isolated from Mount Rittman (Australia). *Syst Appl Microbiol* **29**, 300-307

[530] Poli, A., Romano, I., Caliendo, G., Nicolaus, G., Orlando, P., Falco, A., Lama, L., Gambacorta, A. & Nicolaus, B. (2006b). *Geobacillus toebii* subsp. *decanicus* subsp. nov., a hydrocarbon-degrading, heavy metal resistant bacterium from hot compost. J Gen Appl Microbiol **52**, 223-34

[531] Poli, A., Romano. I., Cordella, P., Orlando, P., Nicolaus, B. & Ceschi Berrini, C. (2009). *Anoxybacillus thermarum* sp. nov., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs, Abano Terme, Italy. Extremophiles **13**, 867-874

[532] Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Mol Biol Evol* **25**, 1253-1256

[533] Postgate, J. (1995). Breathless niches. *Nature* **377**, 26

[534] Prado, B.M., Sombers, S.E., Ismail, B. & Hayes,K.D. (2006). Effect of heat treatment on the activity of inhibitors of plasmin and plasminogen activators in milk. *Int Dairy J* **16**, 593-599

[535] Prakash, O., Kumari, K. & Lal, R. (2007). *Pseudomonas delhiensis* sp. nov., from a fly ash dumping site of a thermal power plant. *Int J Syst Evol Microbiol* **57**, 527-531

[536] Priest, F.G., Barker, M., Baillie, L.W.J., Holmes, E.C. & Maiden, M.C.J. (2004). Population structure and evolution of the *Bacillus cereus* group. *J Bacteriol* **186**, 7959-7970

[537] Priest, F.G., Goodfellow, M., Shute, L.A. & Berkeley, R.C.W. (1987). *Bacillus amyloliquefaciens* sp. nov., nom. rev. *Int J Syst Bacteriol* **37**, 69-71

[538] Priest, F.G., Goodfellow, M. & Todd, C. (1989). A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847-1882

[539] Proom, H. & Knight, B.C.J.G. (1950). Bacillus pantothenticus (n. sp.). J Gen Microbiol 4, 539-541

[540] Psallidas, P.G. & Panagopoulos, C.G. (1975). A new bacteriosis of almond caused by *Pseudomonas amygdali* sp. nov. *Annales de l'Institut Phytopathologique Benaki N.S*. **11**, 94-108

[541] Pungrasmi, W., Lee, H.S., Yokota, A. & Ohta, A. (2008). *Pseudomonas japonica* sp. nov., a novel species that assimilates straight chain alkylphenols. *J Gen Appl Microbiol* **54**, 61-69

[542] Qiu, F., Zhang, X., Liu, L., Sun, L., Schumann, P. & Song, W. (2009). *Bacillus beijingensis* sp. nov. and *Bacillus ginsengi* sp. nov., isolated from ginseng root. *Int J Syst Evol Microbiol* **59**, 729-734

[543] Quiberoni, A., Guglielmotti, D. & Reinheimer, J. (2008). New and classical spoilage bacteria causing widespread blowing in Argentinean soft and semihard cheeses. *Int J Dairy Tech* **61,** 358-363

[544] Quizlet (2011). Independant study site in the US. http://quizlet.com/ Consulted June 2011

[545] Raats, D., Offek, M., Minz, D. & Halpern, M. (2011). Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. *Food Microbiol* **28**, 465-471

[546] Rahman, R.N.Z.R.A., Leow, T.C., Salleh, A.B. & Basri, M. (2007). *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. *BMC Microbiol* **7**, 77

[547] Rainey, F.A., Fritze, D. & Stackebrandt, E. (1994). The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. *FEMS Microbiol Lett* **115**, 205-212

[548] Ralston, E., Palleroni, N.J. & Doudoroff, M. (1973). Pseudomonas pickettii, a new species of clinical origin related to Pseudomonas solanacearum. Int J Syst Bact 23, 15-19

[549] Rasko, D.A., Altherr, M.R., Han, C.S. & Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms, *FEMS Microbiol Rev* **29**, 303-329

[550] Reddy, G.S.N., Matsumoto, G.I., Schumann, P., Stackebrandt, E. & Shivaji, S. (2004). Psychrophilic pseudomonads from Antarctica: *Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov. and *Pseudomonas proteolytica* sp. nov. *Int J Syst Evol Microbiol* **54**, 713-719

[551] Reddy, G.S.N., Uttam, A. & Shivaji, S. (2008). *Bacillus cecembensis* sp. nov., isolated from the Pindari glacier of the Indian Himalayas. *Int J Syst Evol Microbiol* **58**, 2330-2335

[552] Redfearn, M.S., Palleroni, N.J. & Stanier, R.Y. (1966). A comparative study of *Pseudomonas pseudomallei* and *Bacillus mallei*. *J Gen Microbiol* **43**, 293

[553] Reva, O.N., Smirnov, V.V., Pettersson, B. & Priest, F.G. (2002). *Bacillus endophyticus* sp. nov., isolated from the inner tissues of cotton plants (*Gossypium* sp.). *Int J Syst Evol Microbiol* **52**, 101-107

[554] Rheims, H., Frühling, A., Schumann, P., Rohde, M. & Stackebrandt, E. (1999). *Bacillus silvestris* sp. nov., a new member of the genus *Bacillus* that contains lysine in its cell wall. *Int J Syst Bacteriol* **49**, 795-802

[555] Rhuland, L.E., Work, E., Denman, R.F. & Hoare, D.S. (1955). The behavior of the isomers of α, ε diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844-4846

[556] Richardson, B.C. & Te Whaiti, I.E. (1978). Partial characterization of heat-stable extracellular proteases of some psychrotrophic bacteria of dairy origin. *New Zeal J Dairy Sci* **13**, 172-176

[557] RIVM (2011). RijksInstituut voor Volksgezondheid en Milieu. http://www.rivm.nl./ Consulted August 2011

[558] Rilfors, L., Wieslander, A. & Stahl, S. (1978). Lipid and protein composition of membranes of *Bacillus megaterium* variants in the temperature range 5 to 70°C. *J Bacteriol* **135**, 1043-1052

[559] Robbs, C.F. (1956). Uma nova doenca bacteriana do mamoeiro. *Revista da Sociedade Brasileira de Agronomia* **12**, 73-76

[560] Roberts, S.J., Eden-Green, S.J., Jones, P. & Ambler, D.J. (1990). *Pseudomonas syzygii* sp. nov., the cause of Sumatra disease of cloves. *Syst Appl Microbiol* **13**, 34-43

[561] Roberts, M.S., Nakamura, L.K. & Cohan, F.M. (1994). *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and difference in fatty acid composition. *Int J Syst Bacteriol* **44**, 256-264

[562] Roberts, M.S., Nakamura, L.K. & Cohan, F.M. (1996). *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. *Int J Syst Bacteriol* **46**, 470-475

[563] Rohner-Thielen, E. (2008). Agriculture and Fisheries: From grass to glass; a look at the dairy chain. Eurostat, statistics in focus 76/2008

[564] Romano, I., Lama, L., Nicolaus, B., Gambacorta, A. & Giordano, A. (2005a). *Bacillus saliphilus* sp. nov., isolated from a mineral pool in Campania, Italy. *Int J Syst Evol Microbiol* **55**, 159-163

[565] Romano, I., Poli, A., Lama, L., Gambacorta A. & Nicolaus, B. (2005b). *Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov., isolated from the geothermal volcanic environment. *J Gen Appl Microbiol* **51**, 183-189

[566] Romanenko, L.A., Uchino, M., Falsen, E., Frolova, G.M., Zhukova, N.V. & Mikhailov, V.V. (2005a). *Pseudomonas pachastrellae* sp. nov., isolated from a marine sponge. *Int J Syst Evol Microbiol* **55**, 919-924

[567] Romanenko, L.A., Uchino, M., Falsen, E., Lysenko, A.M., Zhukova, N.V. & Mikhailov, V.V. (2005b). *Pseudomonas xanthomarina* sp. nov., a novel bacterium isolated from marine ascidian. *J Gen Appl Microbiol* **51**, 65-71

[568] Romanenko, L.A., Uchino, M., Tebo, B.M., Tanaka, N., Frolova, G.M. & Mikhailov, V.V. (2008). *Pseudomonas marincola* sp. nov., isolated from marine environments. *Int J Syst Evol Microbiol* **58**, 706-710

[569] Rooney, A.P., Price, N.P.J., Ehrhardt, C., Swezey, J.L. & Bannan, J.D. (2009). Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. *Int J Syst Evol Microbiol* **59**, 2429-2436

[570] Rossello-Mora, R. (2005). Updating prokaryotic taxonomy. *J Bacteriol* **187**, 6255-6257

[571] Rosello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39-67

[572] Rowan, N.J. & Anderson, J.G. (1998). Diarrhoeal enterotoxin production by psychrotrophic *Bacillus cereus* present in reconstituted milk-based infant formulae (MIF). *Lett Appl Microbiol* **26**, 161-165

[573] Rueckert, A., Ronimus, R.S. & Morgan, H.W. (2004). A RAPD-based survey of thermophilic bacilli from milk powders. *Int J Food Microbiol* **96**, 263-272

[574] Rueckert, A., Ronimus, R.S. & Morgan, H.W. (2005). Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. *J Microbiol Meth* **60**, 155-167

[575] Rueckert, A., Ronimus, R.S. & Morgan, H.W. (2006). Development of a real-time PCR assay targeting the sporulation gene, *spo0A*, for the enumeration of thermophilic bacilli in milk powder. *Food Microbiol* **23**, 220-230

[576] Rüger, H.J. (1983). Differentiation of *Bacillus globisporus*, *Bacillus marinus* comb. nov., *Bacillus aminovorans*, and *Bacillus insolitus*. *Int J Syst Bacteriol* **33**, 157-161

[577] Rüger, H.J. & Richter, G. (1979). Bacillus globisporus subsp. marinus subsp. nov. Int J Syst Bact 29, 196-203

[578] Ruiz-Garcia, C., Béjar, V., Martinez-Checa, F., Llamas, I. & Quesada, E. (2005a). *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain. *Int J Syst Evol Microbiol* **55**, 191-195

[579] Ruiz-Garcia, C., Quesada, E., Martinez-Checa, F., Llamas, I, Urdaci, M.C. & Bejar, V. (2005b). *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain. *Int J Syst Evol Microbiol* **55**, 1279-1285

[580] Saha, R., Spröer, C., Beck, B. & Bagley, S. (2010). *Pseudomonas oleovorans* subsp. *lubricantis* subsp. nov., and reclassification of *Pseudomonas pseudoalcaligenes* ATCC 17440^T as later synonym of *Pseudomonas oleovorans* ATCC 8062^T. *Curr Microbiol* **60**, 294-300

[581] Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C., Honkanen-Buzalski, T. & Scoging, A.C. (1999). Toxigenic strains of *Bacillus licheniformis* related to food poisoning. *Appl Environ Microbiol* **65**, 4637-4645

[582] Santini, J.M., Streimann, I.C.A. & Vanden Hoven, R.N. (2004). *Bacillus macyae* sp. nov., an arsenate-respiring bacterium isolated from an Australian gold mine. *Int J Syst Evol Microbiol* **54**, 2241-2244

[583] Sarkar, S.F. & Guttman, D.S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl Environ Microbiol* **70**, 1999-2012

[584] Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. MIDI technical note 101

[585] Satomi, M., La Duc, M.T. & Venkateswaran, K. (2006). *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *Int J Syst Evol Microbiol* **56**, 1735-1740

[586] Satomi, M., Kimura, B., Hamada, T., Harayama, S. & Fujii, T. (2002). Phylogenetic study of the genus *Oceanospirillum* based on 16S rRNA and *gyrB* genes: emended description of the genus *Oceanospirillum*, description of *Pseudospirillum* gen. nov., *Oceanobacter* gen. nov. and *Terasakiella* gen. nov. and transfer of *Oceanospirillum jannaschii* and *Pseudomonas stanieri* to *Marinobacterium* as *Marinobacterium jannaschii* comb. nov. and *Marinobacterium stanieri* comb. nov. *Int J Syst Evol Microbiol* **52**, 739-747

[587] Savulescu, T. (1947). Contribution à la classification des bacteriacées phytopathogènes. *Analele Academiei Romane* **4**, 1-26

[588] Scanlan, R.A., Sather, L.A. & Day, E.A. (1965). Contribution of free fatty acids to the flavor of rancid milk. *J Dairy Sci* **48**, 1582-1584

[589] Scarlett, C.M., Fletcher, J.T., Roberts, P. & Lelliott, R.A. (1978). Tomato pith necrosis caused by *Pseudomonas corrugata* n. sp. *Ann Appl Biol* **88**, 105-114

[590] Schaad, N.W., Postnikova, E., Sechler, A., Claflin, L.E., Vidaver, A.K., Jones, J.B., Agarkova, I., Ignatov, A., Dickstein, E. & Ramundo, B.A. (2008). Reclassification of subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad *et al*. 2009*,* 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Syst Appl Microbiol* **31**, 434-446

[591] Schleifer, K.H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bact Rev* **36**, 407-477

[592] Schäffer, C., Franck, W.L., Scheberl, A., Kosma, P., McDermott, T.R. & Messner, P. (2004). Classification of isolates from locations in Austria and Yellowstone National Park as *Geobacillus tepidamans* sp. nov. *Int J Syst Evol Microbiol* **54**, 2361-2368

[593] Schardinger, F. (1905). *Bacillus macerans*, ein Aceton bildender Rottebacillus. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **14**, 772-781

[594] Scheldeman, P., Herman, L., Foster, S. & Heyndrickx, M. (2006). *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *J Appl Microbiol* **101**, 542-555

[595] Scheldeman, P., Herman, L., Goris, J., De Vos, P. & Heyndrickx, M. (2002). Polymerase chain reaction identification of *Bacillus sporothermodurans* from dairy sources. *J Appl Microbiol* **92**, 983-991

[596] Scheldeman, P., Pil, A., Herman, L., De Vos, P. & Heyndrickx, M. (2005). Incidence and diversity of potentially highly heat resistant spores isolated at dairy farms. *Appl Environ Microbiol* **71**, 1480-1494

[597] Scheldeman, P., Rodriguez-Diaz, M., Goris, J., Pil, A., De Clerck, E., Herman, L., De Vos, P., Logan, N.A. & Heyndrickx, M. (2004). *Bacillus farraginis* sp. nov., *Bacillus fortis* sp. nov. and *Bacillus fordii* sp. nov., isolated at dairy farms. *Int J Syst Evol Microbiol* **54**, 1355-1364

[598] Schenk, A. & Aragno, M. (1979). *Bacillus schlegelii* a new species of thermophilic, facultatively chemolithoautotrophic bacterium oxidizing molecular hydrogen. *J Gen Microbiol* **115**, 333-341

[599] Scholz, T., Demharter, W., Hensel, R. & Kandler, O. (1987). *Bacillus pallidus* sp. nov., a new thermophilic species from sewage. *Syst Appl Microbiol* **9**, 91-96

[600] Scholz, T., Demharter, W., Hensel, R. & Kandler, O. (1988). Validation list N° 24. *Int J Syst Bacteriol* **38**, 136-137

[601] Schröder, M.J.A. (1984). Origin and levels of post pasteurization contamination of milk in the dairy industry and the effects on the keeping quality. *J Dairy Res* **51**, 59-67

[602] Schütz, M., Jülicher, B. & Wiesner, H.U. (1990). Use of a bactofuge for reducing spore content of cheese milk. *Deutsche Milchwirtschaft* **41**, 678-681

[603] Scott, S.A., Brooks, J.D., Rakonjac, J., Walker, K.M.R. & Flint, S.H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *Int J Dairy Tech* **60**, 109-117

[604] Segers, P., Vancanneyt, M., Pot, B., Torck, U., Hoste, B., Dewettinck, D., Falsen, E., Kersters, K. & De Vos. P. (1994). Classification of *Pseudomonas diminuta Leifson* and *Hugh* 1954 and *Pseudomonas vesicularis Büsing, Döll* and *Freytag* 1953 in *Brevundimonas* gen. nov. as *Brevundimonas diminuta* comb. nov. and *Brevundimonas vesicularis* comb. nov., respectively. *Int J Syst Bacteriol* **44**, 499-510

[605] Seldin, L., Van Elsas, J.D. & Penido, E.G.C. (1984). *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. *Int J Syst Bacteriol* **34**, 451-456

[606] Setlow, P. (2006). Review article: Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* **101**, 514-525

[607] Seubert, W. (1960). Degradation of isoprenoid compounds by microorganisms. I. Isolation and characterization of an isoprenoid-degrading bacterium, Pseudomonas citronellolis n. sp. J Bact 79, 426-434

[608] Severini, G. (1913). Una bacteriosi dell' *Ixia maculata* e del *Gladiolus colvilli*. *Annali di Botanica (Roma)* **11**, 413-424

[609] Sharp, R. J., Bown K. J. & Atkinson, A. (1980). Phenotypic and genotypic characterization of some thermophilic species of *Bacillus*. *J Gen Microbiol* **117**, 201-210

[610] Shearer, J.K., Bachman, K.C. & Boosinger, J. (1992). The production of quality milk. University of Florida, IFAS Extension, DS61 (http://edis.ifas.ufl.edu/pdffiles/DS/DS11200.pdf)

[611] Shelobolina, E.S., Avakyan, Z.A., Bulygina, E.S., Turova, T.P., Lysenko, A.M., Osipov, G.A. & Karavaiko, G.I. (1997). Description of a new species of mucilaginous bacteria, *Bacillus edaphicus* sp. nov., and confirmation of the taxonomic status of *Bacillus mucilaginosus* Avakyan *et al*. 1986 based on data from phenotypic and genotypic analysis. *Microbiol* **66**, 681-689

[612] Shida O., Takagi, H., Kadowaki, K. & Komagata, K. (1996). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* **46**, 939-946

[613] Shida O., Takagi, H., Kadowaki, K, Nakamura, L.K. & Komagata, K. (1997). Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *Int J Syst Bacteriol* **47**, 289-298

[614] Shida O., Takagi, H., Kadowaki, K, Udaka, S. & Komagata, K. (1994a). *Bacillus galactophilus* is a later subjective synonym of *Bacillus agri*. *Int J Syst Bacteriol* **44**, 172-173

[615] Shida O., Takagi, H., Kadowaki, K, Udaka, S. Nakamura, L.K. & Komagata, K. (1995). Proposal of *Bacillus reuszeri* sp. nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., nom. rev. *Int J Syst Bacteriol* **45**, 93-100

[616] Shida O., Takagi, H., Kadowaki, K, Yano, H., Abe, M., Udaka, S. & Komagata, K. (1994b). *Bacillus aneurinolyticus* sp. nov., nom. rev. *Int J Syst Bacteriol* **44**, 143-150

[617] Shipe, W.F., Basette, R., Deane, D.D., Dunkley, W.L., Hammond, E.G., Harper, W.J., Kleyn, D.H., Morgan, M.E., Nelson, J.H. & Scanlan, R.A. (1978). Off flavors in milk: nomenclature, standards and bibliography. *J Dairy Sci* **61**, 856-869

[618] Shivaji, S., Chaturvedi, P., Begum, Z., Pindi, P.K., Manorama, R., Padmanaban, D.A., Shouche, Y.S., Pawar, S., Vaishampayan, P., Dutt, C.B.S., Datta, G.N., Manchanda, R.K., Rao, U.R., Bhargava, P.M. & Narlikar, J.V. (2009). *Janibacter hoylei* sp. nov., *Bacillus isronensis* sp. nov. and *Bacillus aryabhattai* sp. nov., isolated from cryotubes used for collecting air from the upper atmosphere. *Int J Syst Evol Microbiol* **59**, 2977-2986

[619] Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S.N., Dutt, C.B.S., Wainwright, M., Narlikar, J.V. & Bhargava, P.M. (2006). *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int J Syst Evol Microbiol* **56**, 1465-1473

[620] Shivaji, S., Suresh, K., Chaturvedi, P., Dube, S. & Sengupta, S. (2005). *Bacillus arsenicus* sp. nov., an arsenic-resistant bacterium isolated from a siderite concretion in West Bengal, India. *Int J Syst Evol Microbiol* **55**, 1123-1127

[621] Sijderius, R. (1946). Heterotrophe bacterien, die thiosulfaat oxyderen. Thesis, University Amsterdam

[622] Sikorski, J., Stackebrandt, E. & Wackernagel, W. (2001). *Pseudomonas kilonensis* sp. nov., a bacterium isolated from agricultural soil. *Int J Syst Evol Microbiol* **51**, 1549-1555

[623] Sim, S.H., Yu, Y., Lin, C.H., Karuturi, R.K.M., Wuthiekanun, V., Tuanyok, A., Chua, H.H., Ong, C., Paramalingam, S.S., Tan, G., Tang, L., Lau, G., Ooi, E.E., Woods, D. & Feil, E. (2008). The core and accessory genomes of *Burkholderia pseudomallei*: Implications for human melioidosis. *PLoS Pathog* **4**, e1000178. doi:10.1371/journal.ppat.1000178.

[624] Simões, M., Simões, L.C. & Vieira, M.J. (2009). Species association increases biofilm resistance to chemical and mechanical treatments. *Water Res* **43**, 229-237

[625] Skerman, V.B.D., McGowan, V. & Sneath, P.H.A. (1980). Approved lists of bacterial names. *Int J Syst Bacteriol* **30**, 225-420

[626] Slabbinck, B., Dawyndt, P., Martens, M., De Vos, P. & De Baets, B. (2008). TaxonGap: a visualisation tool for intra- and inter-species variation among individual biomarkers. *Bioinformatics* **24**, 866-867

[627] Slaghuis, B.A., te Giffel, M.C., Beumer, R.R. & Andre, G. (1997). Effect of pasturing on the incidence of *Bacillus cereus* spores in raw milk. *Int Dairy J* **7**, 201-205

[628] Slonczewski, J.L. & Foster, J.W. (2011). Bacterial culture, growth, and development. In '*Microbiology, an evolving science*'. 2nd ed. Norton Publishers

[629] Smit, G. (2000). Dairy processing: improving quality. Cambridge: Woodhead Publishing Limited. The University of Reading, UK

[630] Smith, E.F. (1911). Bacteria in relation to plant diseases. *Carnegie Institute, Washington* **2**, 1-368

[631] Smith, N.R.S., Gordon, R.E. & Clark, F.E. (1946). Aerobic mesophilic sporeforming bacteria. U.S. Department of Agriculture, misc. pub. **559**, 112

[632] Sørhaug, T. & Stepaniak, L. (1997). Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends Food Sci Tech* **8**, 35-41

[633] Sorokin, A., Candelon, B., Guilloux, K., Galleron, N., Wackerow-Kouzova, N., Dusko, S., Ehrlich, S., Bourguet, D. & Sanchis, V. (2006). Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. *Appl Environ Microbiol* **72**, 1569-1578

[634] Sorokin, I.D., Kravchencko, I.K., Tourova, T.P., Kolganova, T.V., Boulygina, E.S. & Sorokin, D.Y. (2008a). *Bacillus alkalidiazotrophicus* sp. nov., a diazotrophic, low salt-tolerant alkaliphile isolated from Mongolian soda soil. *Int J Syst Evol Microbiol* **58**, 2459-2464

[635] Sorokin, D.Y., Van Pelt, S. & Tourova, T.P. (2008b). Utilization of aliphatic nitriles under haloalkaline conditions by *Bacillus alkalinitrilicus* sp. nov. isolated from soda solonchak soil. *FEMS Microbiol Lett* **288**, 235-240

[636] Spanka, R. & Fritze, D. (1993). *Bacillus cohnii* sp. nov., a new, obligately alkaliphilic, oval-spore-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. *Int J Syst Bacteriol* **43**, 150-156

[637] Spring, S., Wagner, M., Schumann, P. & Kämpfer, P. (2005). *Malikia granosa* gen. nov., sp. nov., a novel polyhydroxyalkanoate- and polyphosphate-accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. *Int J Syst Evol Microbiol* **55**, 621-629

[638] Spröer, C., Lang, E., Hobeck, P., Burghardt, J., Stackebrandt, E. & Tindall, B.J. (1998). Transfer of *Pseudomonas nautica* to *Marinobacter hydrocarbonoclasticus*. *Int J Syst Bacteriol* **48**, 1445-1448

[639] Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kämpfer, P., Maiden, M.C., Nesme, X., Rosello-Mora, R., Swings, J., Truper, H.G., Vauterin, L., Ward, A.C. & Whitman, W.B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043-1047

[640] Stackebrandt E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152-155

[641] Stackebrandt, E. & Swiderski, J. (2002). From phylogeny to systematic: the dissection of the genus *Bacillus*. In '*Applications and Systematics of Bacillus and relatives*'. Eds. Berkeley, R., Heyndrickx, M., Logan, N.A. & De Vos, P. Blackwell publishing, UK, ISBN 0-632-05758-0

[642] Stanier, R.Y., Palleroni, N.J. & Doudoroff, M. (1966). The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol* **43**, 159-271

[643] Staley, J.T. (2006). The bacterial species dilemma and the genomic-phylogenetic species concept. *Phil Trans R Soc* **361**, 1899-1909

[644] Stapp, C. (1928). *Schizomycetes* (Spaltpilze oder Bakterien). In '*Handbuch der Pflanzenkrankheiten*'. Ed. Sorauer. 5^{th} ed. Paul Parey, Berlin

[645] Starr, M.P. & Burkholder, W.H. (1942). Lipolytic activity of phytopathogenic bacteria determined by means of spirit blue agar and its taxonomic significance. *Phytopathology* **32**, 598-604

[646] Stead, D.E., Sellwood, J.E., Wilson, J. & Viney, I. (1991). Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *J Appl Bacteriol* **72**, 315-321

[647] Stenfors, L.P., Arnesen, K., O'Sullivan & Granum, P.E. (2007). Food poisoning potential of *Bacillus cereus* strains from Norwegian dairies. *Int J Food Microbiol* **116**, 292-296

[648] Stevens, F.L. (1925). Plant Disease Fungi. MacMillan Co., New York

[649] Stolz, A., Busse, H.-J. & Kämpfer, P. (2007). *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572-576

[650] Suhren, G. (1989). Producer microorganisms. In '*Enzymes of Psychrotrophs in Raw Food*'. Ed. McKellar, R.C. Boca Raton, Florida, CRC Press Inc

[651] Sumpavapol, P., Tongyonk, L., Tanasupawat, S., Chokesajjawatee, N., Luxananil, P. & Visessanguan, W. (2010). *Bacillus siamensis* sp. nov., isolated from salted crab (*poo-khem*) in Thailand. *Int J Syst Evol Microbiol* **60**, 2364-2370

[652] Sung, M.H., Kim, H., Bae, J.W., Rhee, S.K., Jeon, C.O., Kim, K., Kim, J.J., Hong, S.P., Lee, S.G., Yoon, J.H., Park, Y.H. & Baek, D.H. (2002). *Geobacillus toebii* sp. nov., a novel thermophilic bacterium isolated from hay compost. *Int J Syst Evol Microbiol* **52**, 2251-2255

[653] Sunna, A., Tokajian, S., Burghardt, J., Rainey, F., Antranikian, G. & Hashwa, F. (1997). Identification of *Bacillus kaustophilus, Bacillus thermocatenulatus* and *Bacillus* strain HSR as members of *Bacillus thermoleovorans*. *Syst Appl Microbiol* **20**, 232-237

[654] Suominen, I., Andersson, M.A., Andersson, M.C., Hallaksela, A.M., Kampfer, P., Rainey, F.A. & Salkinoja-Salonen, M. (2001). Toxic *Bacillus pumilus* from indoor air, recycled paper pulp, Norway spruce, food poisoning outbreaks and clinical samples. *Syst Appl Microbiol* **24**, 267-276

[655] Suresh, K., Prabagaran, S.R., Sengupta, S. & Shivaji, S. (2004). *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 1369-1375

[656] Sutherland, A.D. & Murdoch, R. (1994). Seasonal occurrence of psychrotrophic *Bacillus* species in raw milk, and studies on the interactions with mesophilic *Bacillus* sp. *Int J Food Microbiol* **21**, 279-292

[657] Sutra, L., Siverio, F., Lopez, M.M., Hunault, G., Bollet, C. & Gardan, L. (1997). Taxonomy of *Pseudomonas* strains isolated from tomato pith necrosis: emended description of *Pseudomonas corrugata* and proposal of three unnamed fluorescent *Pseudomonas* genomospecies. *Int J Syst Bacteriol* **47**, 1020-1033

[658] Suzuki, Y., Kishigami, T., Inoue, K., Mizoguchi, Y., Eto, N., Takagi, M. & Abe, S. (1983). *Bacillus thermoglucosidasius* sp. nov., a new species of obligately thermophilic bacilli. *Syst Appl Microbiol* **4**, 487-495

[659] Svensson, B., Ekelund, K., Ogura, H. & Christiansson, A. (2004). Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *Int Dairy J* **14**, 17-27

[660] Swings, J., De Vos, P., Van den Mooter, M. & De Ley, J. (1983). Transfer of *Pseudomonas maltophilia Hugh* 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (*Hugh* 1981) comb. nov. *Int J Syst Bacteriol* **33**, 409-413

[661] Switzer Blum, J., Burns Bindi, A., Buzzelli, J., Stolz, J.F. & Oremland, R.S. (1998). *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens*, sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Arch Microbiol* **171**, 19-30

[662] Takagi, H., Shida, O., Kadowaki, K., Komagata, K. & Udaka, S. (1993). Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. *Int J Syst Bacteriol* **43**, 221-231

[663] Tamaoka, J., Ha, D.-M. & Komagata, K. (1987). Reclassification of Pseudomonas acidovorans den Dooren de Jong 1926 and Pseudomonas testosteroni Marcus and Talalay 1956 as Comamonas acidovorans comb. nov. and Comamonas testosteroni comb. nov., with an emended description of the genus Comamonas. Int J Syst Bacteriol 37, 52-59

[664] Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24, 1596-1599

[665] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol available online, doi:10.1093/molbev/msr121.

[666] Tatzel, R., Ludwig, W., Schleifer, K.H. & Wallnöfer, P.R. (1994). Identification of Bacillus strains isolated from milk and cream with classical and nucleic acid hybridization methods. J Dairy Res 61, 529-535

[667] Täubel, M., Kämpfer, P. Buczolits, S., Lubitz, W. & Busse, H.-J. (2003). Bacillus barbaricus sp. nov., isolated from an experimental wall painting. Int J Syst Evol Microbiol 53, 725-730

[668] Tayeb, L.A., Ageron, E., Grimont, F. & Grimont, P.A.D. (2005). Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. *Res Microbiol* **156**, 763-773

[669] Taylor, J.M., Sutherland, A.D., Aidoo, K.E. & Logan, N.A. (2005). Heat-stable toxin production by strains of *Bacillus cereus, Bacillus firmus, Bacillus megaterium, Bacillus simplex* and *Bacillus licheniformis*. *FEMS Microbiol Lett* **242,** 313-317

[670] te Giffel, M.C., Beumer, R.R., Slaghuis, B.A. & Rombouts, F.M. (1995). Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in The Netherlands. *Neth Milk Dairy J* **49**, 125-138

[671] te Giffel, M.C., Wagendorp, T.A., Herrewegh, A. & Driehuis, F. (2002). Bacterial spores in silage and raw milk. *Ant Leeuw* **81**, 625-630

[672] Ten, L.N., Baek, S.H., Im, W.T., Larina, L.L., Lee, J.S., Oh, H.M. & Lee, S.T. (2007). *Bacillus pocheonensis* sp. nov., a moderately halotolerant, aerobic bacterium isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* **57**, 2532-2537

[673] Ten, L.N., Baek, S.H., Im, W.T., Liu, Q.M., Aslam, Z. & Lee, S.T. (2006a). *Bacillus panaciterrae* sp. nov., isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* **56**, 2861-2866

[674] Ten, L.N., Im, W.T, Baek, S.H., Lee, J.S., Oh, H.M. & Lee, S.T. (2006b). *Bacillus ginsengihumi* sp. nov., a novel species isolated from soil of a ginseng field in pocheon Province, South Korea. *J Microbiol Biotechnol* **16**, 1554-1560

[675] Ternström, A., Lindberg, A.M. & Molin, G. (1993). Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus. J Appl Bacteriol* **75**, 25-34

[676] Thomas, S.B. (1966). Sources, incidence and significance of psychrotrophic bacteria in milk. *Milchwissenschaft* **21**, 270-275

[677] Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. & Higgins, D. (1997). The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc Acids Res* **25**, 4876-4882

[678] Tiago, I., Pires, C., Mendes, V., Morais, P.V., Da Costa, M.S. & Verissimo, A. (2006). *Bacillus foraminis* sp. nov., isolated from a non-saline alkaline groundwater. *Int J Syst Evol Microbiol* **56**, 2571-2574

[679] Tindall, B.J. (1990a). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199-202

[680] Tindall, B.J. (1990b). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128-130

[681] Tindall, B.J., Rosello-Mora, R., Busse H.-J., Ludwig, W. & Kämpfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* **60**, 249-266

[682] Tindall, B.J., Sikorski, J., Smibert, R.M. & Krieg, N.R. (2007). Phenotypic characterization and the principles of comparative systematics. In *Methods for General and Molecular Mircobiology.* 3^{rd} ed. Eds. Reddy, C.A., Beveridge, T.J., Breznak, J.A., Mazluf, G., Schmidt, T.M. & Snyder, L.R. ASM Press, Washington DC, USA

[683] Tomimura, E., Zeman, N.W., Frankiewicz, J.R. & Teague, W.M. (1990). Description of *Bacillus naganoensis* sp. nov. *Int J Syst Bacteriol* **40**, 123-125

[684] Tominaga, T., An, S.Y., Oyaizu, H. & Yokota, A. (2009). *Oceanobacillus soja* sp. nov. isolated from soy sauce production equipment in Japan. *J Gen Appl Microbiol* **55**, 225-232

[685] Toora, S., Singh, G., Tiwari, R.P. & Singh, G. (1989). Drug resistance and lecithinase activity of *Yersinia enterocolitica* isolated from buffalo milk. *Int J Food Microbiol* **9**, 167-171

[686] Tourova, T.P., Korshunova, A.V., Mikhailova, E.M., Sokolova, D.Sh., Poltaraus, A.B. & Nazina, T.N. (2010). Application of *gyrB* and *parE* sequence similarity analyses for differentiation of species within the genus *Geobacillus*. *Microbiol* **79**, 376-389

[687] Tvrzova, L., Schumann, P., Spröer, C., Sedlacek, I., Pacova, Z., Sedo, O., Zdrahal, Z., Steffen, M. & Lang, E. (2006). *Pseudomonas moraviensis* sp. nov. and *Pseudomonas vranovensis* sp. nov., soil bacteria isolated on nitroaromatic compounds, and emended description of *Pseudomonas asplenii*. *Int J Syst Evol Microbiol* **56**, 2657-2663

[688] Uchino, M., Shida, O., Uchimura, T. & Komagata, K. (2001). Recharacterization of *Pseudomonas fulva* Iizuka and Komagata 1963, and proposals of *Pseudomonas parafulva* sp. nov. and *Pseudomonas cremoricolorata* sp. nov. *J Gen Appl Microbiol* **47**, 247-261

[689] Urakami, T., Araki, H., Oyanagi, H., Suzuki, K.-I. & Komagata, K. (1992).Transfer of Pseudomonas aminovorans (den Dooren de Jong 1926) to Aminobacter gen. nov. as Aminobacter aminovorans comb. nov. and description of Aminobacter aganoensis sp. nov. and Aminobacter niigataensis sp. nov. Int J Syst Bacteriol 42, 84-92

[690] Urakami, T., Ito-Yoshida, C., Araki, H., Kijima, T., Suzuki, K.-I. & Komagata, K. (1994). Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderia vandii* sp. nov. *Int J Syst Bacteriol* **44**, 235-345

[691] Vacheyrou, M., Normand, A.-C., Guyot, P., Cassagne, C., Piarroux, R. & Boubon, Y. (2011). Cultivable microbial communities in raw cow milk and potential transferss from stables of sixteen French farms. *Int J Food Microbiol* **146**, 253-262

[692] Vaerewijck, M.J.M., De Vos, P., Lebbe, L., Scheldeman, P., Hoste, B. & Heyndrickx, M. (2001). Occurrence of *Bacillus sporothermodurans* and other aerobic spore-forming species in feed concentrate for dairy cattle. *J Appl Microbiol* **91**, 1074-1084

[693] Vaishampayan, P., Probst, A., Krishnamurthi, S., Ghosh, S., Osman, S., McDowall, A., Ruckmani, A., Mayilraj, S. & Venkateswaran, K. (2010). *Bacillus horneckiae* sp. nov., isolated from a spacecraft-assembly clean room. *Int J Syst Evol Microbiol* **60**, 1031-1037

[694] Vaishampayan, P., Miyashita, M., Ohnishi, A., Satomi, M., Rooney, A., La Duc, M.T. & Venkateswaran, K. (2009). Description of *Rummeliibacillus stabekisii* gen. nov., sp. nov. and reclassification of *Bacillus pycnus* Nakamura *et al.* 2002 as *Rummeliibacillus pycnus* comb. nov. *Int J Syst Evol Microbiol* **59**, 1094-1099

[695] Vancanneyt, M., Witt, S., Abraham, W.-R., Kersters, K. & Fredrickson, H.L. (1996a). Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: a taxonomic evaluation. *Syst Appl Microbiol* **19**, 528-540

[696] Vancanneyt, M., Torck, U., Dewettinck, D., Vaerewijck, M. & Kersters, K. (1996b). Grouping of pseudomonads by SDS-PAGE of whole-cell proteins. *Syst Appl Microbiol* **19**, 556-568

[697] Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407-438

[698] Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M., Kersters, K. & Govan, J.R.W. (1997). Occurence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**, 1188-1200

[699] Van Damme, P.A., Johannes, A.G., Cox, H.C. & Berends, W. (1960). On toxoflavin, the yellow poison of *Pseudomonas cocovenenans*. *Recueil des Travaux Chimiques des Pays-Bas* **79**, 255-267

[700] Vaneechoutte, M., Kämpfer, P., De Baere, T., Falsen, E. & Verschraegen, G. (2004). *Wautersia* gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts *et al*. 1990) comb. nov. *Int J Syst Evol Microbiol* **54**, 317-327

[701] Van Hall, C.J.J. (1902). Bijdragen tot de kennis der Bakterieele Plantenziekten. Inaugural Dissertation Amsterdam

[702] Van Heddeghem, A. & Vlaemynck, G. (1992). Sources of contamination of milk with *B. cereus* on the farm and in the factory. *Bulletin of the International Dairy Federation* **275,** 19-22

[703] Van Netten, P., Van de Moosdijk, A., Van Hoensel, P., Mossel, D.A.A. & Perales, I. (1990). Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *J Appl Bacteriol* **69**, 73-79

[704] Vanparys, B., Heylen, K., Lebbe, L. & De Vos, P. (2006). *Pseudomonas peli* sp. nov. and *Pseudomonas borbori* sp. nov., isolated from a nitrifying inoculum. *Int J Syst Evol Microbiol* **56**, 1875-1881

[705] Vargas, V.A., Delgado, O.D., Hatti-Kaul, R. & Mattiasson, B. (2005). *Bacillus bogoriensis* sp. nov., a novel alkaliphilic, halotolerant bacterium isolated from a Kenyan soda lake. *Int J Syst Evol Microbiol* **55**, 899-902

[706] Vauterin, L., Yang, P., Hoste, B., Vancanneyt, M., Civerolo, E. L., Swings, J. & Kersters, K. (1991). Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. *Int J Syst Bacteriol* **41**, 535-542

[707] Vedder, A. (1934). Bacillus alcalophilus n. sp. Ant Leeuw J Microbiol Serol 1, 141-147

[708] Vela, A.I., Gutierrez, M.C., Falsen, E., Rollan, E., Simarro, I., Garcia, P., Dominguez, L., Ventosa, A. & Fernandez-Garayzabal, J.F. (2006). *Pseudomonas simiae* sp. nov., isolated from clinical specimens from monkeys (*Callithrix geoffroyi*). *Int J Syst Evol Microbiol* **56**, 2671-2676

[709] Venkateswaran, K., Kempf, M., Chen, F., Satomi, M., Nicholson, W. & Kern, R. (2003). *Bacillus nealsonii* sp. nov., isolated from a spacecraft-assembly facility, whose spores are γ-radiation resistant. *Int J Syst Evol Microbiol* **53**, 165-172

[710] Ventosa, A., Garcia, M.T., Kamekura, M., Onishi, H. & Ruiz-Berraquero, F. (1989). *Bacillus halophilus* sp. nov., a moderately halophilic *Bacillus* species. *Syst Appl Microbiol* **12**, 162-165

[711] Verbaendert, I., Boon, N., De Vos, P. & Heylen, K. (2011). Denitrification is a common feature in members of the genus *Bacillus*. *Syst Appl Microbiol* **34**, 385-391

[712] Verdier-Metz, I., Michel, V., Delbès, C. & Montel, M.C. (2009). Do milking practices influence the bacterial diversity of raw milk? *Food Microbiol* **26**, 305-310

[713] Verhille, S., Baïda, N., Dabboussi, F., Hamze, M., Izard, D. & Leclerc, H. (1999a). *Pseudomonas gessardii* sp. nov. and *Pseudomonas migulae* sp. nov., two new species isolated from natural mineral waters. *Int J Syst Bacteriol* **49**, 1559-1572

[714] Verhille, S., Baïda, N., Dabboussi, F., Izard, D. & Leclerc, H. (1999b). Taxonomic study of bacteria isolated from natural mineral waters: proposal of *Pseudomonas jessenii* sp. nov. and *Pseudomonas mandelii* sp. nov. *Syst Appl Microbiol* **22**, 45-58

[715] Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K. & Balandreau, J. (1998). *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of *Pseudomonas phenazinium*, *Pseudomonas pyrrocinia* and *Pseudomonas glathei* as *Burkholderia*. *Int J Syst Bacteriol* **48**, 549-563

[716] Vissers, M.M.M., te Giffel, M.C., Driehuis, F., De Jong, P. & Lankveld, J.G.M. (2007). Minimizing the level of *Bacillus* spores in farm tank milk. *J Dairy Sci* **90,** 3286-3293

[717] Waes, G. (1976). Aerobic mesophilic spores in raw milk. *Milchwissenschaft* **31**, 521-525

[718] Wainø, M., Tindall, B.J., Schumann, P. & Ingvorsein, K. (1999). *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int J Syst Bacteriol* **49**, 821-831

[719] Wakabayashi, H. & Egusa, S. (1972). Characteristics of a *Pseudomonas* sp. from an epizootic of pond-cultured eels (*Anguilla japonica*). *Bulletin of the Japanese Society of Scientific Fisheries* **38**, 577-587

[720] Walker, P.D. & Wolf, J. (1971). The taxonomy of *Bacillus stearothermophilus*. In '*Spore Research*'. Eds. Barker, A.N., Gould, G.W. & Wolf, J. Academic Press, London

[721] Walstra, P. (1979). The voluminosity of bovine casein micelles and some of its implications. *J Dairy Sci* **46**, 317-322

[722] Wang, L.-T., Lee, F.-L., Tai, C.-J. & Kasai, H. (2007a). Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA-DNA hybridisation in the *Bacillus subtilis* group. *Int J Syst Evol Microbiol* **57**, 1846-1850

[723] Wang, L.T., Lee, F.L., Tai, C.J. & Kuo, H.P. (2008). *Bacillus velezensis* is a later heterotypic synonym of *Bacillus amyloliquefaciens*. *Int J Syst Evol Microbiol* **58**, 671-675

[724] Wang, L.T., Lee, F.L., Tai, C.J., Yokota, A. & Kuo, H.P. (2007b). Reclassification of *Bacillus axarquiensis* Ruiz-García *et al*. 2005 and *Bacillus malacitensis* Ruiz-García *et al*. 2005 as later heterotypic synonyms of *Bacillus mojavensis* Roberts *et al*. 1994. *Int J Syst Evol Microbiol* **57**, 1663-1667

[725] Wang, L.T., Tai, C.J., Wu, Y.C., Chen, Y.B. & Wang, S.L. (2010). *Pseudomonas taiwanensis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **60**, 2094-2098

[726] Wang, Q.F., Li, W., Liu, Y.L., Cao, H.H., Li, Z. & Guo, G.Q. (2007c). *Bacillus qingdaonensis* sp. nov., a moderately haloalkaliphilic bacterium isolated from a crude sea-salt sample collected near Qingdao in eastern China. *Int J Syst Evol Microbiol* **57**, 1143-1147

[727] Ward, W.R., Hughes, J.W., Faull, W.B., Cripps, P.J., Sutherland, J.P. & Sutherst, J.E. (2002). Observational study of temperature, moisture, pH and bacteria in straw bedding, and faecal consistency, cleanliness, and mastitis in cows in four dairy herds. *Vet Rec* **151**, 199-206

[728] Webb, B.H., Johnson, A.H. & Alford, J.A. (1974). Fundamentals of Dairy Chemistry. 2^{nd} ed. AVI Publishing Co., Westport, CT

[729] Wen, A., Fegan, M., Hayward, C., Chakraborty, S. & Sly, L.I. (1999). Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka *et al.* 1987) gen. nov., comb. nov. *Int J Syst Bact* **49**, 567-576

[730] Weng, F.Y., Chioun, C.S., Lin, P.H.P. & Yang, S.S. (2009). Application of *recA* and *rpoB* sequence analysis on phylogeny and molecular identification of *Geobacillus* species. *J Appl Microbiol* **107**, 452-464

[731] Weon, H.Y., Kim, B.Y., Yoo, S.H., Baek, Y.K., Lee, S.Y., Kwon, S.W., Go, S.J. & Stackebrandt, E. (2006). *Pseudomonas pohangensis* sp. nov., isolated from seashore sand in Korea. *Int J Syst Evol Microbiol* **56**, 2153-2156

[732] Westhoff, D.C. & Dougherty, S.L. (1981). Characterization of *Bacillus* species isolated from spoiled ultrahigh temperature processe milk. *J Dairy Sci* **64**, 572-580

[733] White, D., Sharp, R.J. & Priest, F.G. (1993). A polyphasic taxonomic study of thermophilic bacilli from a wide geographical area. *Ant Leeuw* **64**, 357-386

[734] WHO (2011). World Health Organization. http://www.who.int/en/ Consulted August 2011

[735] Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R. & Boor, K.J. (2000). Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Appl Environ Microbiol* **66**, 2085-2095

[736] Wieser, M., Worliczek, H., Kämpfer, P. & Busse, H.-J. (2005). *Bacillus herbersteinensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 2119-2123

[737] Willems, A., Busse, H.-J., Goor, M., Pot, B., Falsen, E., Jantzen, E., Hoste, B., Gillis, M., Kersters, K., Auling, G. & De Ley, J. (1989). *Hydrogenophaga*, a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and "*Pseudomonas carboxydoflava*"), and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). *Int J Syst Bacteriol* **39**, 319-333

[738] Willems, A., De Ley, J., Gillis, M. & Kersters, K. (1991). Notes: *Comamonadaceae*, a new family encompassing the acidovorans rRNA complex, including Variovorax paradoxus gen. nov. comb. nov., for *Alcaligenes paradoxus* (Davis, 1969). *Int J Syst Evol Microbiol* **41**, 445-450

[739] Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA-DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315-1322

[740] Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., Vandamme, P., Gillis, M., Kersters, K. & De Ley, J. (1990). *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) Group 13, EF Group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *Int J Syst Bacteriol* **40**, 384-398

[741] Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K. & De Ley, J. (1992). Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli, Acidovorax avenae* subsp. *cattleyae,* and *Acidovorax konjaci. Int J Syst Bact* **42**, 107-119

[742] Winslow, C.E.A., Broadhurst, J., Buchanan, R.E., Krumwiede, C., Rogers, L.A. & Smith, G.A. (1920). The families and genera of bacteria. Final report of the committee of the society of American Bacteriologists on characterization and classification of bacterial types. *J Bact* **5**, 191-229

[743] Wisotzkey, J.D., Jurtshuk Jr., P., Fox, G.E., Deinhard, G. & Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int J Syst Bact* **42**, 263-269

[744] Wolfgang, W.J., Coorevits A., Cole, J.A., De Vos, P., Dickinson, M.C., Hannett, G.E., Jose, R., Nazarian, E.J., Schumann, P., Van Landschoot, A., Wirth, S.E. & Musser, K.E. (2011). Description of *Sporosarcina newyorkensis* sp. nov. recovered from clinical specimens in the state of New York and from raw cow's milk in Belgium. *Int J Syst Evol Microbiol*, in press (doi 10.1099/ijs.0.030080-0)

[745] Wolterink, A.F.W.M., Jonker, A.B., Kengen, S.W.M. & Stams A.J.M. (2002). *Pseudomonas chloritidismutans* sp. nov., a non-denitrifying, chlorate-reducing bacterium. *Int J Syst Evol Microbiol* **52**, 2183-2190

[746] Xiao, Y.P., Hui, W., Wang, Q., Roh, S.W., Shi, X.Q., Shi, J.H. & Quan, Z.X. (2009). *Pseudomonas caeni* sp. nov., a denitrifying bacterium isolated from the sludge of an anaerobic ammonium-oxidizing bioreactor. *Int J Syst Evol Microbiol* **59**, 2594-259

[747] Xie, C.H. & Yokota, A. (2005). Reclassification of *Alcaligenes latus* strains IAM 12599^T and IAM 12664 and *Pseudomonas saccharophila* as *Azohydromonas lata* gen. nov., comb. nov., *Azohydromonas australica* sp. nov. and *Pelomonas saccharophila* gen. nov., comb. nov., respectively. *Int J Syst Evol Microbiol* **55**, 2419-2425

[748] Xin, Y.H., Zhang, D.C., Liu, H.C., Zhou, H.L. & Zhou, Y.G. (2009). *Pseudomonas tuomuerensis* sp. nov., isolated from a bird's nest. *Int J Syst Evol Microbiol* **59**, 139-143

[749] Xue, Y., Ventosa, A., Wang, X., Ren, P., Zhou, P. & Ma, Y. (2008). *Bacillus aidingensis* sp. nov., a moderately halophilic bacterium isolated from Ai-Ding salt lake in China. *Int J Syst Evol Microbiol* **58**, 2828-2832

[750] Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251-1275. Erratum in *Microbiol Immunol* **37**, 335

[751] Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. & Nishiuchi, Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Qmith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol Immunol* **39**, 897-904

[752] Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T. & Yamamoto, H. (1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* **34**, 99-119

[753] Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104-1109

[754] Yamamoto, S. & Harayama, S. (1998a). Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyrB, rpoD* and 16S rRNA genes. *Int J Syst Bacteriol* **48**, 813-819

[755] Yamamoto, S. & Harayama, S. (1998b).PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104-1109

[756] Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Vivian, A. & Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiol* **146**, 2385-2394

[757] Yamamoto, S., Bouvet, P.J.M. & Harayama, S. (1999). Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA-DNA hybridization. *Int J Syst Bacteriol* **49**, 87-95

[758] Yamamura, S., Yamashita, M., Fujimoto, N., Kuroda, M., Kashiwa, M., Sei, K., Fujita, M. & Ike, M. (2007). *Bacillus selenatarsenatis* sp. nov., a selenate- and arsenate-reducing bacterium isolated from the effluent drain of a glass-manufacturing plant. *Int J Syst Evol Microbiol* **57**, 1060-1064

[759] Yan, L., Langlois, B.E., O'Leary, J. & Hicks,C. (1983). Effect of storage conditions of grade A raw milk on proteolysis and cheese yield. *Milchwissenschaft* **38**, 715-719

[760] Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.-H., Glöckner, F.O. & Rosello-Mora, R. (2010). Update of the all-species living tree project based on 16S and 23S rRNA sequence analyses. *Syst Appl Microbiol* **33**, 291-299

[761] Yoon, J.H., Kang, S.S., Lee, K.C., Kho, Y.H., Choi, S.H., Kang, K.H. & Park, Y.H. (2001a). *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. *Int J Syst Evol Microbiol* **51**, 1087-1092

[762] Yoon, J.H., Kang, S.S., & Oh, T.K. (2007). Reclassification of *Marinococcus albus* Hao *et al.* 1985 as *Salimicrobium album* gen. nov., comb. nov. and *Bacillus halophilus* Ventosa *et al.* 1990 as *Salimicrobium halophilum* comb. nov., and description of *Salimicrobium luteum* sp. nov. *Int J Syst Evol Microbiol* **57**, 2406-2411

[763] Yoon, J.H., Kim, H., Kang, K.H., Oh, T.K. & Park, Y.H. (2003a). Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. *Int J Syst Evol Microbiol* **53**, 1357-1361

[764] Yoon, J.H., Kim, I.G., Kang, K.H., Oh, T.K. & Park, Y.H. (2003b). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **53**, 1297-1303

[765] Yoon, J.H., Kim, I.G., Kang, K.H., Oh, T.K. & Park, Y.H. (2004a). *Bacillus hwajinpoensis* sp. nov. and an unnamed *Bacillus* genomospecies, novel members of *Bacillus* rRNA group 6 isolated from sea water of the East Sea and the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **54**, 803-808

[766] Yoon, J.H., Lee, C.H. & Oh, T.K. (2005a). *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **55**, 733-736

[767] Yoon, J.H., Lee, K.C., Weiss, N., Kho, Y.H., Kang, K.H., Park, Y.H. (2001b). *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of the genus *Sporosarcina*. *Int J Syst Evol Microbiol* **51**, 1079-1086

[768] Yoon, J.H. & Oh, T.K. (2005). *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**, 1945-1948

[769] Yoon, J.H., Oh, T.K. & Park, Y.H. (2004b). Transfer of *Bacillus halodenitrificans* Denariaz *et al*. 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. *Int J Syst Evol Microbiol* **54**, 2163-2167

[770] Yoon, J.H., Weiss, N., Lee, K.C., Lee, I.S., Kang, K.H. & Park, Y.H. (2001c). *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Rüger 1983 as *Marinibacillus marinus* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 2087-2093

[771] Yoon, J.H., Kang, S.J., Schumann, P. & Oh, T.K. (2010). *Jeotgalibacillus salarius* sp. nov., isolated from a marine saltern, and reclassification of *Marinibacillus marinus* and *Marinibacillus campisalis* into the genus *Jeotgalibacillus* as *Jeotgalibacillus marinus* comb. nov. and *Jeotgalibacillus campisalis* comb. nov., respectively. *Int J Syst Evol Microbiol* **60**, 15-20

[772] Young, J.M. (1970). Drippy gill: a bacterial disease of cultivated mushrooms caused by *Pseudomonas agarici* n. sp. *New Zeal J Agr Res* **13**, 977-990

[773] Yumoto, I., Kusano, T., Shingyo, T., Nodasaka, Y., Matsuyama, H. & Okuyama, H. (2001a). Assignment of *Pseudomonas* sp. strain E-3 to *Pseudomonas psychrophila* sp. nov., a new facultatively psychrophilic bacterium. *Extremophiles* **5**, 343-349

[774] Yumoto, I., Hirota, K., Goto, T., Nodasaka, Y. & Nakajima, K. (2005). *Bacillus oshimensis* sp. nov., a moderately halophilic, non-motile alkaliphile. *Int J Syst Evol Microbiol* **55**, 907-911

[775] Yumoto, I., Hirota, K., Yamaga, S., Nodasaka, Y., Kawasaki, T., Matsuyama, H. & Nakajima, K. (2004). *Bacillus asahii* sp. nov., a novel bacterium isolated from soil with the ability to deodorize the bad smell generated from short-chain fatty acids. *Int J Syst Evol Microbiol* **54**, 1997-2001

[776] Yumoto, I., Yamaga, S., Sogabe, Y., Nodasaka, Y., Matsuyama, H., Nakajima, K. & Suemori, A. (2003). *Bacillus krulwichiae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and *m*-hydroxybenzoate. *Int J Syst Evol Microbiol* **53**, 1531-1536

[777] Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Suemori, A., Nakajima, K., Inoue, N. & Kawasaki, K. (2001b). *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. *Int J Syst Evol Microbiol* **51**, 349-355

[778] Yumoto, I., Yamazaki, K., Sawabe, T., Nakano, K., Kawasaki, K., Ezura, Y. & Shinano, H. (1998). *Bacillus horti* sp. nov., a new Gram-negative alkaliphilic bacillus. *Int J Syst Bacteriol* **48**, 565-571

[779] Zarilla, K. & Perry, J.J. (1987). *Bacillus thermoleovorans*, sp. nov., a species of obligately thermophilic hydrocarbon utilizing endospore-forming bacteria. *Syst Appl Microbiol* **9**, 258-264

[780] Zavarzina, D.G., Tourova, T.P., Kolganova, T.V., Boulygina, E.S. & Zhilina, T.N. (2009). Description of *Anaerobacillus alkalilacustre* gen. nov., sp. nov. Strictly anaerobic diazotrophic bacillus isolated from soda lake and transfer of *Bacillus arseniciselenatis*, *Bacillus macyae*, and *Bacillus alkalidiazotrophicus* to *Anaerobacillus* as the

new combinations *A. arseniciselenatis* comb. nov., *A. macyae* comb. nov., and *A. alkalidiazotrophicus* comb. nov. *Microbiol* **78**, 723-731

[781] Zeigler, D.R. (2005). Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* **53**, 1893-1900

[782] Zhang, T., Fan, X., Hanada, S., Kamagata, Y. & Fang, H.H.P. (2006). *Bacillus macauensis* sp. nov., a long-chain bacterium isolated from a drinking water supply. *Int J Syst Evol Microbiol* **56**, 349-353

[783] Zhang, L., Wang, Y., Dai, J., Tang, Y., Yang, Q., Luo, X. & Fang, C. (2009). *Bacillus korlensis* sp. nov., a moderately halotolerant bacterium isolated from a sand soil sample in China. *Int J Syst Evol Microbiol* **59**, 1787-1792

[784] Zhang, J., Wang, J., Fang, C., Song, F., Xin, Y., Qu, L., & Ding, K. (2010). *Bacillus oceanisediminis* sp. nov., isolated from marine sediment. *Int J Syst Evol Microbiol* **60**, 2924-2929

[785] Zhang, L., Xu, Z. & Patel, B.K.C. (2007). *Bacillus decisifrondis* sp. nov., isolated from soil underlying decaying leaf foliage. *Int J Syst Evol Microbiol* **57**, 974-978

[786] Zhou, Y., Wei, W., Che, Q., Xu, Y., Wang, X., Huang, X. & Lai, R. (2008). *Bacillus pallidus* sp. nov., isolated from forest soil. *Int J Syst Evol Microbiol* **58**, 2850-2854

[787] Zhou, Y., Xu, J., Xu, L. & Tindall, B.J. (2009). *Falsibacillus pallidus* to replace the homonym *Bacillus pallidus* Zhou *et al*. 2008. *Int J Syst Evol Microbiol* **59**, 3176-3180

[788] Zolg, W. & Ottow, J.C.G. (1975). *Pseudomonas glathei* sp. nov., a new nitrogen-scavenging rod isolated from acid lateritic relicts in Germany. *Zeitschrift fur Allgemeine Mikrobiologie* **15**, 287-299

Curriculum vitae

Personalia

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Born December 23, 1982, Vilvoorde

Educational background

2006 - present	PhD - University College Ghent, Faculty of Applied Engineering Sciences; Ghent University, Faculty of Sciences Dissertation "Pseudomonads and bacilli as important spoilage organisms in the dairy industry - a taxonomic study" Promotors: Prof. Dr. A. Van Landschoot & Prof. Dr. P. De Vos
2005 - 2006	Dehousse scholarship — Ghent University, Faculty of Sciences IWT-project 030846 "Invloed van de bedrijfsvoering op de schadelijke aërobe sporenvormende bacteriële flora in rauwe melk: biologische versus conventionele melkveehouderij" Promotors: Prof. Dr. M. Heyndrickx & Prof. Dr. P. De Vos
2000 - 2005	Master of Biotechnology (graduated cum laude) - Ghent University Dissertation "Biodiversiteit van aërobe sporenvormers uit rauwe melk afkomstig van biologische en conventionele melkveebedrijven" Promotors: Prof. Dr. M. Heyndrickx & Prof. Dr. P. De Vos
1994 - 2000	Koninklijk Atheneum Ronse (Latijn/Wiskunde)

Scientific output

a1-publications

1. De Jonghe, V., **Coorevits, A.**, Vandroemme, J., Heyrman, J., Herman, L., De Vos, P. & Heyndrickx, M. (2007). Intraspecific genotypic diversity of Bacillus species from raw milk. *Int Dairy J* **18**, 496-505

2. **Coorevits, A.**, De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from conventional and organic dairy fams. *Syst Appl Microbiol* **31**, 126-140

3. Marchand, S., Vandriesche, G., **Coorevits, A.**, Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M. & De Block, J. (2009). Heterogeneity of heat resistant proteases from milk spoiling Pseudomonas species. *Int J Food Microbiol* **133**, 68-77

4. De Jonghe, V., **Coorevits, A.**, De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., De Vos, P. & Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *Int J Food Microbiol* **136**, 318-325

5. Stals, I., Samyn, B., Sergeant, K., White, T., Hoorelbeke, K., **Coorevits, A.**, Devreese, B., Claeyssens, M. & Piens, K. (2010). Identification of a gene coding for a deglycosylating enzyme in *Hypocrea jecorina*. *FEMS Microbiol Lett* **303**, 9-17

6. Dinsdale, A.E., Halket, G., **Coorevits, A.**, Van Landschoot, A., Busse, H.-J., De Vos, P. & Logan, N.A. (2011). Emended descriptions of *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*. *Int J Syst Evol Microbiol* **61**, 1802-1810

7. De Jonghe, V., **Coorevits, A.**, Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2011). Influence of storage conditions on the growth of Pseudomonas species in refrigerated raw milk. *Appl Environ Microbiol* **77**, 460-470

8. **Coorevits, A.**, Logan, N.A., Dinsdale, A., Scheldeman, P., Heyndrickx, M., Schumann, P., Van Landschoot, A. & De Vos P. (2011). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int J Syst Evol Microbiol* **61**, 1954-1961

9. Heyndrickx, M., **Coorevits, A.**, Scheldeman, P., Lebbe, L., Schumann, P., Rodriguez-Diaz, M., Forsyth, G., Dinsdale, A., Heyrman, J., Logan, N.A. & De Vos, P. (2011). Emended descriptions of *Bacillus sporothermodurans* and *Bacillus oleronius* with the inclusion of dairy farm isolates of both species. *Int J Syst Evol Microbiol*, in press (doi:10.1099/ijs.0.026740-0)

10. **Coorevits, A.**, Dinsdale, A.E., Heyrman, J., Schumann, P., Van Landschoot, A., Logan, N.A. & De Vos P. (2011). *Lysinibacillus macroides* sp. nov., nom. rev., comb. nov. *Int J Syst Evol Microbiol*, in press (doi:10.1099/ijs.0.027995-0)

11. Wolfgang, W.J., **Coorevits, A.**, Cole, J.A., De Vos, P., Dickinson, M., Hannett, G.E., Jose, R., Nazarian, E.J., Schumann, P., Van Landschoot, A., Wirth, S.E. & Musser, K.A. (2011). Description of *Sporosarcina newyorkensis* sp. nov., recovered from clinical specimens in the State of New York and raw cow's milk from Belgium. *Int J Syst Evol Microbiol*, in press (doi:10.1099/ijs.0.030080-0)

12. **Coorevits, A.**, Dinsdale, A.E., Halket, G., Lebbe, L., De Vos, P., Van Landschoot A. & Logan, N.A. (2011). Taxonomic revision of the genus *Geobacillus*: emendation of *Geobacillus, G. stearothermophilus, G. jurassicus, G. toebii, G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly "*thermoglucosidasius*"); transfer of *Bacillus thermantarcticus*

to the genus as *G. thermantarcticus*; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. *Int J Syst Evol Microbiol*, in press (doi:10.1099/ijs.0.030346-0)

b2-publications

13. **Coorevits, A.**, De Jonghe, V., Vandroemme, J., Van Landschoot, A., Heyndrickx, M. & De Vos, P. (2010). How can the type of dairy farming influence the bacterial flora in milk? In "*Organic Farming and Peanut Crops*", pp. 123-136. Eds. D.C. Grossman, T.L. Barrios. Nova Science Publishers, Inc., New York. ISBN: 978-1-60876-187-6

14. De Jonghe, V., **Coorevits, A.**, Marchand, S., Van Landschoot, A., De Block, J., De Vos, P. & Heyndrickx, M. (2011). Microbial contamination and spoilage of consumer milk – facts and fiction. In "*Raw milk: production, consumption and health effects*". Eds. Momani, J. & Natsheh, A. Nova Science Publishers, Inc., New York, ISBN: 978-1-61470-751-6

oral presentation

15. **Coorevits, A.**. Taxonomic situation of the genus *Pseudomonas*. 2009. Symposium "Houdbaarheid en Stabiliteit van Zuivelproducten", ILVO & Food2Know

poster presentations and abstracts

16. **Coorevits, A.**, De Jonghe, V., Vandroemme, J., Heyrman, J., De Vos, P. & Heyndrickx, M. (2006). Characterization of aerobic spore-forming bacteria in raw milk from conventional and organic dairy farms. Symposium of the Belgian Society for Microbiology - Novel Compounds & strategies to combat pathogenic micro-organisms. November 24, 2006. Brussel, Belgium

17. **Coorevits, A.**, De Gelder, L., Stals, I., Taghon, M., van den Berg, S., Vanderputten, D., Vandoorne, S. & Van Landschoot, A. (2007). Microbiologie voor voeding. Food2Know Tweede Intern Networking Event. January 11, 2007. Gent, Belgium

18. **Coorevits, A.**, De Jonghe, V., Heyndrickx, M., Van Landschoot, A. & De Vos, P. (2007). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from conventional and organic dairy farms. Doctoraatssymposium Faculteit Wetenschappen, Ugent. April 24, 2007. Gent, Belgium

19. **Coorevits, A.**, De Jonghe, V., Van Landschoot, A., Heyndrickx, M. & De Vos, P. (2007). Diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. 12th Conference on Food Microbiology. June 21-22, 2007. Luik, Belgium

20. De Jonghe, V., **Coorevits, A.**, De Block, J., Van Coillie, E., Grijspeerdt, K., De Vos, P. & Heyndrickx, M. (2007). Toxicity and spoilage potentials of aerobe spore-forming isolates from raw milk. 12th Conference on Food Microbiology. June 21-22, 2007. Luik, Belgium

21. **Coorevits, A.**, De Jonghe, V., Heyndrickx, M., Van Landschoot, A. & De Vos P. (2007). Preliminary identification of isolated aerobic spore-forming bacteria from raw milk. Symposium of the "Belgian Society for Microbiology" – Evolution in the microbial world. November 23, 2007. Brussel, Belgium

22. Van Landschoot, A., **Coorevits, A.**, De Vos, P. & Villa, A. (2007). Hen egg white lysozyme as an antibacterial agent in the brewing industry. International Conference on Environmental, Industrial and Applied Microbiology. November 28 - December 1, 2007. Sevilla, Spain

23. Marchand, S., Vandriesche, G., **Coorevits, A.**, Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M. & De Block, J. (2008). Conservation of the *aprX* gene within *Pseudomonas* milk isolates. NZMS Conference. November 18-21, 2008. Christchurch, New Zealand

24. **Coorevits, A.**, Reekmans, R., Van Landschoot, A. & De Vos, P. (2008). Real-time PCR for the detection of aerobic spore-formers in milk. Symposium "Levensmiddelenmicrobiologie in de 21^{e} eeuw – Heden en toekomst". May 29, 2008. Gent, Belgium

25. De Jonghe, V., **Coorevits, A.**, De Vos, P. & Heyndrickx, M. (2008). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Food Micro. September 1-4, 2008. Aberdeen, Schotland

26. **Coorevits, A.**, Samyn, E., Ruiz de la Haba, R., Cottyn, B., Van Landschoot, A. & De Vos, P. (2008). Improvement of the identification of *Pseudomonas syringae* group: a molecur approach. Symposium of the Belgian Society for Microbiology - Stress responses in the microbial world. December 12, 2008. Brussel, Belgium

27. **Coorevits, A.**, De Jonghe, V., Vandroemme, J., Van Landschoot, A. & Heyndrickx, M. (2009). Identification of aerobic spore-forming bacteria in raw milk and characterization of their harmful enzymatic properties. International Congress on Spore-forming bacteria in food. June 15-17, 2009. Quimper, France

28. Marchand, S., Vandriesche, G., **Coorevits, A.**, Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M. & De Block, J. (2009). Heterogeneity of heat resistant proteases of milk spoiling *Pseudomonas* spp. BioMicroWorld Conference. December 2-4, 2009. Lissabon, Portugal

29. **Coorevits, A.**, De Vos, P., Van Landschoot, A. (2009). Bacterial diversity of milk powder. Research seminar - Food2Know. February 24, 2009. Gent, Belgium

30. **Coorevits, A.**, Van Landschoot, A. & De Vos, P. (2009). Real-time PCR for the detection of aerobic spore-formers in milk. Doctoraatssymposium Faculteit Wetenschappen, UGent. April 28, 2009. Gent, Belgium

31. De Jonghe, V., **Coorevits, A.**, Marchand, S., De Block, J., Van Coillie, E., Van Landschoot, A., De Vos, P. & Heyndrickx, M. (2010). The role of *Pseudomonas* and *Bacillus* s.l. in bacterial spoilage of milk products. Fifteenth Conference on Food Microbiology. September 16-17, 2010. Gent, Belgium

conferences and workshops

32. Real-time PCR workshop. RocheDiagnostics. November 21, 2006. Leuven, Belgium

33. Symposium of the Belgian Society for Microbiology - Novel compounds and strategies to combat pathogenic micro-organisms. November 24, 2006. Brussel, Belgium

34. qPCR course, TATAA BioCenter. June 6-7, 2007. Le Perray en Yvelines, France
35. Twelfth Conference on Food Microbiology. University of Luik. June 21-22, 2007. Luik, Belgium
36. UNIX course: Linux in a nutshell & Bio-linux. Department of Applied Mathematics and Computer Sciences, Ghent University. July 6-13, 2007. Gent, Belgium
37. Symposium "Novelties in quality control of raw milk and heat processed dairy products". Institute for Agricultural and Fisheries Research. September 28, 2007. Melle, Belgium
38. Symposium of the Belgian Society for Microbiology - Evolution in the Microbial World. November 23, 2007. Brussel, Belgium
39. Second International Conference on Environmental, Industrial and Applied Microbiology. November 25 - December 1, 2007. Sevilla, Spain
40. Symposium "Levensmiddelenmicrobiologie in de 21^{e} eeuw – heden en toekomst". May 29, 2008. Gent, Belgium
41. Benelux qPCR symposium. Ugent, UZ-Gent, ULB. October 6, 2008. Gent, Belgium
42. Follow-up workshop on BioNumerics and Gelcompar II. Applied Maths. September 22-23, 2008. St-Martens Latem, Belgium
43. Symposium of the Belgian Society for Microbiology - Stress responses in the Microbial World. December 12, 2008. Brussel, Belgium
44. Research seminar Food2Know. February 24, 2009. Gent, Belgium
45. qPCR symposium. March 8-11, 2009. Munchen, Germany
46. qPCR workshop. March 12-13, 2009. Munchen, Germany
47. Symposium "Houdbaarheid en Stabiliteit van Zuivelproducten". Institute for Agricultural and Fisheries Research. April 29, 2009. Melle, Belgium
48. International Congress Spore-forming Bacteria in Food. June 15-17, 2009. Quimper, France
49. arb workshop. Max-Planck Institut, Technical University Munich. November 30 – December 3, 2010. Bremen, Germany