UNIVERSITEIT GENT

Denitrification in Gram-positive bacteria, with focus on members of the *Bacillaceae*

Ines Verbaendert

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Sciences, Biotechnology

LM-UGent

Ines Verbaendert – Denitrification in Gram-positive bacteria, with focus on members of the *Bacillaceae*

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ISBN-number: 987-94 -6197 -232 -3

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Printed by University Press | www.universitypress.be

Ph.D. thesis, Faculty of Sciences, Ghent University, Ghent, Belgium.

Publicly defended in Ghent, Belgium, October 23rd 2014

… voor mijn twee liefste schatten

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Merci!!

Doctoreren is… een onverwachte droom in vervulling zien gaan. Soms een beetje een droom die aanvoelde als een nachtmerrie, maar ook een droom vol hoogtepunten. Een beetje zoals de ene dag in weer en wind moeizaam met de fiets de lange Citadellaan bergop naar de Ledeganck trotseren en de andere dag vlotjes het gaspedaal van de auto induwen. Hoewel ik nooit had gepland om te doctoreren, is het beëindigen van dit doctoraat nu écht een droom die werkelijkheid wordt.

Tussen droom en werkelijkheid lag een hobbelig parcours, maar met de inzet en het enthousiasme van héél wat mensen was veel mogelijk. Velen hebben mij geholpen om deze droom in vervulling te doen gaan!

Tussen droom en daad staat in de eerste plaats mijn promotor Prof. Dr. Paul De Vos, zonder wie ik nooit zover gekomen was. Ook al heeft mijn doctoraat af en toe aan een zijden draadje gehangen, jij bent mij steeds blijven steunen en je bent steeds in mij blijven geloven!! Bedankt voor de professionele bemoediging, de kritische verbeteringen, de constructieve gesprekken en de gekregen zelfstandigheid tijdens mijn doctoraat, zeker tijdens de eindspurt! Voor diezelfde zaken moet ik ook mijn co-promotor Prof. Dr. Nico Boon bedanken. Jouw enthousiaste en leerrijke toelichtingen en de - altijd zeer snelle - kritische verbeteringen van papers zal ik niet snel vergeten.

Finishing my PhD has almost fully preoccupied my mind for at least the last 6 months. During these months I read, re-read and adapted my manuscript over and over again. So I am very grateful to the Examination Committee for the helpful final suggestions, constructive remarks and fruitful discussion that helped me mould my manuscript into the shape it has righ now.

Tussen droom en daad staan nog mensen die allemaal op hun eigen manier hebben bijgedragen tot dit werk. Het lot van een assistent is veel mensen zien toekomen en veel mensen weer zien gaan, maar is ook fijne banden opbouwen met de anciens. In de eerste plaats wil ik dan ook iedereen van onze 'PDV' groep bedanken voor hulp bij allerlei en om de ambiance er in te houden. Liesbeth, Emly en An, de zotte dozen van de groep ☺, merci voor de leuke sfeer en de soms niet-zo-figuurlijke schouder. Caroline, Jeroen en Stefanie – al lang gevlogen, maar niet vergeten - en Joke, merci voor jullie hulp met allerlei praktische labo-zaken. En ondanks dat we na de verhuis naar de 2^{e} fase – en uiteindelijk zelfs 3^{e} fase – allemaal wat versnipperd zaten in verschillende lokalen, was het altijd leuk om eens tegen elkaar 'te zagen' ☺.
Bureaugenootjes, zonder jullie zou ik me verveeld hebben alleen in mijn kot, al was de productiviteit soms toch wat lager bij volle bezetting.... ☺. Jonas en Sven, de 'PDV-tweeling', wees maar zeker dat de 'Ines-app' ook op jullie kon rekenen! Jullie wisten me trouwens ook altijd op te beuren met allerhande domme mopjes, filmpjes, filosofieën en soms diepgaande discussies… merci daarvoor! Joanna, mijn tweede bureau-maatje, merci voor alle fun (en soms ook kommer en kwel ;-)) die samengaat met het delen van een bureau! Bram en Helen, het laatste jaar heb ik véél aan jullie gehad (professioneel en minder professioneel), hele dikke dankjewel daarvoor en veel succes met het laatste jaar van jullie doctoraat!
Maar er zijn nog meer mensen die hun steentje bijgedragen hebben tot dit doctoraat en de fijne werksfeer en zonder wie alles veel trager en moeilijker zou verlopen zijn… Jeanine – merci voor de jarenlange service als stock-, afwas- en nieuwtjes-lady; Margo en Bart H. – heel erg bedankt voor de vaak ad-hoc hulp bij problemen allerhande; Maarten – mijn practicumbuddy, merci om mee een beetje orde te scheppen in de materiaalchaos die het practicum op het gelijkvloers was en om de miserie van inplanning van de begeleiding mee op te vangen, zeker de laatste keer; Isabel – merci om de laatste loodjes tijdens het indienproces samen door te maken (vooral bij elkaar dubbel-checken bleek belangrijk ☺); en nog zoveel anderen – bedankt!!

Mijn familie en vrienden wil ik bedanken om te zorgen voor de nodige afleiding en het amusement wanneer ik het nodig had en een dikke merci voor het luisterende oor wanneer ik daar echt nood aan had. Delphine, Pieternel en Hilde, er is zoveel waar ik jullie voor zou moeten bedanken… het voelt goed te weten dat ik steeds bij jullie terecht kan! Love you girlz! En vanaf nu zeg ik op alle voorstellen 'ja'! ☺

Tussen droom en daad ligt een hele weg en deze weg zou er nooit gekomen zijn zonder de hulp van twee héél belangrijke mensen die mij erg nauw aan het hart liggen. Zonder hen zou ik hier nooit gestaan hebben. Bedankt

mama en papa om mij al die jaren te steunen, bedankt voor jullie geduld, jullie liefde, en vooral, jullie steun en aanmoedigingen!! Nick, al was mijn doctoraat iets ver-van-je-bed, toch mercikes om gewoon altijd je nuchtere zelf en mijn lieve broer te blijven.

Een speciaal plaatsje in mijn hart en bijgevolg ook in dit dankwoord is er voor mijn kleine Julieke. Veel sneller dan verwacht werd je werkelijkheid! En ondanks de chronische oorontstekingen en de bijhorende korte nachten de laatste twee-en-half jaar, kon je me steeds doen stralen met je lieve glimlach en je fonkelende oogjes en deed je me vaak even de dagelijkse labobeslommeringen vergeten!

Tot slot wil ik mijn tweede liefste schat bedanken… Aan het begin van mijn doctoraat gingen we met zijn tweeën heel onbezorgd door het leven ☺. Stilaan zijn we de laatste jaren volwassen geworden met al wat er voor ons bijhoorde: samenwonen, een huisje kopen, trouwen, een kindje maken. En nu het beëindigen van mijn doctoraat. Lieve schat, Dominique, merci om samen met mij deze droom en hopelijk nog vele andere waar te maken. Bedankt om het leven naast de wetenschap ook de moeite waard te maken! Bedankt om me tot rust te laten komen. Of toch een verdienstelijke poging gedaan hebt tot…☺ Deze droom maak ik ook een beetje waar voor ons drietjes…

All things are difficult before they are easy – Dr. Thomas Fuller

Ines, oktober 2014

List of acronyms

A

AA	amino acid
AAI	average amino acid identity
AFLP	amplified fragment length polymorphism
AL	alkaline lysis
amoA	ammonium monooxygenase
ATP	adenosine tri phosphate

B

BCCM/LMG	Belgian co-ordinated collections of microorganisms/Laboratory of Microbiology Ghent university
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin

C

cbaA	gene encoding nitric oxide reductase qCu_ANOR
cd1-NiR	*cd1*-containing nitrite reductase
cNOR	nitric oxide reductase accepting electrons from heme *c*
cnorB	gene encoding nitric oxide reductase cNOR
CO_2	carbon dioxide
CuNiR	copper-containing nitrite reductase
cycA	gene encoding cytochrome c554
cytP	gene encoding cytochrome c' alpha
cytS	gene encoding cytochrome c' beta

D

DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate reduction to ammonium
DNR	dissimilatory nitrate reducers/reduction
dNTP	deoxynucleotide triphosphate

E

EcoRI	restriction endonuclease enzyme isolated from strains of *E. coli*
EDTA	ethhylenediaminetetraacetic acid
EMBL	European molecular biology laboratory

F

FASTA	fast all
fdp	gene encoding flavo-di-iron protein

G

g	gram or g-force
GC	gas chromatography
GES	guanidiniumthyocyanate EDTA sarkosyl

H

H_2	hydrogen gas
hao	hydroxylamine oxidoreductase
HCO	heme copper oxidases
HGT	horizontal gene transfer
hh	hydrazine hydrolase
His	histidine
hzo	hydrazine oxidizing enzyme
hzs	hydrazine synthase

K

KCl	potassium chloride

L

LMG	laboratory for microbiology, Ghent University

M

MEGA	molecular evolutionary genetics analysis
ML	maximum likelihood
µL	microliter
mL	milliliter
µM	micromolar
µmol	micromole
mM	millimolar
MM	mineral medium
mMM	modified mineral medium
mmol	millimole
MPN	most probable number
MseI	restriction endonuclease enzyme isolated from *Micrococcus* species

N

N	nitrogen
N_2	nitrogen gas or dinitrogen
N_2O	nitrous oxide
N_2OR	nitrous oxide reductase
NaOH	sodium hydroxide
n/a	not applicable
n/d	not determined
Nap	periplasmic nitrate reductase
napA(B)	genes encoding periplasmic nitrate reductase (Nap)
$NaPO_3$	sodium phosphate buffer
Nar	membrane-bound nitrate reductase
narG	gene encoding membrane-bound nitrate reductase (Nar)
nas	nitrate reductase (assimilatory)
nBLAST	nucleotide BLAST
NCBI	national center for biotechnology information
NEB	New England Biolab
NH_4^+	ammonium
nifH	nitrogenase
NiR	nitrite reductase
nirB	nitrite reductase (assimilatory)
nirK	gene encoding copper-containing nitrite reductase (CuNiR)
nirS	gene encoding *cd1*-containing nitrite reductase (*Cd1*-NiR)
NJ	neighbor joining
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
nod	gene encoding nitric oxide dismutase
NOR	nitric oxide reductase
N_2OR	nitrous oxide reductase
norVW	gene encoding flavorubredoxin
nosZ	gene encoding nitrous oxide reductase (N_2OR)
nrfA	gene encoding dissimilatory nitrite reductase involved in DNRA
nxr	nitrite oxidoreductase
n°	number

O

OD	optical density

P

pBLAST	protein BLAST
PCR	polymerase chain reaction
pH	power of hydrogen
pmol	picomole
PSI-BLAST	Position iterated BLAST

Q

qCu_ANOR	nitric oxide reductase accepting electrons from menaquinol and cytochrome *c*
qNOR	nitric oxide reductase accepting electrons from quinols
qnorB	gene encoding nitric oxide reductase accepting electrons from quinols (qNOR)

R

R-	research
RAPD	random amplified polymorphic DNA
RAxML	randomized axelerated maximum likelihood
RDP	ribosomal database project
rpm	rounds per minute

S

sec	secretion
s.l.	*sensu lato*
s.s.	*sensu stricto*
SDS	sodium dodecyl sulphate
sNOR	synonym for qCu_ANOR

T

Ta	annealing temperature
TAE	buffer solution containing Tris base, acetic acid and EDTA
TAT	twin-arginine translocation
TCD	thermal conductivity detector
T_{DEN}	number of bacteria capable of denitrification
tDNA	template DNA
T_{NR}	number of bacteria capable of nitrate reduction
TSA	tryptone soya agar
TSB	tryptone soya broth

U

UPGMA	unweighted pair group method with arithmetic averages
UV	ultra violet

V

vol	volume
VitB12	vitamin B12

W

WAG	Whelan and Goldman substitution model
WAG+G+I	WAG substitution model with gamma-distributed rate variation & site variation
WAG+G+I+F	WAG + G + I with observed amino acid frequencies
WGS	whole genome sequencing
wt	weight
16S rRNA	16S ribosomal ribonucleic acid
6-FAM	6-Fluorescein Amidite

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Part I

Introduction and summary

Preamble

Nitrogen (N) is a common element in the Universe: it is estimated to be the seventh most abundant element in our galaxy and solar system. On Earth, the element is primarily found as a gas molecule with 80% of Earth's atmosphere made up of nitrogen gas (N_2). Nitrogen is essential for all organisms, primarily for the synthesis of amino acids (thus proteins) and of nucleic acids (thus DNA and RNA), the two most important polymers of life. As a results, organisms – big and small – need and use fixed nitrogen for growth and energy, making them part of the global nitrogen cycle.

The nitrogen cycle describes the movement of nitrogen from the air, into the biosphere and organic compounds, and then back into the atmosphere. For the past 10.000 years the planet's environment and its nitrogen cycle have been very stable. Nitrogen compounds were exchanged within the cycle by the numerous (micro-) organisms on our planet, sustaining a balanced situation between fixed nitrogen and atmospheric nitrogen. Over the past two centuries however, increased human activities have become an important driver of global environmental change. Nowadays, we are all aware of the rapidly growing reliance on fossil fuels and its effect on the carbon cycle, more specifically on rising carbon dioxide (CO_2) emissions. However, the growing global demand for food and the use of fertilizer in industrial forms of agriculture, has also disrupted the nitrogen cycle. Hence, anthropogenic activities have caused not only increasing atmospheric CO_2-concentrations that add to the greenhouse gas effect, but also have caused global nitrogen pollution leading to (i) hypoxia and eutrophication in water ways and coastal ecosystems, (ii) accumulation of nitrogen in land systems, and (iii) increased atmospheric concentrations of nitrous oxide (N_2O), one of the most important non-CO_2 greenhouse gases. In short, excess reactive nitrogen threatens the quality of air, soil and water and affects ecosystems, biodiversity and the delicate equilibrium of greenhouse gases.

Bacteria that use nitrogen compounds for energy and growth live in all kinds of environments all over our planet. The denitrifier guild of these populations is responsible for nitrogen-removal in wastewater treatment and nitrogen-loss in agriculture, but also for production of N_2O. However, the past two decades, in-depth characterization of denitrifiers has mainly been performed on a small group of Gram-negative bacteria. Genes of Gram-positive denitrifiers are not detected in environmental surveys, enzyme topology and their associated electron transfer pathways are not well comprehended and their phenotype - and environmental factors and regulatory features influencing this phenotype – are not well understood. Hence, it is unknown to what extent Gram-positive bacteria may contribute to denitrification in certain environments. Elucidating this may allow scientists to better model nitrogen losses and nitrous oxide emissions from e.g. soils.

Scope & outline

The research was performed during the course of a junior assistantship at the Laboratory of Microbiology (LM-UGent), Faculty of Sciences, Ghent University. Central research topics of this lab are diversity, classification and identification of a wide variety of bacteria, including food-associated, animal- and human-associated and environmental strains.

This thesis examined the microbial processes of denitrification – and to a lesser extent – dissimilatory nitrate reduction to ammonium (DNRA) in Gram-positive bacteria belonging to the *Bacillaceae*. It has been assumed that *Bacillaceae* may contribute to denitrification and DNRA in terrestrial ecosystems, but phenotypic and molecular tools lack comprehensiveness and cannot identify the contribution of this group of bacteria in these processes. Thus, the present research aimed at (i) evaluation of the information available on Gram-positive denitrification and emphasizing that this group of bacteria is understudied because of specific obstacles hampering accurate phenotypic and molecular detection, (ii) revealing the potential importance of members of the genus *Bacillus* in the denitrification process and the influence of the growth medium on detection of the trait, (iii) development of primers for *nirK*, *qnorB* and *nosZ* genes, encoding enzymes involved in the denitrification process of Gram-positive bacteria, with focus on *Geobacillus*, (iv) disclosing the incidence of these uncharted denitrification genes in a set of denitrifying *Bacillus* and *Geobacillus* strains and, (v) genetic investigation of the co-occurrence of genes involved in denitrification and DNRA in a *Bacillus* strain set and the potential associated environmental implications.

A general introduction and a summary to this work can be found in **Part I** – **Background** and **Summary/Samenvatting**. The literature overview (**Part II – Chapter 1**) presents an overall introduction to the process of denitrification in Gram-positive bacteria highlighting the knowledge gaps with respect to physiology, molecular detection and general importance of this group of bacteria. The experimental work performed can be found in **Part III**. The presented data in this part is divided over the following chapters:

Chapter 2 – An isolation campaign from luvisol soil revealed that *Bacillus* spp. were one of the most abundant retrieved denitrifiers next to members of the *Rhizobiaceae* family and the genus *Cupriavidus*. Additional screening of 180 representatives of the genus *Bacillus* for dissimilatory reduction of nitrate or nitrite and the influence of different electron donors and acceptors on the denitrification phenotype was performed. The latter revealed the potential for denitrification in

many *Bacillus* species and differences in phenotypes depending on the culture conditions. These results indicated that bacilli may be potential important contributors to denitrification processes in soils and possibly other ecosystems.

Chapter 3 – Sequence comparison of primer target sites with frequently used primers in environmental monitoring exposed their general unsuitability to pick up denitrification genes in particular in Gram-positive bacteria. Hence, novel primers for denitrifiers of the family of Gram-positive *Bacillaceae* were developed by genomic mining and tested on phylogenetically closely related geobacilli. To deal with both genetic as well as phenotypic variation in this *Geobacillus* strain panel, AFLP (Amplification Fragment Length Polymorphism) fingerprints and phenotypic denitrification aptitude in complex growth media and liquid defined growth medium were evaluated in parallel with functional gene sequence analyses. The successful primer design led to unique *nirK, qnorB* and *nosZ* type sequences detected in all strains, however *nosZ* was absent in some strains, which was in agreement with phenotypic testing. This study showed that closely related Gram-positive denitrifiers may differ in denitrification phenotype and genotype. But foremost, novel primers targeting very divergent *nirK*, *qnorB* and *nosZ* gene sequences of Gram-positive denitrifiers are now available for cultivation-independent environmental surveys.

Chapter 4 – PCR screening of pure culture denitrifying *Bacillaceae*, with the focus on members of the genus *Bacillus,* with frequently used primers and novel primers for *nirK*, *qnorB* and *nosZ* revealed the preference for distinct variants of *nirK*, *qnorB* and *nosZ* genes by sequence and phylogenetic analyses of the retrieved genes, as observed for *Geobacillus*. However, amplification of *nirK* and *qnorB* was difficult, suggesting the presence of other mechanisms for nitrite reduction and N_2O and/or N_2 production in *Bacillus*. Additional PCR screening for *nrfA* as a proxy for DNRA (Dissimilatory Nitrate Reduction to Ammonium) and screening with newly developed primers for *cbaA*, the gene encoding qCu_ANOR and for which no primers were yet available, revealed the presence of *cbaA* and *nrfA* in many strains, often together with the presence of *qnorB* and *nosZ*. This observation may explain the reduction of nitrate to N_2O (with acetylene inhibition of N_2O reduction to N_2) in complex and defined medium for strains described in Chapter 2 and warrants further in-depth characterization of these microorganisms.

At the end of each chapter hindsight reflections on the work performed are included, emphasizing a number of aspects that – with current knowledge and understanding – would have improved the research performed. Interesting lines of further research are also included in these sections. General conclusions of and remarks on the work performed are given in **Part IV – Chapter 5.**

Summary

Nitrogen – in all its forms – is an important element in the global nitrogen cycle. Due to human activities such as intense agriculture, excess reactive nitrogen is added to the environment leading to global environmental changes, such as (i) nitrogen pollution of water and land ecosystems, and (ii) rising nitrous oxide (N_2O) concentrations in the atmosphere. Denitrifiers are microorganisms that can remove this nitrogen, both with beneficial and detrimental effects, but that – unfortunately – can also produce the greenhouse gas N_2O. Gram-positive denitrifiers have been understudied the last two decades and since Gram-positive bacteria constitute a major group of soil microbiological communities, it is important to (i) understand their contribution to denitrification in the environment, (ii) recognize the environmental factors influencing their phenotype and, (ii) know how to detect these organisms and their potential activities in terrestrial habitats.

This thesis focused on Gram-positive denitrifiers mainly belonging to the genera *Bacillus* and *Geobacillus*, which have been implied in denitrification in a wide variety of environments, such as soils, rhizospheres, waste water treatment plants, composting plant material, hot springs… etc. Although a number of Gram-positive strains have been reported to be 'denitrifiers', comprehensive characterization of all aspects of denitrification has been focused on a few Proteobacterial Gram-negative bacteria. This includes their contribution to denitrification in certain ecosystems, the responsible genes and available molecular tools to detect the latter, their enzymes and their regulation, and environmental drivers that influence their phenotype. Since the build-up of the bacterial cell wall is markedly different between Gram-negative and Gram-positive bacteria, also the enzyme organization of the involved reductases and their encoding gene sequences ought to be different and responses to changing environmental conditions may differ.

Within the frame of this PhD thesis, initially, information on Gram-positive denitrifiers and the methodology used for assessment of the denitrification ability was evaluated. The research revealed that this group of bacteria is underexplored because of specific obstacles hampering accurate phenotypic and molecular detection of denitrification and that many gaps exist in knowledge on Gram-positive denitrifiers with respect to physiology, molecular detection and general importance of this group of bacteria. Secondly, the potential importance of *Bacillaceae*, with focus on *Bacillus*, in the denitrification process in soils and possible other environments was investigated. Results showed that members of the genus *Bacillus* may be indeed important soil denitrifiers or

nitrate ammonifiers and that strains can display different phenotypes depending on the test conditions - either variation in electron donor, electron acceptor or other parameters – and thus can possible remain unidentified as potential denitrifying strains when one single medium is applied. Thirdly, the lack of primers targeting Gram-positive denitrification genes presented an ideal opportunity to develop novel primers for *nirK*, *qnorB* and *nosZ* genes involved in the denitrification process in Gram-positive denitrifiers. For the latter, research was focused on members of the genus *Geobacillus* since they are also very abundant soil inhabitants and hence presented a suited target for assessment of the novel primers. The results revealed that Gram-positive denitrifiers of the genus *Geobacillus* harbor very divergent *nirK*, *qnorB* and *nosZ* gene sequences and established primers have been excluding this group of bacteria when used in PCR-based microbial community analyses. This finding has implications for ecology studies since these genes are frequently used as molecular markers for the detection and quantification of denitrification. As a result, quantifications of denitrification genes are likely to be underestimates. In addition, a PCR-screening of pure culture denitrifying *Bacillaceae*, with the focus on members of the genus *Bacillus*, with frequently used primers and novel primers for genes involved in denitrification and improved primers for DNRA (Dissimilatory Nitrogen Reduction to Ammonium) was performed. The results showed the remarkable co-occurrence of genes involved in denitrification and DNRA in a number of strains, highlighting the flexible physiology of various members of the genus *Bacillus* regarding dissimilatory nitrate reducing processes and its intermediates or by-products. These observations may have possible environmental implications regarding N_2O emissions, especially in environments with a high abundance of members of this genus. In addition, the unsuccessful use of degenerate primers designed for denitrification genes of *Geobacillus*, a genus closely related to *Bacillus*, suggest that broad-range primers for genes involved in dissimilatory nitrate reduction processes are probably unlikely ever to be succesfully developed, even for specific phyla (e.g. *Bacillaceae*) or specific groups of phylogenetically related gene sequences.

In conclusion, studying the potential contributions of denitrifying members of the Gram-positive genera *Bacillus* and *Geobacillus* and the presence of genes involved in denitrification and DNRA in these genera has brought us somewhat closer to understanding the possible importance of Gram-positive bacteria in N-cycling, especially for denitrification and – to a lesser extent – nitrate ammonification or DNRA. However, many aspects need to be further investigated. Complementary with in-depth studies that thoroughly characterize reference strains, it also appears to be useful to screen many strains in parallel for desired features such as denitrification and DNRA and this to further investigate the functional redundancy and diversity over different taxa, amongst which the *Bacillaceae*.

Samenvatting

Stikstof – in al zijn vormen – is een belangrijk element in de wereldwijde stikstofcyclus. Menselijke activiteiten zoals intensieve landbouw dragen bij aan de overmaat aan reactieve stikstof die zo terecht komt in het milieu. Dit leidt tot wereldwijde milieuvervuiling, zoals verontreiniging van aquatische en terrestrische ecosystemen met allerhande stikstofhoudende verbindingen en stijgende concentraties aan distikstofmonoxide (N_2O) in onze atmosfeer. Denitrificeerders zijn micro-organismen die dit stikstof kunnen verwijderen, zowel met positieve als negatieve effecten, maar die jammer genoeg ook het broeikasgas N_2O kunnen produceren. Gram-positieve denitrificeerders zijn de laatste twintig jaar amper bestudeerd en omdat Gram-positieve bacteriën een groot deel uitmaken van microbiële gemeenschappen in de bodem, is het belangrijk om (i) hun bijdrage aan denitrificatie in het milieu te begrijpen, (ii) de omgevingsfactoren die hun fenotype beïnvloeden te herkennen en (iii) om te weten hoe ze samen met hun mogelijke activiteit kunnen gedetecteerd worden in de natuur.

In deze thesis werden vooral Gram-positieve denitrificeerders, voornamelijk behorend tot de genera *Bacillus* en *Geobacillus*, bestudeerd. Van deze genera is er van verschillende leden beschreven dat ze betrokken zouden zijn in denitrificatie en dit in een waaier aan omgevingen, zoals in de bodem, in de rhizosfeer, in waterzuiveringsinstallaties, in composterend plantaardig materiaal, in heetwaterbronnen… enz. Niettegenstaande dat er dus reeds een aantal 'denitrificerende' Gram-positieve stammen werden gerapporteerd, is grondige karakterisering van alle aspecten van denitrificatie nog altijd gericht op een beperkt aantal Gram-negatieve *Proteobacteria*. En dit gaat van hun bijdrage aan denitrificatie in bepaalde ecosystemen, de verantwoordelijke genen en de beschikbare moleculaire tools om deze te detecteren, hun enzymen en de regulatie van deze enzymen, tot de omgevingsfactoren die hun fenotype beïnvloeden. Omdat de opbouw van de bacteriële celwand opvallend verschillend is tussen Gram-negatieve en Gram-positieve bacteriën, kan het niet anders dat ook de enzym organisatie van de betrokken reductases en hun coderende gensequenties anders zijn en dat reacties op fluctuerende omgevingsfactoren zullen verschillen.

Binnen het kader van dit doctoraatsonderzoek werd initieel de beschikbare informatie rond Gram-positieve denitrificeerders en de methodologie die gebruikt wordt voor de beoordeling van de denitrificatie capaciteit geëvalueerd. Het onderzoek toonde aan dat deze groep van bacteriën niet voldoende is onderzocht door specifieke hindernissen die accurate fenotypische en moleculaire

detectie van denitrificatie bemoeilijken. Bovendien is er onvoldoende kennis omtrent Gram-positieve denitrificeerders, zowel op fysiologisch als moleculair vlak en op vlak van algemeen belang van deze groep van bacteriën. Ten tweede werd het belang van de *Bacillaceae*, met de focus op *Bacillus*, voor het denitrificatieproces in bodem en mogelijks andere omgevingen onderzocht. De resultaten suggereerden dat leden van het genus *Bacillus* inderdaad belangrijke denitrificeerders of DNRA bacteriën in bodem kunnen zijn en dat stammen verschillende fenotypes kunnen vertonen afhankelijk van de omstandigheden waaronder ze getest worden – hetzij variatie in elektrondonor, de elektronacceptor of andere parameters – en dus mogelijks niet opgepikt worden als potentieel denitrificerende stammen bij het gebruik van één enkel groeimedium. Ten derde, het gebrek aan primers voor denitrificatiegenen van Gram-positieve bacteriën leek ons de ideale kans om nieuwe primers te ontwikkelen voor *nirK, qnorB* en *nosZ* genen betrokken in het denitrificatieproces. Voor dat laatste gedeelte werd de nadruk gelegd op leden van het genus *Geobacillus*. Wegens hun hoge abundantie in bodem beschouwden we leden van dit genus als een geschikt doelwit voor de beoordeling van de nieuwe primers. De resultaten toonden aan dat Gram-positieve denitrificeerders van het genus *Geobacillus* erg divergente *nirK, qnorB* en *nosZ* gensequenties bevatten en dat frequent gebruikte primers uit de literatuur deze groep van bacteriën niet detecteert als ze gebruikt worden voor PCR-gebaseerde analyse van microbiële gemeenschappen. Deze bevinding heeft implicaties voor ecologische studies, want deze genen worden frequent gebruikt als moleculaire merkers voor denitrificatie en heeft als resultaat dat bijvoorbeeld de kwantificering van denitrificatiegenen vaak de realiteit onderschat. Daarnaast werd ook een PCR-screening uitgevoerd op reinculturen van denitrificerende *Bacillaceae*, met focus op *Bacillus*. En dit met vaak gebruikte primers en nieuwe primers voor genen betrokken in denitrificatie en DNRA (Dissimilatieve Nitraat Reductie naar Ammonium). In verschillende stammen werden genen voor denitrificatie en DNRA vaak samen aangetroffen. Dit onderstreept de veelzijdige fysiologie van verschillende leden van het genus *Bacillus* wat betreft dissimilatieve nitraat reducerende processen en de bijbehorende intermediaire componenten of nevenproducten. Deze observaties impliceren de potentiële invloed op ons milieu wat betreft N_2O emissies, zeker in omgevingen waar leden van dit genus talrijk zijn. De gedegenereerde primers ontworpen voor de denitrificatie genen van *Geobacillus*, een genus dat nauw verwant is aan *Bacillus*, waren niet succesvol waardoor het er op lijkt dat de ontwikkeling van primers met een breed fylogenetisch bereik moeilijk is, zelfs voor specifieke fyla (vb. de *Bacillaceae*) of specifieke groepen van fylogenetisch gerelateerde gensequenties.

Om te besluiten: het bestuderen van de potentiële bijdrage aan denitrificatie door Gram-positieve bacteriën behorend tot de genera *Bacillus* en *Geobacillus* en van de aanwezigheid van de genen betrokken in denitrificatie en DNRA, is een stap voorwaarts voor het begrijpen van het

mogelijk belang van Gram-positieve bacteriën in de stikstofcyclus, in het bijzonder voor denitrificatie en – in mindere mate – nitraat ammonificatie of DNRA. Veel aspecten moeten echter nog grondig bestudeerd worden. Complementair aan diepgaand onderzoek dat een aantal referentiestammen nauwgezet karakteriseert, lijkt het tevens nuttig om voldoende verschillende stammen in parallel te screenen op zoek naar de gewenste metabolische kenmerken zoals denitrificatie en DNRA en dit om de functionele redundantie en diversiteit over verschillende taxa, waaronder de *Bacillaceae*, verder te kunnen onderzoeken.

Part II

Literature overview

Redrafted from:

Verbaendert, I., and De Vos, P. (2011) Studying denitrification by aerobic endospore-forming bacteria in soil. In Endospore-forming soil bacteria. Logan, N.A., and De Vos, P. (eds): Springer, pp. 271-285.

Verbaendert, I., De Vos, P., Boon, N., and Heylen, K. (2011a) Denitrification in Gram-positive bacteria: an underexplored trait. Biochemical Society Transactions 39: 254-258.

Author's contributions:

PDV and IV proofread and commented on the manuscript for the book section. IV and KH wrote and PDV, NB, KH and IV proofread and commented on the manuscript for Biochemical Society Transactions.

Chapter 1

Denitrification in Gram-positive bacteria

1.1 Denitrification

1.1.1 Definition of denitrification

Denitrification is one of the major processes in the global nitrogen cycle (Fig. 1. 2) and refers to the dissimilatory reduction of nitrates (NO_3^-) or nitrites (NO_2^-) over nitric oxide (NO) to nitrous oxide (N_2O) or nitrogen gas (N_2) allowing respiration (Conrado & Stuart, 1998) in low oxygen or anoxic environments (Gayon & Dupetit, 1886, Payne, 1981). Electrons (e^-) are transferred to the oxidized nitrogen compounds – that are used as alternative electron acceptors – creating an electrochemical gradient along the cytoplasmic membrane. Energy is conserved, like in aerobic respiration, via the generation of a proton motive force across the bacterial membrane driving ATP (Adenosine Tri Phosphate) synthesis. A series of sequentially expressed metalloproteins that can be found in the periplasm and cytoplasmic membrane (also see section 1.1.6, Fig. 1. 4), known as NO_3^--, NO_2^--, NO- and N_2O- reductase and encoded by specific genes, catalyze the process (Fig. 1. 1).

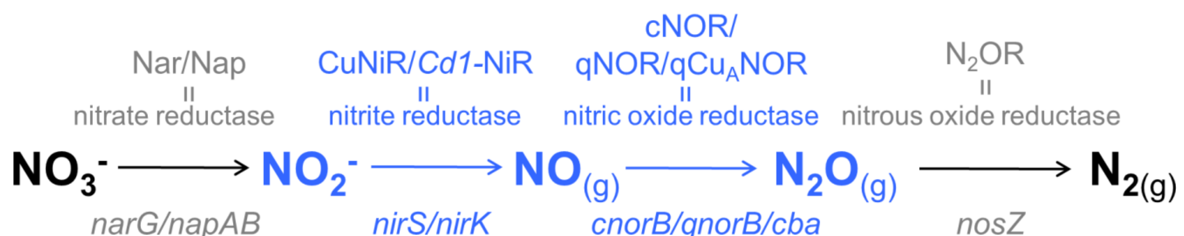


Fig. 1. 1 Sequential transformations from nitrate (NO_3^-) to nitrogen gas (N_2) are catalyzed by proteins encoded by specific genes. NO, N_2O and N_2 are marked with (g), indicating the gaseous nature of these compounds. Abbreviations: Nar, membrane bound nitrate reductase encoded by *narG*; Nap, periplasmic nitrate reductase encoded by *napAB*; CuNiR, copper-containing nitrite reductase encoded by *nirK*; *Cd1*-NiR, cytochrome *cd1*-containing nitrite reductase encoded by *nirS*; cNOR, nitric oxide reductase accepting electrons from heme c and encoded by *cnorB*; qNOR, nitric oxide reductase accepting electrons from quinols and encoded by *qnorB*; qCu_ANOR, nitric oxide reductase from *Bacillus azotoformans* NCCB 100003 encoded by *cba*; N_2OR, nitrous oxide reductase encoded by *nosZ.* Denitrification *sensu stricto* has been indicated in blue.

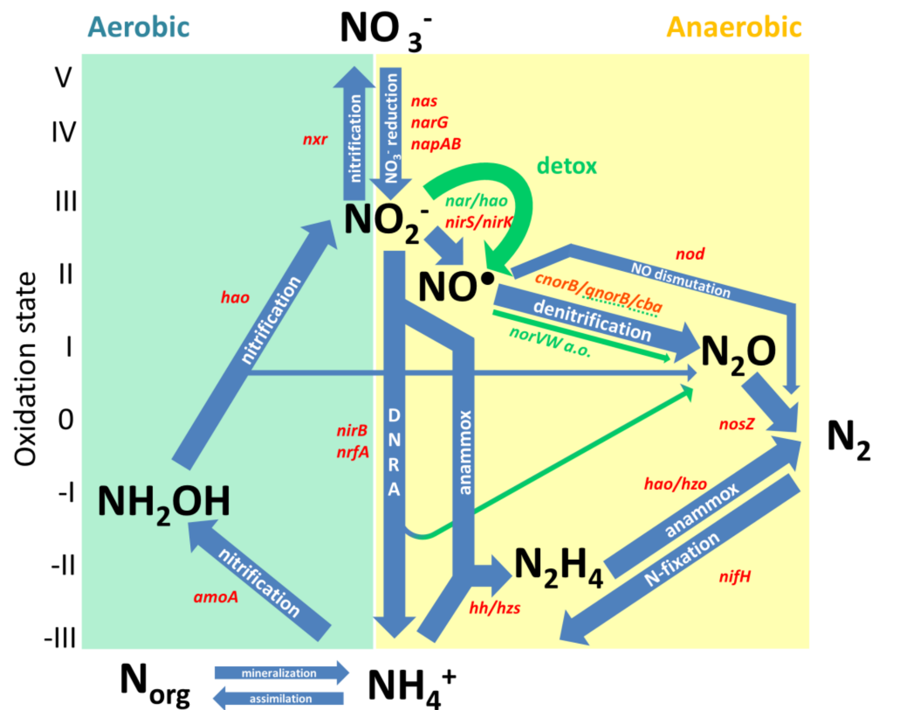
In this facultative and respiratory mechanism, NO_2^-- reductase (NiR) and NO reductase (NOR) are the enzymes that perform the conversion of fixed nitrogen into gaseous nitrogen that is released into the atmosphere. Therefore NO_2^-- and NO- respiration, the involved reductases and corresponding genes are considered to be key elements of denitrification. This part of the process is also called denitrification *sensu stricto* (Tiedje, 1988, Zumft, 1997) (Fig. 1. 1). As pre-genomic evidence revealed, partial denitrification does occur in bacteria, e.g. starting denitrification from NO_2^- (Ettwig *et al.*, 2010) or ending it at N_2O (Denariaz *et al.*, 1989, Shoun *et al.*, 1998), and – with the introduction of genome sequencing – appears to be widespread (Shapleigh, 2013). These truncated versions of the denitrification pathway, makes defining a bacterium as a denitrifier sometimes difficult. In addition, in response to environmental factors such as temperature, oxygen, partial pressure and pH (Wallenstein *et al.*, 2006, Bergaust *et al.*, 2010) variable quantities of intermediates (NO_2^-, NO, N_2O) may accumulate.

1.1.2 Significance of denitrification

The interest in microbial denitrification exists for several reasons. Firstly, it is known as a beneficial process (i) in the natural removal of excess reactive nitrogen (NO_3^-), especially in riparian buffer zones of rivers and streams and during subsoil denitrification of NO_3^- leached from agricultural soils (Addy *et al.*, 1999, Martin *et al.*, 1999), (ii) in the conversion of ammonium to N_2 by coupled nitrification/denitrification (Maeda *et al.*, 2011), (iii) in wastewater treatment from which anthropogenic discharged nitrogen is effectively removed (Choi *et al.*, 2002, Rajakumar *et al.*, 2008, Kampschreur *et al.*, 2009, Park & Yoo, 2009), and (iv) in the anoxic degradation of other organic pollutants, such as hydrocarbons derived from petroleum (naphthalene, benzene, toluene, phenol, etc…) or heavy metals (Lovley & Lonergan, 1990, Galushko *et al.*, 1999, Coates *et al.*, 2001, Maugeri *et al.*, 2002, Liang *et al.*, 2007, Park *et al.*, 2007), and can thus provides important ecosystem services for the environment.

Secondly, it is a process with important environmental implications. It is a detrimental process for agriculture since it causes nitrogen depletion in (rural) soils and loss of fertilizer nitrogen (Mulvaney *et al.*, 1997, Philippot *et al.*, 2007), in its turn leading to excess use of nitrogen-containing fertilizer in agricultural activities. Much of this nitrogen enters the environment, polluting ground and surface waters with nitrate (Rockström *et al.*, 2009, Canfield *et al.*, 2010). It is also a mechanism that adds N_2O to the atmosphere, where N_2O is involved in the stratospheric degradation of ozone and acts as a potent greenhouse gas, with a global warming potential 298 times that of carbon dioxide (CO_2)(IPCC, 2013). Because nitrification also produces N_2O as an intermediate (Fig. 1. 2), the contribution of waste water systems (Prendez & Lara-Gonzalez, 2008, Kampschreur *et al.*, 2009) and agricultural soils (Environmental Protection Agency, 2010) (Mosier *et al.*, 1998) to rising

concentrations of atmospheric N_2O is of particular concern. Although the agricultural impact of denitrification continues to be an important topic in research efforts, focus has essentially shifted towards the environmental consequences of denitrification with the associated molecular and regulatory mechanisms of the process (Shapleigh, 2013).



Denitrification and/or DNRA
narG nitrate reductase
membrane- bound – respiratory (denitrification)
napAB nitrate reductase
periplasmic – respiratory/dissimilatory (denitrification/DNRA)
nrfA nitrite reductase
membrane-bound – respiratory (DNRA)
nirS/nirK nitrite reductase
periplasmic – respiratory (denitrification)
cnorB nitric oxide reductase
membrane-bound – respiratory (denitrification)
qnorB nitric oxide reductase
membrane-bound – quinol-dependent & respiratory (denitrification)
cba (or qCu_ANor/sNor) nitric oxide reductase
membrane-bound – menaquinol/cyt c-dependent & respiratory (denitrification)
nosZ nitrous oxide reductase
periplasmic – respiratory (denitrification and other N_2O reducers)
nod nitric oxide dismutase
membrane-bound (?) – respiratory (methanotrophic denitrifiers)

Detoxification
qnorB nitric oxide reductase
membrane-bound – quinol-dependent & dissimilatory
cba (or qCu_ANor/sNor) nitric oxide reductase
membrane-bound – menaquinol/cyt c-dependent & dissimilatory
norVW a.o. flavorubredoxin
cytoplasmic – dissimilatory
a.o. = Fdp flavo-di-iron proteins
CycA cytochrome c554
CytS cytochrome *c'*- beta
CytP cytochrome *c'* - alpha

Anammox
hh/hzs hydrazine hydrolase/hydrazine synthase
hao/hzo hydroxylamine oxidoreductase/hydrazine-oxidizing enzyme
nifH nitrogenase

Nitrification
amoA ammonium monooxygenase
hao hydroxylamine oxidoreductase
nxr nitrite oxidoreductase

N-assimilation
nas nitrate reductase
nirB nitrite reductase

N_{org}
Amino acids, N-containing carbohydrates, amino sugars, nucleic acids, purine and pyrimidine bases, vitamins,...etc.

Fig. 1. 2 Major pathways of the prokaryotic N-cycle. Major biological transformation pathways with their associated enzymes and genes, based on (Canfield *et al.*, 2010, Kraft *et al.*, 2011, Stein & Klotz, 2011, Simon & Klotz, 2013). Shown are the various microbial processes that respire, assimilate or detoxify nitrogenous compounds. The name of each process is indicated. The caption for processes important to this thesis have been particularized.

Thirdly, denitrification is one of the main microbial processes in the global nitrogen cycle, in which, together with the anammox process, it closes the cycle by returning N_2 to the atmosphere. However, it became clear in the past two decades that the so-called nitrogen cycle - comprised of different pathways - is actually more like a network (Kraft *et al.*, 2011) and more modular than previously thought (Simon & Klotz, 2013) (Fig. 1. 2). Horizontal gene transfer (HGT) complicates assignment of genes to a particular process or organism (Heylen *et al.*, 2007, Jones *et al.*, 2008) and multiple evolutionary unrelated enzymes convert the same molecule in different processes, e.g. NO reduction to N_2O in denitrification, in detoxification and in NO dismutation (Simon & Klotz, 2013). Moreover, as mentioned before, anthropogenic activities of the last decades have an impact on denitrification rates and occurrence and therefore the global nitrogen cycle. The on-going perturbation of the N-cycle is altering biogeochemical processes of natural ecosystems, their trophic dynamics and biological diversity (Elser *et al.*, 2009) and active intervention and vigilant management strategies are warranted (Rockström *et al.*, 2009, Canfield *et al.*, 2010).

1.1.3 Bacterial denitrifiers

Since its discovery in 1886 by Gayon & Dupetit, the ability to use nitrogen oxides as alternative electron acceptors has been described in a broad variety of physiological and taxonomic groups, ranging from *Bacteria* and *Archaea* (Shapleigh, 2006) to *Eukaryota* as fungi (Shoun & Tanimoto, 1991) and foraminifers (Risgaard-Petersen *et al.*, 2006), creating the potential for denitrification in a wide spectrum of habitats and ecosystems.

The work in this thesis focuses on bacteria, which can be divided into two major groups: Gram-negative and Gram-positive bacteria. The original distinction between them was based on the Gram-stain, a staining procedure that visualizes differences in cell wall structure (Fig. 1. 3).

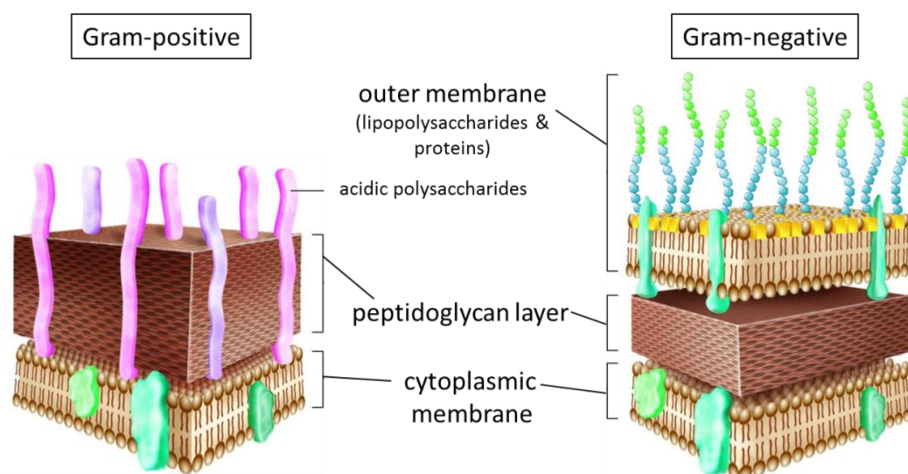


Fig. 1. 3 Gram-positive and Gram-negative bacterial cell walls. Adapted from figure on http://biology-forums.com.

Gram-negative bacteria have a multi-layered cell wall consisting of an outer membrane (with proteins and lipopolysaccharides), a periplasm containing a thin layer of peptidoglycan and the cytoplasmic membrane. Gram-positive bacteria have a much thicker cell wall of which almost 90% is peptidoglycan and a cytoplasmic membrane. Most of the characterized bacterial denitrifiers belong to the phylum *Proteobacteria*. This is a group of Gram-negative microorganisms that is subdivided in six classes (alpha-, beta-, gamma-, delta-, epsilon- and zeta-*Proteobacteria*) of which only the zeta-class has not been reported to harbor denitrifiers. However, the work in this thesis focuses specifically on Gram-positive denitrifiers that belong to the endospore-forming genera *Bacillus* and *Geobacillus*.

Ferdinand Cohn, a German botanist, was fascinated by heat-resistant forms of bacteria. He described the process of endospore formation and renamed the organism "*Vibrio subtilis*" (Ehrenberg 1835) as *Bacillus subtilis* (1872). This species was the first member of the very large and diverse genus that is part of the phylum *Firmicutes* and the family *Bacillaceae*. In contrast, the genus *Geobacillus* was proposed in the year 2001 by Nazina and co-workers (Nazina *et al.*, 2001). However, several strains of this group of microorganisms were already, on the basis of 16S rRNA gene sequence information, described as belonging to *Bacillus* group V (Ash *et al.*, 1991). *Bacillus* (now *Geobacillus*) *stearothermophilus*, a well-known and extensively studied thermophilic organism, became the type species of this new genus and since then a number of related thermophilic species have been transferred to and described within the genus *Geobacillus* (Nazina *et al.*, 2001, Sung *et al.*, 2002, Banat *et al.*, 2004, Schäffer *et al.*, 2004). The *Bacillaceae* family's salient characteristic is the production of endospores, which are formed within bacterial cells by a process called sporulation, and may be oval, spherical or cylindrical. These spores can remain dormant for exceptionally long periods, and are extremely heat-resistant and resistant to other physical (e.g. desiccation and UV-radiation) and various chemical (e.g. acids and disinfectants) agents. Endospores are easily detected using a phase contrast microscope, because of their highly refractile nature, as well as with a mere Gram-stain, because the spore remains unstained while the vegetative cells or the vegetative parts of the cells will stain (Slepecky & Hemphill, 2006).

Thus, the genera *Bacillus* and *Geobacillus* belong to the endospore-forming, low-GC Gram-positive *Bacillaceae*. These rod-shaped organisms follow the biological cycle of spore-formers, namely from vegetative cell to spore and from spore to vegetative cell through a very complex series of events in cellular differentiation. Although most endospore-forming bacteria seem to share a common ecological characteristic (*i.e.* being associated with [agricultural] soils), they form a phylogenetically heterogeneous group within the *Firmicutes*. Within this phylum, the family *Bacillaceae* encompasses the strictly aerobic or facultatively anaerobic endospore-formers that were, until the early 1990s, accommodated within the single genus *Bacillus*. Since then, a major taxonomic

reshuffling has taken place, reflecting the vast variety in (1) physiology, (2) ecology, (3) genetics, (4) morphology (mainly in the size and position of the endospore within the vegetative cell), (5) nutrition, and (6) growth characteristics (Madigan et al. 2003). As a consequence, the genus *Bacillus sensu stricto* is now limited to the members phylogenetically closest to *Bacillus subtilis* (the type species) and a few others that are awaiting reclassification. The genus *Geobacillus,* on the other hand, is limited to strains phylogenetically closest to *Geobacillus stearothermophilus*.

1.1.4 Energetic aspects of denitrification

From a theoretical, thermodynamic point of view all denitrification reduction steps and the overall denitrification reaction allow a sufficiently large change of free energy to be coupled to electron transport phosphorylation (Table 1. 1), the production of a proton motive force and thus the generation of ATP (Zumft, 1997, van Spanning *et al.*, 2007). However, enzymatically, it seems that not all steps are indeed electrogenic (see section 1.1.6).

Table 1. 1 Single reactions of complete denitrification.
Adapted from (Gottschalk, 1985, Zumft, 1997, Suharti *et al.*, 2001, Philippot *et al.*, 2007, Stein *et al.*, 2007).

Separate reactions	**$\Delta G_0'$ (for each reaction)**	**Enzymes**	**Genes encoding catalytic subunits**
(a) $2\ NO_3^- + 4\ e^- + 4\ H^+ \rightarrow 2\ NO_2^- + 2\ H_2O$	-161.1 kJ/mol	Membrane-bound nitrate reductase: Nar	*narGHI*
		Periplasmic nitrate reductase: Nap	*napAB*
(b) $2\ NO_2^- + 2\ e^- + 4\ H^+ \rightarrow 2\ NO + 2\ H_2O$	-76.2 kJ/mol	Cu nitrite reductase: CuNiR	*nirK*
		Cd1 nitrite reductase: *Cd1*-NiR	*nirS*
(c) $2\ NO + 2\ e^- + 2\ H^+ \rightarrow N_2O + H_2O$	-306.3 kJ/mol	Cytochrome *c* nitric oxide reductase: cNOR	*cnorB*
		Quinol nitric oxide reductase: qNOR	*qnorB*
		qCu_ANOR/sNOR	*cbaA*
(d) $N_2O + 2\ e^- + 2\ H^+ \rightarrow N_2 + H_2O$	-339.5 kJ/mol	Nitrous oxide reductase: N_2OR	*nosZ*
Overall reaction			
$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$	-2,670 kJ/mol	n/a[a]	n/a

[a]n/a, not applicable

In addition, until recently, it was assumed that microorganisms could only be capable of one single anaerobic respiration process, either denitrification, DNRA (Dissimilatory Nitrate Reduction to Ammonium, also called nitrate ammonification), anammox or NO dismutation (Simon & Klotz, 2013). However, it seems that within some bacteria both denitrification and DNRA are processes that may be competing for nitrate or nitrite available in the environment. Nevertheless, so far only three *Bacillus* strains (Heylen & Keltjens, 2012, Mania *et al.*, 2014), *Opitutus terrae* strain PB90-1, *Marivirga tractuosa* DSM 4126 and *Shewanella loihica* PV-4 (Sanford *et al.*, 2012) and *Wolinella succinogenes* (Simon *et al.*, 2004) have been reported to have (parts of) the particular gene inventory for both. But from a bioenergetical perspective, the questions rise then (i) when and why would an organism favor one of the processes and/or (ii) are they maybe intertwined?

Respiration of NO_3^- to N_2 (denitrification) or to ammonium (DNRA) are the highest yielding respiratory energy processes (Gottschalk, 1985, Strohm *et al.*, 2007) after oxygen respiration.

Calculation and comparison of the free energy of oxygen respiration ($\Delta G_0'$ = -2,870 kJ/mol glucose), denitrification ($\Delta G_0'$ = -2,670 kJ/mol glucose) and DNRA ($\Delta G_0'$ = -1,870 kJ/ mol glucose) points to a higher amount of free energy for denitrification versus DNRA. On the basis of these calculations, one would expect that ATP generation, and thus growth yields, in denitrification would come close to that of aerobic respiration, whereas DNRA would generate less ATP, and thus lower growth yields. However, growth yield measurements showed that ATP synthesis in denitrification was far lower than expected from calculated free energy changes and even lower than in DNRA (Strohm *et al.*, 2007), as expected by Smith and Zimmerman (Smith & Zimmerman, 1981). Moreover, although denitrification theoretically provides microorganisms with more energy, the preferred process in anoxic environments with limited nitrate supply and strongly reducing conditions, appears to be DNRA (Bleakley & Tiedje, 1982, Tiedje *et al.*, 1982, Tiedje, 1988). Under these conditions a shortage of electron acceptors is most likely limiting microbial growth and DNRA has the advantage over denitrification that more electrons can be transferred per mole NO_3^- (Tiedje *et al.*, 1982, Rütting *et al.*, 2011). However, there is evidence that DNRA is not a strictly anaerobic process but can also occur in the presence of O_2 (Morley & Baggs, 2010, Rütting *et al.*, 2011), whereas completely anoxic environments may be more advantageous for denitrification (Baggs, 2011). Hence, the environmental conditions under which DNRA or denitrification within the same organism is preferred still warrants further experimental research.

1.1.5 Genes involved in denitrification

As can be understood from above (Fig. 1. 1, Fig. 1. 2, Table 1. 1), the collection of reductases involved in denitrification may be encoded by different types of genes and may sometimes have different localizations and biochemical features.

Two distinct classes of nitrate reductases exist (also see section 1.1.6): the membrane-bound nitrate reductase (Nar) encoded by the *nar* gene cluster *narGHI* and the periplasmic-located nitrate reductase (Nap) encoded by the *nap* gene cluster *napEDABC*.

Also two distinct classes of nitrite reductases exist: cytochrome *cd1*-containing nitrite reductase (*cd1*-NiR) encoded by *nirS*, whereas *nirK* encodes for the copper-containing nitrite reductase (CuNiR). Up till now there is no organism identified that harbors both classes of nitrite reductase. *NirK* sequences are more heterogeneous than *nirS* sequences. Based on sequence similarity, *nirK* may be divided into at least two subtypes, namely: (1) group I *nirK*, probably encoding a soluble periplasmic protein, that is most frequently detected in denitrifiers (Jones *et al.*, 2008) and, (2) group II *nirK*, probably encoding outer-membrane lipoproteins and carrying an additional domain at the N-terminus (Nojiri *et al.*, 2009). However, a third *nirK* variant has been revealed by database searches as well (Philippot, 2002, Ellis *et al.*, 2007). The fact that group I *nirK* seems to be most frequently

detected in denitrifiers, is mostly due to limitations in the detection process that relies on the available primers (see section 1.3.2).

The nitric oxide reductases cNOR and qNOR are encoded by *cnorB* and *qnorB* and the recently revealed *cbaA* genes that encode for qCu_ANOR (Fig. 1. 1, Table 1. 1). Thus far, the *cnorB* gene seems to be detected almost only in denitrifiers, whereas *qnorB*-encoded enzymes may also play a role in detoxifying processes, since they are often present in bacteria that do not denitrify. The functional role of *qnorB* and *cba* encoded enzymes – and especially qCu_ANOR – or their ecological importance has not been studied comprenhensively and their structural and functional characterization is an interesting target for further research.

Nitrous oxide reductase is encoded by *nosZ* of which three genetic variants have been described. Typical 'true denitrifier' *nosZ* (Z-type) and atypical *nosZ* (A-type) both have distinctive regulatory and functional components and encode for a 'TAT-dependent N_2OR' and a 'Sec-dependent N_2OR', respectively. The latter protein form lacks the recognizable twin-arginine translocation (TAT) signal at the N-terminus (Sanford *et al.*, 2012, Pauleta *et al.*, 2013) and its features still remain largely unresolved. Non-denitrifying bacteria that have a more diverse N-metabolism, such as nitrate ammonifiers and bacteria missing *nirK*/*nirS* (Sanford *et al.*, 2012, Mania *et al.*, 2014), may also harbor this atypical *nosZ* as a protective mechanism against the toxic effects of N_2O (Sullivan *et al.*, 2013) and may thus act as a sink of N_2O in natural ecosystems. However, the ability to reduce N_2O has so far only been detected in very few nitrate ammonifying strains (Simon *et al.*, 2004, Sanford *et al.*, 2012, Mania *et al.*, 2014) and is thought to be rare in organisms performing DNRA. Recent analyses revealed that atypical *nosZ* genes outnumber typical *nosZ* genes in soil metagenomes (Orellana *et al.*, 2014) and suggested niche differentiation or even competitive interactions between organisms with different *nosZ* types (Jones *et al.*, 2014), nevertheless the exact functional or ecological role of this atypical *nosZ* and associated enzyme still needs clarification. The nitrate ammonifying *Wollinella succinogenes* possesses an H-type N_2O reductase (Zumft & Körner, 2007).

With the advent of whole genome sequencing (WGS) it is becoming clear that denitrification genes are scattered among diverse groups of microorganisms and that microbial genomes do not always contain the full set of genes, but often only have one or two of the denitrification modules present. Consequently, horizontal gene transfer (HGT) seems to have played an important role in the exchange of denitrification genes between strains. HGT describes the unique ability of bacteria to acquire DNA from their surroundings, either the environment or their neighbors, and incorporate it into their genome. The mechanisms of HGT include (i) transformation, in which 'raw' DNA is taken up from the environment, (ii) conjugation, in which DNA is transferred via cell-cell contact, most often by mobile elements called plasmids, but also by other mobile gene elements (e.g. mobilizable islands, integrative conjugative or transposable elements), (iii) transduction, in which DNA is acquired after

phage-infection, (iv) gene transfer agents that are phage-like DNA-vehicles that are released to the environment and (v) nanotubes, of which the mechanism is still unknown (Popa & Dagan, 2011, Rankin *et al.*, 2011). Acquired DNA from unrelated or distantly related bacterial strains may differ in nucleotide composition (e.g. GC-content) and/or the preferential use of one of the several codons that encode the same amino acid (codon usage). However, there is no definite proof for HGT of the denitrification genes since – in most cases – the genes do not reside on apparent mobile elements nor do they have anomalous codon usage or GC-content (Jones *et al.*, 2008, Shapleigh, 2013).

1.1.6 Enzymes encoded by denitrification genes

A number of similarities and differences in the organization of the involved enzymes in denitrification exist or are predicted between Gram-negative and Gram-positive denitrifiers.

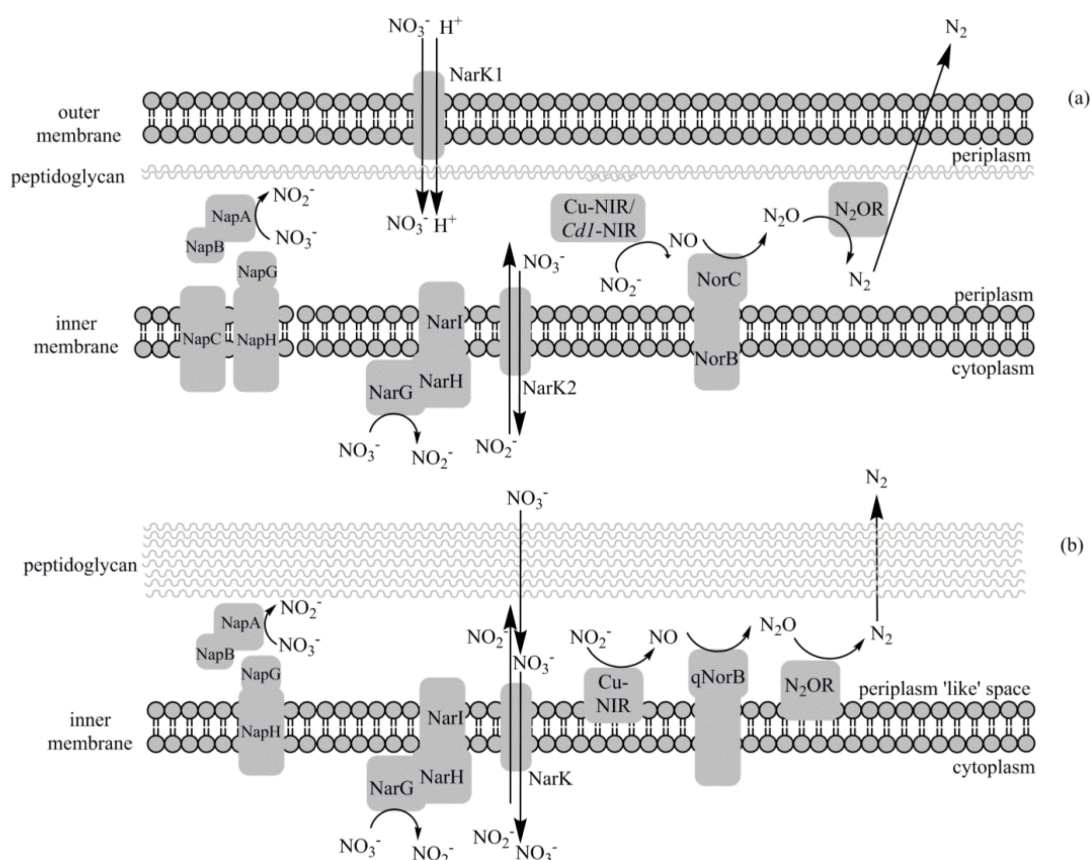


Fig. 1. 4 Bacterial enzymes involved in denitrification. In (a) Gram-negative bacteria (model organism: *Pseudomonas stutzeri*) and (b) Gram-positive bacteria (model organisms: *Bacillus azotoformans*, *Bacillus bataviensis*, *Geobacillus thermodenitrificans, Geobacillus kaustophilus*). Electron transport is omitted for clarity, as well as the nitric oxide dismutase enzyme complex for Gram-negative denitrifying methanotrophs and the possibility of both cNorB and qNorB as possible nitric oxide reductases in Gram-negative denitrifiers. Adapted from (Suharti & de Vries, 2005, Kraft *et al.*, 2011, Simon & Klotz, 2013) with info from (Denariaz *et al.*, 1991, Urata & Satoh, 1991, Ho *et al.*, 1993, Feng *et al.*, 2007, Fukuda *et al.*, 2011, Heylen & Keltjens, 2012)

The most notable difference being that Gram-positive bacteria lack the periplasmic compartment and that all enzymes have to be membrane-bound, either by presenting as integral membrane proteins, by association with membrane-bound enzymes or by lipid-attachment (Suharti & de Vries,

2005, Heylen & Keltjens, 2012)(Fig. 1. 4).In both groups, nitrate is transported to the cytoplasm via transmembrane transporters NarK1 (a nitrate and proton uniporter) and/or NarK2 (nitrate/nitrite antiporter) (Feng *et al.*, 2007, Kraft *et al.*, 2011, Heylen & Keltjens, 2012, Simon & Klotz, 2013) in oxygen-low or anoxic conditions and in the presence of nitrate.

In Gram-negative bacteria, respiratory nitrate reductase complexes involved in denitrification may belong to the Nar- or the Nap- type (Fig. 1. 4, a). The membrane-bound nitrate reductase (Nar) consists of three subunits. The α-subunit (NarG) and β-subunit (NarH) form a complex that is attached to the cytoplasmic side of the membrane by the γ-subunit or membrane anchor NarI. The α-subunit contains a [4Fe-4S] cluster and harbors the catalytic site of nitrate reduction (Kraft *et al.*, 2011). Electrons are transferred to NarG and next to nitrate in the cytoplasm, usually via ubiquinol located in the membrane (Richardson *et al.*, 2007). For each pair of electrons two protons (H^+) are translocated across the membrane, hence this three-subunit enzyme complex is involved in the generation of ATP (Chen & Strous, 2013). Alternatively, the periplasmic-located nitrate reductase (Nap) consists of two periplasmic subunits, NapA and NapB. The [4Fe-4S] cluster resides in NapA, which is the catalytic subunit. NapA forms a periplasmic complex with the smaller subunit NapB that receives electrons via membrane-bound NapC, NapH and NapG and transfers them to NapA. However, the latter enzyme complex is most likely not involved in ATP generation and is probably implicated in nitrogen assimilation and/or nitrate-dependent regeneration of quinone from quinol and thus redox homeostasis of the cells (Kim *et al.*, 2012, Shapleigh, 2013, Simon & Klotz, 2013). However, some denitrifiers do only possess Nap for their nitrate reduction (Delgado *et al.*, 2003). The genomes of Gram-positive *Bacillus azotoformans*, *Bacillus bataviensis* and *Geobacillus thermodenitrificans* all encode for the membrane-bound NarGHI (Suharti & de Vries, 2005, Feng *et al.*, 2007, Heylen & Keltjens, 2012), but the *Bacillus azotoformans* genome also encodes for a periplasmic NapAB complex (combined with NapH/NapG) (Heylen & Keltjens, 2012) (Fig. 1. 4, b). It has been suggested that presence of both enzyme complexes may point to different functions within the cell: energy conservation with Nar and redox homeostasis, transition of oxic to anoxic conditions or aerobic denitrification with Nap (Zumft, 1997, Hartsock & Shapleigh, 2011, Kraft *et al.*, 2011). However, this needs to be substantiated for Gram-positive bacteria.

Subsequently, nitrite is transported into the periplasm by NarK and the periplasmic copper-containing (CuNiR) or cytochrome *cd1*-containing nitrite reductase (*Cd1*-NiR) catalyzes reduction of nitrite into nitric oxide in Gram-negative bacteria. CuNiR contains two distinct Cu-centers, a type 1 and a type 2 Cu-center (Rinaldo & Cutruzzola, 2007). Nitrite is bound to the type 2 Cu-center and is reduced to NO with electrons delivered from a.o. *c*-type cytochromes via the type 1 Cu-center. Next to its main product NO, CuNiR can also produce small amounts (i.e. 3 to 6%) of N_2O if NO accumulates (Rinaldo & Cutruzzola, 2007). Each subunit of *Cd1*-NiR contains heme *c* and a unique

heme *d1*. Nitrite is bound to the heme *d1* and is reduced to NO with electrons delivered via heme *c* from *c*-type cytochromes. In *Bacillus firmus*, *Bacillus azotoformans*, *Geobacillus thermodenitrificans*, *Geobacillus stearothermophilus*, *Geobacillus kaustophilus*, *Geobacillus thermoglucosidasius* and *Virgibacillus halodenitrificans,* a membrane-bound copper-containing nitrite reductase (Denariaz *et al.*, 1991, Urata & Satoh, 1991, Ho *et al.*, 1993, Suharti & de Vries, 2005, Feng *et al.*, 2007, Fukuda *et al.*, 2011, Heylen & Keltjens, 2012) is involved in nitrite reduction. And although some *nirS* genes have been PCR-amplified from Gram-positive denitrifiers, it seems that Gram-positive denitrifiers favor the Cu-type nitrite reductase (Heylen & Keltjens, 2012, Verbaendert *et al.*, 2014). In both Gram-positive as Gram-negative bacteria, Cu-NiR and *cd1*-NiR probably do not to contribute to a proton motive force directly because of their periplasmic or periplasmic-oriented localization (Chen & Strous, 2013, Simon & Klotz, 2013).

Nitric oxide is then reduced to nitrous oxide by nitric oxide reductases which are integral membrane proteins with the active site in the periplasm. Diverse enzyme complexes involved in nitric oxide reduction are present in the N-cycle (Fig. 1. 2). The enzymes involved in denitrification are phylogenetically related to cytochrome oxidases, more specifically the heme copper oxidases (HCO). They can be divided in three distinct groups: (1) cNOR, in which the NorB subunit spans the cytoplasmic membrane and the NorC subunit forms the periplasmic catalytic site (Fig. 1. 4, a), and, that accepts electrons from e.g. cytochrome *c*, (2) qNOR, with a similar primary structure to cNOR, but actually a single subunit enzyme, that accept electrons from quinols (Fig. 1. 4, b), and (3) qCu_ANOR that contains a Cu_A site and may accept electrons from both menaquinol as cytochrome c_{551}, and has also been labeled sNOR in various nitrifiers (Stein *et al.*, 2007, Heylen & Keltjens, 2012). However, other versions of NOR that differ in their critical residues have been reported, such as gNOR (Sievert *et al.*, 2008). Neither of these nitric oxide reductases are experimentally proven to be involved in ATP generation (Chen & Strous, 2013), however, involvement of qNOR in production of a proton motive force cannot be excluded (Matsumoto *et al.*, 2012, Salomonsson *et al.*, 2012, Simon & Klotz, 2013). A novel nitric oxide transforming enzyme, NO dismutase, has recently been discovered in anaerobic denitrifying methanotrophs, producing N_2 and O_2 directly from NO, but also does not seem to be directly involved in ATP production (Ettwig *et al.*, 2010, Chen & Strous, 2013, Simon & Klotz, 2013). So far, cNOR has only been found in denitrifying bacteria, whereas qNOR is found in both denitrifying and non-denitrifying pathogenic bacteria. However, although some *cnorB* genes have been PCR-amplified from Gram-positive denitrifiers, it seems that Gram-positive denitrifiers favor quinol-dependent NOR (Heylen & Keltjens, 2012).

As a last step, reduction of nitrous oxide to dinitrogen is catalyzed by nitrous oxide reductase (N_2OR). This soluble enzyme resides in the periplasm in Gram-negative bacteria, but in Gram-positive bacteria it has to be associated with the membrane by a lipid anchor while facing the periplasm

(Suharti & de Vries, 2005, Heylen & Keltjens, 2012), like in Archaea. The N_2OR enzyme contains two copper centers, Cu_A and Cu_Z, in which the latter contains the catalytic site and the [4Cu-4S] cluster where N_2O binds (Zumft & Kroneck, 2007, Pauleta *et al.*, 2013). Cu_A is the entering site for electrons. The Z-type N_2OR receives electrons from cytochrome *c* whereas the A-type N_2OR receives electrons from a still unknown carrier (Zumft & Körner, 2007). The reduction of N_2O to N_2 by N_2OR is reported to not be electrogenic and hence does not generate ATP (Chen & Strous, 2013, Simon & Klotz, 2013). However, since the cytochrome bc_1 complex is involved in electron transfer to the Z-type N_2OR, coupling of proton transfer across the cytoplasmic membrane may be possible (Tavares *et al.*, 2006, Zumft & Körner, 2007, Kraft *et al.*, 2011). In addition, *Anaeromyxobacter* spp. N_2OR has been implicated in energy conservation and may function with greater efficiency than the respiratory machinery of a full-fledged denitrifier (Sanford *et al.*, 2012).

Bioenergetically, all denitrification enzymes have been reported to depend on the presence of quinones/quinols and cytochromes *b* and/or *c* for electron transport and [Fe-S] or [Cu-S] centers are obligatory to the majority of the enzymes. It seems though that only the Nar enzymes have been experimentally proven to couple substrate conversion directly to the generation of ATP via the electron transport chain, although NapAB, qNOR and N_2OR may also be involved in the generation of a proton motive force or energy (Matsumoto *et al.*, 2012, Salomonsson *et al.*, 2012, Simon & Klotz, 2013).

1.1.7 Nitrate ammonification or DNRA, a competitive pathway for denitrification

As mentioned above, dissmilatory nitrate reduction to ammonium (DNRA) or ammonification is a process that competes with denitrification for the use of the electron acceptors nitrate or nitrite present in the environment. The process reduces nitrate to nitrite by means of membrane-bound nitrate reductase Nar (catalytic subunit encoded by the *narG* gene) or periplasmic nitrate reductase Nap (catalytic subunit encoded by the *napA* gene). Nitrite is then further reduced to ammonium (NH_4^+) by the nitrite reductase NrfA (encoded by the *nrfA* gene) that is located in the periplasm in Gram-negative bacteria and is bound to the cytoplasmic membrane, but facing the periplasm, in Gram-positive bacteria. Worth mentioning is that althought nitrate reduction is often a prerequisite for subsequent reduction of nitrite, ammonification *sensu stricto* actually only entails the reduction of nitrite to ammonium.

The *nrfA* gene is found in a diverse group of micoorganisms with variable phylogenetic affiliations (Mohan *et al.*, 2004, Smith *et al.*, 2007, Welsh *et al.*, 2014) and has ocassionally been used as a proxy for the presence of DNRA in bacterial strains, mixed cultures or environmental samples (Mohan *et al.*, 2004, Streminska *et al.*, 2012, Welsh *et al.*, 2014). Although the NrfA enzyme has been purified and characterized from a number of different bacteria and nitrate ammonification in

members of the genus *Bacilus* has been repeatedly suggested, reports on the presence and investigation of *nrfA* or the complete DNRA process in Gram-positive bacteria – and in particular in *Firmicutes* and *Bacillaceae* – are rare. Although growing in number due to whole genome sequencing (WGS), the number of available *nrfA* sequences from Gram-positive bacteria is still limited and more *nrfA* sequences should be obtained from isolates that have been confirmed as nitrate ammonifying strains. The limited range of available *nrfA* genotypes has its repercussions on the development of 'universal' molecular tools for the detection of *nrfA* and their use in environmental surveys, similar as for the denitrification genes (Mohan *et al.*, 2004, Kraft *et al.*, 2011, Welsh *et al.*, 2014)(see 1.3.2).

1.2 Diversity of Gram-positive denitrifiers

As mentioned above, the work in this thesis focuses specifically on Gram-positive denitrifiers that belong to the endospore-forming genera *Bacillus* and *Geobacillus*. However, in the last two decades, in-depth biochemical and molecular denitrification research of bacteria mainly focused on Gram-negative bacteria, while mostly overlooking Gram-positive denitrifiers. Information on which Gram-positive organisms are mutually responsible for the denitrification activities observed in certain systems is still virtually non-existent.

Since denitrification is a widespread trait among prokaryotes, some authors have tried to record described denitrifying bacterial species. In 1981, William J. Payne (Payne, 1981) listed a considerable number of genera that contain denitrifying members. One of these genera was the Gram-positive genus *Bacillus*, with denitrifying strains belonging to *Bacillus licheniformis*, *Bacillus azotoformans* and *Bacillus* (now *Geobacillus*) *stearothermophilus*. A decade later Zumft (Zumft, 1992) compiled a list of almost 130 denitrifying bacterial species, however the majority of them being Gram-negative bacteria. Gram-positive denitrifying bacterial genera included in this survey are now members of *Bacillus*, *Brevibacillus*, *Geobacillus*, *Paenibacillus*, *Virgibacillus*, *Sporosarcina*, *Corynebacterium*, *Gemella*, *Jonesia*, *Propionibacterium* and *Tsukamurella*. Shapleigh (2006) also assembled a list of Gram-positive species that were suggested to contain denitrifying strains. He added genera described by Shoun *et al*. (Shoun *et al.*, 1998) and the genus *Frankia* to Zumft's list. In 2007, Philippot and co-workers (Philippot *et al.*, 2007) also included *Paenibacillus terrae*. Similarly, Table 1. 2 is an updated heuristic annotated list of strains that denitrify or were once claimed to, the way denitrification was assessed and current status of their denitrification ability.

Since Gram-positives, other than *Bacillus,* were classically considered as not containing true denitrifiers (Tiedje, 1988), it is no surprise that the majority of the publications on Gram-positive denitrifying taxa focuses on members of the *Firmicutes*, the majority being *Bacillales*. For instance, Table 1. 2 contains several denitrifying strains belonging the genus *Bacillus*, such as strains of *Bacillus*

stearothermophilus (now *Geobacillus*) (Garcia, 1977b), *Bacillus licheniformis* (Pichinoty *et al.*, 1978), *Bacillus circulans* and *Bacillus cereus* (Manucharova *et al.*, 2000), *Bacillus* (now *Geobacillus*) *thermodenitrificans* (Manachini *et al.*, 2000, Feng *et al.*, 2007) and strains that now belong to the genus *Paenibacillus* (de Barjac & Bonnefoi, 1972). The *Actinomycetales* are a second group in which a wide variety of Gram-positive denitrifying bacteria have been found, with a large proportion of members of the genus *Streptomyces* (Shoun *et al.*, 1998, Chèneby *et al.*, 2000). In addition, a small number of non-spore-forming denitrifying Gram-positive bacteria have been reported as well, such as strains belonging to *Corynebacterium* (Flores-Mireles *et al.*, 2007) and *Enterococcus* (Heylen *et al.*, 2006). Besides description of the phenotypic denitrifying capacities of strains belonging to the *Bacillales* and *Actinomycetales,* Gram-positive denitrification reductases have also been described e.g. in *Bacillus* (now *Virgibacillus*) *halodenitrificans* (Denariaz *et al.*, 1991), *Bacillus firmus* (Urata & Satoh, 1991) and *Bacillus azotoformans* (Suharti *et al.*, 2001) (Table 1. 2). As evident from Table 1. 2 various Gram-positive species and strains are/were thought to denitrify, but in general the denitrification trait, enzymes and genes in Gram-positive bacteria remain underexplored.

1.3 Reasons why denitrification in Gram-positive bacteria is overlooked

Although the denitrifying capacity of Firmicute isolates has long been recognized (Table 1. 2), (1) most published data on Firmicute denitrifiers is rather old, (2) only very few denitrifying endospore-formers have so far been phenotypically, biochemically and genomically characterized as true denitrifiers, and, (3) limited reliable information is available on the occurrence and distribution of the trait and responsible enzymes and genes within this group of microorganisms. More importantly, accurate phenotypic and molecular detection of denitrification in Gram-positive bacteria has been and is still being hampered by specific obstacles.

1.3.1 Phenotypic detection of denitrification

In general practice, NO_3^- or NO_2^- is added to tubes or serum vials containing inoculated defined (Stanier *et al.*, 1966, Fazzolari *et al.*, 1990, Bergaust *et al.*, 2008, Bergaust *et al.*, 2011) or complex (Pichinoty *et al.*, 1976, Pichinoty *et al.*, 1978, Smith & Zimmerman, 1981, Bleakley & Tiedje, 1982, Fazzolari *et al.*, 1990, Mahne & Tiedje, 1995, Ishii *et al.*, 2011) liquid growth medium. The most commonly used concentrations of NO_3^- or NO_2^- in literature roughly vary from <1 to 10mM

Table 1. 2 Overview of Gram-positive bacteria once claimed to denitrify and current status of their denitrification capacity.

This table is an updated heuristic annotated list of strains that denitrify or were once claimed to, the way denitrification was assessed and current status of their denitrification ability. Denitrification status is given as √, denitrification; -, no denitrification; ?, not clear

Species	Denitrification assessment	Current knowledge	Status
(a) (Endo) spore-forming Gram-positive bacteria: *Firmicutes*, *Bacillales*			
Anoxybacillus pushinoensis (Yamamoto et al., 2006) (AT-1 & AT-2)	Presence of NO_3^- was tested using a modified Griess reagent, gas samples from the head space were analyzed with gas chromatography. Observations: ability to reduce NO_3^- and NO_2^- , >99% of the composition of the gases was N_2.	More recent literature not available.	√
Bacillus azotoformans (Pichinoty et al., 1976, Pichinoty et al., 1983) (CIP R925)	Isolation by N_2O enrichment, NO_3^-, NO_2^- and N_2O are used anaerobically, considerable quantities of N_2 are produced during reduction of NO_3^-, NO_2^- and N_2O, NO_3^- reductase, NO_2^- reductase, NO-reductase and N_2O reductase are present. Methods are not mentioned.	Purification and biochemical characterization of qCu_ANOR of *Bacillus azotoformans* (NCCB 100003); all denitrification genes were described in 2012 (Suharti *et al.*, 2001, Suharti *et al.*, 2004, Suharti & de Vries, 2005, Heylen & Keltjens, 2012).	√
Bacillus cereus (Hackenthal, 1966) (ATCC 8035)	Biochemical comparison of enzymatic biosynthesis was performed. Observation: appearance of NO_3^- and NO_2^- reductase activity in cell suspensions from cells of anaerobic cultures.	Maximal denitrifying activity of strains isolated from soddy podzolic soil has been determined by gas chromatographic analysis of N_2O evolved from NO_3^- with the acetylene inhibition method (AIM) (Manucharova *et al.*, 2000). *Bacillus cereus* (PK-5) is involved in aerobic denitrification with conversion of NO_3^- to N_2 (Kim *et al.*, 2005).	√
Bacillus circulans (Manucharova et al., 2000) (no strain number)	Observation: Maximal denitrifying activity was determined by acetylene inhibition method and gas chromatographic analysis of N_2O evolved from NO_3^-.	More recent literature not available.	√
Bacillus firmus (Urata & Satoh, 1991) (NIAS 237)	Enzyme analysis was performed by cell fractionation and proton translocation measurements. Observation: dissimilatory NO_2^- reductase is located on the cytoplasmic membrane, its reducing side is on the inner side of this membrane.	More recent literature not available.	√
Bacillus licheniformis (Pichinoty *et al.*, 1978) (multiple strains)	NO_2^- was analyzed colorimetrically, identification and measurement of NO, N_2O and N_2 was established by gas chromatography. Enzymatic extracts were used to detect NO_3^- and NO_2^- reductase activity. Observations: NO_3^- and NO reductases enzymes were detected in enzymatic extracts, but not the NO_2^- and N_2O reductases. N_2O and N_2 production from NO_3^- are slow and weak.	*Bacillus licheniformis* (PK-16) is involved in aerobic denitrification with conversion of NO_3^- to N_2 (Kim *et al.*, 2005).	√
"Bacillus nitritollens" (Delaporte, 1972) (multiple strains)	Simple phenotypic testing[b]: Observations: gas is produced under anaerobic conditions and NO_2^- is produced from NO_3^- and it rapidly disappears.	Recent literature not available.	-
Bacillus subtilis (Sakai et al., 1996) (I-41)	Nitrite concentration was studied with ion chromatography, the presence of nitrate was assayed by reducing nitrate with zinc powder. N_2 and N_2O in the gaseous phase were analyzed by gas chromatography. Observations: significant levels of N_2O and N_2 were observed in the gas phase, NO_2^- is reduced.	These *B. subtilis* strains require more detailed descriptions.	√
***Bacillus* sp.**	Denitrification activity was screened for strain R22 after anaerobic growth with NO_3^- as	More recent literature not available.	√

Species	Denitrification assessment	Current knowledge	Status
	terminal electron acceptor, confirmed with AIM and N_2O accumulation measured by gas chromatography. *nirS* (AJ626841) gene sequenced (Goregues *et al.*, 2005). For strain R-13: *nirS* (AF335924) gene sequenced. *nirS* containing strains were tested for their ability to remove NO_3^- and NO_2^- (Lee *et al.*, 2005). For strain TSA4w: *nirS* (AB542306) gene sequenced. For strain R-32546 and R-31856: *nirK* gene sequenced (AM404294 and AM404293). For strains R-33773 (AM778674), R-32656 (AM778673), R-31770 (AM778672), R-31841 (AM778671), R-32702 (AM778670), R-33820 (AM778669), R-32709 (AM778667), R-32715 (AM404295), R-32526 (AM403579) and strain SH3 to SH63 (EU374113 to EU374135): *qnorB* genes sequenced. For strain R-32694: *qnorB* and *cnorB* (AM778668, AM403581) sequenced. Only gene info available on the NCBI website[c]. For strains ULT-41 to ULT-816: probable phenotypic denitrification to N_2 and presence of *nosZ* genes (GU733381-GU733415)		
Brevibacillus ginsengisoli (Baek et al., 2006) (Gsoil 3088^T)	Anaerobic growth in 'denitrifying conditions' only in the presence of NO_3^-, with reduction of NO_3^- to NO_2^-. Only reduction of NO_3^- was analyzed, what is meant with 'denitrifying conditions' is not clear.	More recent literature not available.	-
Brevibacillus laterosporus (de Barjac & Bonnefoi, 1972) (multiple strains)	Simple phenotypic testing[b] was performed. Observations: reduction of NO_3^- to NO_2^- and weak gas formation from NO_3^- in anaerobic conditions.	More recent literature not available.	-
Geobacillus kaustophilus (Takami et al., 2004) (HTA426)	WGS contain *nirK* and *qnorB* sequences, but no *nar* or *nos* genes.	More recent literature not available.	?
***Geobacillus* sp.** (Mishima *et al.*, 2009) (multiple strains)	For strain TDN01: Reduction of NO_3^- and NO_2^- were evaluated using the Griess-Romijn reagent and ion-chromatography, the amount of produced N_2 and N_2O was analyzed by gas chromatography. Observation: production of N_2O and N_2 gas from nitrate. For strain G11MC16: all denitrification genes present in WGS.	For strain TDN01: further functional analysis of this thermophilic denitrifying bacterium was performed (Nara *et al.*, 2009).	√
Geobacillus stearothermophilus (Garcia, 1977a) ($TnBA_1$)	N_2O and N_2 were studied with Warburg respirometry and gas chromatography using AIM, enzyme activity was measured for all nitrogenous reductases. Observations: denitrification from NO_2^- is much faster than from NO_3^- and the process produces N_2O and N_2.	Description of the membrane-bound denitrification enzymes of strain ATCC 12016, except N_2O reductase (Ho *et al.*, 1993). No denitrification in *Geobacillus stearothermophilus* ATCC 12980^T and DSM 22^T (Manachini et al., 2000, Nazina et al., 2001). *nirK* and *qnorB* gene are found in the genome of a non-denitrifying *G. stearothermophilus* strain (Heylen, 2007).	√
Geobacillus subterraneus (Nazina et al., 2001) (strain Sam, strain K, strain 34^T)	Occurrence of denitrification was measured with gas chromatography. Observations: anaerobic growth, reducing NO_3^- and NO_2^- to N_2.	More recent literature not available.	√
Geobacillus thermodenitrificans (Manachini et al., 2000) (DSM 465^T, DSM 466, strains TH6A, TH8A, TH4B, TH45A, TH33A, TH35A, TH51A, TH61A, BI5A, TU6F3)	Reduction of NO_3^- was examined with the Griess reagent. NO_3^- and NO_2^- reduction to gas and anaerobic production of gas from NO_3^-. Methods are not mentioned.	Anaerobic growth of DSM 466, reducing NO_3^- and NO_2^- to N_2 (measurement with gas chromatography) (Nazina *et al.*, 2001). Identification of the genes for a complete denitrification pathway and a	√

Species	Denitrification assessment	Current knowledge	Status
		whole genome sequencing approach yielded a complete novel N_2O reductase gene in strain NG80-2 (Feng *et al.*, 2007), which was functionally characterized (Liu *et al.*, 2008).	
Geobacillus thermoleovorans (Nazina et al., 2001) (DSM 5366[T])	Occurrence of denitrification was measured with gas chromatography. The strain grew anaerobically, reducing NO_3^- to N_2.	No denitrification in strain DSM 5366[T] (Manachini *et al.*, 2000).	√
Kyrpidia tusciae *(*Klenk *et al., 2011)* (DSM 2912)	Presence of *qnorB* gene (CP002017) in WGS.	More recent literature not available.	?
Paenibacillus larvae **subsp.** ***pulvifaciens*** (de Barjac & Bonnefoi, 1972) (multiple strains)	Simple phenotypic testing[b] was performed. Observations: reduction of NO_3^- to NO_2^- and gas formation from NO_3^- in anaerobic conditions.	NO_3^- reduced to NO_2^-, established by API test (Heyndrickx *et al.*, 1996), but actual denitrification capacity is not tested properly yet.	-
Paenibacillus macerans (de Barjac & Bonnefoi, 1972) (multiple strains)	Simple phenotypic testing[b] was performed. Observations: reduction of NO_3^- to NO_2^- and weak gas formation from NO_3^- in anaerobic conditions.	More recent literature not available.	-
Paenibacillus polymyxa (de Barjac & Bonnefoi, 1972) (multiple strains)	Simple phenotypic testing[b] was performed. Observations: reduction of NO_3^- to NO_2^- and weak gas formation from NO_3^- in anaerobic conditions.	Maximal denitrifying activity of strains isolated from soddy podzolic soil has been determined by gas chromatographic analysis of N_2O evolved from NO_3^- with AIM (Manucharova *et al.*, 2000).	√
Paenibacillus **spp.** (Behrendt *et al.*, 2010) (multiple strains)	Reduction of NO_3^- and NO_2^- to N_2 or N_2O under anaerobic culture conditions was measured by helium atmosphere incubation method. Presence of functional genes coding for *nirK*, *nirS* and *nos* was tested.	More recent literature not available.	√
Paenibacillus terrae (Horn et al., 2005) (MH72 & AM141[T])	Reduction of NO_3^- to NO_2^- and gas chromatographic analysis of N_2 and N_2O.	Are the *Paenibacillus terrae* strains fermentative or true denitrifying strains?	?
Sporosarcina pasteurii (de Barjac & Bonnefoi, 1972)	Simple phenotypic testing[b] was performed. Observations: reduction of NO_3^- to NO_2^- and weak gas formation from NO_3^- in anaerobic conditions.	NO_3^- reduction to NO_2^- in *Sporosarcina* (Yoon et al., 2001).	-
Virgibacillus halodenitrificans (Denariaz *et al.*, 1989, Denariaz *et al.*, 1991) (ATCC 49067)	Gas chromatographic analysis of the products of the denitrification reaction was performed. Observations: product of denitrification is N_2O, the isolate lacks N_2O reductase activity and NO_3^- and NO_2^- support anaerobic growth (Denariaz *et al.*, 1989). Purification of a copper-containing NO_2^- reductase (Denariaz *et al.*, 1991).	Growth occurs under anaerobic conditions on marine agar, only in the presence of NO_3^-, which is reduced to NO_2^- (Yoon *et al.*, 2004); no further specification of denitrification.	√
Virgibacillus pantothenticus (de Barjac & Bonnefoi, 1972) (multiple strains)	Simple phenotypic testing[b] was performed. Observations: weak reduction of NO_3^- to NO_2^- and weak gas formation from NO_3^- in anaerobic conditions.	NO_3^- reduction to NO_2^- (API test) (Heyndrickx *et al.*, 1998). Growth occurs under anaerobic conditions on marine agar, only in the presence of NO_3^-, which is reduced to NO_2^- (Yoon *et al.*, 2004); no further specification of denitrification.	-
(b) (Endo) spore-forming Gram-positive bacteria: *Firmicutes*, other			
Desulfitobacterium hafniense (Bouchard *et al.*, 1996, Christiansen & Ahring, 1996) (DCB-2[T] & PCP-1[T])	Only phenotypic reduction of NO_3^- is tested, NO_3^- is reduced to NO_2^-.	Genome encodes the cytochrome *bc*-type NO reductase, *norB* (Zumft, 2005), it reveals a *nosCZ•orf•nosDLFY* cluster and is predicted to encode the Z-type N_2OR of Gram-negative bacteria (Zumft & Kroneck, 2007).	?
Symbiobacterium thermophilum (Ueda et al., 2004) (IAM14863)	WGS contains a *nirK* and *nar* gene, but it lacks *nor* (Shapleigh, 2013).	More recent literature not available.	-
Symbiobacterium toebii (Rhee et al., 2002)	WGS contains a *nar* gene, but it lacks all other denitrification genes (Shapleigh, 2013).	More recent literature not available.	-

Species	Denitrification assessment	Current knowledge	Status
(SC-1)			
Thermaerobacter subterraneus (NCBI website[c]) (DSM 13965)	WGS contains *nirK*, but lack *nar*/*nap* and *nor* (Shapleigh, 2013).	More recent literature not available.	-
(c) (Endo) spore-forming Gram-positive bacteria: *Actinomycetales*			
Actinomyces coleocanis (NCBI website[c]) (DSM 15436)	WGS contains *nar* and *nir* genes (Shapleigh, 2013).	More recent literature not available.	?
Actinomyces odontolyticus (NCBI website[c]) (ATCC 17982)	WGS contains *nar* and *nir* genes (Shapleigh, 2013).	More recent literature not available.	?
***Arthrobacter* sp.** (Lee *et al.*, 2005) (N6)	*nirS* (AF335922) gene sequenced. *nirS* containing strains were tested for their ability to remove NO_3^- and NO_2^-.	More recent literature not available.	?
***Arthrobacter* sp.** (Flores-Mireles *et al.*, 2007) (61k)	Denitrification was presumed positive if neither NO_2^- or NO_3^- could be detected or showed only traces in the supernatant after isolate incubation. The final criteria for considering an isolate a denitrifier were detection of *nirS* or *nirK* by PCR followed by BLAST sequence analysis. *nirK* (EU035283) gene sequenced.	More recent literature not available.	√
***Arthrobacter* sp.** (TSA68)	*nirS* (AB542303) gene sequenced. Only gene info available on the NCBI website[c].		?
Dactylosporangium aurantiacum (Shoun *et al.*, 1998) (JCM 3041)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from both NO_3^- and NO_2^- under O_2 limited conditions only.	More recent literature not available.	√
Dermatophilus congolensis (Shoun et al., 1998) (JCM 3081)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions.	More recent literature not available.	√
Frankia (Lensi et al., 1990) (no strain number)	Screen for denitrification originated in a number of denitrifying strains. N_2O in the gas phase was analyzed by gas chromatography and AIM.	More recent literature not available.	√
Kocuria varians (Wang & Skipper, 2004) (DN16)	Nitrate and nitrite reduction tested with Griess reagents, *nirS* & *nirK* en *nosZ* genes amplified, *nirS* (AY345246) gene sequenced.	*Kocuria varians* DN16 was identified by GC-FAME as a member of the genus *Kocuria*, but 16S rRNA analysis and BLAST identified it as a member of the **Gram-negative** genus ***Acinetobacter*** (Wang & Skipper, 2004).	√
Kineosporia aurantiaca (JCM 3230) ***Nocardia salmonidica*** (JCM 4826) ***Saccharomonospora caesia*** (JCM 3098) (Shoun *et al.*, 1998)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions.	More recent literature not available.	√
Microtetraspora glauca (Shoun et al., 1998) (JCM 3300)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observations: N_2O evolution from NO_3^- under anaerobic conditions and N_2O evolution from NO_2^- under anaerobic	More recent literature not available.	√

Species	Denitrification assessment	Current knowledge	Status
	and O_2 limited conditions.		
Pilimelia anulata (Shoun et al., 1998) (JCM 3090)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions.	More recent literature not available.	√
Saccharothrix australiensis (Shoun et al., 1998) (JCM 3370)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observations: N_2O evolution from both NO_3^- and NO_2^- under O_2 limited conditions and N_2O evolution from NO_2^- under anaerobic conditions.	More recent literature not available.	√
Spirillospora albida (Shoun et al., 1998) (JCM 3041)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observations: N_2O evolution from NO_2^- under anaerobic and O_2 limited conditions and N_2O evolution from NO_3^- under O_2 limited conditions.	More recent literature not available.	√
Streptomyces akiyoshiensis (JCM 7970) ***Streptomyces aureofaciens*** (JCM 4624) ***Streptomyces cavourensis* subsp. *cavourensis*** (JCM 4555) ***Streptomyces cinnamoneus*** (JCM 4633) ***Streptomyces coelicolor*** (JCM 4357) ***Streptomyces endus*** (JCM 4636) ***Streptomyces glaucus*** (JCM 6922) ***Streptomyces lavendulae* subsp. *lavendulae*** (JCM 4664) ***Streptomyces zelensis*** (JCM 5024) (Shoun et al., 1998)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions.	Isolation of denitrifying Gram-positive *Streptomyces* strains from a soil denitrifying community (Chèneby *et al.*, 2000), but the strains were not identified. Analysis of N_2O evolved from NO_3^- was performed with gas chromatography.	√
Streptomyces antibioticus (Kumon et al., 2002) (B-546)	N_2 and N_2O in the gas phase were analyzed by gas chromatography. Observation: evolves N_2 and some N_2O from NO_3^-. Presence of denitrification together with co-denitrification[a]**.**	More recent literature not available.	√
Streptomyces flavotricini (Shoun et al., 1998) (JCM 4371)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observations: N_2O evolution from NO_3^- under anaerobic and O_2 limited conditions and N_2O evolution from NO_2^- only under anaerobic conditions.	Isolation of denitrifying Gram-positive *Streptomyces* strains from a soil denitrifying community (Chèneby *et al.*, 2000), but the strains were not identified. Analysis of N_2O evolved from NO_3^- was performed with gas chromatography.	√
Streptomyces thioluteus (Shoun et al., 1998) (JCM 4844)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observations: N_2O evolution from both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions, denitrification is accompanied by distinctive cell growth. Purification of CuNiR and azurin.	Isolation of denitrifying Gram-positive *Streptomyces* strains from a soil denitrifying community (Chèneby *et al.*, 2000), but the strains were not identified. Analysis of N_2O evolved from NO_3^- was performed with gas chromatography.	√
Micromonospora chalcea (JCM 3031) ***Streptosporangium roseum*** (JCM 3005)	N_2O in the gas phase was analyzed by gas chromatography and gas chromatography spectrometry (with ^{15}N-nitrate and ^{15}N-nitrite tracers). Observation: N_2O evolution from	More recent literature not available.	√

Species	Denitrification assessment	Current knowledge	Status
(Shoun *et al.*, 1998)	both NO_3^- and NO_2^- under anaerobic and O_2 limited conditions.		
(d) Non-(endo)spore-forming Gram-positive bacteria			
"Corynebacterium nephridii" (Renner & Becker, 1970)	Denitrification by resting cells was measured with Warburg respirometry and gas measurements were performed by gas chromatography. Only 1 strain is isolated, N_2O is the end product of denitrification.	The "*Corynebacterium nephridii"* strain was reported to be gram-positive, but **the strain is actually gram-negative** (Hart *et al.*, 1965). Tiedje (1988) mentions that the strain probably is an ***Alcaligenes sp.***	√
Corynebacterium diptheriae (Cerdeño-Tárraga et al., 2003) (NCTC 13129)	WGS contains *nar*, *nirK* and *qnorB*, but lacks *nosZ* (Shapleigh, 2013).	More recent literature not available.	√
Corynebacterium efficiens (NCBI website[c]) (YS-314)	WGS contains nitrate reductase and *nirK*, but lacks al other nitrogen oxide reductases (Shapleigh, 2013).	More recent literature not available.	?
Corynebacterium pseudogenitalium (NCBI website[c]) (ATCC 33035)	WGS contains *nirK*, but lacks al other nitrogen oxide reductases (Shapleigh, 2013).	More recent literature not available.	?
***Corynebacterium* sp.** (Flores-Mireles *et al.*, 2007) (12a)	Denitrification was presumed positive if neither NO_2^- or NO_3^- could be detected or showed only traces in the supernatant after isolate incubation. The final criteria for considering an isolate a denitrifier were detection of *nirS* or *nirK* by PCR followed by BLAST sequence analysis. *nirS* (EU035284) gene was sequenced.	More recent literature not available.	√
***Corynebacterium* sp.** (Flores-Mireles *et al.*, 2007) (63k)	Denitrification was presumed positive if neither NO_2^- or NO_3^- could be detected or showed only traces in the supernatant after isolate incubation. The final criteria for considering an isolate a denitrifier were detection of *nirS* or *nirK* by PCR followed by BLAST sequence analysis. *nirK* (EU035280) gene sequenced.	More recent literature not available.	√
***Enterococcus* sp.** (Heylen *et al.*, 2006) (R-25205)	NO_3^- and NO_2^- reduction tested with Griess reagents in combination with presence of a pH shift. Confirmation with gas chromatography of N_2O and AIM. *nirK (*AM230873*)* gene sequenced.	More recent literature not available.	√
***Enterococcus* sp.** (Heylen *et al.*, 2006, Heylen *et al.*, 2007) (R-24626)	Nitrate and nitrite reduction tested with Griess reagents in combination presence of a pH shift. Confirmation with gas chromatography of N_2O and AIM. *nirK (*AM230813*) and cnorB* (AM284330) genes sequenced.	More recent literature not available.	√
Gemella haemolysans (Berger & Pervanidis, 1986)	Only simple phenotypic testing[b] with observation of gas formation was performed. Observation: reduces low concentrations of NO_2^-, earlier studies reported 'denitrification'.	More recent literature not available.	-
Jonesia denitrificans (Rocourt et al., 1987)	Technique for denitrification is not mentioned in this species description. Single isolate.	Observation of nitrates reduced to nitrites (Seeliger & Jones, 1986). WGS contains a membrane-bound Nar and a CuNiR, but lacks *nor* and *nos* genes (Shapleigh, 2013).	-
***Lactobacillus* sp.** (NCBI website[c]) (multiple strains)	WGS contains abbreviated denitrification pathways containing *qnorB*, but no *nir* or *nos* (Shapleigh, 2013).	More recent literature not available.	-
Mycobacterium avium (104)	WGS contain *qnorB*, but no other denitrification genes (Shapleigh, 2013).	More recent literature not available.	-

Species	Denitrification assessment	Current knowledge	Status
Mycobacterium intracellulare (ATCC 13950)	WGS contain *qnorB*, but no other denitrification genes (Shapleigh, 2013).	More recent literature not available.	-
Mycobacterium parascrofulaceum (ATCC BAA-614)	WGS contain *qnorB*, but no other denitrification genes (Shapleigh, 2013).	More recent literature not available.	-
Propionibacterium acidi-proprionici (Van Gent-Ruijters *et al.*, 1975)	Gas production from NO_3^- was measured by Warburg manometry. Denitrification (reduction from NO_3^- to gaseous nitrogen (N_2O or N_2)) is strain dependent).	It was shown that *Propionibacterium acidi-proprionici* strains did not have respiratory denitrifying ability, nor did they produce N_2O at a rate typical for respiratory denitrifiers, but they did reduce NO_2^- to N_2O in nearly stoichiometric amounts (Tiedje, 1988), maybe as a detoxifying process (Zumft, 1992).	-
Propionibacterium acnes (NCBI website[c]) (multiple strains)	Several strains with WGS: all of the strains contain *nar*, *nirK* and *qnorB*, but lack *nosZ* (Shapleigh, 2013).	Isolates of this species have been found to grow in the presence of nitrate and nitrous oxide is the final denitrification product (Allison & McFarlan, 1989)	√
***Staphylococcus* sp.** (Lee et al., 2005) (N23)	*nirS* containing strains were tested for their ability to remove NO_3^- and NO_2^-. *nirS* (AF335923) gene sequenced.	More recent literature not available.	√
***Staphylococcus* sp.** (Heylen et al., 2006) (R-25050)	NO_3^- and NO_2^- reduction tested with Griess reagents in combination with presence of a pH shift. Confirmation with gas chromatography of N_2O and AIM. *nirK* (AM230813) gene sequenced.	More recent literature not available.	√
Tsukamurella paurometabola (Collins et al., 1988)	NO_2^- is utilized in some strains, assessment of the denitrification capacity was not performed.	More recent literature not available	-

[a] Co-denitrification is the process in which a hybrid N_2 or N_2O molecule is formed by combining two nitrogen atoms, one from NO_3^- and one from other nitrogen sources. The Actinomycetal hybrid N_2 species appears to be formed from NO_2^- (or NO) and not directly from NO_3^-, since the formation continues long after NO_3^- is consumed.

[b] Simple phenotypic testing stands for the observation of gas bubbles in Durham tubes and/or performance of Griess reduction tests or similar techniques.

[c] http://ncbi.nlm.nih.gov

(Tiedje, 1988, Mahne & Tiedje, 1995, Goregues *et al.*, 2005, Yamamoto *et al.*, 2006, Liang *et al.*, 2007, Bergaust *et al.*, 2010). The medium should also provide required trace elements and vitamins. Mineral media (Stanier *et al.*, 1966) complemented with succinate, ethanol or acetate as carbon sources have proven to be successful in the isolation of denitrifying strains, including aerobic endospore-formers (Kim *et al.*, 2005, Heylen, 2007, Park *et al.*, 2007). However, anaerobic cultivation of e.g. *Bacillus* in mineral medium is not always straightforward due to more complex growth requirements (Pichinoty *et al.*, 1976, Pichinoty *et al.*, 1978). The inoculum is a standard amount of bacterial cells (Bergaust *et al.*, 2011), e.g. 0.1 to 1ml suspension with a standard turbidity. This standard turbidity of the cell suspension may be 0.5 McFarland, an optical density at 600 nm (OD_{600}) of 0.05,… etc. and also depends on the organism under scrutiny. Next, the vials are sealed with butyl rubber bottle stoppers and aluminum crimp seals, the oxygen in the headspace is replaced in a sterile way by argon (Manucharova *et al.*, 2000, Nazina *et al.*, 2001, Kumon *et al.*, 2002) or helium (Mahne & Tiedje, 1995, Shoun *et al.*, 1998, Morley *et al.*, 2008) through repeated evacuating and flushing of the headspace. Subsequently, denitrification is tested through quantitative measurement of NO_3^- and/or NO_2^- (Weier & Macrae, 1992, Wang & Skipper, 2004, Mishima *et al.*, 2009, Bergaust *et al.*, 2010) and of N_2O and/or N_2 after (Weier & Macrae, 1992, Yamamoto *et al.*, 2006, Dandie *et al.*, 2007) or during (Molstad *et al.*, 2007, Bergaust *et al.*, 2010, Yoon *et al.*, 2013) anaerobic incubation and at strain-related suitable temperatures. Since the planet's atmosphere contains almost 80% N_2, often 10% acetylene (C_2H_2), is added to the headspace to block the reduction of N_2O to N_2 and N_2O is measured, although this step can already be circumvented by use of an automated incubation system allowing the study of gas kinetics of denitrifiers (Molstad *et al.*, 2007). Other headspace percentages of C_2H_2 have also been reported (Chèneby *et al.*, 2000, Manucharova *et al.*, 2000). The gas chromatographic analyses are, as mentioned above, automatic or start by taking a gas sample (0.1 to 1 ml, depending on the gas chromatographic set-up) with an air-tight syringe, that may additionally be flushed with the same gas that was used to flush the headspace (Mahne & Tiedje, 1995) and inject it into the gas chromatograph. A system with a dual-column system (e.g. a Hayesep Q [80-100-mesh] and a Chromatopac column at 25°C, with helium as carrier gas) and equipped with a thermal conductivity detector (TCD) or an electron capture detector (ECD) may be used. Other successful column combinations and carrier gases have been used as well (Gamble *et al.*, 1977, Weier & Macrae, 1992, Sakai *et al.*, 1996, Nazina *et al.*, 2001, Dandie *et al.*, 2007, Bergaust *et al.*, 2010). Although in 2011 guidelines were published (Bergaust *et al.*, 2011) for the characterization of denitrifying bacteria, some problems with phenotypic determination of the denitrification trait still persist.

Large amounts of novel taxa of cultured microorganisms are described yearly, with an average of 727 novel taxa per year for the last decade (Fig. 1. 5). Unfortunately, only a limited mandatory minimum characterization of phenotypic traits is demanded for valid description of novel microbial taxa. So although 'Minimal standards' are formulated as guidelines for many taxa, often diverse and non-standardized methodologies are used to assess the same biological traits leading to unreliable results.

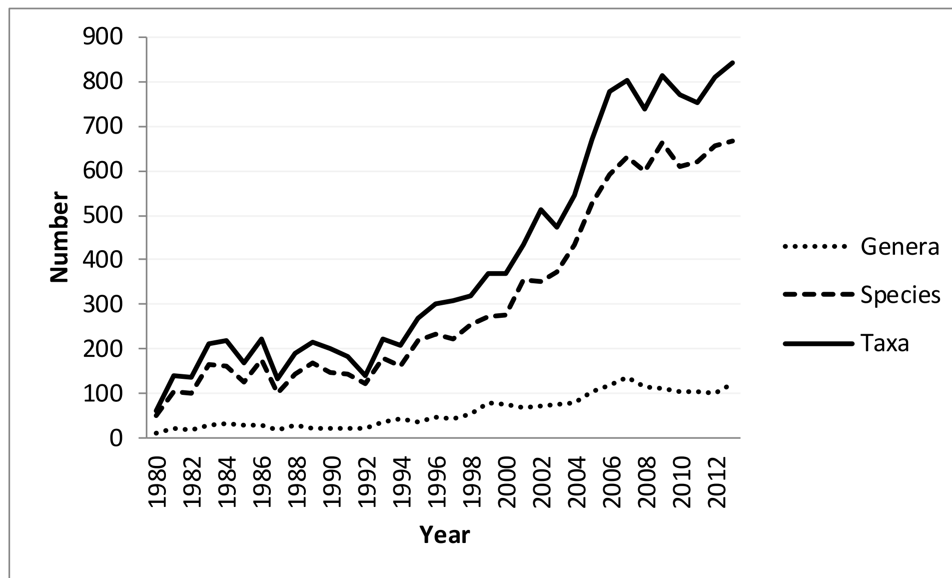


Fig. 1. 5 Number of described cultivable microorganisms (www.bacterio.cict.fr).

Hence, when the dissimilatory nitrogen metabolism (including NO_3^- reduction, dissimilatory nitrate reduction to ammonium (DNRA) or denitrification) is investigated in novel species, often phenotypes are inaccurately deduced from miniaturized test panels using e.g. only a colorimetric NO_3^-/NO_2^- reduction test (Fig. 1. 6). In addition, when the type strain is not a denitrifier, characterization of the dissimilatory nitrogen metabolism in other strains of the same species is often not considered, despite of its strain-dependent nature (Shapleigh, 2006). For Gram-positive bacteria this effect was also enhanced by the general assumption that denitrification in Gram-positive bacteria is limited to some representatives of the genus *Bacillus* (Tiedje, 1988). As a result, the actual prevalence of denitrification in described and cultivated bacteria is highly unknown and hence underestimated.

Another factor in the underestimation of occurrence of denitrification in pure cultures is the ambiguous assessment of the ability of an organism to denitrify. Quick, cheap and easy tests, such as colorimetric NO_3^- and NO_2^- reduction tests (Fig. 1. 6), gas formation in Durham tubes (in which the gas produced is often not identified) and detection of pH increase with a dye indicator in the growth medium

(Mazoch & Kucera, 2002), are seldom specific for detection of denitrification but are still widely applied. Exclusive use of the aforementioned tests for assessment of the denitrification capacity in e.g. *Bacillus* would prove complex, since some strains only perform the first step of denitrification (which should then be regarded as nitrate reduction) (Fig. 1. 1 & Fig. 1. 2), some strains have the potential to perform both DNRA and denitrification (Heylen & Keltjens, 2012, Sanford *et al.*, 2012) or even use denitrification, DNRA and detoxification modules at the same time depending on the conditions (Mania *et al.*, 2014). Of course, when studying denitrifying (Gram-positive) bacteria, mere nitrate reduction and DNRA should be ruled out. Currently, general consensus exists on the two main phenotypic criteria for an organism to be named a true respiratory denitrifier (Mahne & Tiedje, 1995), namely (1) gaseous nitrogen species, principally N_2O and N_2, are products from NO_3^- and/or NO_2^- reduction, and (2) the process is coupled to a significant growth yield increase. Using these criteria, denitrifiers were considered to be distinguished easily from non-denitrifiers, in particular from those that express NO reductase as a protection against nitrosative stress (Philippot, 2005, Streminska *et al.*, 2012). Furthermore, other processes such as DNRA, anammox or even detoxification processes can also generate N_2O or N_2 from NO_3^- or NO_2^- (Tiedje, 1988, Kartal *et al.*, 2007, Stein & Klotz, 2011, Simon & Klotz, 2013)(Fig. 1. 2), while new denitrifiers, such as the recently described *Methylomirabilis oxyfera* (Ettwig *et al.*, 2010), can follow alternative enzymatic pathways and may only produce known intermediates in trace amounts. Thus, gas chromatographic analyses are also not incontestable as sole investigative tool to demonstrate denitrification.

It should also be mentioned that independent of the method used, results will be dependent on the test conditions. For example, some bacteria can only start denitrification from NO_2^- and not from NO_3^- (Zumft, 1992, Shapleigh, 2013), although mostly only NO_3^- is tested as electron acceptor. In addition, use of other growth media with different carbon sources or different pH and different incubation conditions such as temperature, amount of oxygen, etc. can influence the denitrifying ability of the tested organism as well (Tiedje, 1988, Bergaust *et al.*, 2008). As a result, many NO_3^--respiring NH_4^+-producing isolates have incorrectly been considered denitrifiers in routine testing (Philippot *et al.*, 2007), whereas true denitrifiers that start from NO_2^- have been overlooked. At present, the current criteria for denitrifiers may not need updating, but correct interpretation of phenotypic observations to assess denitrification certainly deserves more attention.

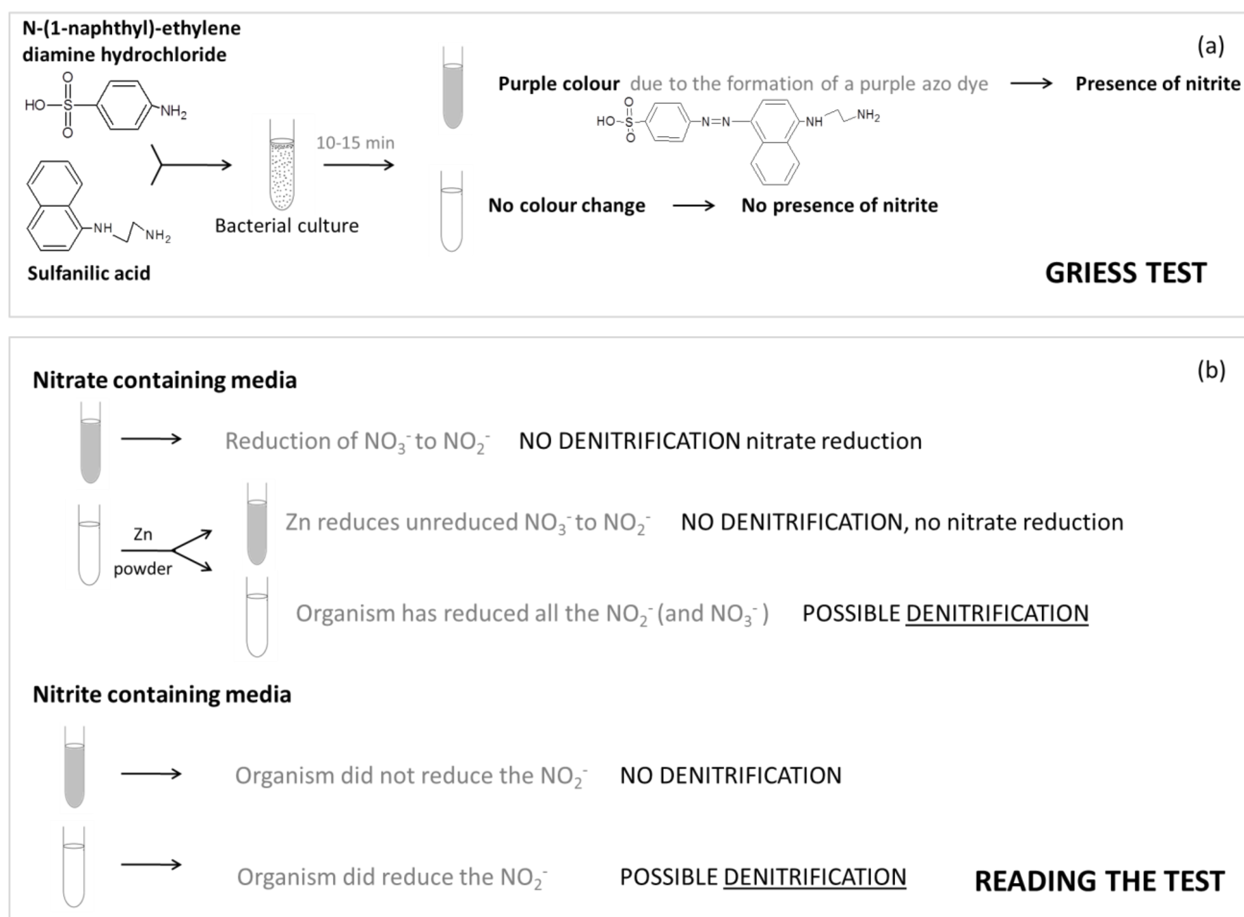


Fig. 1. 6 Colorimetric NO_3^- and NO_2^- reduction with the Griess reaction. (a) Chemistry: after addition of equal amounts of N-(1-naphthyl)-ethylene diamine hydrochloride and sulfanilic acid to the bacterial culture, the colour change can be read after 10 to 15 minutes. If a purple colour appears, nitrite is present in the bacterial culture; if not, nitrite is absent. (b) Interpretation of the Griess reaction according to the medium used. Depending on the original nitrogen source in the medium, a colour change reveals whether or not denitrification has occurred.

1.3.2 Molecular detection of denitrification

Although above-mentioned issues with phenotypic detection of denitrification are relevant for all bacteria and not solely for Gram-positive bacteria, molecular detection of the involved genes is more specifically a problem for Gram-positive denitrification.

Gene sequence analyses of key denitrification genes have been used to confirm isolates as denitrifiers (Mahne & Tiedje, 1995, Wang & Skipper, 2004, Flores-Mireles *et al.*, 2007) or, in culture-independent research, to monitor influence of different physico-chemical parameters on abundance and diversity of denitrifying populations *in-situ* (Henry *et al.*, 2004, Philippot, 2005, Liu *et al.*, 2010, Mao *et al.*, 2011). The majority of gene sequences available in the public databases encoding catalytic subunits of key denitrifying reductases, namely *nirS*, *nirK*, *qnorB* and *cnorB* (see section 1.1.5, Fig. 1. 1 & Table 1. 1), or even N_2O-producing nitrous oxide reductase (*nosZ*) are retrieved from either Gram-negative bacteria that are phylogenetically closely related or from uncultured bacteria (Fig. 1. 7). As a result, most

available molecular tools for *nir*, *norB* and *nosZ* gene detection (e.g. described by Braker *et al*. (Braker *et al.*, 1998), Hallin & Lindgren (Hallin & Lindgren, 1999), Braker & Tiedje (Braker & Tiedje, 2003), Goregues *et al*. (Goregues *et al.*, 2005), Casciotti & Ward, Scala & Kerkhof (Scala & Kerkhof, 1998, Casciotti & Ward, 2001, Casciotti & Ward, 2005) and Flores-Mireles *et al*. (Flores-Mireles *et al.*, 2007)) only target Gram-negative bacteria. These primers have been used on some denitrifying strains of the genus *Bacillus* (Green *et al.*, 2010) and *Paenibacillus* (Behrendt *et al.*, 2010), but without success.

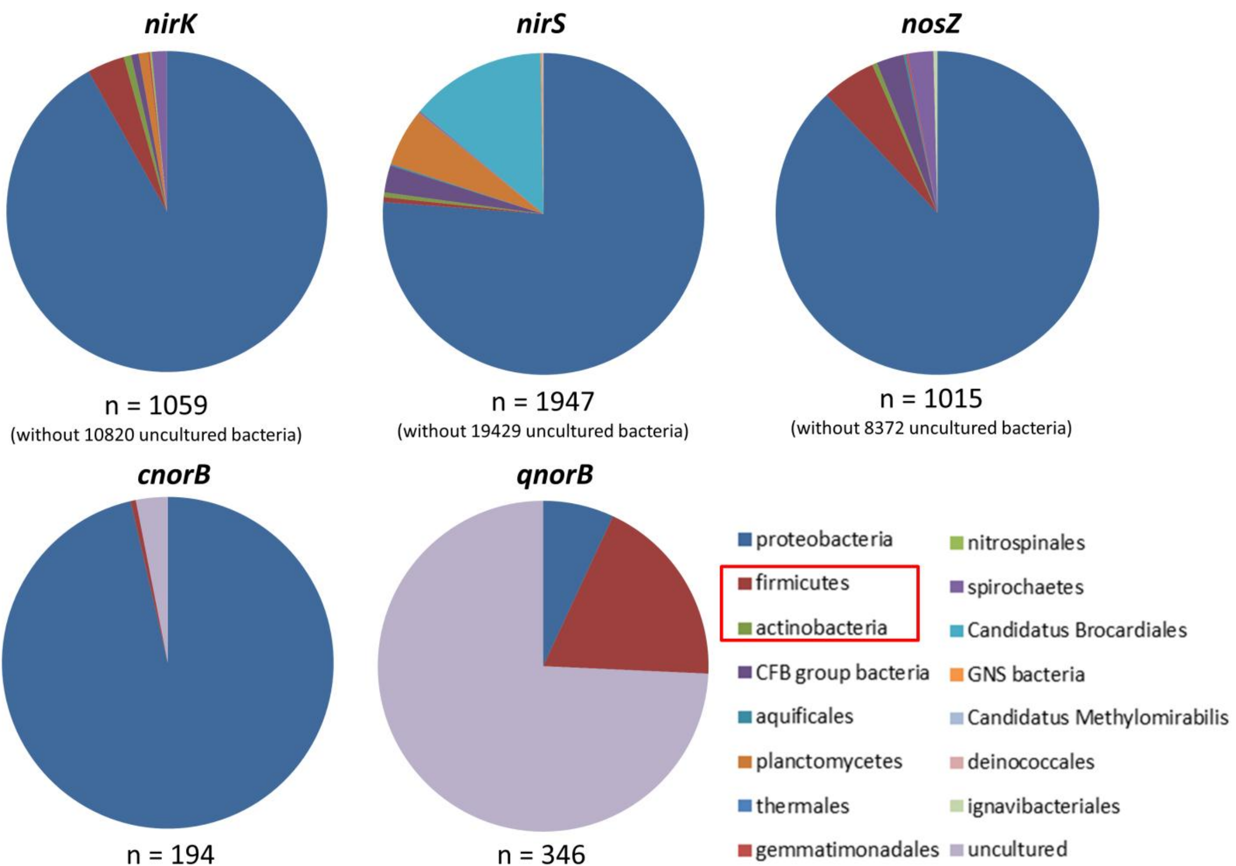


Fig. 1. 7 Number of available denitrification genes sequences. Data collected from the National Center for Biotechnology Information Nucleotide database, March 2014 (update on figure from 2010 (Verbaendert *et al.*, 2011a) (http://www.ncbi.nlm. nih.gov/nucleotide/). Only a few *nir, nor* and *nos* sequences of Gram-positive denitrifying bacteria are available. Genes annotated with the term *nirS*, *nirK*, *cnorB, qnorB* and *nosZ* were included in the dataset. CFB group, *Cytophaga*/*Flavobacterium*/*Bacteroides* group; GNS bacteria, Green Non-sulfur Bacteria

When denitrification genes are occasionally detected in Gram-positive denitrifiers, their sequence phylogeny is very closely related to that of genes from Gram-negative denitrifiers (Heylen *et al.*, 2006, Jones *et al.*, 2008). This observation is in disagreement with the phylogeny of gene sequences from WGS. Jones *et al*. (Jones *et al.*, 2008) showed that concatenated denitrification gene sequences from

Geobacillus thermodenitrificans - one of the only few confirmed Gram-positive denitrifiers of which the whole genome is currently available - were only distantly related to other known sequences. This sequence divergence obviously has consequences: a nucleotide-based BLAST (Basic Local Alignment Search Tool) search with a denitrification gene sequence of *Geobacillus thermodenitrificans* as query will not find any homologous sequences; only the use of derived amino acid sequences will result in identification of the correct protein domain and retrieval of homologous sequences. Because of this, relevant denitrification gene sequences were probably overlooked, e.g. in metagenomic data, and therefore efforts are necessary to generate more whole genomes from Gram-positive or other confirmed denitrifiers (Green *et al.*, 2010), or even large fosmid libraries from the environment (Bartossek *et al.*, 2010), to expand the knowledge on denitrification gene sequences which are not targeted by current primers. This information can then be used to design specific primers which target certain groups of gene sequences, e.g. members of the *Firmicutes*. Unfortunately, although the last years an overwhelming amount of data from genomes and environmental metagenomes became available, currently only few genomes of phenotypically Gram-positive denitrifiers have been sequenced (www.genomesonline.org). This still limited phylogenetic coverage of Gram-positive denitrifiers in public sequence databases may not represent their real functional N-reducing/denitrifying capabilities.

Lack of suitable primers and very divergent sequences can certainly explain the underestimation of Gram-positive denitrifiers in the last two decades, in which research was dominated by molecular analyses. However, another explanation could be the presence of new denitrification genes coding for unknown NO_2^- and NO reductases. This hypothesis is supported by many recently discovered enzymes that have been attributed to microorganisms from less studied phyla (Simon & Klotz, 2013), e.g. the discoveries of the qCu_ANOR/sNOR enzyme purified from *Bacillus azotoformans* and *Nitrosomonas eutropha* (Suharti *et al.*, 2001, Stein *et al.*, 2007), encoded by the newly described *cbaA* genes (Heylen & Keltjens, 2012), the gNOR gene (Sievert *et al.*, 2008), encoding a hitherto unknown nitric oxide reductase in *Sulfurimonas denitrificans,* and presence of an NO dismutase catalyzing conversion of two NO compounds to N_2 and O_2 in a denitrifying aerobic methane oxidizer (Ettwig *et al.*, 2010). These new discoveries suggest that the denitrification pathway is more redundant than previously thought. It seems that despite intensive research for over many decades, our knowledge of denitrification is still far from complete and the existence of currently unknown, new denitrification enzymes is very plausible.

1.4 Importance of Gram-positive denitrifiers

As described above, the denitrification pathway may be a trait of many Gram-positive genera and the sections above may have unraveled why they are overlooked in current research. But is Gram-positive denitrification important from an ecological point of view? Unfortunately, molecular tools need to be available before its relevance, more specifically numerical importance and activity, *in situ* can be investigated. However, a range of culture-dependent denitrification studies already isolated Gram-positive denitrifiers from different types of NOx (nitrogen oxides) reducing ecosystems, although these reports are very limited. Main habitats of denitrifying bacteria are (1) sediments and the water column of aquatic systems such as oceans, estuaries, rivers, lakes and lagoons, (2) parts of wastewater treatment systems, such as activated sludge, and (3) terrestrial systems, such as soil and the rhizosphere associated with it. Aquatic denitrifying *Corynebacterium* spp. and an *Arthrobacter* sp. have been isolated from around mangrove roots (Flores-Mireles *et al.*, 2007) and *Bacillus* spp. from lagoon sediments (Goregues *et al.*, 2005). Gram-positive denitrifiers have also been isolated from wastewater treatments systems (Lee *et al.*, 2005, Harbi *et al.*, 2010, Zhao *et al.*, 2010) and *Bacillus* spp. are even essential for some specific wastewater treatments (Choi *et al.*, 2002, Park *et al.*, 2007). In terrestrial systems, denitrifying members of the genus *Bacillus and Paenibacillus* have already been found in rice soils and rice plant rhizospheres (Garcia, 1977b, Roussos *et al.*, 1980), bent grass and Bermuda grass (Wang & Skipper, 2004), in soddy podzolic soil (Manucharova *et al.*, 2000) and in a Korean night soil treatment system (Kim *et al.*, 2005), whereas denitrifying *Actinomycetes* were already isolated from agricultural soils (Shoun *et al.*, 1998, Chèneby *et al.*, 2000). In addition, microbial composition of terrestrial systems has been studied globally, in many types of soil and rhizospheres, and Gram-positive bacteria are found to be abundant in these soil ecosystems (Chèneby *et al.*, 2000, McMullan *et al.*, 2004, Dong & Reddy, 2010, Zeigler, 2014).

Gram-positive microorganisms have often been reported to terminate denitrification with N_2O (Hart *et al.*, 1965, Renner & Becker, 1970, Denariaz *et al.*, 1989, Shoun *et al.*, 1998, Zumft & Kroneck, 2007, Mishima *et al.*, 2009). This truncated version of the denitrification process may perhaps be of importance in N_2O emission hot-spots containing relatively higher numbers of (Gram-positive) denitrifiers that lack the last step of the process. In addition, driving forces that may influence N_2O emission from Gram-positive denitrifiers, such as pH (Liu *et al.*, 2010), water content, carbon and nitrate availability (Wallenstein *et al.*, 2006) and selection pressure (Nadeem *et al.*, 2013), influencing end-product stoichiometry (N_2O:N_2 ratio) and expression of N_2O reductase, still need to be elucidated. Hence,

Gram-positive denitrifiers may contribute to the often observed large spatial variability of soil N_2O emissions (Scala & Kerkhof, 1998, Takaku *et al.*, 2006) and the global greenhouse gas effect.

Moreover, the Gram-positive bacteria that were investigated in this work – members of the genera *Bacillus* and *Geobacillus* – may form resistant spores or have vegetative cells that are resistant to environmental stress. This has ecological implications since in these inactive states they do not directly contribute to ecosystem processes of a given environment at any one time (Prosser *et al.*, 2007). Denitrifying *Bacillaceae* may thus belong to the large portion of bacteria that remain in a 'dormant' spore-state as part of the seed bank from which different traits can be resuscitated (Krause *et al.*, 2014) and may be important for the flexibility of communities on the advent of perturbation or under changing environmental conditions.

1.5 Current status

Denitrification is a trait of global ecological importance, in which Gram-positive bacteria could play a key role. Denitrification in all its aspects, from phenotypic observation of nitrogen conversion to molecular detection of the encoding genes, has been underexplored in this large group of microorganisms. There is a need for more stringent phenotypic characterization of the process in the description of novel taxa, creating an elaborate and reliable inventory of the capacity to denitrify among cultivated and identified microorganisms, which makes the latter publically available for generation of more whole genomes. In addition, failure of currently available molecular tools to detect denitrification genes in cultivated denitrifiers and the high divergence in phylogeny between PCR amplified gene sequences and those retrieved from whole genome sequencing suggest (1) incomplete and inaccurate *in-situ* monitoring of the denitrification process and (2) incomplete and inaccurate modeling of denitrification nitrogen fluxes in natural environments. In its turn this is leading to a defective management of denitrifying ecosystems with regard to its ecological implications. This underlines the necessity of new efforts in primer development and the need for other approaches to identify predominant denitrifying microbial taxa in environmental monitoring studies, as some researchers already endeavored (Stepanauskas & Sieracki, 2007, Green *et al.*, 2010, Hoshino & Schramm, 2010, Stepanauskas, 2012).

Part III

Experimental work

Redrafted from:

Verbaendert, I., Boon, N., De Vos, P., and Heylen, K. (2011). Denitrification is a common feature in members of the genus *Bacillus*. Systematic and Applied Microbiology 34: 385 - 391.

Author's contributions:

IV, KH and PDV designed the experiments. IV and KH performed the experiments and analyzed the data. Data on isolation campaign and subsequent analyses were already available in PhD thesis of KH (Heylen, 2007). Screening of the reference strain set was performed by IV. IV and KH wrote the manuscript. NB, PDV, KH and IV proofread and commented on the manuscript.

Chapter 2

Denitrification is a common feature among members of the genus *Bacillus*

Summary

Although several Gram-positive denitrifiers have been characterized in the past, there is still uncertainty about the occurrence of the denitrification trait among these bacteria. In an isolation campaign from a luvisol soil, *Bacillus* spp. were among the most abundant retrieved cultured denitrifiers next to members of *Rhizobiaceae* family and genus *Cupriavidus*. Subsequent screening of 180 representatives of the genus *Bacillus* (encompassing more than half of the current validly described species diversity in *Bacillus*) was performed and demonstrated the potential for dissimilatory reduction of nitrogen compounds in 45 of the 87 investigated species, with 19 species containing denitrifying members. The influence of several electron donors and acceptors was tested. The use of more than one electron acceptor, e.g. both nitrate and nitrite, was crucial to detect the denitrification potential of reference strains. Complex electron donors, most suitable for aerobic growth, were ideal for denitrification testing, while retrieval of denitrifiers from the environment was facilitated by the use of defined electron donors, due to less interference of other anaerobic growers. The outcome of the isolation campaign and screening of reference strain set suggest that bacilli may be potential contributors to denitrification in terrestrial and possibly other ecosystems.

2.1 Introduction

Denitrification is a widespread process of global importance, due to consumption of bio-available fixed nitrogen and emission of greenhouse gases (Duxbury *et al.*, 1982, Chapuis-Lardy *et al.*, 2007). Denitrification is a modular process with sequential reduction of nitrate, nitrite, nitric oxide and nitrous oxide. Although it is often considered redundant – it is carried out by a variety of microorganisms and the first three steps can be catalyzed by functionally similar but structurally different enzymes – the microbial diversity performing denitrification *in-situ* is functionally significant (Cavigelli & Robertson, 2000). Elucidating denitrifier community structure therefore may be important for understanding, modeling and managing denitrification in specific ecosystems. Because of the lack of interrelatedness between denitrifying organisms (Payne, 1981), which e.g. does exist for ammonia oxidizers (Prosser, 2007) and anammox (Jetten *et al.*, 2009), denitrifier community structure is preferably analyzed with molecular tools that target key denitrification proteins and/or encoding functional genes (Philippot & Hallin, 2005, Philippot *et al.*, 2009, Liu *et al.*, 2010, Morales *et al.*, 2010). However, phylogeny of these functional markers is not related to 16S rRNA gene phylogeny (Philippot, 2002, Heylen *et al.*, 2006, Heylen *et al.*, 2007, Jones *et al.*, 2008), making most culture-independent approaches less suited for identification of microorganisms involved in denitrification. Although community structure cannot be unraveled in culture-dependent research due to cultivation bias (Ritz, 2007), isolation and study of cultivated denitrifiers is still important for expanding knowledge on denitrifier diversity (Goregues *et al.*, 2005, Nichols, 2007, Alain & Querellou, 2009, Green *et al.*, 2010).

The actual known diversity of denitrifiers is thus far limited to sporadic reports in members of less than 100 prokaryote genera (Shapleigh, 2006). During the last two decades, denitrification in newly described microorganisms other than *Proteobacteria*, e.g. in Gram-positive bacteria, was mostly overlooked. Denitrifiers belonging or related to the genus *Bacillus*, such as strains of *Bacillus stearothermophilus* (now *Geobacillus stearothermophilus)* (Garcia, 1977b), *Bacillus licheniformis* (Pichinoty *et al.*, 1978), *Bacillus azotoformans* (Pichinoty *et al.*, 1983), *Bacillus halodenitrificans* (now *Virgibacillus halodenitrificans*) (Denariaz *et al.*, 1989), *Bacillus firmus* (Urata & Satoh, 1991) and strains that now belong to the genus *Paenibacillus* (de Barjac & Bonnefoi, 1972), were already described decades ago. Other Gram-positives were classically considered as not containing true denitrifiers (Tiedje, 1988), but more recently, denitrification was also demonstrated in members of the genus *Streptomyces* (Shoun *et al.*, 1998) and some non-spore-forming Gram-positives such as *Corynebacterium* (Flores-Mireles *et al.*, 2007). Despite these reports, bacteria belonging to Gram-positive taxa are rarely considered to be denitrifiers due to (i) the taxon-unrelated and strain-dependent nature of the process

(Shapleigh, 2006), (ii) the inadequate screening of dissimilatory nitrogen metabolism, and (iii) the lack of Gram-positive denitrification gene sequences in public databases, as a result of which available molecular tools almost only target denitrification gene sequences from Gram-negative bacteria. Their application has proven unsuccessful when tested on denitrifying members of *Bacillus* (Green *et al.*, 2010) and *Paenibacillus* (Behrendt *et al.*, 2010).

To investigate the denitrifier diversity present in a luvisol soil, we performed an isolation campaign applying different electron donors and acceptors to retrieve a wide diversity of cultivated denitrifiers. A high number of retrieved denitrifiers surprisingly belonged to the genus *Bacillus*. Because of the limited knowledge on Gram-positive denitrification, we subsequently investigated how common the ability to denitrify was in a large set of representative strains, covering half of the currently described species diversity within *Bacillus*. Moreover, a better insight in the effect of different electron donors and acceptors on phenotypic detection of denitrification was obtained.

2.2 Materials and methods

2.2.1 Sample, Most Probable Number (MPN) enumeration and isolation campaign

A soil sample was taken from a luvisol test field in Melle, Belgium. The texture class of the test field is sand-loam (composition 8.6% clay/11.6% loam/75.8% fine sand/4% rough sand). The soil was suspended in a sterile solution of 1% $NaPO_3$ buffer (3g soil in 300ml) by stirring at room temperature (Chèneby *et al.*, 2000). MPN enumeration was performed in fivefold on a dilution series (10^{-2} to 10^{-10}) using four different growth media: (i) Tryptone Soya Broth (TSB, Oxoid) supplemented with 10mM potassium nitrate and 10µM phenol red (incubated at 37°C), and (ii) three defined growth media, based on the mineral medium described before (Stanier *et al.*, 1966). The defined growth media were supplemented with 10µM phenol red and with either (i) 18mM potassium nitrate and 22.5mM ethanol (at pH 7.5, incubated at 20°C), (ii) 3mM potassium nitrite and 15mM sodium succinate (at pH 7, incubated at 37°C), and (iii) 3mM potassium nitrate and 15mM sodium succinate (at pH 7, incubated at 37°C), as described before (Heylen *et al.*, 2006). After two weeks of incubation in anoxic conditions (composition gas mixture 8%CO_2/8%H_2/84%N_2 of which 10% was replaced with acetylene), tubes were scored for growth, nitrate (NO_3^-) consumption and nitrous oxide (N_2O) gas production. In parallel with MPN enumeration, 0.1ml of the same dilution series was spread plated on agar plates of the four above-mentioned media. From each growth medium, visibly different colony types were randomly picked and further purified by sub-culturing under isolation conditions.

2.2.2. DNA extraction, diversity assessment and identification

Genomic diversity of all newly isolated denitrifiers was assessed with random amplification of polymorphic DNA (RAPD) fingerprinting and representatives of each RAPD fingerprint were assigned at the genus level, based on 16S rRNA gene sequence analysis. For DNA extraction, one colony was suspended in an Eppendorf tube with 20µl of lysis buffer (2.5ml 10% SDS; 5ml 1M NaOH; 92.5ml MilliQ water). After 15 min at 95°C, 180ml MilliQ water was added, the tube was centrifuged for 5 min at 13,000 g and the supernatant was transferred to a new tube. RAPD-PCR fingerprinting was performed with primers RAPD-270 (5'-TGCGCGCGGG-3') and RAPD-272 (5'-AGCGGGCCAA-3') as described previously (Mahenthiralingam *et al.*, 1996, Coenye *et al.*, 2002). Gels were normalized and analyzed with BioNumerics 4.5 (Applied Maths, St-Martens-Latem, Belgium). Similarity matrices of densitometric curves of the gel tracks were calculated with the Pearson's product-moment correlation coefficient. Cluster analyses of similarity matrices were performed by unweighted pair group method with arithmetic averages (UPGMA). Two cluster significance tools were applied to express the stability of the error at each branching level and the overall quality of the cluster analysis: (i) cluster cut-off method, which delimitates relevant clusters at different similarity levels and corresponds to the lowest similarity value within the entries of a dendrogram; (ii) the cophenetic correlation, which allows to distinguish reliable from unreliable subclusters. Reproducibility was assessed by generating a three-fold RAPD of five isolates. Similarity indices between these replicates ranged from 74 - 91% (average ± standard deviation, 81.8% ± 6.7%), which was similar to that obtained by Coenye and colleagues (Coenye *et al.*, 2002). All isolates with RAPD-PCR fingerprint patterns above 80% similarity and/or with visually identical fingerprints were assigned to the same RAPD type (Fig. 2. 1).

16S rRNA gene amplification and sequencing was performed as described previously (Mahenthiralingam *et al.*, 1996, Coenye *et al.*, 2002). Partial 16S rRNA gene sequences (first 300-500 bp) were assembled using the BioNumerics 4.5 software. A reliable identification was obtained in two steps: (i) query in "Classifier" program of Ribosomal Database Project II (Cole *et al.*, 2005) of the 16S rRNA gene sequence of an isolate, (ii) all type strains of all species of all genera mentioned in the Classifier report were compared in an exhaustive pair wise manner with the query sequence of each isolate in BioNumerics 4.5. The isolates were assigned to a genus based on the obtained pairwise 16S rRNA gene sequence similarity (Table 2. 4).

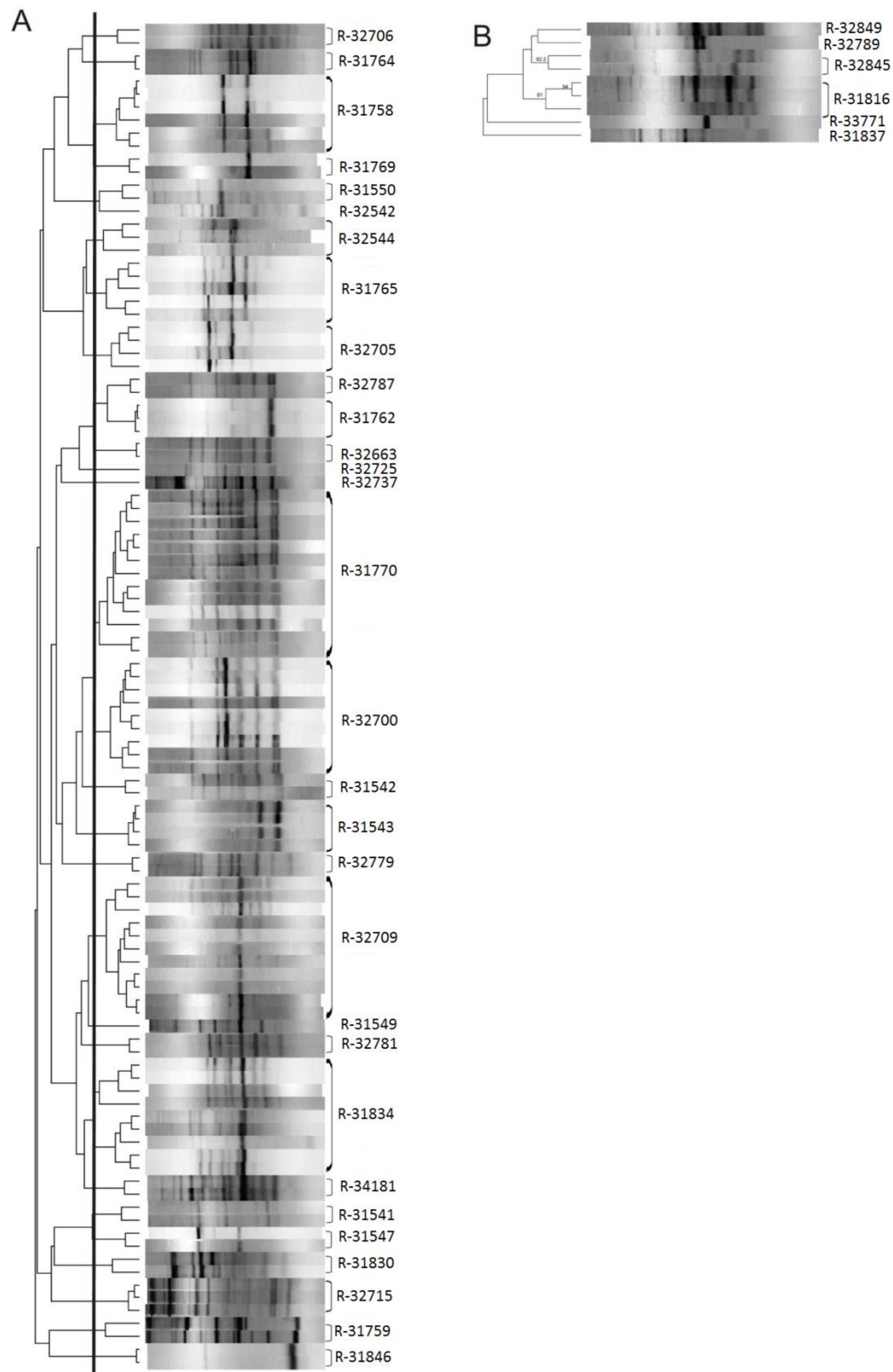


Fig. 2. 1 Genomic diversity of denitrifiers isolated from soil. Assessment with RAPD fingerprinting, UPGMA cluster analysis and Pearson's product-moment correlation coefficient. Strain numbers of cluster representatives are given next to the according profile. (**A**) Fingerprints generated with primer RAPD-270. Calculated 80% similarity cut-off to delineate clusters is indicated by a vertical black bar. Digital fingerprints of members of delineated clusters were also inspected visually to confirm or overrule cut-off value, especially when cluster delineations bordered on the cut-off value. Two exceptions were made (i.e. R-32542 was excluded from cluster with R-31550 as representative and cluster containing R-32787 and R-31762 was split in two clusters, each represented by one of these isolates) and later confirmed as valid by 16S rRNA gene sequencing (Table 2.1). (**B**) Fingerprints generated with primers RAPD-272 (used when no profile could be obtained with primer RAPD-270). Relevant similarity values used for cluster delineation are given on the branches.

Table 2. 1 Identification of denitrifiers retrieved from soil representing a RAPD cluster.
Assignment to genus level was based on 16S rRNA gene sequence analysis. Accession number of 16S rRNA gene of each isolate is given, as well as information on the most similar type strain based on pairwise 16S rRNA gene sequence comparison.

	strain nr	16S rRNA gene sequence		Type strain with highest 16S rRNA gene sequence similarity to query sequence			
Genus designation		length (bp)	accession n°	species name	strain n°	accession n°	% sequence similarity
Bacillus sp.	R-32706	261	AM403585	*B. licheniformis*	LMG 12363[T]	X68416	100%
	R-31769	258	AM403640				100%
	R-31770	407	AM403618	*B. bataviensis*	LMG 21833[T]	AJ542508	99,00%
	R-31834	424	AM403624				98,80%
	R-32700	365	AM403610				98,60%
	R-32709	432	AM403631				99,10%
	R-32779	424	AM403650				99,00%
	R-32781	422	AM403651				99,00%
	R-32787	409	AM403623				99,00%
	R-32845	412	AM403642				99,00%
	R-32715	414	AM403611	*B. soli*	LMG 21838[T]	AJ542513	99,10%
	R-32849	441	AM403635				98,80%
	R-31541	447	AM403630	*B. drentensis*	LMG 21831[T]	AJ542506	100%
	R-31547	424	AM403583				100%
	R-31550	437	AM403602				100%
	R-31846	421	AM403645				99%
	R-32663	419	AM403646				100%
	R-32705	437	AM403634				99%
	R-32789	426	AM403637				99%
	R-31830	424	AM403609	*B. pseudomycoides*	LMG 18993[T]	AF013121	99,50%
Staphylococcus sp.	R-33771	438	AM403643	*S. warneri*	LMG 13354[T]	L37603	100%
	R-34181	437	AM403638				100%
Sinorhizobium sp.	R-31759	343	AM403647	*S. morelense*	LMG 21331[T]	AY024335	100%
	R-31764	398	AM403648				100%
	R-31816	356	AM 403653				100%
	R-32542	376	AM403606				100%
	R-32737	371	AM403632				100%
Rhizobium sp.	R-31549	1455	AM403621	*R. radiobacter*	LMG 140[T]	AJ389904	98,50%
	R-31762	1451	AM403584				98,30%
	R-31837	1400	AM403614				98,50%
	R-32725	359	AM403605				100%
Ensifer sp.	R-32544	361	AM403593	*E. medicae*	A321[T]	Z78204	100%
Pseudomonas sp.	R-31758	424	AM403600	*P. kilonensis*	DSM 13547[T]	AJ292426	100%
	R-31765	425	AM403604				100%
Cupriavidus sp.	R-31542	416	AM403597	*C. necator*	LMG 8453[T]	AF191737	99,30%
	R-31543	416	AM403599				99,30%

2.2.3 Reference strain set & growth conditions

A total number of 180 *Bacillus* reference strains was selected from the BCCM/LMG[1] Bacteria Collection (Table 2. 2). Throughout the paper, reference strains are referred to by their species assignment as available in the BCCM/LMG Bacteria Collection (i.e. no additional identification tests were performed on reference strains in this study).

[1] BCCM/LMG : Belgian Co-ordinated Collections of Microorganisms/LMG Bacteria collection; Laboratory of Microbiology Ghent University

Denitrifying ability of reference strains was screened after incubation in six different liquid growth media at incubation temperatures recommended by the BCCM/LMG Bacteria Collection. Similar mineral media as mentioned above were used, supplemented with (i) 15mM sodium succinate and 3mM potassium nitrate, (ii) 15mM sodium succinate and 3mM potassium nitrite, (iii) 22.5mM ethanol and 18mM potassium nitrate, and (iv) 22.5mM ethanol and 18mM potassium nitrite. In addition, complex growth media recommended by the BCCM/LMG Bacteria Collection (specified in Table 2. 2) supplemented with 10µM phenol red were used with either 10mM potassium nitrate or potassium nitrite.

Table 2. 2 Overview of all *Bacillus* reference strains included in the study.
Growth medium (medium) and temperature (temp) are specified.

Species	LMG strain n°	Medium[a], temp
Bacillus agaradhaerens	17948[T]	M253, 28°C
Bacillus alcalophilus	7120[T]	M53, 37°C
Bacillus altitudinis	LMG 24750[T] (t1 and t2)	M14, 30°C
Bacillus amyloliquefaciens	9814[T], 12325, 12329, 12331, 12384, 17599, 17600, 17601	M1, 30°C
Bacillus aquimaris	23073[T] t1	M14, 28°C
Bacillus asahii	LMG 24728[T]	M207, 30°C
Bacillus atrophaeus	16797[T], 8198, 8199 (t1 and t2, t1+t2), 17795 (t1 and t2, t1+t2)	M51 or M185, 30°C
Bacillus azotoformans	9581[T], 15445, 15449	M1, 30°C
Bacillus badius	7122[T], 18004, 18005	M1, 30°C
Bacillus barbaricus	23067[T]	M14, 28°C
Bacillus bataviensis	21833[T], 21832	M14, 28°C
Bacillus benzoevorans	20225[T]	M1, 30°C
Bacillus bogoriensis	22234[T]	M292, 37°C
Bacillus carboniphilus	18001[T]	M1, 30°C
Bacillus cereus	6923[T]	M1, 30°C
Bacillus circulans	13261[T]	M51, 30°C
Bacillus clarkii	17947[T]	M253, 28°C
Bacillus clausii	17945[T]	M253, 28°C
Bacillus coagulans	6326[T]	M1, 37°C
Bacillus coahuilensis	24687[T]	M14, 30°C
Bacillus cohnii	16678[T]	M53, 30°C
Bacillus decolorationis	19507[T]	M185, 28°C
Bacillus drentensis	21831[T]	M14, 28°C
Bacillus endophyticus	21715[T]	M14, 28°C
Bacillus farraginis	22081[T]	M207 or M14, 37°C
Bacillus fastidiosus	12406, 12403, 12404, 12405	M54, 30°C
Bacillus firmus	7125[T]	M1, 30°C
Bacillus flexus	11155[T]	M1, 30°C
Bacillus fordii	22080[T]	M207 or 14, 37°C
Bacillus fortis	22079[T]	M207 or 14, 37°C
Bacillus fumarioli	19448[T], 17492, 18409, 18418	M241, 52°C
Bacillus galactosidilyticus	17892[T], 12353, 12396	M14, 28°C
Bacillus gelatini	21880[T]	M185, 50°C
Bacillus gibsonii	17949[T]	M253, 28°C
Bacillus halmapalus	17950[T]	M253, 28°C
Bacillus halodurans	7121[T]	M263, 28°C
Bacillus horti	18497[T]	M185, 28°C
Bacillus humi	22167[T], 22168	M14, 28°C
Bacillus hwajinpoensis	LMG 24749[T]	M14, 30°C
Bacillus indicus	22858[T]	M14, 28°C
Bacillus infantis	LMG 24756[T]	M114 + 5% sheep blood, 37°C
Bacillus insolitus	17757[T]	M1 or M51, 15°C
Bacillus lehensis	LMG 24751[T]	M14, 30°C

Species	LMG strain n°	Medium[a], temp
Bacillus lentus	16798[T]	M1 or M185, 26°C
Bacillus licheniformis	12363[T], 6934, 7558, 7559, 7560, 7561, 7562, 7626, 7627, 7628, 7633, 7634, 7636, 7637, 17334, 17335, 17336, 17337, 17339, 17340, 17341, 17651, 17652, 17653, 17654, 17655, 17657, 17658, 17659, 17660, 17661, 17662, 17663, 18685, 19409	M1, 37°C
Bacillus luciferensis	18422[T], 21400	M185, 30°C
Bacillus marisflavi	23072[T]	M185, 28°C
Bacillus megaterium	7127[T], 9300, 12252	M1, 30°C
Bacillus methanolicus	LMG 24730[T]	M14, 45°C
Bacillus mojavensis	17797[T], 17798, 22476, 22477	M14, 30°C
Bacillus muralis	20238[T]	M185, 28°C
Bacillus mycoides	7128[T], 9680, 12256, 12410	M1, 30°C
Bacillus niacini	16677[T]	M1, 30°C
Bacillus novalis	21837[T]	M14, 28°C
Bacillus odysseyi	24110[T]	M14, 28°C
Bacillus okuhidensis	22468[T]	M308, 45°C
Bacillus oleronius	17952[T]	M1, 30°C
Bacillus panaciterrae	23408[T]	M193, 28°C
Bacillus patagoniensis	23070[T]	M14, 28°C
Bacillus plakortidis	LMG 24732[T] (t1 and t2)	M14, 30°C
Bacillus pseudalcaliphilus	17951[T]	M253, 28°C
Bacillus pseudofirmus	17944[T]	M253, 28°C
Bacillus psychrodurans	23063[T]	M14, 25°C
Bacillus psychrosaccharolyticus	9580[T]	M1, 15°C
Bacillus psychrotolerans	23062[T]	M14, 25°C
Bacillus pumilus	18928[T]	M1, 30°C
Bacillus ruris	22866[T], 22867	M207 or M185, 37°C
Bacillus schlegelii	7133[T]	M85, 65°C
Bacillus shackletonii	18435[T]	M242, 30°C
Bacillus simplex	11160[T]	M1, 30°C
Bacillus siralis	22467[T]	M14, 28°C
Bacillus smithii	12526[T], 6327, 17534, 17535 t2	M1, 55°C
Bacillus soli	21838[T], 21839, 21840	M14, 28°C
Bacillus sonorensis	21636[T]	M1, 28°C
Bacillus sporothermodurans	17668[T], 17895, 17896, 18460, 18461, 18466	M207, 37°C
Bacillus subtilis	12379, 3589, 12262, 12264, 17725 t1, 20037,23370	M1, 30°C
Bacillus subtilis subsp. *spizizenii*	19156[T]	M1, 30°C
Bacillus subtilis subsp. *subtilis*	7135[T]	M1, 30°C
Bacillus thermoamylovorans	18084[T]	M14, 50°C
Bacillus thermantarcticus	23032[T]	M14, 52°C
Bacillus thuringiensis	7138[T], 12256, 12266, 12418	M1, 30°C
Bacillus tusciae	17940[T]	M255, 50°C
Bacillus vallismortis	18725[T], 17800	M14, 28°C
Bacillus vedderi	17954[T]	M253, 30°C
Bacillus vietnamensis	LMG 24742[T] (t1 and t2)	M14, 30°C
Bacillus vireti	21834[T]	M14, 28°C
Bacillus weihenstephanensis	18989[T]	M14, 30°C

[a] **Specifications growth media:**

M1: Nutrient Agar (NA, Oxoid); **M14:** Tryptone Soya Agar (TSA, Oxoid); **M51:** NA supplemented with 10g glucose (pH 7.4); **M53:** NA supplemented with 1M sterile Na-sesquicarbonate solution to achieve a pH of 9.7; **M85:** 9g $Na_2HPO_4.12H_2O$; 1.5g KH_2PO_4; 1g NH_4Cl; 10mg $MnSO_4.H_2O$; 10mg $CaCl_2.2H_2O$; 0,2g $MgSO_4.7H_2O$; 5mg ferric ammonium citrate; 1.5g sodium pyruvate, 3ml trace element solution, distilled water up to 1 liter, pH 7.1. Distribute in 30-50ml amounts in Erlenmeyer flasks and sterilize. Incubate without agitation. If needed, add 30 g agar per liter medium; **M114:** Columbia Blood Agar Base (Oxoid); **M185:** Trypticase Soy Broth (TSB, Oxoid); **M253:** NA supplemented with 1M sterile Na-sesquicarbonate solution to achieve a pH of 9,7 and with 0,5% NaCl; **M193:** R2A Agar Medium (Difco); **M207:** Brain Heart Infusion agar (BHI, Oxoid) supplemented with 100µl (per liter) of 1% vitamin B12 solution after sterilization; **M241:** TSA, adjusted to pH 5.5; **M242:** TSA adjusted to pH 6.5; **M255:** 5g yeast extract and 1 liter distilled water adjusted to pH 5.0 with 1N HCl; **M263:** Alkaline *Bacillus* medium: 10g glucose, 5g yeast extract, 10g peptone, 1g K_2HPO_4, 15g agar, 900ml distilled water; cooled to 50°C after sterilization and supplemented with 100ml of filter-sterilized 1% Na_2CO_3 solution; **M292:** 5g yeast extract, 5g peptone, 5g glucose, 15g agar, 1L distilled water and adjusted to a final pH of 10 with Na_2CO_3; **M308:** M207, adjusted to pH 10 with Na_2CO_3.

2.2.4 Screening of dissimilatory nitrogen metabolism

For detection of their denitrifying ability, all new isolates and reference strains were evaluated for reduction of nitrate (NO_3^-) and/or nitrite (NO_2^-) using Griess reagents (Smibert & Krieg, 1994) and for N_2O gas production with the acetylene inhibition method. All cultures were anaerobically grown in 10 ml liquid medium in 50-ml culture flasks with rubber stoppers. The headspace of the vials was replaced with filter-sterilized argon by evacuating and refilling six times. Acetylene (10%) was added to stop the reduction of N_2O to N_2. After a 2-week incubation period, a gas sample (1 ml) was taken with a gas-tight syringe and N_2O was measured with a gas chromatograph (Shimadzu GC-14B) equipped with a TCD (thermal conductivity detector), a pre-column (1m), a HayeSep Q column (2m, 80-100 mesh), and C-R8A Chromatopac V1.07 integrator. Cultures were scored positive for denitrification when ≥ 80% of fixed nitrogenous compound (NO_3^-/NO_2^-) was converted to N_2O.

2.2.5 Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study have been deposited in GenBank/EMBL/DDBJ with accession numbers AM403583 to AM403585, AM403593, AM403597, AM403599, AM403600, AM403602, AM403604 to AM403606, AM403609 to AM403611, AM403614, AM403618, AM403621, AM403623, AM403624, AM403630 to AM403632, AM403634, AM403635, AM403637, AM403638, AM403640, AM403642, AM403643, AM403645 to AM403648, AM403650, AM403651, AM403653.

2.3 Results

2.3.1 Enumeration and isolation of soil denitrifiers

An enumeration and isolation campaign was set up in parallel to quantify and retrieve cultured denitrifying soil bacteria able to grow on complex and defined electron donors (ethanol or succinate) and with NO_3^- or NO_2^- as electron acceptor. MPN enumeration of the soil sample (Table 2. 3) demonstrated the presence of 1.45 to 3.65 x 10^5 cultured denitrifiers per gram soil, dependent on the growth conditions. Abundance of cultured nitrate reducers was about one order of magnitude higher (0.36 to 2.44 x 10^6/g soil), suggesting that 15-40% of cultured nitrate reducers in the soil sample were able to denitrify. The ratio of total denitrifiers to total nitrate reducers was 100% for medium with ethanol/nitrate as electron donor/electron acceptor combination.

Table 2. 3 Enumeration of soil bacteria capable of nitrate reduction (T_{NR}) and denitrification (T_{DEN}).
Mean values are given, 95% confidence interval between brackets.

Functional groups	Growth conditions[a]			
	complex / nitrate	succinate / nitrite	ethanol / nitrate	succinate / nitrate
T_{NR} (x 10^6 CFU/ g soil)	2,44 (0,90 - 6,64)	ND[b]	0,36 (0,09 - 1,42)	1,52 (0,44 - 5,27)
T_{DEN} (x 10^5 CFU/ g soil)	ND[b]	3,65 (0,94 - 14,25)	3,65 (0,94 - 14,25)	1,45 (0,44 - 4,79)

[a] only electron donor and electron acceptor are given
[b] total number of denitrifying bacteria was not determined because of interference of dissimilatory nitrate reduction to ammonium (DNRA)

In the isolation campaign a total of 249 isolates was picked, of which 112 were confirmed as denitrifiers when tested in isolation medium with Griess reagents and for N_2O production with the acetylene inhibition method. These denitrifiers were assessed for their genetic diversity through RAPD fingerprinting (Fig. 2. 1), and representatives of each obtained cluster (36 in total) were selected for further identification. Analysis of the 16S rRNA gene (Table 2. 1 and Table 2. 4) revealed that the characterized denitrifiers belonged to 5 different genera/families within two different phyla. The majority of isolates (22 out of 36 clusters) were Gram-positives, belonging to the phylum *Firmicutes*. Herein most isolates showed highest 16S rRNA gene sequence similarities to type strains of *Bacillus bataviensis* and *Bacillus drentensis* (Table 2. 1 and Table 2. 4). The environmental sequences most similar to the 16S rRNA gene of these denitrifying bacilli were recovered from a metagenome from a bioreactor with green-waste compost and switch grass (87-90% similarity with GenBank accession n° ADGO01079332 and ADGO01073475, respectively). All other isolates belonged to the *Proteobacteria*, with a second major group of denitrifiers assigned to *Rhizobiaceae* family (10 out of 36 clusters). In this taxon, most isolates showed highest 16S rRNA gene sequence similarities to type strains of *Sinorhizobium morelense* and *Rhizobium radiobacter* (Table 2. 1 and Table 2. 4). The environmental sequences most similar to the 16S rRNA gene of these denitrifying *Rhizobiaceae* isolates were recovered from the metagenome sludge of an enhanced phosphorous removal bioreactor (96% similarity with GenBank accession number AATO01014156) and a marine metagenome from North Pacific Subtropical Gyre at 4000m depth (93-97% similarity with GenBank accession number ABEF01051492), respectively. When correlating the characterized denitrifiers to dilution of the sample from which they were isolated (through dilution-plating, data not shown), isolates with highest 16S rRNA genes sequence similarities to type strains of *Bacillus drentensis*, *Sinorhizobium morelense* and *Cupriavidus necator* represented the most abundant cultured denitrifiers in the studied soil sample, present at 10^5 CFU/g soil, which is in accordance with the enumeration results (Table 2. 3).

Table 2. 4 Identification of cultured denitrifying diversity retrieved from soil.
Assignment to genus or family level based on 16S rRNA gene sequence analysis, with specification of isolation conditions and number of isolates per genus/family.

Genus/family *class or phylum*	**Type strain with highest 16S rRNA gene sequence similarity to query sequence**				**Isolation conditions**		**Isolate n°**
	species name	**strain n°**	**% seq. simil.**	**Acc. n°**	**e^- donor**	**e^- acceptor (mM)**	
Bacillus	*Bacillus licheniformis*	LMG 12363^T	100%	X68416	succinate	NO_2^- (3mM)	2
Firmicutes	*Bacillus bataviensis*	LMG 21833^T	98,6 - 99,1%	AJ542508	succinate	NO_2^- (3mM)	6
					succinate	NO_3^- (3mM)	2
	Bacillus soli	LMG 21838^T	98,8 - 99,1%	AJ542513	succinate	NO_2^- (3mM)	2
	Bacillus drentensis	LMG 21831^T	98,7 - 100%	AJ542506	succinate	NO_2^- (3mM)	1
					succinate	NO_3^- (3mM)	5
					complex	NO_3^- (10mM)	1
	Bacillus pseudomycoides	LMG 18993^T	99,50%	AF013121	succinate	NO_3^- (3mM)	1
Staphylococcus *Firmicutes*	*Staphylococcus warneri*	LMG 13354^T	100%	L37603	succinate	NO_2^-(3mM)	2
Rhizobiaceae *Alphaproteobacteria*[a]	*Sinorhizobium morelense*	LMG 21331^T	100%	AY024335	ethanol	NO_3^- (18 mM)	5
	Rhizobium radiobacter	LMG 140^T	98,3 - 100%	AJ389904	ethanol	NO_3^- (18 mM)	2
					succinate	NO_3^- (3mM)	2
	Ensifer medicae	A321^T	100%	Z78204	ethanol	NO_3^- (18 mM)	1
Pseudomonas *Gammaproteobacteria*	*Pseudomonas kilonensis*	DSM 13547^T	100%	AJ292426	ethanol	NO_3^- (18 mM)	2
Cupriavidus *Betaproteobacteria*	*Cupriavidus necator*	LMG 8453^T	99,30%	AF191737	complex	NO_3^- (10 mM)	2

[a] different genera of the family *Rhizobiaceae* were taken together to highlight the high relatedness of these genera

2.3.2 Screening *Bacillus* reference strains

In the isolation campaign the majority of denitrifying isolates belonged to the Gram-positive genus *Bacillus*, which were also among the most abundant cultured denitrifiers in the studied soil sample under applied cultivation conditions. Motivated by these observations and by limited recent descriptions of denitrification in members of this genus, a large-scale screening of the denitrifying ability in *Bacillus* strains from various origins and belonging to various species was performed. For this, all *Bacillus* species available in the BCCM/LMG Bacteria Collection (November - December 2007) were included. Each species was represented by the type strain and, when available, also by other non-type strains assigned to the species and retrieved from different origins (all tested strains are listed in Table 2.2). In total 180 *Bacillus* strains belonging to 87 species were screened, covering a large part (54.4%, calculated June 2010) of the currently cultivated and validly described species diversity with the genus *Bacillus* (for a list of validly described taxa, we refer to http://www.bacterio.cict.fr/). Of these, 21 species were represented by the type strain only. The species identification of tested strains was adopted from the BCCM/LMG Bacteria Collection; no new identification tests were performed.

All strains were screened for their ability to denitrify in different growth conditions. Strains were scored for denitrification (i.e. consumption of NO_3^- and/or NO_2^- with N_2O production), NO_3^- reduction (i.e. consumption of NO_3^- to NO_2^- without N_2O production) and NO_2^- reduction (i.e. consumption of NO_2^- without N_2O production). As denitrification is a strain-dependent trait and thus type strains do not account for the metabolic diversity within a given species, the involved species was assigned the poten-

Table 2. 5 Overview of observed dissimilatory reduction of nitrogenous compounds in strain set with 180 *Bacillus* reference strains.

Species identification of strains were taken from the BCCM/LMG Bacteria Collection. See text for specific definition of each functional group. Reduction of nitrate and/or nitrite and denitrification have been tested in 6 different growth conditions with different e^- donors and e^- acceptors. The results are summarized in this table.

Species	n°[a]	Reduction of only			Denitrification from			No reduction
		NO_3^-	NO_2^-	NO_3^- and NO_2^-	NO_3^-	NO_2^-	NO_3^- and NO_2^-	
B. agaradhaerens	1	-[b]	+	-	-	-	-	-
B. altitudinis	1	-	-	-	-	+	-	-
B. amyloliquefaciens	8	+/- (1)	+/- (3)	+/- (1)	-	-	-	+/- (3)
B. aquimaris	1	+	-	-	-	-	-	-
B. asahii	1	-	-	-	-	+	-	-
B. atrophaeus	5	+/- (3)	-	-	-	-	+/- (1)	+/- (1)
B. azotoformans	3	-	-	-	-	-	+	-
B. badius	3	+/- (1)	-	+/- (1)	-	-	-	+/- (1)
B. bataviensis	2	-	-	+/-(1)	-	+/- (1)	-	-
B. cereus	1	-	-	-	-	+	-	-
B. clarkii	1	-	+	-	-	-	-	-
B. fastidiosus	4	+/- (2)	-	-	-	-	-	+/- (2)
B. fumarioli	4	+/-(3)	-	-	-	-	-	+/- (1)
B. galactosidilyticus	3	+/-(2)	-	-	-	-	-	+/- (1)
B. humi	2	-	-	+	-	-	-	-
B. hwajinpoensis	1	-	-	-	+	-	-	-
B. indicus	1	-	-	-	+	-	-	-
B. infantis	1	-	-	-	-	-	+	-
B. lehensis	1	-	-	-	-	-	+	-
B. licheniformis	35	-	+/- (1)	+/- (8)	+/- (17)	+/- (4)	+/- (3)	+/- (2)
B. luciferensis	2	+/- (1)	-	-	-	-	-	+/- (1)
B. megaterium	3	+/- (1)	-	-	+/- (1)	-	-	+/- (1)
B. methanolicus	1	-	-	-	+	-	-	-
B. mojavensis	4	+/- (2)	-	-	+/- (1)	-	-	+/- (1)
B. mycoides	4	+/- (1)	-	-	+/- (1)	-	-	+/- (2)
B. niacini	1	-	+	-	-	-	-	-
B. panaciterrae	1	-	-	+	-	-	-	-
B. plakortidis	1	-	-	-	-	-	+	-
B. psychrotolerans	1	+	-	-	-	-	-	-
B. ruris	2	-	+/- (1)	-	-	-	-	+/- (1)
B. shackletonii	1	+	-	-	-	-	-	-
B. siralis	1	-	-	+	-	-	-	-
B. smithii	4	+/- (1)	-	-	-	-	-	+/- (3)
B. soli	3	-	+/- (1)	-	-	-	-	+/- (2)
B. sonorensis	1	-	-	+	-	-	-	-
B. sporothermodurans	6	+/- (3)	-	-	-	-	-	+/- (3)
B. subtilis	7	+/- (4)	-	-	-	-	-	+/- (3)
B. thermantarcticus	1	+	-	-	-	-	-	-
B. thuringiensis	4	+/- (1)	+/- (1)	-	-	+/- (1)	+/- (1)	-
B. vallismortis	2	+	-	-	-	-	-	-
B. vietnamensis	1	-	-	-	-	-	+	-
B. vireti	1	-	-	-	+	-	-	-

[a] N° is number of strains tested; type strain always tested, except for *Bacillus fastidiosus*
[b] '+' = positive for all strains tested, '-' = negative for all strains tested
'+/-' = positive for some strains tested (number given between parentheses)

tial to denitrify when at least one tested strain of the species scored positive in one of the tested growth conditions (Table 2. 5). The denitrification potential was demonstrated in 45 strains (45/180 or 25% of all tested strains) belonging to 19 *Bacillus* species (19/87 or 21.8% of all tested species). Nitrate reduction not followed by nitrite reduction and nitrite reduction not followed by denitrification or preceded by nitrate reduction occurred in several strains belonging to the same species. This was observed in 12 and 5 *Bacillus* species respectively, while 6 species demonstrated the potential to reduce NO_3^- and NO_2^- (not always in the same growth conditions) but were not able to denitrify (i.e. produce N_2O) in any of the tested growth conditions. In total, 42 of all included *Bacillus* species (48.3%) were not able to contribute to a dissimilatory reduction of NOx (nitrous oxides).

2.3.3 Influence of growth conditions on denitrification

All reference strains of *Bacillus* were screened for their ability to denitrify in six different growth conditions, using NO_3^- or NO_2^- as electron acceptors combined with defined (succinate or ethanol) or complex electron donors, the latter based on optimal species-specific growth conditions recommended by the BCCM/LMG Bacteria Collection (Table 2. 2). We checked the influence of different electron donors and acceptors on the ability to phenotypically denitrify (Fig. 2. 2).

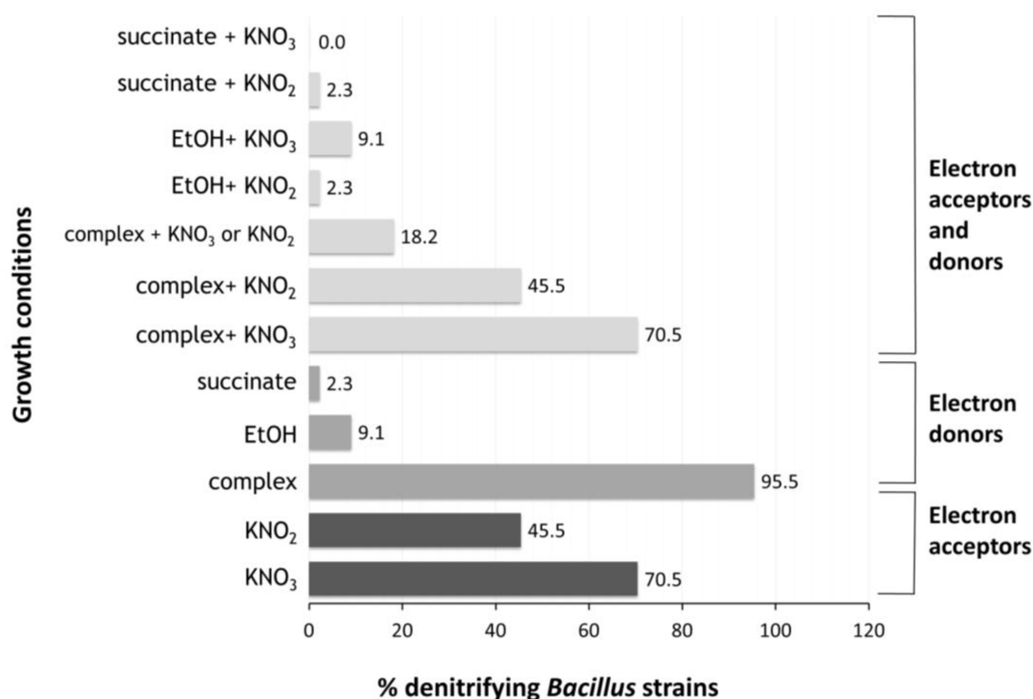


Fig. 2. 2 Influence of growth conditions on denitrifying capability of *Bacillus* reference strains.
Given: type of electron donor, electron acceptor and combination of both.

The majority of denitrifiers (70.5%) were able to start denitrification from nitrate as electron acceptor, almost half (45.5%) could start from nitrite, and a minority (18.2%) could start from both electron acceptors. Almost all denitrifying strains (95.5%) were able to start denitrification from complex electron donors, as opposed to only few strains that could use ethanol (9.1%) and succinate (2.3%). Denitrification with succinate was only coupled to nitrite as electron acceptor, while ethanol could be combined with both nitrate and nitrite. It should be noted that a large fraction of the denitrifying *Bacillus* reference strains were members of *Bacillus licheniformis* (Table 2. 2). Nevertheless, similar trends were observed when data of non-*Bacillus licheniformis* strains were evaluated without *Bacillus licheniformis* results (data not shown).

2.4 Discussion

This study demonstrated that the dissimilatory nitrogen metabolism, more specifically denitrification, is a common trait among members of the genus *Bacillus*. Although the nitrogen metabolism is strain-dependent and only *Bacillus* strains from a single bacteria collection – although harbouring a wide variety of *Bacillus* strains – and single isolation campaign were tested here, these strains were spread over more than half of the currently described species (180 *Bacillus* strains belonging to 87 species). In total, 19 species were shown to contain denitrifying representatives. To our knowledge, only four of the investigated species (Table 2. 5) were previously reported to include strains with a denitrifying activity, namely *Bacillus licheniformis* (Pichinoty *et al.*, 1978), *Bacillus azotoformans* (Pichinoty *et al.*, 1983, Suharti *et al.*, 2001), *Bacillus bataviensis* (Heyrman *et al.*, 2004, Fan *et al.*, 2006, Green *et al.*, 2010) and *Bacillus vireti* (Heyrman *et al.*, 2004, Fan *et al.*, 2006), with the remaining 15 species documented here for the first time (Table 2. 5). Twelve species contained only nitrate reducers, 5 only nitrite reducers and six species contained strains able to perform the reduction of NO_3^- and NO_2^- without the production of N_2O. The latter observation was not investigated further, but alternative microbial mediated metabolic processes, such as dissimilatory nitrogen reduction to ammonium (DNRA) (Burgin & Hamilton, 2007) can explain NO_3^- or NO_2^- removal from the medium. The ability of bacilli to perform DNRA has been observed previously, e.g. in *Bacillus subtilis* and *Bacillus licheniformis* (Tiedje, 1988). However, this screening did not intend to elucidate the different possible processes for the nitrate and nitrite reducing *Bacillus* strains and we therefore cannot assure presence or absence of either dissimilatory pathway.

An isolation strategy and characterization of pure cultures was used to study denitrification. Other similar studies (older and recent) have also identified *Bacillus* as potential denitrifiers in soil

(Gamble *et al.*, 1977, Garcia, 1977b, Pichinoty *et al.*, 1979, Weier & Macrae, 1992, Chèneby *et al.*, 2000, Wang & Skipper, 2004). Nevertheless, it is generally accepted that conclusions from such approaches cannot infer anything about denitrifier community structure in a certain ecosystem (Ritz, 2007). Moreover, molecular studies of denitrifying communities did not yet identify Gram-positive denitrifiers, or more specifically denitrifying bacilli, as significant players of denitrification in soil. However, at the moment, it is very difficult to assess *in-situ* abundance of denitrifiers other than *Proteobacteria* because suitable molecular tools are lacking. Since denitrification is regarded as a weak selector of the microbial community (Tiedje, 1988, Philippot *et al.*, 2009), abundance of microbiota in a certain ecosystem (regardless of their functional guild) can shed some light on potential denitrifier diversity. Based on cultivation work, *Firmicutes* families *Bacillaceae* and *Clostridiaceae* were in the past considered as abundant terrestrial inhabitants. Recent molecular studies only assigned a mean of 2% of total clone library composition to these Gram-positive taxa (Janssen, 2006), although this observation could be explained by problems with lysis of cells or formation of spores (Dunbar *et al.*, 1999, Janssen, 2006). Here, the isolated denitrifying bacilli were highly related to *Bacillus drentensis*, *Bacillus bataviensis* and *Bacillus soli* (Heyrman *et al.*, 2004). Members of these species have been identified as generally dominant active bacteria in the Drentse A grassland soils (Felske *et al.*, 1998), demonstrated with rRNA quantification and the combination of denaturing gradient gel electrophoresis (DGGE) and a 16S rRNA gene clone library. Thus, bacilli may have a role in terrestrial denitrification.

Other abundantly retrieved denitrifying isolates in this study belonged to known terrestrial denitrifiers *Sinorhizobium* and *Cupriavidus*. The denitrification trait is probably widespread among rhizobia (O'Hara & Daniel, 1985, Monza *et al.*, 2006, Fernandez *et al.*, 2008). They were already described as numerically dominant among isolated denitrifiers from the rhizosphere (Chèneby *et al.*, 2004) but not from bulk soils (O'Hara & Daniel, 1985). The retrieval of rhizobia in our study may be attributed to the choice of growth media, as rhizobia do not grow well on general complex media (Tiedje, 1988), and were only isolated here on defined growth media. *Cupriavidus* is a fairly recently described genus, created to accommodate some atypical members of the genus *Alcaligenes*. It harbors members that have been recognized as dominant denitrifying bacteria in soil (Gamble *et al.*, 1977, Weier & Macrae, 1992). In similar isolation campaigns on denitrification, members of the genus *Pseudomonas* were numerically always well represented (Gamble *et al.*, 1977, Weier & Macrae, 1992, Wang & Skipper, 2004). Here, only two unique isolates, each representing an RAPD cluster, were retrieved.

Criteria and methodologies for the identification of denitrifiers have already been described (Mahne & Tiedje, 1995), but guidelines on growth conditions to test denitrification in pure cultures are

lacking. Based on our data, complex media are most appropriate for aerobic heterotrophic growth and well-suited for testing denitrification, after addition of an electron acceptor. Only succinate and ethanol were investigated here as electron donors in defined media, but other carbon sources could be more generally suitable. Their relative success in the isolation campaign compared to trypticase soy agar can probably be attributed to suitability of the latter for non-denitrifying growth. Almost 27% of all tested denitrifying strains were able to start denitrification from both nitrate and nitrite, 53% could only use nitrate and 20% only nitrite. Nitrite is generally considered as toxic for bacteria, and not often supplemented to growth media for isolation or denitrification testing purposes. In addition, in (commercial) miniaturized biochemical panels such as API (Analytical Profile Index), only a test for nitrate reduction is included. Our data suggests that a significant proportion of true denitrifiers is neglected or discarded when nitrite is not separately tested as electron acceptor. Although it is generally known for many years that some bacteria can only respire nitrite (Garcia, 1977b, Pichinoty *et al.*, 1979, Zumft, 1992), it is necessary to underline the usefulness of testing both electron acceptors for future pure culture characterizations. For isolation purposes, use of nitrite also has its benefits, with retrieving a different denitrifying diversity from the environment. Indeed, in this study, representatives of some species, namely *Bacillus licheniformis* and *Bacillus soli*, would not have been isolated from soil when only nitrate would have been used - and also avoids the high background of nitrate reducers. It should be noted that a minority of strains favored one medium above the other to start denitrification, e.g. only NO_2^--reduction on the defined media and denitrification from NO_3^- on complex medium. Our results demonstrate that strains can display different phenotypes depending on the test conditions - either electron donor, electron acceptor or other parameters not varied in this study – and thus can possible remain unidentified as potential denitrifying strains.

Although the denitrification process has already been extensively studied in soil and other environments and it is generally accepted that the process is widespread among microbial life, identification of major players remains difficult. Here, we demonstrate that denitrifying bacteria are well-represented among members of the genus *Bacillus*. As such, this study provides information on the nitrogen metabolism of publicly available *Bacillus* strains, facilitating future whole genome sequencing efforts necessary to generate new functional gene sequence data. We believe that cultivation studies and good characterization of nitrogen metabolism of new microbial taxa are necessary for archiving the microbial diversity capable of denitrification. Therefore, we recommend (i) inclusion of both nitrate and nitrite for isolation and denitrification testing and (ii) use of optimal growth medium allowing aerobic growth for denitrification testing.

2.5 Acknowledgements

This work was partially supported by the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005). The authors wish to acknowledge Pascal Boeckx for kindly providing acetylene and lab technician Denis Demeyer for technical assistance.

Reflection and discussion

Because of cultivation-based research the Firmicute family *Bacillaceae* was in the past considered to belong to one of the most abundant populations in terrestrial habitats. Members of these families, e.g. *Bacillus* (Tzeneva, 2006) and *Geobacillus* (Zeigler, 2014), are still frequently cultivated from and detected in many soils. However, more recent molecular studies and soil cell extraction methods only assigned a mean of 2% of total clone library composition (Janssen, 2006) and 1-5% of the total cells obtained (Holmsgaard *et al.*, 2011) to these Gram-positive taxa, although, that observation could be explained by problems with cell lysis, formation of spores or strong attachment to soil particles (Dunbar *et al.*, 1999, Janssen, 2006, Holmsgaard *et al.*, 2011). In addition, in a number of studies *Bacillus* spp. have been identified as potential denitrifiers in soil (Gamble *et al.*, 1977, Garcia, 1977b, Pichinoty *et al.*, 1979, Weier & Macrae, 1992, Chèneby *et al.*, 2000, Wang & Skipper, 2004). Unfortunately, detailed functional analysis – on all facets - of denitrification is still largely missing in this group of bacteria.
Therefore, this chapter reports on (1) the retrieval of an abundance of denitrifying *Bacillus* spp. from luvisol soil in an isolation strategy, (2) subsequent characterization of the potential for denitrification in 180 pure *Bacillus* cultures, and (3) the influence of different electron donors and acceptors on the presence of this trait. The screening of *Bacillus* reference strains demonstrated the potential for dissimilatory reduction of nitrogen compounds in more than half of the investigated species, with 19 species containing denitrifying members. Moreover, using both nitrate and nitrite as electron acceptors and complex electron donors was crucial to detect the denitrification potential in the reference strain set. Conversely, the use of defined electron donors enabled the recovery of environmental denitrifiers. Results of both strategies suggest that bacilli may be potential important contributors to denitrification in terrestrial and possibly other ecosystems and that denitrification phenotype strongly depends on the test conditions.

Although both the isolation campaign and the screening advocates the presence of dissimilatory reduction of NOx, e.g. denitrification, in many members of the genus *Bacillus,* a number of aspects regarding detection could have been improved:

The screening for denitrification of the *Bacillus* reference strain set – regardless on which medium – comprised the detection of the removal of nitrite with Griess reagents and the gas

chromatographic detection of N_2O with the acetylene inhibition technique. We assumed that when ≥ 80% of the fixed nitrogenous compound (NO_3^-/NO_2^-) was present as N_2O in the headspace of liquid cultures after 14 days of incubation, denitrification had occurred. However, some other causes for this N_2O build-up in the headspace are plausible:

In the treatment with ethanol as a carbon-source, 18mM of nitrate or nitrite was added to mineral medium. Nitrite is known to be toxic to bacterial cells, although nitrite-toxicity seems to be strain-dependent (Bollag & Henninger, 1978). Several enzymatic routes exist in bacterial cells for the reductive detoxification to nitrite over NO to N_2O, such as the reduction of nitrite to NO by NarG and detoxification of NO by flavohaemoglobin or flavorubredoxin, the latter widespread in dissimilatory nitrate-reducing bacteria (Gilberthorpe & Poole, 2008). Provided that cells did not perish, accumulation of nitrite (via reduction of nitrate without denitrification) or the supplemented nitrite itself may have led to detoxification, yielding N_2O. Ideally, when provided with optimal growth conditions, most known denitrifiers are able to convert nitrogen compounds within 4-5 days (in our case to N_2O) during the exponential phase of growth. On the other hand, N_2O production is also possible when actively growing cells reduce nitrate to nitrite and detox the accumulating nitrite during the stationary phase to N_2O. Since we performed end-point assessment of the denitrification ability after a two-week incubation period, it cannot be ruled out that measured N_2O was (partly) due to detox of reduced nitrate.

Microbial mediated DNRA is a dissimilatory process reducing nitrate to ammonium that competes with denitrification in terrestrial environments. The gene inventory for (part of) both processes can be present in one single organism (Heylen & Keltjens, 2012, Sanford *et al.*, 2012, Mania *et al.*, 2014) and DNRA organisms have been reported to produce N_2O (Bleakley & Tiedje, 1982, Vine & Cole, 2011, Rowley *et al.*, 2012). In the used complex growth media, we cannot fully exclude DNRA as N_2O producing mechanism due to the following issues:

(1) With phenol red as pH indicator, we considered a decrease in pH indicative of DNRA and an increase in pH indicative of denitrification, however, the colour change of the indicator in the complex media was visually not always as clear-cut .

(2) At that time, it was not possible to measure ammonium after incubation in complex media due to cross-reaction or inhibition by certain components , most likely amino acids.

(3) We evaluated end-point presence or absence of nitrite after the incubation of the cultures in complex medium supplemented with nitrate and associated the initial nitrate concentrations and the nitrite removal to amounts of produced N_2O as a measure for denitrification. However, in DNRA, nitrite accumulation after the respiratory reduction of nitrate to nitrite

may make N_2O a significant by-product due to detoxification of nitrite over NO to N_2O (Streminska *et al.*, 2012, Mania *et al.*, 2014).

Another observation we made was that only few strains were able to use ethanol or succinate for denitrification while incubated in mineral medium. However, growth of the cultures was only visually inspected and it was obvious that mineral medium was not (fully) supporting growth of the bacilli. At first, we assumed that this was due to the inability of *Bacillus* to denitrify on the specific combinations and concentrations of electron donor and/or acceptor, however, it seems that some *Bacillus* strains may have additional nutritional requirements (e.g. certain amino acids or casamino acids) that surpass the kind of nutrients present in mineral medium. Alternatively, the cease of growth in the treatments with ethanol could have been caused due to the high concentrations of nitrate/nitrite causing toxicity to bacterial cells.

Thus, the processes responsible for N_2O formation in *Bacillus* remain partially unresolved. In general, shorter incubation times, measurement of ammonium, growth rate measurements with simultaneous monitoring of nitrate and nitrite, and addition of specific growth factors to the mineral media would have allowed to more accurately pinpoint which *Bacillus* strains were able to reduce nitrate and nitrite to N_2O as denitrifiers or produce N_2O as nitrate ammonifiers via detoxification. However, this would have been an immense task for 180 strains. Assessment has become even more complex since a patchwork of metabolisms can sometimes be present in one organism. For example, the combined occurrence of respiratory ammonification and the expression of nitrous oxide reductase (*nosZ*) – the latter reducing N_2O originating from detoxification of nitrite – in *Wolinella succinogenes* and *Bacillus vireti* (Simon *et al.*, 2004, Mania *et al.*, 2014), with *Bacillus vireti* even performing a denitrification-like production of N_2O and N_2.

In this chapter, we advocate an improved characterization of the N-metabolism of new taxa to aid archiving of the (cultivable) microbial diversity capable of denitrification. However, denitrification should definitely not be considered as a taxonomical differential characteristic since (i) there is no overall interrelatedness between denitrifying microorganisms and (ii) gene phylogenies of the denitrification genes are significantly different from 16S rRNA gene phylogenies – with the seeming exception of *nosZ*. Notwithstanding these dissimilarities, we do believe that identification of properly characterized denitrifiers or vice versa – correct characterization of denitrification in identified strains – entails more

than only expansion of knowledge on diversity. Indeed, one can argue that the identity of an organism performing denitrification is not relevant, as long as the process can be monitored and understood. However, environmental parameters that influence denitrification such as pH, temperature, salinity,… etc. are linked with the growth characteristics of the microorganisms present, e.g. of the dominant denitrifiers. Thus far well-documented denitrification (regulatory) phenotypes in a small number of model organisms, however, do not suggest any systematic relationship between phylogeny and the various phenotypic characteristics (Bakken *et al.*, 2012). Nevertheless, because both phylogenetic identity and biodiversity affect ecosystem functioning and because organism identity – if maybe only at higher taxonomic ranks – is often linked to specific growth characteristics or ecology (Philippot *et al.*, 2010, Wessén *et al.*, 2010), identification of the present (and active) denitrifiers may make us better understand and manage denitrification, and often associated N_2O emissions, in certain ecosystems. This will not be straightforward though, since species-specific effects depend on often changing environmental conditions and are therefore difficult to predict (Philippot *et al.*, 2013).

The work in this chapter focused on a.o. isolation of functional denitrifiers with traditional isolation techniques. Isolation and cultivation allows for more detailed taxonomic, physiological and biochemical as well as genetic characterization of strains belonging to a certain functional guild. The bottle-neck of isolation strategies, however, often is the amount of laborious culturing work, skewing assessment of the denitrifier diversity from soil samples.

With the advent of the robotized colony picker at LM-UGent, a whole range of high-throughput possibilities now become available for tapping and analyzing the Gram-positive denitrifier diversity from all kinds of samples with so-called miniaturized 'microbial culturomics' (Lagier *et al.*, 2012). Combined with high-throughput bacterial identification tools, miniaturized colorimetric assays and gas chromatographic analyses of NOx compounds, this approach may constitute a revolutionary cultivation technique and has the potential to generate a large number of Gram-positive denitrifiers. For example, denitrifying spore-forming *Firmicutes*, e.g. from soil, can be selectively isolated and grown in pure cultures by (i) subjecting a sample to 80°C for 10 to 30 minutes (pasteurization), (ii) subsequent spread plating of the heat-resistant endospores, (iii) colony picking and transfer to 96-multiwell plates with broth media fit for denitrification, (iv) incubation under anaerobic denitrifying conditions, and (v) further dereplication, identification and functional analyses for detection of nitrogenous compounds. Thereupon a better microbial archiving of the cultivable Gram-positive denitrifier diversity – both on a phylogenetic as on a phenotypic level – may become feasible, allowing for selection and/or manipulation of

microbiota that can provide ‘ecosystem services’, e.g. the removal of N_2O (Calvo *et al.*, 2013) as part of an improved microbial resource management.

Redrafted from:

Verbaendert, I., Hoefman, S., Boeckx, P., Boon, N., De Vos, P. (2014). Primers for overlooked *nirK*, *qnorB* and *nosZ* genes of thermophilic Gram-positive denitrifiers. FEMS Microbiology Ecology, 89 (1): 162-180.

Author's contributions:

IV, PDV & NB designed the experiments. IV performed the experiments, analyzed the data and wrote the manuscript. SH & PB assisted with initial installation and calibration of the compact-GC. PB initially also provided acetylene gas. PDV, NB, PB and IV proofread and commented on the manuscript.

Chapter 3

Primers for overlooked *nirK*, *qnorB* and *nosZ* genes of thermophilic Gram-positive denitrifiers

Summary

Although efforts have been made the past few years, knowledge on genomic and phenotypic diversity and occurrence of the denitrification ability in Gram-positive bacteria is still fragmentary. Many environmental monitoring approaches have used *nir*, *nor* and *nos* genes as marker genes for detection of denitrification or denitrifying bacteria. However, primers used in these methods often fail to detect the genes in specific bacterial taxa, such as Gram-positive denitrifiers. In this study, novel primer sets specifically targeting *nirK, qnorB* and *nosZ* genes of the Firmicute genus *Geobacillus* were developed by genomic mining and tested in parallel with commonly used primers on a set of phylogenetically closely related denitrifying geobacilli. Novel *nirK* and *qnorB* sequences were recovered from all strains tested, whereas *nosZ* was detected in part of the strain set, which was in agreement with phenotypic testing. Inter-species and modest intra-species variations in AFLP (Amplified Fragment Length Polymorphism) patterns were observed, verifying presence of genomic variation within the strain set. Our study shows that closely related Gram-positive denitrifiers may differ in denitrification phenotype and genotype. But foremost, novel primers targeting very divergent *nirK*, *qnorB* and *nosZ* gene sequences of Gram-positive denitrifiers are now available for cultivation-independent environmental surveys.

3.1 Introduction

Nitrate and nitrite are employed by denitrifiers as alternative electron acceptors in micro-oxic or anoxic environments (Knowles, 1982). For that, different sequentially induced enzymes catalyze a four-step modular process: nitrate (NO_3^-) reductase, nitrite (NO_2^-) reductase, nitric oxide (NO) reductase and nitrous oxide (N_2O) reductase. This denitrification process converts fixed nitrogen into gaseous N_2O and/or nitrogen gas (N_2) that are emitted into the atmosphere, hence contributing to nitrogen loss in agricultural soils (Cameron *et al.*, 2013), the greenhouse effect and destruction of the ozone layer (Ravishankara *et al.*, 2009).

In-depth studies of only a limited number of organisms lie at the basis of the current knowledge of denitrification (Bergaust *et al.*, 2011), but organism-based research has proven to provide novel insights on all aspects of the process (Green *et al.*, 2010, Jones *et al.*, 2011, Heylen & Keltjens, 2012) and comparison of individual denitrifying strains can provide new insights on denitrifier diversity (Jones *et al.*, 2011). Despite the fact that the denitrification trait has been observed in several Gram-positive strains (Shapleigh, 2006, Jones *et al.*, 2011, Verbaendert *et al.*, 2011a, Verbaendert *et al.*, 2011b), the fundamentals of denitrification within this group, such as biochemistry, majors drivers of end-product stoichiometry and DRP (denitrification regulatory phenotype) (Bergaust *et al.*, 2011), are still underexplored. Furthermore, molecular community analyses of denitrifiers in the environment fail to detect this group of organisms because most available PCR primers for nitrite reductase (*nir*), nitric oxide reductase (*nor)* and nitrous oxide reductase (*nosZ*) genes have been designed on genes of Gram-negative *Proteobacteria* (Braker *et al.*, 1998, Scala & Kerkhof, 1998, Hallin & Lindgren, 1999, Casciotti & Ward, 2001, Kloos *et al.*, 2001, Braker & Tiedje, 2003, Thröback *et al.*, 2004, Goregues *et al.*, 2005, Henry *et al.*, 2006, McGuiness *et al.*, 2006, Liang *et al.*, 2007). Together with the lack of knowledge on the presence and contribution of Gram-positive denitrifiers in nature, both molecular and physiological characterization of denitrification in these organisms therefore still needs to be tackled.

A genomic study of *Geobacillus thermodenitrificans* NG80-2 revealed the presence of complete gene clusters for denitrification (Feng *et al.*, 2007), putting the process in the Gram-positive Firmicute genus *Geobacillus* on the map. *Geobacillu*s is a phenotypically and phylogenetically coherent genus of thermophilic sporeformers with high interspecies and intraspecies 16S rRNA gene sequence similarity (Coorevits *et al.*, 2012) and members of this genus are present in various terrestrial and marine environments (Zeigler, 2014) and have been isolated on every continent from both geothermal areas and temperate regions (McMullan *et al.*, 2004) and from both natural hot environments as well as artificial, man-made thermal environments (Obojska *et al.*, 2002, Prakash *et al.*, 2012). Surprisingly however, cool

terrestrial environments also seem a reservoir for large numbers of viable cells of these supposedly obligate thermophilic bacteria (Marchant & Banat, 2010). Although *Firmicutes*, a.o. *Geobacillus*, are soil inhabitants that have been reported to be the most abundant and/or dominant active group of bacteria in certain ecosystems (Felske *et al.*, 1998, Rahman *et al.*, 2004, Janssen, 2006), studies on functional - physiological and ecological – roles, in particular on nitrogen transformations, of these and other thermophilic organisms in both hot as cold environments are virtually nonexistent. Apart from a number of earlier reports on phenotypic denitrification and denitrification enzymes in *Geobacillus* (Garcia, 1977b, Ho *et al.*, 1993, Manachini *et al.*, 2000, Nazina *et al.*, 2001), in-depth research on thermophilic denitrifying bacteria has only been started recently (Pavlostathis *et al.*, 2006, Mishima *et al.*, 2009, Nara *et al.*, 2009, Fukuda *et al.*, 2011, Matsumoto *et al.*, 2012, Salomonsson *et al.*, 2012, Jung *et al.*, 2013).

Primers for functional genes are still often used to measure denitrification and to investigate denitrifier abundance and diversity in the environment. However, currently available primers for *nir* and *nor* do not target Gram-positive denitrification genes and only few do for *nos*, leading to inaccurate modeling of denitrifier community structures and nitrogen fluxes. Therefore the main goals of this study were to (i) design *nirK*, *qnorB* and *nosZ* primer sets for thermophilic Gram-positive denitrifying geobacilli, (ii) to examine the relationship of obtained *nirK, qnorB* and *nosZ* sequences to the large number of publicly available sequences, and (iii) to understand, at a genetic level, denitrification pathways within the strain set. In order to also explore overall genetic variability and phenotypic denitrification potential within a set of closely related *Geobacillus* strains from various origins, AFLP (Amplification Fragment Length Polymorphism) fingerprinting in combination with measurement of N_2O and N_2 production under denitrifying conditions was performed.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

Twenty-one strains belonging to *Geobacillus kaustophilus, Geobacillus toebii*, *Geobacillus stearothermophilus* and *Geobacillus thermodenitrificans* were selected from the BBCM/LMG bacteria collection and from the LM-UGent Research collection (Table 3. 3). They were grown in 120mL glass serum bottles containing growth medium, with a final liquid phase volume of 50mL. Both TSB (Trypticase Soy Broth, Oxoid) and 1/10 TSB, supplemented with 10mM potassium nitrate (KNO_3), and mineral medium (Stanier *et al.*, 1966), supplemented with 10mM sodium succinate and 10mM KNO_3 (MM), were used as growth media. Culture vessels were sealed with black butyl rubber stoppers, secured with airtight aluminum crimps and were sterilized by autoclaving for 20 min at 121°C. Vitamin solutions

(Widdel & Bak, 1992, Kniemeyer *et al.*, 1999) and trace metal solutions (Widdel & Bak, 1992) were added from filter-sterilized stocks to the vials with MM through the stoppers with sterile needles and syringes. Before inoculation, headspace atmospheres were replaced by filter-sterilized helium (He) in a 4-cycle procedure of evacuation and He addition. Culture vessels received acetylene (10% [vol/vol] of the headspace) and 1% (vol/vol) inoculum (with a density of McFarland 1) with a slight overpressure of 0.3 bar present after inoculation. Acetylene was used to block conversion of N_2O to N_2, enabling measurement of denitrified nitrogen as N_2O by gas chromatography. One % (vol/vol) TSB was added to additional cultures on MM, supplemented with vitamins and trace elements, to provide growth requirements of the strains creating modified MM (mMM). All cultures were incubated at 55°C and shaken at 150 rpm for 7 days. An additional similar experiment on mMM without acetylene was performed to test for N_2 production. Growth experiments and blanks (i.e. medium without inoculum) were performed in triplicate. Nitrate was omitted from control cultures to test for fermentative growth.

3.2.2 Analytical methods

After the incubation period and equilibration of the flasks to room temperature, gas pressure was measured by a pressure gauge attached to a needle and a gas sample (5mL) was taken through the rubber stopper with a gas-tight syringe. The gas sample was analyzed for N_2O and/or N_2 with a gas chromatograph (CompactGC, InterScience) equipped with an TCD (Thermal Conductivity Detector) and a capillary (Molsieve 5A. 7m*0.32mm) and Rt-QBond (3m*0.32mm) column. The operating conditions were as follows: carrier gas He (5mL/min), injector temperature 65°C, column and oven temperature 40°C and detector temperature 110°C. The chromatograph was calibrated using N_2O and N_2 standard gases; results were analyzed with the EZChrome Elite Software. Production of N_2O and N_2 was calculated on the basis of measured headspace concentration and pressure and corrected for dissolved gas, using the ideal gas law and Henry correction (Sander, 1999).

End-point sampling of cultures (500 µL) was performed. To extract inorganic nitrogen, samples were pre-treated with 2.5 mL KCl, shaken at room temperature for one hour and filtered through a 2µM cellulose filter (Keeney & Nelson, 1987). Nitrate concentrations were determined by chemical reduction of nitrate to nitrite using active cadmium (Cataldo *et al.*, 1975, Navarro-Gonzálves *et al.*, 1998), nitrite concentrations were determined with Griess reagents (Smibert & Krieg, 1994) and pH was established before and after incubation with a calibrated pH meter.

3.2.3 Primer design

The novel primers used in this study are shown in Table 3. 1. The nucleotide sequence of the *nirK* gene from *Geobacillus thermodenitrificans* NG80-2 (CP000557) was used as query sequence in nBLAST and pBLAST to retrieve homologous sequences from annotated whole genome sequencing projects from the EMBL sequence database. Cut-off for e-values of homologous genes was set at 1e-5. Only sequences from Gram-positive and from presumably denitrifying strains were retained; for this purpose, denitrification was assumed when both a gene encoding a nitrite reductase (either *nirK* or *nirS*) and a gene encoding a nitric oxide reductase (*qnorB* or *cnorB*) were present in the genome. Genes that did not have definite annotation in published genomes were examined by comparison of conserved positions using PSI-BLAST (Altschul *et al.*, 1997) to confirm gene identity. DNA-based alignments were made using MEGA version 5 (Tamura *et al.*, 2011) under amino acid settings. Degenerated *nirK* primers were designed for the family *Bacillaceae* with focus on *Geobacillus,* targeting the same conserved gene regions as used previously (Braker *et al.*, 1998, Hallin & Lindgren, 1999, Casciotti & Ward, 2001, Goregues *et al.*, 2005, Liang *et al.*, 2007)(Fig. 3.1,, A).

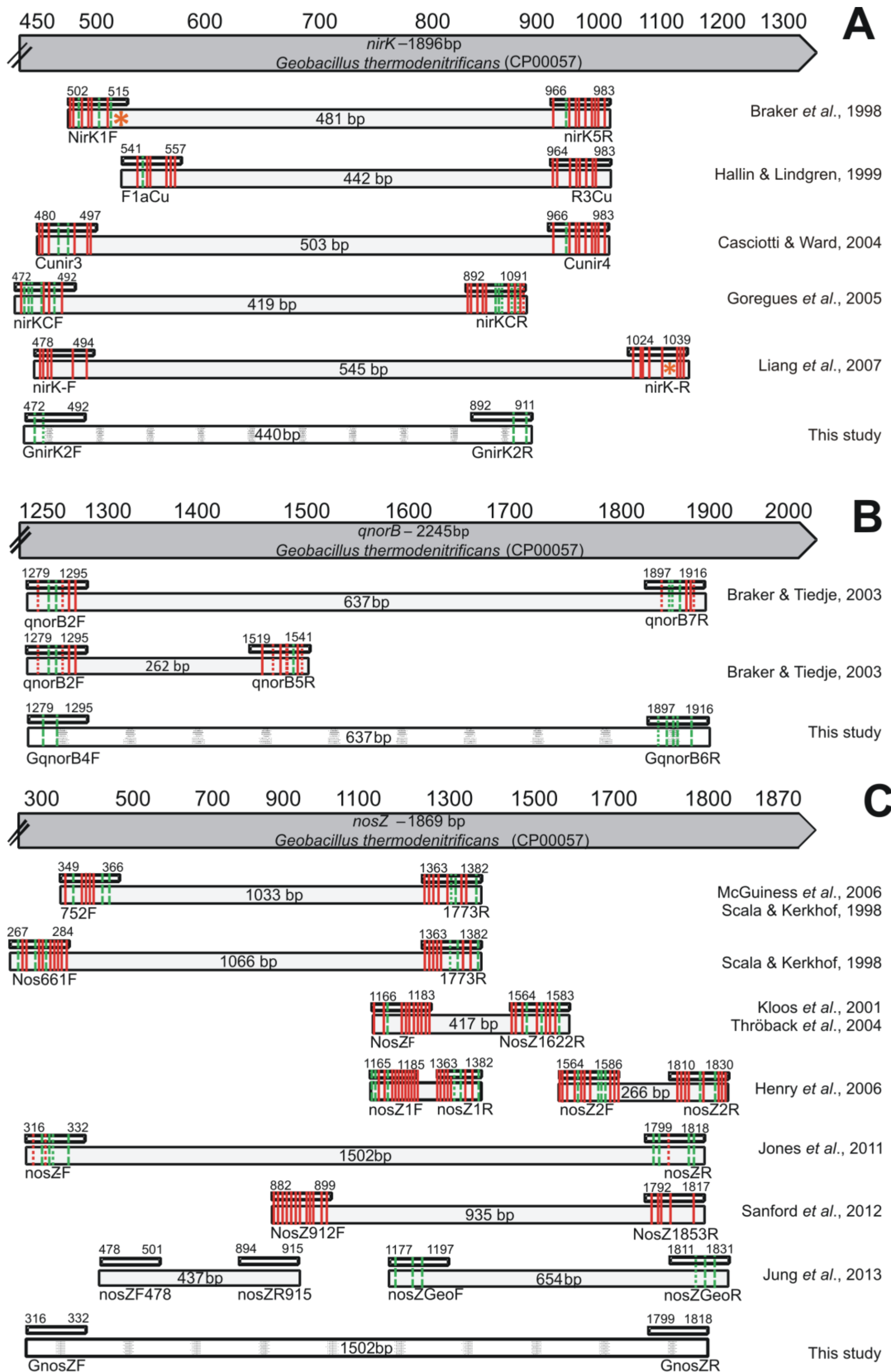
For *qnorB* and *nosZ* primers a similar approach for retrieval and alignment of sequences of *Bacillaceae* genomes and genomes from Gram-positive bacteria was adopted, resulting in the design of degenerated *qnorB* and *nosZ* primers for the family *Bacillaceae* with focus on *Geobacillus*, targeting conserved gene regions as used before (Braker & Tiedje, 2003, Jones *et al.*, 2011)(Fig. 3.1, B and C).

Table 3. 1 Novel primers for amplification of *nirK*, *qnorB* and *nosZ* genes in *Geobacillus*.

Gene	Primer[a]	Position (nt)	Sequence (5'-3')[b]	Degeneracy[c]
nirK	GnirK2F	472 - 492	GGK GTV TTT ATG TAC CAT TGC	6
	GnirK2R	892 - 912	SCC GCT YGC CGG AAG CAT CAC	4
qnorB	GqnorB4F	1279 - 1295	GGC CAY CAA GGY TGG GA	4
	GqnorB6R	1916 - 1897	GGC AAR TTG ATW ARR AAV CC	16
nosZ	GnosZF	316 - 332	TGG GGA GAT TTG CAT CA	0
	GnosZR	1798 - 1817	GAA CAG AAG TTC GTG CAA TA	0

[a]Primers are named by *nirK*, *qnorB* or *nosZ* targeting genes for copper-containing nitrite reductase, quinol-oxidizing nitric oxide reductase and nitrous oxide reductase; forward and reverse primers are indicated by F and R as the last letter, respectively.
[b] Positions in *nirK*, *qnorB* and *nosZ* genes of *G. thermodenitrificans* NG80-2 (CP000557).
[c] The overall degeneracy of the primer equals multiplication of the degeneracies at different positions present in the primer sequence, with the degeneracy at a specific nucleotide position being dependent on the used base. E.g. primer GnirK2F contains a K (standing for either one of the bases G or T) and a V (standing for either one of the bases A, C or G), leading to a degeneracy of 2*3=6)

Fig. 3. 1 (next page) Schematic illustrations showing comparison of primer positions in *Geobacillus thermodenitrificans* NG80-2 *nirK* (A), *qnorB* (B), and *nosZ* (C) gene sequences (CP00057). Primers are represented by black-framed rectangles. Mismatches and 4-fold, 3-fold and 2-fold degeneracies with *Geobacillus thermodenitrificans* nucleotide positions are indicated with red lines, dotted red lines, dotted green lines and dashed green lines, respectively. Three-bp insertions (deletions) in the nirK-R and nirK1F target region are indicated with *. Amplicon size is shown in PCR-products of matching primer sets.



A
450 500 600 700 800 900 1000 1100 1200 1300
nirK –1896bp
Geobacillus thermodenitrificans (CP00057)
502 515 966 983
481 bp
NirK1F nirK5R
Braker et al., 1998
541 557 964 983
442 bp
F1aCu R3Cu
Hallin & Lindgren, 1999
480 497 966 983
503 bp
Cunir3 Cunir4
Casciotti & Ward, 2004
472 492 892 1091
419 bp
nirKCF nirKCR
Goregues et al., 2005
478 494 1024 1039
545 bp
nirK-F nirK-R
Liang et al., 2007
472 492 892 911
440bp
GnirK2F GnirK2R
This study
B
1250 1300 1400 1500 1600 1700 1800 1900 2000
qnorB – 2245bp
Geobacillus thermodenitrificans (CP00057)
1279 1295 1897 1916
637bp
qnorB2F qnorB7R
Braker & Tiedje, 2003
1279 1295 1519 1541
262 bp
qnorB2F qnorB5R
Braker & Tiedje, 2003
1279 1295 1897 1916
637bp
GqnorB4F GqnorB6R
This study
C
300 500 700 900 1100 1300 1500 1700 1800 1870
nosZ –1869 bp
Geobacillus thermodenitrificans (CP00057)
349 366 1363 1382
1033 bp
752F 1773R
McGuiness et al., 2006
Scala & Kerkhof, 1998
267 284 1363 1382
1066 bp
Nos661F 1773R
Scala & Kerkhof, 1998
1166 1183 1564 1583
417 bp
NosZF NosZ1622R
Kloos et al., 2001
Thröback et al., 2004
1165 1185 1363 1382 1564 1586 1810 1830
266 bp
nosZ1F nosZ1R nosZ2F nosZ2R
Henry et al., 2006
316 332 1799 1818
1502bp
nosZF nosZR
Jones et al., 2011
882 899 1792 1817
935 bp
NosZ912F NosZ1853R
Sanford et al., 2012
478 501 894 915 1177 1197 1811 1831
437bp 654bp
nosZF478 nosZR915 nosZGeoF nosZGeoR
Jung et al., 2013
316 332 1799 1818
1502bp
GnosZF GnosZR
This study

The *in-silico* efficiency of previously described primers (Braker *et al.*, 1998, Scala & Kerkhof, 1998, Hallin & Lindgren, 1999, Casciotti & Ward, 2001, Kloos *et al.*, 2001, Braker & Tiedje, 2003, Thröback *et al.*, 2004, Goregues *et al.*, 2005, Henry *et al.*, 2006, McGuiness *et al.*, 2006, Liang *et al.*, 2007, Jones *et al.*, 2011, Sanford *et al.*, 2012, Jung *et al.*, 2013) was examined by alignment of the primers with primer binding sites in the genome of *Geobacillus thermodenitrificans* NG80-2 (Fig. 3.1) and of a set of *Bacillaceae* (Table 3. 3). A subset of the primers was also experimentally tested with total genomic DNA from *Geobacillus thermodenitrificans* LMG 17532^T, *Geobacillus kaustophilus* LMG 9819^T *Geobacillus stearothermophilus* LMG 6939^T, *Achromobacter denitrificans* LMG 1231^T, *Alcaligenes faecalis* LMG 1229^T, *Cupriavidus necator* LMG 1201, *Ochrobactrum anthropi* LMG 2136 and *Paracoccus denitrificans* LMG 4049^T (Table 3. 2).

Table 3. 2 Comparison qualitative PCR performance for established and novel primers for a selection of *Geobacillus* and non-*Geobacillus* reference strains.

		PCR products[a]											
		nirK						*qnorB*			*nosZ*		
		nirK1F-nirK5R[b]	F1aCu-R3Cu[c]	Cunir3-Cunir4[d]	nirKCF-nirKCR[e]	nirK-F-nirK-R[f]	GnirK2F-GnirK2R[g]	qnorB2F-qnorB7R[h]	qnorB2F-qnorB5R[i]	GqnorB4F-GqnorB6R[j]	NosZ-F-NosZ1622R[k]	nosZ-F-nosZ-R[l]	GnosZF-GnosZR[m]
Species	**Strain**	(514)	(472)	(538)	(475)	(600)	(440)	(637)	(262)	(606)	(1600)	(1502)	(1502)
A. denitrificans[p]	LMG 1231^T	+	+	-	n/d	+	-	+	+	-	n/d	-	-
A. faecalis[q]	LMG 1229^T	-	+	-	n/d	+	-	+	+	-	n/d	-	-
C. necator[r]	LMG 1201^T	-[n]	-[n]	-[n]	-[n]	-[n]	-[n]	+	+	-	+	-	-
O. anthropi[s]	LMG 2136	+	+	-	n/d	+	-	-[o]	-[o]	-[o]	n/d	-	-
P. denitrificans[t]	LMG 4049^T	-[n]	-[n]	-[n]	-[n]	-[n]	-[n]	+	+	-	n/d	-	-
G. thermo[u]	LMG 17532^T	-	-	-	-	-	+	-	-	+	-	+	+
G. kaustophilus	LMG 9819^T	-	-	-	-	-	+	-	-	+	-	+	+
G. stearo	LMG 6939^T	-	-	-	-	-	+	-	-	+	-	-	-
G. thermo	R-32614	-	-	-	-	-	+	-	-	+	-	-	+

[a] +, PCR product of expected size, -, no PCR amplification. Expected size of PCR product is shown in parentheses.
[b] to [m] Primer pairs described in (Braker *et al.*, 1998), (Hallin & Lindgren, 1999), (Casciotti & Ward, 2001), (Goregues *et al.*, 2005), (Liang *et al.*, 2007), this study, (Braker & Tiedje, 2003), (Braker & Tiedje, 2003), this study, (Kloos *et al.*, 2001, Thröback *et al.*, 2004) , (Jones *et al.*, 2011) and this study, respectively.
[n] organisms harboring *nirS* (Heylen *et al.*, 2006)
[o] organisms harboring *cnorB* (Heylen *et al.*, 2007)
[p] to [u] Abbreviated genus names stand for *Achromobacter*, *Alcaligenes*, *Cupriavidus*, *Ochrobactrum*, *Paracoccus* and *Geobacillus*, respectively and *thermo* and *stearo* refer to species indication *thermodenitrificans* and *stearothermophilus*
n/d not determined

Table 3. 3 (next pages). Alignment of selected *nirK*, *qnorB* and *nosZ* sequences of Bacillaceae with previously and currently developed denitrifier-specific *nirK*, *qnorB* and *nosZ* gene-targeted primers.
Primer sequences are shown on the top line with specific primer names shown above the arrows. The color code indicates mismatches and 2-, 3- and 4-fold degeneracy at nucleotide positions with the respective primers. Primers developed in this study are indicated in blue. Combined presence of (1) gaps within, (2) multiple mismatches with and, (3) a high degree of degeneracy with primer binding sites, with all hitherto developed primers suggest that they would not amplify *Geobacillus* denitrification genes (with the exception of the very recently described primers of Jung *et al.*, 2013).

MM, mismatch
***, 4-fold degeneracy
**, 3-fold degeneracy
*, 2-fold degeneracy

NirK

FORWARD

Organism name and accession number	Primer site nirKCF (Goregues *et al*., 2005))	MM	*
	G G C R Y S T T Y G T C T A Y C A C T G C		
Geobacillus thermodenitrificans NG80-2 (CP000557)	G G T G T G T T T A T G T A C C A T T G C	4	5
Geobacillus kaustophilus HTA426 (BA000043)	G G T G T G T T T A T G T A C C A T T G C	4	5
Geobacillus sp. Y4.1MC1 (NC_014650)	G G G G T A T T T A T G T A C C A T T G C	5	4
Geobacillus sp. C56-T3 (CP002050)	G G C G T G T T T A T G T A C C A T T G C	3	5
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	G G G G T A T T T A T G T A C C A T T G C	5	4
Geobacillus sp. G11MC16 (NZ_ABV01000001)	G G T G T G T T T A T G T A C C A T T G C	4	5
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	G G G G T A T T T A T G T A C C A T T G C	5	4
Bacillus azotoformans LMG 9581T (NZ_AJLR01000038)	G G T G T A T T T A T G T A T C A C T G T	5	4
Bacillus bataviensis LMG 21833 (NZ_AJLS01000042)	G G C G T G T T C A T G T A T C A T T G C	3	5
5' GnirK2F	G G K G T V T T T A T G T A C C A T T G C		
	~472 … ~492		

Organism name and accession number	Primer site nirK-F (Liang *et al*., 2007)	MM
	T T C G T C T A C C A C T G C G C	
Geobacillus thermodenitrificans NG80-2 (CP000557)	T T T A T G T A C C A T T G C G G	5
Geobacillus kaustophilus HTA426 (BA000043)	T T T A T G T A C C A T T G C G G	5
Geobacillus sp. Y4.1MC1 (NC_014650)	T T T A T G T A C C A T T G C G G	5
Geobacillus sp. C56-T3 (CP002050)	T T T A T G T A C C A T T G C G G	5
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	T T T A T G T A C C A T T G C G G	5
Geobacillus sp. G11MC16 (NZ_ABV01000001)	T T T A T G T A C C A T T G C G G	5
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	T T T A T G T A C C A T T G C G G	5
Bacillus azotoformans LMG 9581T (NZ_AJLR01000038)	T T T A T G T A T C A C T G T G G	5
Bacillus bataviensis LMG 21833 (NZ_AJLS01000042)	T T C A T G T A T C A T T G C G G	4
	~478 … ~494	

Organism name and accession number	Primer site Cunir3 (Casciotti *et al*., 2001)	MM	*	**
	C G T C T A Y C A Y T C C G C V C C			
Geobacillus thermodenitrificans NG80-2 (CP000557)	T A T G T A C C A T T G C G G T A C	6	2	0
Geobacillus kaustophilus HTA426 (BA000043)	T A T G T A C C A T T G C G G T A C	6	2	0
Geobacillus sp. Y4.1MC1 (NC_014650)	T A T G T A C C A T T G C G G T A C	6	2	0
Geobacillus sp. C56-T3 (CP002050)	T A T G T A C C A T T G C G G C A C	5	2	1
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	T A T G T A C C A T T G C G G C A C	5	2	1
Geobacillus sp. G11MC16 (NZ_ABV01000001)	T A T G T A C C A T T G C G G T A C	6	2	0
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	T A T G T A C C A T T G C G G T A C	6	2	0
Bacillus azotoformans LMG 9581T (NZ_AJLR01000038)	T A T G T A T C A C T G T G G A A C	6	2	1
Bacillus bataviensis LMG 21833 (NZ_AJLS01000042)	C A T G T A T C A T T G C G G A A C	4	2	1
	~480 … ~497			

Organism name and accession number	Primer site nirK1F (Braker *et al*., 1998)	MM	*	gaps
	G G M A T G G T K C C S T G G C A			
Geobacillus thermodenitrificans NG80-2 (CP000557)	C C A G T C T T G C A G - - - C A	6	3	3
Geobacillus kaustophilus HTA426 (BA000043)	C C A G T C T T G C A G - - - C A	6	3	3
Geobacillus sp. Y4.1MC1 (NC_014650)	C C G G T G C T C G C C - - - C A	7	1	3
Geobacillus sp. C56-T3 (CP002050)	C C G G T G T T G C A G - - - C A	6	2	3
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	C C G G T G C T C G C C - - - C A	7	1	3
Geobacillus sp. G11MC16 (NZ_ABV01000001)	C C A G T C T T G C A G - - - C A	6	3	3
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	C C G G T G C T C G C C - - - C A	7	1	3
Bacillus azotoformans LMG 9581T (NZ_AJLR01000038)	C C T G T G T T A G C A - - - C A	8	0	3
Bacillus bataviensis LMG 21833 (NZ_AJLS01000042)	C C A G T G T T A T C C - - - C A	6	2	3
	~502 … ~515			

Organism name and accession number	Primer site F1aCu (Hallin and Lindgren, 1999)	MM	*
	A T C A T G G T S C T G C C G C G		
Geobacillus thermodenitrificans NG80-2 (CP000557)	A T C A T T G T C A A G C C A A A	6	1
Geobacillus kaustophilus HTA426 (BA000043)	A T C A T T G T C A A G C C A A A	6	1
Geobacillus sp. Y4.1MC1 (NC_014650)	A T G A T C G T C A A G C C G A A	6	1
Geobacillus sp. C56-T3 (CP002050)	A T C A T T G T C A A G C C G A A	5	1
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	A T G A T C G T C A A G C C G A A	6	1
Geobacillus sp. G11MC16 (NZ_ABV01000001)	A T C A T T G T C A A G C C A A A	6	1
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	A T G A T C G T C A A G C C G A A	6	1
Bacillus azotoformans LMG 9581T (NZ_AJLR01000038)	A T T A T T G T A A A A C C G A A	8	1
Bacillus bataviensis LMG 21833 (NZ_AJLS01000042)	A T T A T C G T C A A A C C A A C	8	1
	~541 … ~557 3'		

Table 3.3

MM, mismatch
***, 4-fold degeneracy
**, 3-fold degeneracy
*, 2-fold degeneracy

NirK

REVERSE

Organism name and accession number	Primer site nirKCR (Goregues *et al* ., 2005)	MM	*	**	***
	← TGGTTYATYCSBGGBGGYDCN				
Geobacillus thermodenitrificans NG80-2 (CP000557)	GTGATGCTTCCGGCGAGCGGC	8	3	3	1
Geobacillus kaustophilus HTA426 (BA000043)	GTGATGCTTCCGGCGAGCGGC	8	3	3	1
Geobacillus sp. Y4.1MC1 (NC_014650)	GTGATGCTTCCGGCAAGCGGG	9	3	2	1
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	GTGATGCTTCCGGCAAGCGGG	9	3	2	1
Geobacillus sp. G11MC16 (NZ_ABV01000001)	GTGATGCTTCCGGCGAGCGGC	8	3	3	1
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	GTGATGCTTCCGGCAAGCGGG	9	3	2	1
Bacillus azotoformans LMG 9581^T	GTAATGTTACCTGCCAGCGGC	10	2	3	1
Bacillus bataviensis LMG 21833^T (NZ_AJLS01000042)	GTCATGCTTCCGCGGAGCGGT	7	3	2	1
5'	**GTGATGCTTCCGGCRAGCGGS**				
	~892 **GnirK2R** ~911				

Primer site nirK5R (Braker *et al* ., 1998) and Cunir4 (Casciotti & Ward, 2001)	MM	*
← CCAYAAYCTGATCGAGGC		
TCATCAGTTTAATCATGC	9	1
TCATCAGTTTAATCATGC	9	1
CCATCAATTCAACCATGC	7	1
CCATCAATTCAACCATGC	7	1
TCATCAGTTTAATCATGC	9	1
CCATCAATTCAACCATGC	7	1
ACACCAATTTAACCATGT	9	1
CCACCAATTTAACCATGC	7	1
~966 ~983		

Organism name and accession number	Primer site R3Cu (Hallin and Lindgren, 1999)	MM
	← AACCACAAYCTGATCGAGGC	
Geobacillus thermodenitrificans NG80-2 (CP000557)	ACTCATCAGTTTAATCATGC	8
Geobacillus kaustophilus HTA426 (BA000043)	ACTCATCAGTTTAATCATGC	8
Geobacillus sp. Y4.1MC1 (NC_014650)	ACCCATCAATTCAACCATGC	6
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	ACCCATCAATTCAACCATGC	6
Geobacillus sp. G11MC16 (NZ_ABV01000001)	ACTCATCAGTTTAATCATGC	8
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	ACCCATCAATTCAACCATGC	6
Bacillus azotoformans LMG 9581^T	ACACACCAATTTAACCATGT	7
Bacillus bataviensis LMG 21833^T (NZ_AJLS01000042)	ACCCACCAATTTAACCATGC	6
	~964 ~983	

Primer site nirK-R (Liang *et al* ., 2007)	MM	gaps
← TGGAACGACGATCTGATGA		
GGCGAAGACGAC---GGCA	8	3
GGCGAAGACGAC---GGCT	9	3
GGCGAAGATGAC---GGTG	10	3
GGCGAAGATGAC---GGTG	10	3
GGCGAAGACGAC---GGCA	8	3
GGCGAAGATGAC---GGTT	10	3
GGTACAGATGAC---GGTT	10	3
GGTCATGATGAT---GGCA	8	3
~1024 ~1039		**3'**

Table 3. 3 (continued)

MM, mismatch
***, 4-fold degeneracy
**, 3-fold degeneracy
*, 2-fold degeneracy

QnorB

FORWARD & REVERSE

Organism name and accession number	Primer site qnorB2F (Braker and Tiedje, 2003)	MM	*	***
	G G N C A Y C A R G G N T A Y G A →			
Geobacillus thermodenitrificans NG80-2 (CP000557)	G G C C A T C A A G G T T G G G A	2	2	2
Geobacillus sp. Y4.1MC1 (NC_014650)	G G C C A T C A A G G C T G G G A	2	2	2
Geobacillus sp. C56-T3 (CP002050)	G G C C A C C A A G G T T G G G A	2	2	2
Geobacillus sp. G11MC16 (NZ_ABV01000001)	G G C C A T C A A G G T T G G G A	2	2	2
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	G G C C A T C A A G G C T G G G A	2	2	2
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	G G C C A T C A A G G C T G G G A	2	2	2
Geobacillus kaustophilus HTA426 (BA000043)	G G C C A C C A A G G T T G G G A	2	2	2
Geobacillus sp. Y412MC52 (NC_014915)	G G C C A C C A A G G T T G G G A	2	2	2
Geobacillus stearothermophilus (AB450501)	G G C C A T C A A G G T T G G G A	2	2	2
Geobacillus thermoleovorans CCB_US3_UF5 (CP003125)	G G C C A C C A A G G T T G G G A	2	2	2
Geobacillus sp. Y412MC61 (CP001794)	G G C C A C C A A G G T T G G G A	2	2	2
Bacillus azotoformans LMG 9581' (NZ_AJLR01000004)	G G A C A T C A A G G T T T T G A	1	3	2
Bacillus azotoformans LMG 9581' (NZ_AJLR01000048)	G G A C A T A A T G G T T G G G A	4	1	2
Bacillus licheniformis ATCC 14580 (CP000002)	G G C C A T C A A G G A A T G G A	3	2	2
Bacillus sp. BT1B_CT2 (NZ_ACW01000002)	G G C C A T C A A G G A A T G G A	3	2	2
Bacillus sp. 1NLA3E (NZ_AGJ000003)	G G G C A T C A A G G T T G G G A	2	2	2
	5' G G C C A Y C A A G G Y T G G G A			
	~1279 **qnorB4F** ~1295			

Organism name and accession number	Primer site qnorB5R (Braker and Tiedje, 2003)	MM	*	**
	← T G G T G G G T N G T N C A Y C T N T G G G T			
Geobacillus thermodenitrificans NG80-2 (CP000557)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Geobacillus sp. Y4.1MC1 (NC_014650)	T G G T G G A T T A T C C A T T T G T G G G T	3	1	3
Geobacillus sp. C56-T3 (CP002050)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Geobacillus sp. G11MC16 (NZ_ABV01000001)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	T G G T G G A T T A T C C A T T T G T G G G T	3	1	3
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	T G G T G G A T T A T C C A T T T G T G G G T	3	1	3
Geobacillus kaustophilus HTA426 (BA000043)	T G G T G G A T C A T C C A C T T G T G G G T	3	1	3
Geobacillus sp. Y412MC52 (NC_014915)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Geobacillus stearothermophilus (AB450501)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Geobacillus thermoleovorans CCB_US3_UF5 (CP003125)	T G G T G G A T C A T C C A C T T G T G G G T	3	1	3
Geobacillus sp. Y412MC61 (CP001794)	T G G T G G A T C A T C C A C T T A T G G G T	3	1	3
Bacillus azotoformans LMG 9581' (NZ_AJLR01000004)	T G G T G G A T T G T C C A C T T A T G G G T	2	1	3
Bacillus azotoformans LMG 9581' (NZ_AJLR01000048)	T G G T G G A T T A T C C A C T T A T G G G T	3	1	3
Bacillus licheniformis ATCC 14580 (CP000002)	T G G T G G A T C A T C C A T C T T T G G G T	2	1	3
Bacillus sp. BT1B_CT2 (NZ_ACW01000002)	T G G T G G A T C A T C C A T C T T T G G G T	2	1	3
Bacillus sp. 1NLA3E (NZ_AGJ000003)	T G G T G G A T C A T C C A T C T T T G G G T	2	1	3
	~1519 ~1541			

REVERSE

Organism name and accession number	Primer site qnorB7R (Braker and Tiedje, 2003)	MM	*	**	***
	← G G N T T C H T G A T H A A Y C C N C C				
Geobacillus thermodenitrificans NG80-2 (CP000557)	G G T T T C T T G A T C A A C T T G C C	2	1	2	2
Geobacillus sp. Y4.1MC1 (NC_014650)	G G G T T C C T C A T C A A C T T G C C	3	1	2	2
Geobacillus sp. C56-T3 (CP002050)	G G C T T T C T C A T C A A T T T G C C	4	1	2	2
Geobacillus sp. G11MC16 (NZ_ABV01000001)	G G T T T C T T G A T C A A C T T G C C	2	1	2	2
Geobacillus thermoglucosidasius C56-YS93 (CP002835)	G G G T T C C T C A T C A A C T T G C C	3	1	2	2
Geobacillus thermoglucosidans TNO-09.020 (AJJN01000019)	G G G T T C C T C A T C A A C T T G C C	3	1	2	2
Geobacillus kaustophilus HTA426 (BA000043)	G G T T T C C T C A T C A A C T T G C C	3	1	2	2
Geobacillus sp. Y412MC52 (NC_014915)	G G C T T T C T C A T C A A T T T G C C	4	1	2	2
Geobacillus stearothermophilus (AB450501)	G G T T T C T T G A T C A A C T T G C C	2	1	2	2
Geobacillus thermoleovorans CCB_US3_UF5 (CP003125)	G G T T T C C T C A T C A A C T T G C C	3	1	2	2
Geobacillus sp. Y412MC61 (CP001794)	G G C T T T C T C A T C A A T T T G C C	4	1	2	2
Bacillus azotoformans LMG 9581' (NZ_AJLR01000004)	G G A T T C T T T A T T A A C C C G C C	1	1	2	2
Bacillus azotoformans LMG 9581' (NZ_AJLR01000048)	G G A T T C C T A A T C A A C T T A C C	3	1	2	2
Bacillus licheniformis ATCC 14580 (CP000002)	G G C T T T T T G A T C A A C T T G C C	3	1	2	2
Bacillus sp. BT1B_CT2 (NZ_ACW01000002)	G G C T T T T T G A T C A A C T T G C C	3	1	2	2
Bacillus sp. 1NLA3E (NZ_AGJ000003)	G G T T T C C T T A T C A A C T T G C C	3	1	2	2
	5' G G B T T Y Y T S A T C A A Y T T G C C 3'				
	~1897 **qnorB6R** ~1917				

Table 3. 3 (continued)

NosZ

FORWARD

Organism name and accession number	Primer site Nos661F (Scala and Kerkhof, 1998)	MM	*
	CGGYTGG G GSMWKACCA A		
Geobacillus thermodenitrificans NG80-2 (CP000557)	CGGCTAT G GGTGGGATG C	9	3
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	TGGATAT G GATGGGACG A	10	1
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	AGGATAT G GCTGGTCTG A	9	2
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	AGGGTAT G GCTATGACA A	7	3
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	AGGGTAT G GATATGATG A	10	2
Bacillus vireti LMG 21834[T] (ALAN01000089)	GGGCTAT G GTTTTGATG A	9	3
	5' ~267 … ~284		

Organism name and accession number	Primer site nosZ-F (Jones *et al*., 2011)	MM	*	**	***
	TGGGGNGAYNYBCAYCA				
Geobacillus thermodenitrificans NG80-2 (CP000557)	TGGGGAGATTTGCATCA	0	3	1	2
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	TGGGGAGATTTGCACCA	0	3	1	2
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	TGGGGTGACTTCCACCA	0	3	1	2
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	TGGGGTGACCTCCACCA	0	3	1	2
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	TGGGGTGATGCCCACCA	0	3	1	2
Bacillus vireti LMG 21834[T] (ALAN01000089)	TGGGGGGACCTGCACCA	0	3	1	2
GnosZF	**TGGGGAGATTTGCATCA**				
	~316 … ~332				

Organism name and accession number	Primer site 752F (McGuiness *et al.*, 2006)	MM	*
	ACCGAYG G SACCTAYGA Y		
Geobacillus thermodenitrificans NG80-2 (CP000557)	ACAAACG G AGAATATGA C	5	3
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	ACGAACG G AGATTATGA C	5	3
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	ACAAAAG G TGAATATGA C	6	2
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	ACAGATG G AGATTATGA T	4	3
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	ACAAATG G TGATTATGA T	5	3
Bacillus vireti LMG 21834[T] (ALAN01000089)	ACAAAGG G CGATTATGA T	5	3
	~349 … ~366		

Organism name and accession number	Primer site nosZF478 (Jung *et al*., 2013)	MM
	TGTGCCGCGTTTGTGACTGA G AA T	
Geobacillus thermodenitrificans NG80-2 (CP000557)	TGTGCCGCGTTTGTGACTGA G AA T	0
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	TGTGCGGCATTTGTGACGGA A AA C	5
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	TGTGCGGCATTCGTTACTGA T AA T	5
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	TGTGCGGCTTTTGTTACACC G AA T	5
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	TCCGCCGTATTCGTAACACC G AA T	6
Bacillus vireti LMG 21834[T] (ALAN01000089)	TGTGCTGCATTCGTAACGGA A AA T	5
	~478 … ~501	

Organism name and accession number	Primer site NosZ912F (Sanford *et al.*, 2012)	MM
	CGTCCCC G GCCTCGTGT A	
Geobacillus thermodenitrificans NG80-2 (CP000557)	GCATAAA G GTGGCATTT A	12
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	ACATGAA G GCGGCATTT A	13
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	GAACAAA G GTGGAATCT A	15
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	GCATAAG G GAGGTATAT T	15
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	AGCCCCA G GAATCGTTT A	8
Bacillus vireti LMG 21834[T] (ALAN01000089)	ACAAAAA G GCGGTATTT A	14
	~882 … ~899	

Organism name and accession number	Primer site NosZ-F (Kloos *et al*., 2001)	MM	*
	C G YTGTTCMTCGACAGCC		
Geobacillus thermodenitrificans NG80-2 (CP000557)	C G ATGTTTATTTCGTCTG	10	1
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	C G ATGTTCATTTCCTCTG	8	1
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	C G ATGTTCATTTCTTCTG	9	1
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	C G ATGTTTATTTCATCCG	9	1
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	C A AACTTCGTTGAAAGTA	8	0
Bacillus vireti LMG 21834[T] (ALAN01000089)	C A ATGTTCATTTCTTCAG	10	1
	~1166 … ~1183		

Organism name and accession number	Primer site nosZ1F (Henry *et al*., 2006)	MM	*
	WCSYTGT T CMTCGACAG C C A		
Geobacillus thermodenitrificans NG80-2 (CP000557)	ACGATGT T TATTTCGTC T G A	10	3
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	ACGATGT T CATTTCCTC T G A	8	3
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	ACAATGT T CATTTCTTC T G A	10	1
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	ACGATGT T TATTTCATC C G A	9	3
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	ACAAACT T CGTTGAAAG T A C	10	1
Bacillus vireti LMG 21834[T] (ALAN01000089)	ACAATGT T CATTTCTTC A G A	10	2
	~1165 … ~1185		

Organism name and accession number	Primer site nosZGeoF (Jung *et al*., 2013)	MM	*
	TCRTCTGAAGTYGTRAAA T GG		
Geobacillus thermodenitrificans NG80-2 (CP000557)	TCGTCTGAAGTCGTGAAA T GG	0	3
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	TCCTCTGAAGTCGTCAAA T GG	2	1
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	TCTTCTGAAATCGTTAAG T GG	4	1
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	TCATCCGAGATTGTTAAA T GG	4	2
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	GAAAGTACCGTTGCAAAA T GG	8	3
Bacillus vireti LMG 21834[T] (ALAN01000089)	TCTTCAGAAGTCGTAAAA T GG	2	2
	~1177 … ~1197		

Organism name and accession number	Primer site nosZ2F (Henry *et al*., 2006)	MN	*
	CGCRACG G CAASAAGGT S M S S G T		
Geobacillus thermodenitrificans NG80-2 (CP000557)	GTCTACG G CATCGCGAT G C G T T C	#	4
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	GTATATG G TATTGCGAT G C G T T C	#	3
Bacillus azotoformans LMG 9581[T] (AJLR01000147)	GTCTACG G TATTGCAAT G C G T T C	#	3
Bacillus azotoformans LMG 9581[T] (AJLR0100041)	GTTCGTG G TGTTGCGAT G A G A T C	#	3
Bacillus azotoformans LMG 9581[T] (AJLR01000004)	GTTTATG G AATTGCCGT G C G C T C	#	4
Bacillus vireti LMG 21834[T] (ALAN01000089)	GTTTACG G GATCGCGAT G C G C T C	#	5
	~1564 … ~1586 3'		

Table 3. 3 (continued)

REVERSE

Organism name and accession number	Primer site nosZR915 (Jung *et al*., 2013)	MM
	← C A T T T A T T T G G T A C C G G T C G C C	
Geobacillus thermodenitrificans NG80-2 (CP000557)	C A T T T A T T T G G T A C C G G T C G C C	0
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	C A T T T A T T T T A A T A C C G G T A G C	10
Bacillus azotoformans LMG 9581^T (AJLR01000147)	A A T C T A C T T A G T A C C A G T T G C T	7
Bacillus azotoformans LMG 9581^T (AJLR0100041)	T A T A T T C T T A G T A G C C G C T G A A	11
Bacillus azotoformans LMG 9581^T (AJLR01000004)	C G T T T A T G C A G T A G C C G C T G C A	9
Bacillus vireti LMG 21834^T (ALAN01000089)	T A T T T A T T T A G T G C C G G T C G C T	4
	5' ~894 … ~915	

Organism name and accession number	Primer site nosZ1R (Henry et al., 2006) & 1773R (Scala & Kerkhof, 1	MM	*	**
	← A A C G A V C A G Y T G A T C G A Y A T			
Geobacillus thermodenitrificans NG80-2 (CP000557)	T C G A T G C A G C T C A T T G A C A T	7	2	1
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	T C G A T G C A G T T A A T T G A T A T	7	2	1
Bacillus azotoformans LMG 9581^T (AJLR01000147)	T C C A T G C A G T T A A T C G A C T T	6	2	1
Bacillus azotoformans LMG 9581^T (AJLR0100041)	T C T A T G C A G C T T A T T G A T C T	8	2	1
Bacillus azotoformans LMG 9581^T (AJLR01000004)	A A T A T G C A A T T A A T C G A T A T	5	2	1
Bacillus vireti LMG 21834^T (ALAN01000089)	T C G A T G C A G T T A A T T G A C A T	7	2	1
	~1363 … ~1382			

Organism name and accession number	Primer site NosZ1622R (Thröback *et al*., 2004)	MM	*
	← C G C R A S G G C A A S A A G G T S C G		
Geobacillus thermodenitrificans NG80-2 (CP000557)	G T C T A C G G C A T C G C G A T G C G	7	3
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	G T A T A T G G T A T T G C G A T G C G	11	1
Bacillus azotoformans LMG 9581^T (AJLR01000147)	G T C T A C G G T A T T G C A A T G C G	10	2
Bacillus azotoformans LMG 9581^T (AJLR0100041)	G T T C G T G G T G T T G C G A T G A G	14	1
Bacillus azotoformans LMG 9581^T (AJLR01000004)	G T T T A T G G A A T T G C C G T G C G	11	1
Bacillus vireti LMG 21834^T (ALAN01000089)	G T T T A C G G G A T C G C G A T G C G	9	3
	~1564 … ~1583		

Organism name and accession number	Primer site NosZ1853R (Sanford *et al*., 2012)	MM
	← C C C T A C T A C T G C A C G A A C T T C T G C T C	
Geobacillus thermodenitrificans NG80-2 (CP000557)	C C G T T G T A T T G C A C G A A C T T C T G T T C	5
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	C C A A T T T A T T G C A C A A A C T T T T G T T C	8
Bacillus azotoformans LMG 9581^T (AJLR01000147)	C C A C T A T A T T G T A C A A A C T T C T G T T C	8
Bacillus azotoformans LMG 9581^T (AJLR0100041)	C C A T T C T A C T G T A C A A A C T T C T G C T C	5
Bacillus azotoformans LMG 9581^T (AJLR01000004)	C C A T T C T A C T G T A C A A A C T T C T G T T C	5
Bacillus vireti LMG 21834^T (ALAN01000089)	C C A A T C T A T T G T A C A A A C T T C T G C T C	6
	~1792 … ~1817	

Organism name and accession number	Primer site nosZ-R (Jones *et al*., 2011)	MM	*	***
	← T A Y T G Y A C N A A C T T Y T G Y T C			
Geobacillus thermodenitrificans NG80-2 (CP000557)	T A T T G C A C G A A C T T C T G T T C	0	4	1
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	T A T T G C A C A A A C T T T T G T T C	0	4	1
Bacillus azotoformans LMG 9581^T (AJLR01000147)	T A T T G T A C A A A C T T C T G T T C	0	4	1
Bacillus azotoformans LMG 9581^T (AJLR0100041)	T A T T G C A C A A A C T T C T G C T C	0	4	1
Bacillus azotoformans LMG 9581^T (AJLR01000004)	T A C T G T A C A A A C T T C T G T T C	0	4	1
Bacillus vireti LMG 21834^T (ALAN01000089)	T A T T G T A C A A A C T T C T G C T C	0	4	1
	T A T T G C A C G A A C T T C T G T T C			

Organism name and accession number	Primer site nosZ2R (Henry *et al*., 2006)	MM	*
	← T T C T G C C A Y G C S W T G C A Y W T G		
Geobacillus thermodenitrificans NG80-2 (CP000557)	T T C T G T T C G G C G C T G C A T C A A	8	2
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	T T T T G T T C G G C A T T G C A C C A A	9	2
Bacillus azotoformans LMG 9581^T (AJLR01000147)	T T C T G T T C A G C G C T A C A C C A A	9	2
Bacillus azotoformans LMG 9581^T (AJLR0100041)	T T C T G C T C C G C A T T G C A T C A G	5	3
Bacillus azotoformans LMG 9581^T (AJLR01000004)	T T C T G T T C A G C G C T T C A C C A A	9	2
Bacillus vireti LMG 21834^T (ALAN01000089)	T T C T G C T C A G C C T T A C A C C A G	6	3
	~1810 … ~1830		
	~1799 **GnosZR** ~1818		

Organism name and accession number	Primer site NosZGeoR (Jung *et al*., 2013)	MM	*	**
	← T C T G T T C B G C G Y T G C A Y C A A G			
Geobacillus thermodenitrificans NG80-2 (CP000557)	T C T G T T C G G C G C T G C A T C A A G	0	2	1
Anoxybacillus flavithermus TNO-09.006 (AMCM0100039)	T T T G T T C G G C A T T G C A C C A A G	2	2	1
Bacillus azotoformans LMG 9581^T (AJLR01000147)	T C T G T T C A G C G C T A C A C C A A G	2	2	0
Bacillus azotoformans LMG 9581^T (AJLR0100041)	T C T G C T C C G C A T T G C A T C A A G	2	2	1
Bacillus azotoformans LMG 9581^T (AJLR01000004)	T C T G T T C A G C G C T T C A C C A A G	2	2	0
Bacillus vireti LMG 21834^T (ALAN01000089)	T C T G C T C A G C C T T A C A C C A G G	4	2	0
	~1811 … ~1831 **3'**			

Table 3. 3 (continued)

3.2.4 DNA extraction and PCR conditions

Total genomic DNA from pure cultures was extracted according to the guanidium-thiocyanate-EDTA-sarkosyl method (Pitcher *et al.*, 1989) which was adapted for Gram-positive bacteria with an additional lysozyme step. All PCRs were performed with a Veriti 96-well Thermocycler (Applied Biosystems).

For the novel primers, amplification of *nirK* and *qnorB* fragments from 50ng of pure culture DNA extract was performed in 25-µl reactions containing 1x PCR buffer (Qiagen), 1x Q solution (Qiagen), 12.5 pmol of each primer, 200 µM of each deoxynucleotide triphosphate and 1.25 U of Taq polymerase (Qiagen). Expected amplicon size was about 440 bp and 606 bp for *nirK* and *qnorB*, respectively. Amplification of *nosZ* fragments was performed as described before (Jones *et al.*, 2011) and with novel *nosZ* primers targeting *Geobacillus* (Table 3. 1), with an expected amplicon size of 1502 bp. With latter primers, amplification was performed similarly as for *nirK* and *qnorB*. Specific time-temperature profiles for each gene were established (Table 3. 2).

QnorB, nirK and *nosZ* PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels followed by 30 min of staining with ethidium bromide. Bands were visualized by UV excitation.

Table 3. 2 Time-temperature profile *nirK, qnorB* and *nosZ* touchdown-PCRs.

	nirK			*qnorB*			*nosZ*		
Step	**Temp**	**Time**	**Cycle n°**	**Temp**	**Time**	**Cycle n°**	**Temp**	**Time**	**Cycle n°**
Initial denaturation	95°C	5 min		95°C	5 min		95°C	5 min	
Denaturation	95°C	30 sec		95°C	30 sec		95°C	30 sec	
Annealing	50°C-> 45°C	40 sec	10	62°C-> 53°C	40 sec	15	58°C-> 53°C	40 sec	10
Elongation	72°C	7 min		72°C	1 min		72°C	1 min	
Denaturation	95°C	30 sec		95°C	30 sec		95°C	30 sec	
Annealing	45°C	40 sec	25	56°C	40 sec	25	53°C	40 sec	25
Elongation	72°C	7 min		72°C	1 min		72°C	1 min	
Elongation	72°C	7 min		72°C	10 min		72°C	7 min	
Hold	4°C	until analysis		4°C	until analysis		4°C	until analysis	

3.2.5 Sequencing of amplified *nirK*, *qnorB* and *nosZ* products

Amplicons of the expected size were purified using the Nucleofast®96 PCR clean up membrane system (Macherey-Nagel, Germany). Sequencing reactions were performed in a total volume of 10 µl with 3 µl of purified amplicon, 0.286 µl of BigDyeTM mixture (Terminator Cycle Sequencing Kit version 3.1, Applied Biosystems), 1x sequencing buffer and 1.2 µM of each of the amplification primers used as sequencing primers. The thermal program consisted of 30 cycles (96 °C for 15 s, 35 °C for 1 s, 60 °C for 4 min). Subsequently, sequencing products were purified using the BigDye XTerminator Kit (Applied

Biosystems) and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). For all genes, inspection of chromatograms and assembly of sequences was performed using BioNumerics 5.1 (Applied Maths, Sint-Martens-Latem, Belgium).

3.2.6 Phylogenetic analyses of *nirK*, *qnorB* and *nosZ* genes

3.2.6.1 Datasets

Nucleotide sequences for *nirK*, *qnorB* and *nosZ* genes encoding enzymes in the denitrification pathway were retrieved from the Fungene (http://fungene.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) sequence databases (Table S1, appendices). We limited our data sets to sequences originating from either genome sequencing projects or from cultivation studies and with a length of at least 440 (*nirK*), 600 (*norB*) or 1400 (*nosZ*) bp. All sequences from environmental clones were excluded. Filtering for sequences to be included was also based on the annotation. For *nirK, qnorB* and *nosZ* only sequences with annotations referring to either 'nitrite reductase', 'NirK', 'AniA' and '(conserved) hypothetical protein', 'nitric oxide reductase', 'norB' or '(conserved) hypothetical protein', or nitrous oxide reductase', 'nosZ' or '(conserved) hypothetical protein', respectively, were retained. Sequences containing multiple N-residues or stop codons in all reading frames were excluded. Multiple copies of functional genes were included when found.

3.2.6.2 Analysis

Inferred *nirK*, *norB* and *nosZ* amino acid sequences were aligned using the MEGA 5.0 software (Tamura *et al.*, 2011) using MUSCLE (Edgar, 2004) and resulting amino acid alignments were inspected manually for errors. For each alignment, average percent amino acid identity was calculated to estimate reliability (Thompson *et al.*, 1999). Subsequently, amino acid based Maximum Likelihood phylogenetic trees were constructed using RAxML (Randomized Axelerated Maximum Likelihood) v7.3.5 (Stamatakis, 2006). Best-Fit substitution models were selected based on the AICc criteria calculated by MEGA 5.0. For *nirK* and *nosZ* the WAG substitution model with gamma-distributed rate variation (WAG+G) and site variation (WAG+G+I) was chosen and for *qnorB* the same model modified with observed amino acid frequencies (WAG+G+I+F). Node confidence was determined using 1000 bootstrap replicates. The best scoring ML tree was exported in Newick format and analyzed using MEGA 5.0. For clarity, *nirK*, *qnorB* and *nosZ* trees with a reduced number of sequences were calculated as well (Fig. 3. 3 to Fig. 3. 5), more comprehensive versions of the phylogenetic analyses can be found in figures S1, S2 and S3 (appendices).

3.2.7 Genomic fingerprinting by amplified fragment length polymorphism analysis (AFLP)

Although taxonomic affiliation of the closely related set of strains was known, we also wanted to verify whether the geobacilli also represented a certain genomic variability, especially across *G. thermodenitrificans* strains that embodied the bulk of the strain set. Because of the high 16S rRNA gene sequence similarity level among all *Geobacillus thermodenitrificans* and *Geobacillus stearothermophilus* strains (Coorevits *et al.*, 2012) AFLP fingerprinting was chosen to assess genetic variation between the strains. This technique has proven to be useful for determination of genetic variation amongst strains of the same species (Cleenwerck *et al.*, 2009) and for distinction of highly related strains (Janssen *et al.*, 1996). To account for experimental error when scoring loci analyses were performed in triplicate for each isolate.

Approximately 1 µg of extracted DNA from isolates was double-digested with 5 units of EcoRI and MseI in 10 µl reactions with 1x NEBuffer 4 (New England BioLabs) and 0.1 mg/ml BSA (New England BioLabs) for 30 min at 37°C. Ligation of corresponding double-stranded restriction half-site adaptors to the digested fragments was performed in 10µl reactions consisting of 1x NEBuffer 4, 5mM ATP, 0.2 µM hexadaptor or 2 µM tetra-adaptor, 0.1mg/ml BSA and 40 units of T4 ligase (New England BioLabs) at 37°C for approximately 1h. This template DNA (tDNA) was precipitated in a solution containing 100 µl T0.1E (10mM Tris/HCl, 0.1mM EDTA, pH 8.0) and used for selective PCR amplification with primer combination M00/E02 with labeling of E02 with fluorescent 6-FAM at the 5' end. The selective PCR mixture contained 1.5 µl tDNA, 4µM hexaprimer, 4µM tetraprimer, 1x PCR buffer, 0.2mM dNTPs and 0.06 units of Taq polymerase and reactions were subjected to following temperature program: initial denaturation at 94°C for 2 min, 25 cycles of 94°C for 30s, a step-down annealing temperature of 66°C to 56°C for 40s, and 72°C for 2 min 20 sec, with a final extension step of 60°C for 30min.

An ABI 3130xl analyzer (Applied Biosystems) was used to size fragments between 20 and 600 bp in length and loci were manually binned and scored using Genemapper v.4.0. Tables with fragment information were transferred into BioNumerics 5.1 (Applied Maths) for numerical analysis. Hierarchical clustering was performed using the Dice algorithm with a position tolerance of 0.04% to compensate for technical imperfections and a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA). The zone between 40 bp and 580 bp was used for comparison. Cluster confidence was determined via calculation of cophenetic correlations (Fig. 3. 2).

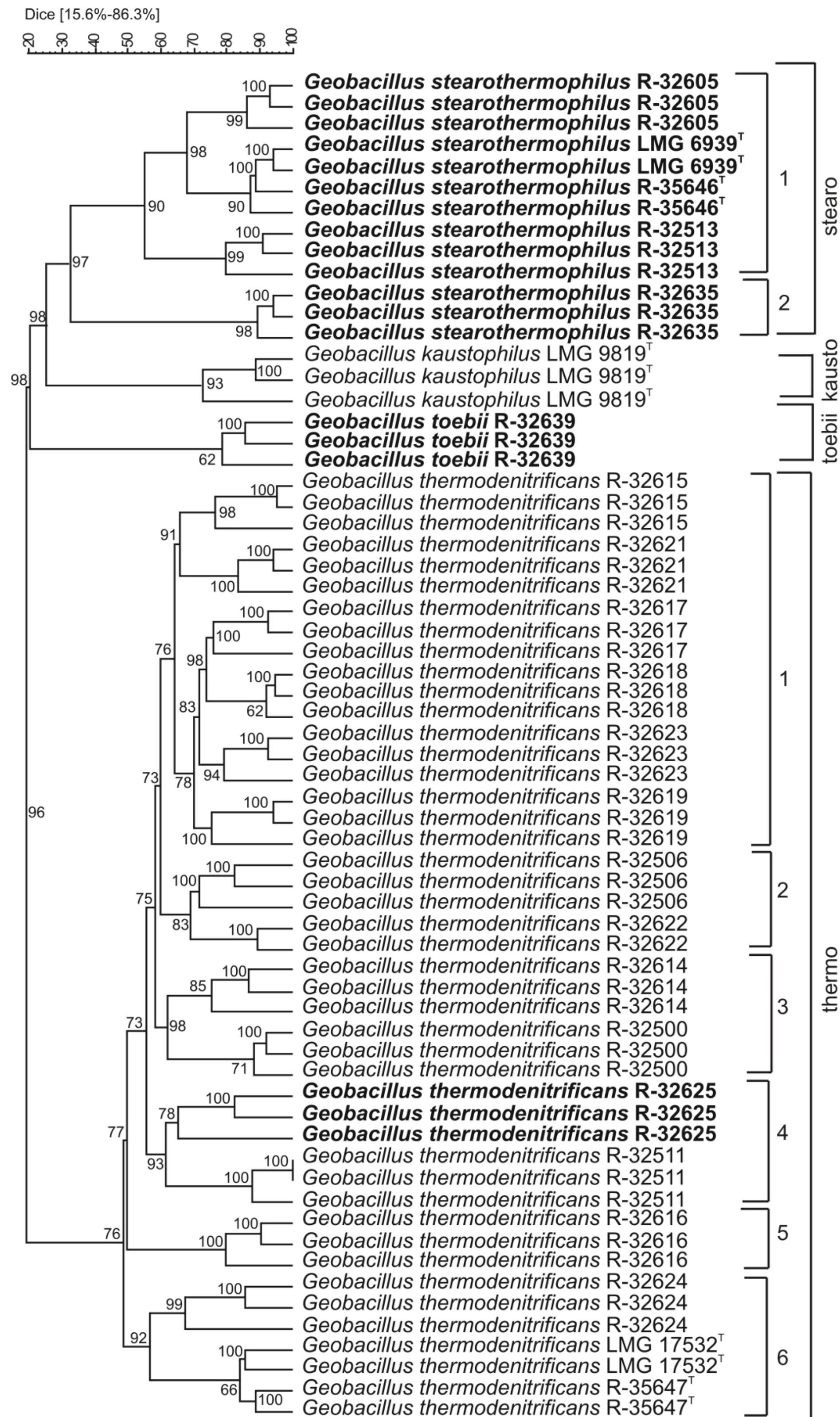


Fig. 3. 2 Phylotypic comparison (AFLP) of N_2O and N_2 producing *Geobacillus* strains. Genetic variation using AFLP fingerprinting. Scales indicate similarity and nodes with cophenetic correlation values lower than 70% were omitted. Strains were analyzed in triplicate. Strains that lack N_2O reductase are indicated in bold.

3.2.8 Nucleotide sequences accession numbers

NirK, *qnorB* and *nosZ* gene sequence data generated in this study have been submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/HG328767-HG328828) under the accession numbers HG328767 to HG328828.

3.3 Results

3.3.1 AFLP

The similarity of AFLP patterns between repeats of the same biological material (= same strain, intra-strain variability) is clearly higher than between different strains (inter-strain variability) of the same species. Furthermore, although for some species only a limited number of strains are included, AFLP patterns also allow a clear separation at the species level of this phylogenetic tight genus (Coorevits *et al.*, 2012). Hence, cluster analysis (Fig. 3. 2) resulted in four species clades of which two with internal separate clusters, indicating inclusion of both inter-species as well as discrete intra-species genetic variation in our strain set.

3.3.2 Growth conditions and denitrification potential

Initial tests were performed on TSB and 1/10 TSB, both supplemented with 10 mM KNO_3, permitting maximum production of 246 ± 10 µmol N_2O and/or N_2. A 7-day incubation period in presence of 10% acetylene (vol/vol) allowed for assessment of the denitrification capability by N_2O measurement. In nitrate-amended TSB batch experiments, three *Geobacillus stearothermophilus* strains and eight *Geobacillus thermodenitrificans* strains were able to reduce between 65.9 ± 3.0% and 99.6 ± 0.2% of the added NO_3^- to N_2O. Other strains, however, reduced lower amounts of NO_3^- (between 6.0 ± 0.2% and 31.9 ± 9.6%) or showed very patchy reduction patterns in different replicates (e.g. 99.5% in one replicate towards 11.5% and 13.0% in the others) (data not shown). On TSB without supplementary NO_3^-, N_2O production was virtually absent (≤ 1.5 µmol N_2O, negligible compared to the theoretic maximum). Similar non-reproducible results were obtained in nitrate-amended 1/10 TSB medium, with even less strains producing N_2O. Only *G. toebii* R-32639 was able to almost completely and consistently reduce added NO_3^- to N_2O. On 1/10 TSB without supplementary NO_3^-, N_2O production amounted to maximum 0.2 µmol, again negligible compared to the theoretic maximum.

Observed anaerobic growth and the results above suggested that the *Geobacillus* strain panel preferred fermentation over denitrification or carried out both processes at the same time. Therefore, mineral medium (Stanier *et al.*, 1966) was tested as well. However, unaltered MM with vitamins and

trace elements was not sufficient to fully support growth of all included *Geobacillus* strains. Thus, MM was supplemented with 1% (v/v) TSB and this mMM supported growth of all strains and allowed for assessment of denitrification ability by measurement of N_2O and N_2 after a 7-day incubation period in presence of 10% acetylene (v/v) and a 5-day incubation period without acetylene, respectively. All strains were capable of converting NO_3^- to gaseous nitrogen species (Table 3. 3). On mMM with acetylene, most strains nearly fully reduced NO_3^- and NO_2^- and all strains introduced a substantial pH increase (Table 3. 3). On mMM without acetylene, prominent differences regarding N_2O and/or N_2 production between strains were observed. The *Geobacillus stearothermophilus* strains, *Geobacillus toebii* R-32639 and *Geobacillus thermodenitrificans* R-32625 produced N_2O as end-point gaseous nitrogen species, whereas *Geobacillus thermodenitrificans* R-32618 produced both N_2O and N_2. All other strains produced N_2 from initial NO_3^- (Table 3. 3). In all cultures, an increased pH was observed. Little or no growth and N_2O production (≤0.17 µmol) was observed in mMM without added NO_3^-.

3.3.3 Primer design

Primer sets were designed for amplification of *nirK*, *qnorB* and *nosZ* genes potentially present in *Bacillaceae*, with focus on thermophilic geobacilli. As little genomic information was available for *nirK*, *qnorB* and *nosZ* in this group of organisms, construction of novel primers was based on primer target regions that were described before in Gram-negative bacteria (Fig. 3.1) and that were verified in available genomes of *Bacillaceae*. This resulted in primers pairs GnirK2F–GnirK2R, GqnorB4F–GqnorB6R and GnosZF–GnosZR for *nirK*, *qnorB* and *nosZ*, respectively (Table 3. 1).

In-silico comparison of primer sequences of established primers and primer binding sites in the genome of *Geobacillus thermodenitrificans* NG80-2 revealed the combined presence of (1) gaps within, (2) multiple mismatches with and, (3) a high degree of degeneracy with primer binding sites, suggesting that they would not amplify *Geobacillus* denitrification genes (Fig. 3.1). Hence, as expected, qualitative PCR evaluation of well-known and novel primers on a set of target and non-target strains clearly showed differences in performance. Amplification of *nirK*, *qnorB* and *nosZ* with established primers was observed in non-*Geobacillus* reference strains only and with novel primers in *Geobacillus* reference strains only (Table 3. 2). Attempts to amplify *nirK* with primers that picked-up *Geobacillus*-like *nirK* (Bartossek *et al.*, 2010) also failed (data not shown). Since the *nosZ* primer pair NosZ-F – NosZ1622R (Kloos *et al.*, 2001, Thröback *et al.*, 2004) did not render amplicons in *Geobacillus* reference strains either (Table 3. 3), additional *nosZ* primers (Scala & Kerkhof, 1998, Henry *et al.*, 2006, McGuiness *et al.*, 2006) targeting the same group of sequences of Gram-negative *Proteobacteria* were not tested experimentally. *NosZ*

primers based on genome data of Gram-positive *Geobacillus thermodenitrificans* NG80-2 and *Desulfitobacterium hafniense* Y-51 (Jones *et al.*, 2011), however, were also not always successful in our geobacilli (Table 3. 1 and Table 3. 3). Hence, in order to expand *nosZ* amplification to the rest of our strain set, we decided to alter these primers (Jones *et al.*, 2011) (Fig. 3.1, C) (prior to the recent publication of another set of *nosZ* primers targeting *Geobacillus* (Jung *et al.*, 2013).

3.3.4 *NirK* – functional genotype

NirK was detected in all *Geobacillus* strains tested, resulting in a fragment of approximately 440 bp (Table 3. 3). *Geobacillus stearothermophilus nirK* sequences were identical based on nucleotide alignments, except for *nirK* of R-32635. *Geobacillus thermodenitrificans nirK* sequences were identical as well. Pairwise comparison of all retrieved sequences resulted in similarities ranging from 78.13% to 100%. pBLAST of derived amino acid sequences indicated similarity to 'copper-containing nitrite reductase' (CuNiR). Average percent amino acid identity (AAI) of the inferred amino acid alignment was calculated to be 45.8%, indicating reliability of the alignment.

The *nirK* dataset consisted of 218 genotypes and RAxML phylogenetic analysis of amino acid sequences inferred from *nirK* genes revealed a phylogeny divided in three clusters (Fig. 3. 3). Cluster III primarily contains Proteobacterial sequences and shows high terminal node support and low bootstrap values at basal nodes (Fig. S1, appendices), congruent with previous observations (Jones *et al.*, 2008). This cluster also contains almost all (partial) *nirK* gene sequences retrieved with commonly used *nirK* primers (Table S1, appendices). Similar node support in basal and terminal regions was observed in cluster I, which contains sequences from an array of taxonomic groups, with a large number of Proteobacterial and Bacteroidetal sequences (Fig. S1, appendices). Cluster II consists of a Firmicute clade (subcluster 3) supported by high node confidence (99%), an Actinobacterial clade (subcluster 1) and several sequences from more unrelated groups, such as *Verrucomicrobia*, *Gemmatimonadetes*, *Chloroflexi*, *Proteobacteria*, and nitrite-oxidizing *Nitrospirales* (subcluster 2). Notably, not all Firmicute *nirK* cluster together, e.g. cluster III harbors *nirK* of *Bacillus* sp. R-32546, cluster I *nirK* of *Symbiobacterium thermophilum* IAM 14863^T, *Thermaerobacter marianensis* DSM 12885^T and *Thermaerobacter subterraneus* DSM 13965^T (Fig. S1, appendices), whereas *nirK* of *Sulfobacillus acidophilus* resides in cluster II away from the Firmicute clade.

Table 3. 3 Genotypic and phenotypic denitrification in *Geobacillus* strains.

Species identification and strain number, origin, results of PCR-based detection of denitrification genes, residual fraction of NO_3^- and NO_2^- and produced N_2O and/or N_2 in µmol and pH (all end-point) for experiments on mMM with and without 10% acetylene (v/v). NO_3^-, NO_2^-, N_2O and pH measurements are means ± standard deviation of n=3. Strains that lack N_2O reductase are indicated in bold. Abbreviations for species names are *thermo, Geobacillus thermodenitrificans; stearo*, *Geobacillus stearothermophilus*; *kausto*, *Geobacillus kaustophilus*; *toebii*, *Geobacillus toebii.*

Species	Isolate	Origin	PCR products[a]				7 days incubation with 10% acetylene (v/v)				5 days incubation without acetylene				
			nirK[b]	*qnorB*[b]	*nosZ*[c]	*nosZ*[b]	NO_3^-	NO_2^-	N_2O from NO_3^-	pH	NO_3^-	NO_2^-	N_2O from NO_3^-	N_2 from NO_3^-	pH
			(440)	(606)	(1502)	(1502)	(µmol)	(µmol)	(µmol)		(µmol)	(µmol)	(µmol)	(µmol)	
thermo	LMG 17532^T	sugar beet juice extraction installations, Austria	+	+	+	+	2.9 ± 4.4	0.0 ± 0.0	236.8 ± 3.9	7.7 ± 0.2	160 ± 1.8	0.0 ± 0.0	0.4 ± 0.2	160.8 ± 3.9	7.7 ± 0.1
thermo	R-35647^T	sugar beet juice extraction installations, Austria	+	+	+	+	0.0 ± 0.0	0.0 ± 0.0	238.4 ± 5.6	7.7 ± 0.1	159.4 ± 2.0	0.0 ± 0.0	0.7 ± 0.2	164.0 ± 3.4	7.6 ± 0.0
stearo	**LMG 6939^T**	**deteriorated canned food**	**+**	**+**	**-**	**-**	**0.0 ± 0.0**	**0.0 ± 0.0**	**244.0 ± 2.7**	**7.5 ± 0.1**	**0.0 ± 0.0**	**99.6 ± 0.0**	**197.5 ± 1.4**	**0.0 ± 0.0**	**7.6 ± 0.0**
stearo	**R-35646^T**	**deteriorated canned food**	**+**	**+**	**-**	**-**	**0.0 ± 0.0**	**0.0 ± 0.0**	**244.0 ± 2.9**	**7.7 ± 0.2**	**0.0 ± 0.0**	**76.7 ± 1.9**	**204.4 ± 3.6**	**0.0 ± 0.0**	**7.7 ± 0.2**
kausto	LMG 9819^T	pasteurized milk	+	+	+	+	0.0 ± 0.0	2.2 ± 1.7	236.6 ± 4.1	7.4 ± 0.0	0.0 ± 0.0	169.6 ± 3.9	0.7 ± 0.0	152.2 ± 4.6	7.5 ± 0.0
thermo	R-32614	Deli People's Commune, India	+	+	+	+	16.7 ± 24.6	0.0 ± 0.0	225.5 ± 0.7	7.6 ± 0.1	158.4 ± 0.4	0.0 ± 0.0	0.7 ± 0.2	164.3 ± 3.4	7.6 ± 0.0
thermo	R-32615	unknown	+	+	-	+	0.0 ± 0.0	72.2 ± 54.0	178.5 ± 1.7	7.5 ± 0.4	0.0 ± 0.0	155.9 ± 2.4	0.7 ± 0.2	165.8 ± 0.0	7.6 ± 0.0
thermo	R-32616	soil, Iceland	+	+	-	+	0.4 ± 0.4	0.0 ± 0.0	243.2 ± 2.7	7.7 ± 0.2	156.5 ± 0.8	0.0 ± 0.0	1.2 ± 0.0	164.5 ± 0.4	7.6 ± 0.04
thermo	R-32617	unknown	+	+	-	+	0.0 ± 0.0	12.7 ± 11.3	234.9 ± 8.5	7.7 ± 0.2	159.4 ± 2.8	0.0 ± 0.0	0.9 ± 0.0	162.8 ± 3.9	7.6 ± 0.01
thermo	R-32618	River Cam, Cambridge, UK	+	+	-	+	111.1 ± 16.2	0.0 ± 0.0	184.0 ± 5.6	7.4 ± 0.1	3.4 ± 4.9	0.0 ± 0.0	31.9 ± 44.2	132.8 ± 38.8	7.5 ± 0.04
thermo	R-32619	unknown	+	(+)	-	+	41.3 ± 37.8	0.0 ± 0.0	175.6 ± 35.4	7.5 ± 0.2	0.0 ± 0.0	153.8 ± 6.8	0.7 ± 0.0	164.3 ± 4.6	7.6 ± 0.00
thermo	R-32621	sugar beet juice, Austria	+	(+)	-	+	0.0 ± 0.0	0.0 ± 0.0	244.5 ± 1.9	7.7 ± 0.2	167.6 ± 0.8	0.0 ± 0.0	0.4 ± 0.2	157.4 ± 2.7	7.6 ± 0.01
thermo	R-32622	unknown	+	(+)	-	+	0.0 ± 0.0	122.5 ± 3.4	182.2 ± 4.6	7.5 ± 0.3	0.0 ± 0.0	160.8 ± 3.4	0.2 ± 0.4	160.8 ± 1.9	7.6 ± 0.01
thermo	R-32623	soil, Italy	+	(+)	-	+	0.4 ± 0.4	0.0 ± 0.0	234.9 ± 9.3	7.7 ± 0.2	153.9 ± 0.4	0.0 ± 0.0	0.4 ± 0.2	165.3 ± 1.9	7.6 ± 0.01
thermo	R-32624	soil, Indonesia	+	+	-	+	0.4 ± 0.4	0.0 ± 0.0	242.5 ± 2.2	7.8 ± 0.2	148.1 ± 0.4	0.0 ± 0.0	0.7 ± 0.0	169.9 ± 5.9	7.6 ± 0.01
thermo	**R-32625**	**soil, Saudi Arabia**	**+**	**+**	**-**	**-**	**0.4 ± 0.4**	**0.0 ± 0.0**	**241.3 ± 5.9**	**7.7 ± 0.2**	**64.4 ± 0.4**	**0.0 ± 0.0**	**211.3 ± 0.2**	**0.0 ± 0.0**	**7.7 ± 0.00**
thermo	R-32500	Ayutthaya, Thailand	+	+	+	+	3.4 ± 4.4	0.0 ± 0.0	238.6 ± 2.7	7.6 ± 0.2	153.0 ± 0.4	0.0 ± 0.0	0.4 ± 0.2	166.7 ± 0.4	7.6 ± 0.00
thermo	R-32506	unknown	+	+	-	+	43.7 ± 15.7	4.9 ± 9.8	210.0 ± 2.2	7.8 ± 0.2	164.8 ± 0.4	0.0 ± 0.0	0.7 ± 0.0	161.6 ± 6.8	7.6 ± 0.01
thermo	R-32511	soil, la Mont St Michel, France	+	+	-	+	0.0 ± 0.0	0.0 ± 0.0	238.6 ± 7.1	7.7 ± 0.2	160.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	162.1 ± 5.1	7.6 ± 0.01
stearo	**R-32513**	**unknown**	**+**	**+**	**-**	**-**	**0.4 ± 0.4**	**0.0 ± 0.0**	**243.7 ± 1.4**	**7.6 ± 0.2**	**0.0 ± 0.0**	**79.7 ± 2.4**	**202.4 ± 3.4**	**0.0 ± 0.0**	**7.6 ± 0.08**
stearo	**R-32605**	**unknown**	**+**	**+**	**-**	**-**	**0.4 ± 0.4**	**0.0 ± 0.0**	**242.8 ± 1.9**	**7.7 ± 0.2**	**0.0 ± 0.0**	**84.0 ± 5.4**	**201.2 ± 0.2**	**0.0 ± 0.0**	**7.7 ± 0.19**
stearo	**R-32635**	**Soil, Wales, UK**	**(+)**	**+**	**-**	**-**	**0.4 ± 0.4**	**0.0 ± 0.0**	**241.8 ± 3.6**	**7.6 ± 0.2**	**0.0 ± 0.0**	**76.2 ± 9.8**	**203.4 ± 4.1**	**0.0 ± 0.0**	**7.5 ± 0.00**
toebii	**R-32639**	**evaporated milk**	**+**	**+**	**-**	**-**	**0.0 ± 0.0**	**0.0 ± 0.0**	**230.7 ± 16.2**	**7.6 ± 0.1**	**72.8 ± 0.4**	**0.0 ± 0.0**	**206.8 ± 0.2**	**0.0 ± 0.0**	**7.6 ± 0.11**

[a] +, PCR product of expected size, - , no PCR amplification, (+) weak PCR amplification. Expected size of product is shown in parentheses.
[b] with primer pair of this study
[c] with primer pair nosZF-nosZR (Jones *et al.*, 2011)

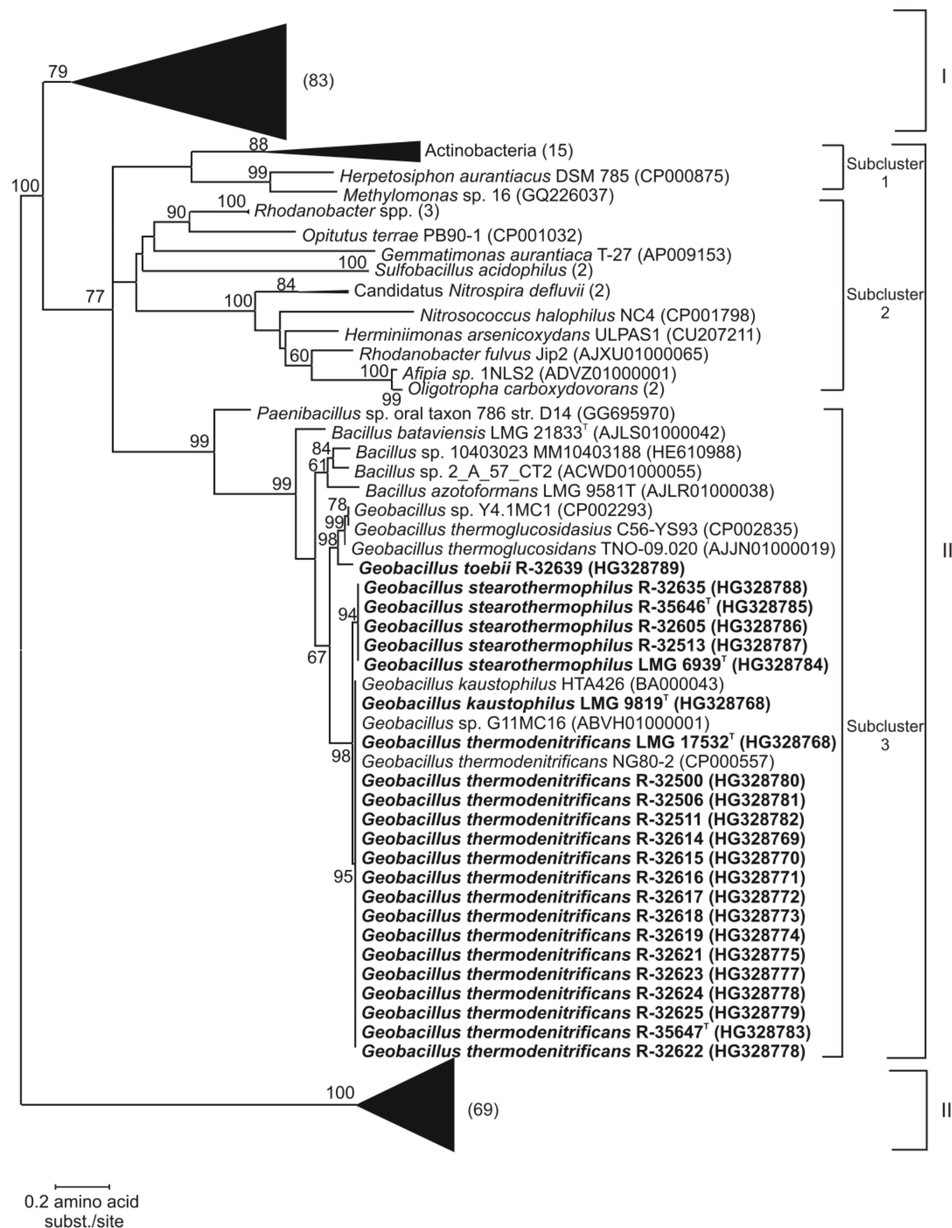


Fig. 3. 3 Maximum likelihood phylogeny of *nirK* amino acid sequences from isolates as well as available genomes in Fungene and GenBank sequence databases. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 218 amino acid sequences and there were a total of 94 positions used in the final dataset. Sequences from this study are given in bold.

3.3.5 *QnorB* – functional genotype

QnorB was detected in all *Geobacillus* strains, resulting in a fragment of approximately 600 bp (Table 3. 3). *Geobacillus thermodenitrificans* LMG 17532^T, R-32500, R-32506, R-32511, R-32614, R-32615, R-32622, R-32523, R-32625 and R-32647^T *qnorB* sequences were identical based on nucleotide alignments. This also applied for *Geobacillus thermodenitrificans* R-32617, R-32618 and R-32619 *qnorB*, for R-32616 and R-32621 *qnorB* and for *Geobacillus stearothermophilus* R-32605 and R-32635 *qnorB*. Pairwise comparison of all retrieved sequences resulted in similarities ranging from

81.8% to 100%. pBLAST of derived amino acid sequences indicated they were all similar to 'nitric oxide reductase large subunit' (NOR). Average percent AAI of the inferred amino acid alignment was calculated to be 49%, indicating reliability of the alignment.

The *qnorB* dataset consisted of 305 genotypes and RAxML phylogenetic analysis of amino acid sequences inferred from the *qnorB* genes revealed 4 distinct clusters (Fig. 3. 4): cluster I, encompassing well-known *qnorB* sequences of denitrifiers and non-denitrifying strains (e.g. *Synechocystis* sp. PCC 6803 and *Corynebacterium* spp.), cluster II, formed by sequences of primarily *Geobacillus*, *Bacillus*, *Lactobacillus* and *Staphylococcus* strains, cluster III, a more deeply branching cluster containing *qnorB* of halophilic *Archaea*, and cluster IV, forming an early-diverging monophyletic clade with Crenaerchaeotal *qnorB* sequences. As for *nirK*, not all Firmicute *qnorB* genes cluster together, e.g. *qnorB* of *Bacillus subtilis* BEST7613 clusters closest with sequences from *Synechocystis* and other Cyanobacteria and *Enterococcus* sp. R-24626 and *Veillonella* spp. sequences are present in a cluster principally containing Beta- and Gammaproteobacterial sequences (subcluster 3; Fig. S2, appendices). Remarkably, *qnorB* of *Bacillus* species isolated from luvisol soil (Verbaendert *et al.*, 2011b) and arable soil (Dandie et al., 2008, direct submission) form a separate clade within cluster I with *qnorB* sequences of soil-derived *Firmicutes* such as *Bacillus bataviensis* LMG 21833^{T}, *Bacillus* sp. 1NLA3E, *Bacillus azotoformans* LMG 9581^{T} and *qnorB* sequences from *Bacteroidetes*. Noteworthy is that *Bacillus azotoformans qnorB* copy A and copy B (Heylen & Keltjens, 2012) and *Bacillus* sp. 1NLA3E *qnorB* copy A and copy B seem very divergent, with one type in cluster I and one in cluster II.

3.3.6 *NosZ* – functional genotype

The *nosZ* primers from this study more robustly amplified the *nosZ* fragment from the *Geobacillus* strains than the *Geobacillus* and *Desulfitobacterium*-based priming set nosZ-F – nosZ-R (Jones *et al.*, 2011)(Table 3. 3). *Geobacillus toebii* R-32639, *Geobacillus kaustophilus* R-32635 and none of the *G. stearothermophilus* strains yielded *nosZ* sequences. It was detected in *Geobacillus kaustophilus* LMG 9819^{T} and in all tested *Geobacillus thermodenitrificans* strains, except in strain R-32625, resulting in a fragment of approximately 1500 bp (Table 3. 3). *Geobacillus thermodenitrificans* LMG 17532^{T}, R-32500 and R-35647 sequences were identical based on nucleotide alignments, as well as sequences of R-32617, R-32618, R-32622, R-32623 and R-32624. Sequence similarity of pairwise compared *nosZ* sequences ranged from 92.38% to 100%. pBLAST analysis indicated that sequences encode for 'Sec-dependent nitrous-oxide reductase' (N_2OR). Average percent amino acid identity of the alignment was calculated to be 48.3%, indicating reliability of the alignment. The *nosZ* dataset consisted of 264 genotypes and RAxML phylogenetic analysis of amino acid residues showed two distinct clusters containing sequences of either

typical/simple Z-type *nosZ* (cluster I) or 'atypical' *nosZ* (cluster II) (Sanford *et al.*, 2012) (Fig. 3. 5; Fig. S3, appendices). As expected, all *Geobacillus* sequences fell within the 'atypical' *nosZ* clade and contributed to a well-supported (98% node confidence) *Firmicutes* clade (subcluster 2). Remarkably, *nosZ* from the thermophile *Anoxybacillus flavithermus* TNO-09.006 is most closely related to the *nosZ*

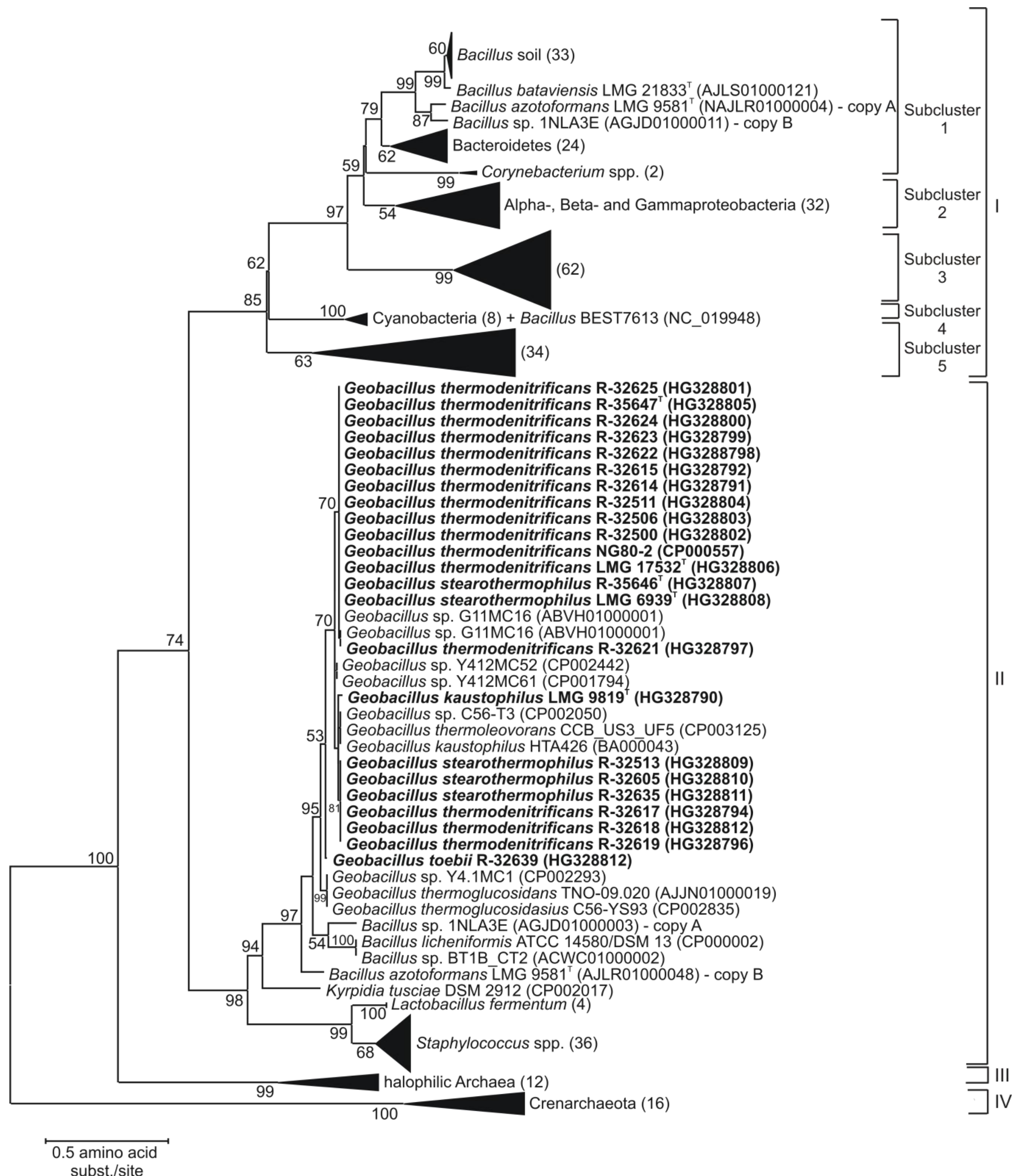


Fig. 3. 4 Maximum likelihood phylogeny of *qnorB* amino acid sequences from isolates as well as available genomes in Fungene and GenBank sequence databases. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I+F) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 305 amino acid sequences and there were a total of 145 positions used in the final dataset. Sequences from this study are given in bold.

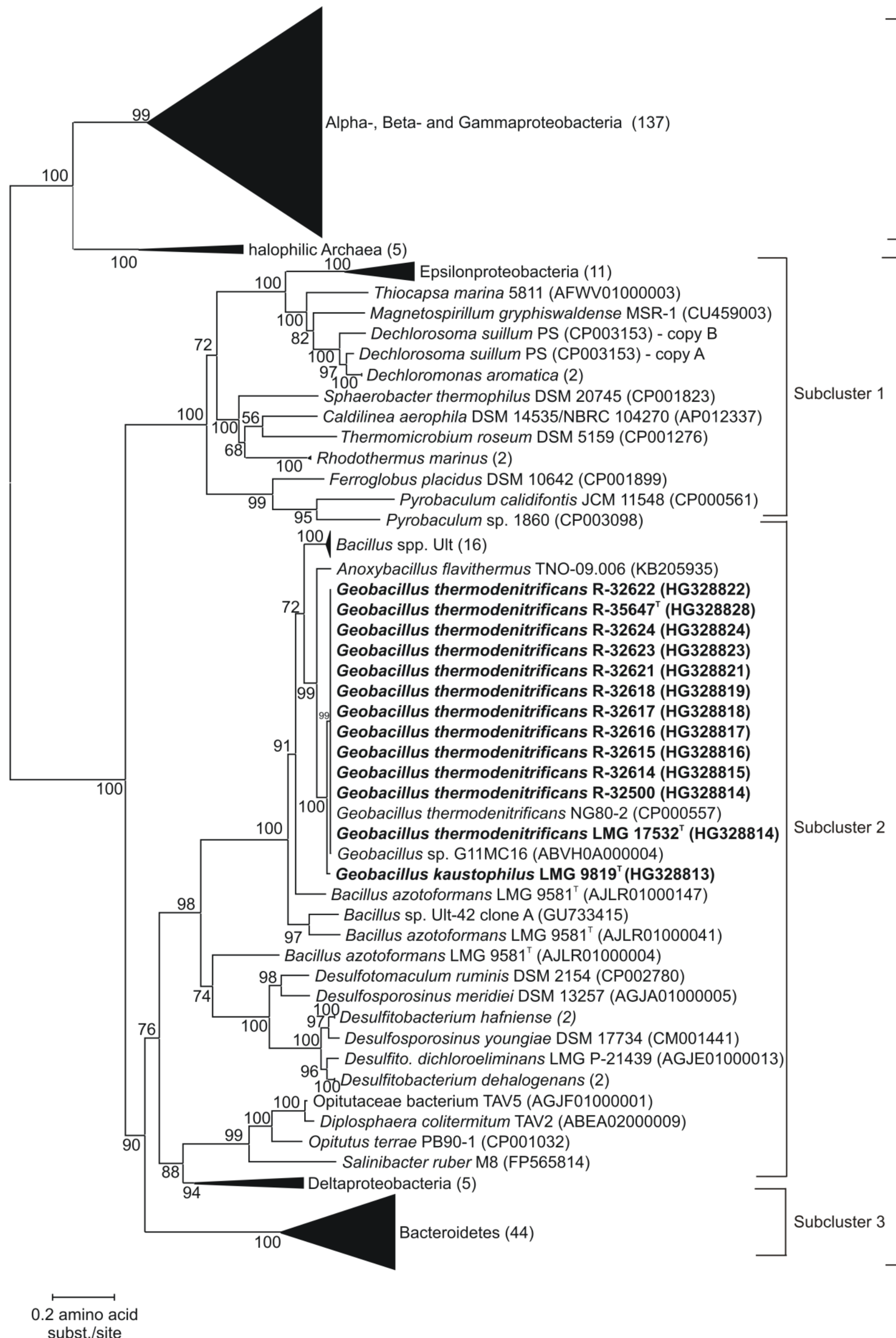


Fig. 3. 5 Maximum likelihood phylogeny of *nosZ* amino acid sequences from isolates as well as available genomes in Fungene and GenBank. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 264 amino acid sequences and there were a total of 407 positions used in the final dataset. Sequences from this study are given in bold.

sequences of thermophilic geobacilli used in this study. Notably, none of the available Firmicute *nosZ* sequences fell outside the *Firmicutes* clade. Close relatives were (1) Bacteroidetal *nosZ* sequences clustering together in a monophyletic clade (subcluster 3, 100 % bootstrap probability) and showing rather high sequence similarity to Firmicute *nosZ* and (2) Verrucomicrobial and Deltaproteobacterial *nosZ* sequences. *NosZ* sequences of halophilic *Archaea* formed a separate, more divergent cluster within typical *nosZ* cluster I as opposed to the presence of *Pyrobaculum* and *Ferroglobus nosZ* sequences in 'atypical' *nosZ* cluster II.

3.4 Discussion

The strain set existed of twenty-one *Geobacillus* strains isolated from various origins. To deal with both genetic as well as phenotypic variation in this *Geobacillus* strain panel, AFLP fingerprints, functional gene sequence analysis and phenotypic denitrification aptitude in complex growth media (Verbaendert *et al.*, 2011b) and liquid defined growth medium (Stanier *et al.*, 1966) were evaluated. Inter-species and discrete intra-species variation in AFLP fingerprints was observed (Fig. 3. 2), as well as phenotypical differences regarding N_2O and N_2 production (Table 3. 3). Absence of *nosZ* was always consistent with N_2O as end-point nitrogen species for a number of *Geobacillus* strains, though did not seem taxon-related. Other Gram-positive microorganisms have been reported to terminate denitrification with N_2O (Denariaz *et al.*, 1989, Shoun *et al.*, 1998, Mishima *et al.*, 2009). As a consequence, these Gram-positive bacteria and the *nosZ* lacking geobacilli of this study may perhaps be of importance in N_2O emission hot-spots containing relatively higher numbers of denitrifiers that lack *nosZ*. The high abundance of geobacilli in soil ecosystems (Rahman *et al.*, 2004, Marchant & Banat, 2010) increases the possibility that a reasonable number of denitrifying members of this genus may lack *nosZ in-situ.* Hence, together with e.g. the fungi (Rütting *et al.*, 2013) they may contribute to the often observed large spatial variability of soil N_2O emissions (Scala & Kerkhof, 1998, Takaku *et al.*, 2006) and the greenhouse gas effect. However, this is still theory because of the bias in molecular surveys towards genes of Gram-negative denitrifiers and the lack of data in literature on phenotypic denitrification in Gram-positive bacteria and on denitrification in thermophilic environments as a whole. Since geobacilli have been isolated from natural hot environments, such as composting plant materials, marine hot springs and hydrothermal vents and subterranean oil fields (Zeigler, 2014), and from artificial thermal environments, such as hot water pipelines, heat exchangers, bioremediation or burning coal refuse biopiles and waste water treatment plants (Obojska *et al.*, 2002, Prakash *et al.*, 2012), it is likely that they thrive as denitrifiers in these ecosystems. The question is whether viable thermophilic geobacilli that are present in large numbers in cool terrestrial environments (Rahman *et al.*, 2004) are also metabolically active and denitrify in

what would appear totally unsuitable growth conditions. For this, geobacilli may either adopt an r-type survival strategy (Pavlostathis *et al.*, 2006)[2] and be opportunistic denitrifiers capable of rapid reproduction under transient thermophilic conditions and high substrate abundance (Zeigler, 2014), e.g. during composting of plant organic matter, or they may grow – and denitrify? – for protracted periods of time at lower temperatures, as was seen for *Geobacillus thermoleovorans* batch experiments after 9 months at 4°C (Marchant & Banat, 2010). In addition, driving forces for thermophilic denitrifiers, such as pH (Liu *et al.*, 2010), water content, carbon and nitrate availability (Wallenstein *et al.*, 2006) and selection pressure (Nadeem *et al.*, 2013), influencing end-product stoichiometry (N_2O:N_2 ratio) and expression of N_2O reductase, also still need to be elucidated.

To date, few primers (Jones *et al.*, 2011, Jung *et al.*, 2013) specifically targeting denitrification genes of Gram-positive denitrifiers have been designed. Nevertheless, previous work (Behrendt *et al.*, 2010, Green *et al.*, 2010) has shown that a high percentage of phenotypically denitrifying Gram-positive bacteria could not render *nir* or *nor* amplicons when applying several commonly used primer sets. When amplification was successful, gene sequences were highly similar to PCR-derived sequences from Gram-negative bacteria (Fig. S1 and S2, appendices). Initial failed attempts to amplify *nirK*, *norB* and *nosZ* from the described *Geobacillus* strain set with well-known primers (Fig. 3.1) and *nirK* primers that picked-up *Geobacillus*-like *nirK* (Bartossek *et al.*, 2010) (data not shown), emphasized the need for primers that can target specific groups of gene sequences, whether or not harbored by taxa that are phylogenetically close. Failure of gene amplification was likely to be sequence divergence at sites targeted by these traditional primers as revealed by alignment of *Geobacillus thermodenitrificans* NG80-2 and other *Bacillaceae nirK*, *qnorB* and *nosZ* sequences with traditional primers (Fig. 3.1 and Table 3.2). *Bacillaceae*, a.o. *Geobacillus,* may possibly have environmental significance as denitrifiers as they are spread in many terrestrial and other (denitrifying) ecosystems (Felske *et al.*, 1998, Rahman *et al.*, 2004, Janssen, 2006, Verbaendert *et al.*, 2011b), but are clearly not targeted by most current primer sets. As a result, we successfully designed *nirK*, *qnorB* and *nosZ* primers to specifically target denitrifiers from this family, with the emphasis on the thermophilic genus *Geobacillus*.

At least three types of *nirK* genes have been hitherto determined: (i) class/group I *nirK*, probably encoding soluble periplasmic proteins, (ii) class/group II *nirK,* probably encoding outer-membrane lipoproteins and (iii) a third type of more divergent *nirK*, present in whole genome sequences of *Geobacillus, Corynebacterium* and *Nitrosomonas* strains (Boulanger & Murphy, 2002, Philippot, 2002, Ellis *et al.*, 2007). It has been established that commonly used *nirK* gene primers

[2] Refers to an ecological survival strategy. When population densities are low, change is dominated by the rate of population increase (populations with higher r). For microorganisms this type of strategy evolution favors a high rate of reproduction. These microorganisms are are also characterized, by their opportunism, by population crashes in resource limited conditions, by extreme fluctuations in populations and by their success in uncrowded communities.

were designed to mainly target class I *nirK*, hence detecting this type most often in denitrifiers (Jones *et al.*, 2008, Green *et al.*, 2010). However, they do not pick up class II *nirK* nor third type *nirK* genes. The inferred *nirK* amino acid alignment revealed well-conserved residues in the Cu-binding sites of *Geobacillus* CuNiR (copper-containing nitrite reductase), e.g. (1) histidine (His) and methionine typically found in the copper type 1 center of nitrite reductases (CX_4HX_4M motif) (Ellis *et al.*, 2007, Bartossek *et al.*, 2010) and (2) copper-binding His in the SSFHV motif specific for class II CuNiR (Jones *et al.*, 2008). Moreover, it also showed the presence of three characteristic loop regions (Boulanger & Murphy, 2002, Ellis *et al.*, 2007, Fukuda *et al.*, 2011), 'linker' loop, 'tower' loop and 'extra' loop, with presence of deletions in the former two which are characteristic for class II CuNiR. They may help bind the enzyme closer to the lipid membrane (Boulanger & Murphy, 2002, Ellis *et al.*, 2007). This is similar to the putative CuNiR lipoproteins of *Bacillus azotoformans* and *Bacillus bataviensis* (Heylen & Keltjens, 2012). Closer examination of conserved residues in Cu-binding sites within the alignment showed that sequences from cluster III (Fig. 3. 3), which received maximum bootstrap probability, contain the canonical TRPHL amino acid motif (Jones *et al.*, 2008) or variants which are characteristic for class I *nirK*. Cluster I (79% bootstrap probability) (Fig. 3. 3) generally contains sequences with the conventional SSFH (V/I/P) motif (Jones *et al.*, 2008) for class II *nirK*. Cluster II (77% node confidence) (Fig. 3. 3) also contains *nirK* amino acid sequences with the latter motif, but they display quite distinct linker and tower loop deletions dissimilar to characteristic deletions in sequences in cluster I. This confirmed that *Geobacillus nirK* sequences belong to a third type *nirK* that has not been targeted by any primer set so far.

Similar to *nirK* gene variation, different types of *nor* exist: (i) *cnorB*, also called *norB,* and so far almost exclusively detected in denitrifying bacteria, (ii) *qnorB*, found in denitrifiers, non-denitrifying pathogenic bacteria and *Archaea,* and also called *norZ,* and (iii) $qCu_A norB$ from *B. azotoformans,* also recently described as *cba* (Heylen & Keltjens, 2012). Amino acid alignment analysis disclosed presence of three conserved His residues in all inferred protein sequences of *Geobacillus qnorB* which are involved in binding of 2 hemes and a non-heme iron (Fe_B) (Watmough *et al.*, 1999) and/or a non-heme zinc (Zn_B) (Matsumoto *et al.*, 2012). In addition, they also exhibited the highly conserved $HLWVEGX_2E$ and glutamate residue, essential for catalytic activity and specificity of the active site of NOR (Watmough *et al.*, 1999, Matsumoto *et al.*, 2012). Previous phylogenetic analysis of *cnorB* and *norZ* (Philippot, 2002) indicated that *norZ* from Gram-positive bacteria *Bacillus stearothermophilus*, *Synechocystis* and *Corynebacterium diptheriae* and the Archaeon *Pyrobaculum aerophilum* clustered separately from *norZ* belonging to *Burkholderia*, *Ralstonia* and *Neisseria*. As with *nirK*, employed *qnorB* primers have thus far not been able to yield proper *qnorB* sequences from these divergent clusters by direct PCR amplification.

Hence, to our knowledge, this study is the first report of *nirK and qnorB* primers that target *nirK and qnorB* from these separate gene clusters and that are specifically designed for Gram-positive bacteria, with successful retrieval of *Geobacillus nirK* and *qnorB* fragments. Denitrification activity of the tested *Geobacillus* strains underpinned that CuNiR and qNOR reductases encoded by these *nirK* and *qnorB* genes are functional, resolving uncertainties raised on their function (Philippot, 2002, Green *et al.*, 2010). However, functionality of other CuNiR and qNOR enzymes in the particular *nirK* and *qnorB* clusters (Fig. 3. 3 and Fig. 3. 4) has not always been confirmed experimentally. Only *Rhodanobacter denitrificans* (Prakash *et al.*, 2012), *Intrasporangium* sp. 4LS1 (Green *et al.*, 2010), *B. azotoformans*, *B. bataviensis* (Verbaendert *et al.*, 2011b) and *Lactobacillus farciminis* (Hammes, 2012) have been shown to phenotypically denitrify. The other *Firmicutes* in this cluster may use *qnorB* for other processes, e.g. detoxification of NO produced by the human immune system (Hendriks *et al.*, 2000) and disproportionation of NO_3^-/NO_2^- to NO in fermented (food) products (Hammes, 2012). Markedly, although molecular detection of denitrification genes is a more specific problem for Gram-positive bacteria (Verbaendert *et al.*, 2011a), PCR-derived *qnorB* sequences (Table S1, appendices) were only present in subcluster 1 and 3 (Fig. 3. 4) and extensive sequence variation has been uncovered by WGS, both in Gram-positive and Gram-negative organisms. Taking this large sequence diversity into account, development of extra *qnorB* primers targeting other phylogenetic groups will be undoubtedly necessary to detect a more complete *qnorB* diversity present in any given ecosystem.

Hitherto, three *nosZ* types have been described, two of which were present in the *nosZ* phylogenetic clustering: (i) typical 'true denitrifier' *nosZ* and, (ii) 'atypical' *nosZ* (Sanford *et al.*, 2012) with distinctive regulatory and functional components. The derived amino acid sequence alignment of *nosZ* genes disclosed conservation of several His residues important for structure and functionality of N_2O-reductase (Scala & Kerkhof, 1998, Sanford *et al.*, 2012). PCR-derived fragments of 'atypical' *nosZ* from *Anaeromyxobacter* spp. (Sanford *et al.*, 2012) and Gram-positive N_2O- and N_2-producing *Bacillus* (Jones *et al.*, 2011) and *Geobacillus* (Jung *et al.*, 2013) strains have been reported recently. However, since primers NosZF912F – NosZ1853R for atypical *nosZ* (Sanford *et al.*, 2012) do not match primer binding sites in Gram-positive denitrifiers (Fig. 3.1 and Table 3.2), primers nosZF-nosZR (Jones *et al.*, 2011) failed in the *Geobacillus* strain set and because of the interest to obtain almost full-length *nosZ* sequences, novel selective primers for 'atypical' *nosZ* genes were designed successfully for the tested strain panel.

Because of conflicting functional and organism gene phylogenies (Heylen *et al.*, 2006, Jones *et al.*, 2008), culture-dependent studies on denitrification are still essential. By gaining knowledge on all aspects of denitrification in pure cultures, development of molecular tools for environmental surveys is stimulated and creation of more elaborate models for prediction of denitrification

greenhouse gas emissions is aided (Bergaust *et al.*, 2011). Moreover, the absence of N_2O-reductase observed in pure cultures may perhaps partially explain the often observed high spatial variabilities of soil N_2O emissions *in-situ,* where relatively higher numbers of *nosZ* lacking denitrifiers may accumulate in so-called N_2O-emission 'hot-spots'. Our findings also reaffirm the value of combining phenotypic denitrification data of pure cultures with genomic data for future *in-situ* characterization of denitrifying communities. Past years have revealed that primers in PCR-based surveys fail to pick up the extensive sequence variation present in functional denitrification genes (Green *et al.*, 2010, Verbaendert *et al.*, 2011a, Sanford *et al.*, 2012), leading to incomplete and inaccurate *in-situ* monitoring of denitrification and quantification of denitrifiers. *NirK, qnorB* and *nosZ* sequences and primers reported here will help in future design of more complete and accurate PCR tools to re-asses ecological importance, *in-situ* activity, diversity and numerical significance of Firmicute denitrifiers. This may contribute to a more precise understanding of the structure of denitrifying guilds *in-situ* and subsequently more accurate modeling of nitrogen fluxes by denitrification. Nevertheless, validation of these novel tools in environmental samples still needs to be accomplished.

In summary, our study has revealed the presence of functional *nirK*, *qnorB* and *nosZ* sequences substantially different from well-known sequences of denitrification genes. The development of the described primer sets allows for detection of denitrification genes for which no or few detection assays are accessible and for investigation of the not-yet-fully-inquired *nir*, *nor* and *nos* sequence diversity in environmental monitoring studies. Further application of these new primers should serve to advance our knowledge of the microbial ecology of denitrifying bacteria and ought to be used to re-evaluate true diversity and abundance of these micro-organisms in nature.

3.5 Acknowledgements

The authors would like to thank Joke Buyse for her assistance with the AFLP fingerprinting protocol, Prof. Dr. Niall Logan for kindly allowing us to use the *Geobacillus* R-collection strains for our study and Evie De Brandt and Dr. Heylen for optimization of the miniaturized colorimetric assays for nitrate and nitrite determination, and also the anonymous reviewers for their detailed and constructive comments on earlier versions of the manuscript.

Reflection and discussion

The reason for switching to *Geobacillus* for further Gram-positive denitrification research was because geobacilli are very abundant in soil-bound environments. Therefore, *Geobacillus* seemed a suited target for phenotypic denitrification assessment and for the evaluation of established *nirK*, *qnorB* and *nosZ* primers and of newly designed primers, with their design based on *Geobacillus* Whole Genome Sequences (WGS). At that time, a small number of *Geobacillus* WGS and few older reports on phenotypic denitrification and on denitrification enzymes in *Geobacillus* were available. In this chapter, assessment of both genetic as well as phenotypic variation in a *Geobacillus* strain panel with AFLP fingerprinting, functional gene sequence analysis and phenotypic denitrification capacity in complex growth media and liquid defined growth medium, are described. Although the *Geobacillus* strains proved to phenotypically denitrify, detection of the responsible genes within the strain set was not possible with the published primers. Accordingly, the results of qualitative comparison of known primers with available *Bacillaceae* sequence data highlighted their prominent unsuitability to amplify denitrification genes from Gram-positive *Bacillaceae*, especially from *Geobacillus*. The novel primers were clearly more robust in amplification of the different denitrification genes. Thus, we can conclude that the developed primers are a promising addition to the so far used PCR primers sets and are able to pick up hitherto uncharted *nirK*, *qnorB* and *nosZ*.

Although the newly developed primers for denitrification genes in Gram-positive *Geobacillus* were very successful, a number of aspects regarding detection and experimental set-up could have been improved and future research may elucidate other aspects of the *Geobacillus* denitrification trait.

The focus of this research was on a range of pure culture *Geobacillus* strains belonging to four species, of which for *Geobacillus thermodenitrificans*, *Geobacillus stearothermophilus* and *Geobacillus toebii* 'denitrification' capabilities are reported. According to the "List of Prokaryotic names withstanding in nomenclature" (LPSN, www.bacterio-net), there are currently twenty validly described species and four subspecies recognized in the genus *Geobacillus*. To more fully broaden our knowledge on the genotypic denitrification potential and phenotypic denitrification ability in *Geobacillus*, it may have been interesting to include less strains of the *Geobacillus thermodenitrificans* species and more different other species and/or subspecies.

So far, no (genetic) evidence exists that members of the genus *Geobacillus* can perform nitrate ammonification, hence N_2O production on TSB and 1/10 TSB due to detoxification of nitrite to N_2O

during DNRA seems unlikely. However, members of the genus have been shown to perform fermentation (Sung *et al.*, 2002, Pavlostathis *et al.*, 2006, Xiao *et al.*, 2012) and denitrifiers are commonly not considered to be able to ferment, although more denitrifiers may be capable of fermentation than has previously been recognized (Tiedje *et al.*, 1982). In addition, other members of the *Bacillaceae*, e.g. *Bacillus vireti* LMG 21834^T, have been proven to simultaneously (i) reduce NO_3^- to NH_4^+ with concomitant production of N_2O and N_2 (with concentrations of the latter depending on the initial concentration of nitrate) and (ii) ferment the present C-sources in TSB to acetate, formate and lactate) (Mania *et al.*, 2014). Full-strength TSB contains ± 13mM glucose and ± 11 mM succinate as carbon sources (Mania *et al.*, 2014), hence the experiments for *Geobacillus* on TSB almost certainly have caused the probable mixed denitrification and fermentation reactions we observed under anaerobic conditions. However, the adaptation of the conventional mineral medium to modified mineral medium provided us with a good alternative liquid growth medium that accommodated the strains' growth requirements, allowed straight-forward assessment of nitrate, nitrite, N_2O and N_2 concentrations and may prove to be useful for denitrification assessment of other *Bacillaceae*, e.g. *Bacillus*.

Although we – unsuccessfully – tested the developed primers on marine sediment samples that showed N_2O-production and of which 16S rRNA data showed the presence of *Firmicutes* (data not included in Chapter 3), we did not thoroughly investigate their efficacy in environmental samples. Of course, the presence of a gene does not necessarily equate to the expression of that gene or the associated activity in the environment, nor does it allow definite conclusions about the phylogenetic affiliation of the organisms present. Yet, up till now, we do not have a clue whether the genes that are involved in denitrification in Gram-positive bacteria are even present in certain environments, let alone that we know whether the genes are expressed and/or the proteins are active. Therefore, it would be very interesting to test the primers on both thermophilic and mesophilic soil samples and reveal the presence and maybe even relevance of the genes in these environments. This would allow insight into (i) the presence of potential denitrifiers belonging to (thermophilic) Gram-positive bacteria, (ii) the relative numbers of Gram-positive (*Bacillaceae*) denitrification genes vs. Gram-negative (Proteobacterial) denitrification genes.

For sequence analysis of *nirK*, *qnorB* and *nosZ* in this chapter, our data set consisted of both nucleotide sequences from strains isolated from environmental samples and nucleotide sequences from genome sequencing projects (Table S1, appendices). Although (i) it is known that taxonomic designations in public databases are sometimes incongruent with the Ribosomal Database Project (RDP) classifier identification (Jones *et al.*, 2008), and (ii) we may have missed functional genes that

were incorrectly or differently annotated, we had to rely on annotations in the original database entries, both for phylogenetic information as well as for information on functional genes. In spite of this, we observed several remarkable features in the gene sequence sets. Firstly, the complete sequence data set had an overall taxonomic distribution predominated by alpha-, beta- and gamma-*Proteobacteria*. We have used functional gene sequence data from approximately 712 whole genomes and these showed a phylogenetic distribution analogous to that of the complete data set (Fig. 3. 6). Secondly, out of the whole genome data set, only 106 genomes contained a near complete denitrification pathway comprised of *nirK*, *qnorB* and *nosZ* and nearly 100 genomes contained *nirK* and *qnorB,* indicative of a denitrification *sensu stricto* pathway (Fig. 3. 6). In both cases, genomes mainly belonged to *Proteobacteria*. In addition, 78 – again mainly Proteobacterial – genomes contained *qnorB* in combination with *nosZ*. In these cases, a *nirS* gene was frequently present, indicative of the full pathway, but sometimes no nitrite reductases involved in denitrification could be found. Similarly, 28 genomes contained *nirK* in combination with *nosZ,* with the sporadic presence of a *cnorB*. All three genes were also found unaccompanied by either of the other examined genes, with genomes containing only *qnorB* outnumbering the others (Fig. 3. 6). This may be explained by the propensity of qNOR to – seemingly often – be used by bacteria as a detoxifying enzyme to cope with NO-stress in the environment. These genomes also had a more diverse phylogenetic origin, with more Gram-positive *Firmicutes* and *Actinobacteria* represented. Genomes containing only *nirK* mostly belonged to the *Proteobacteria*, whereas genomes containing only *nosZ* showed a more even distribution between *Proteobacteria* and *Cyanobacteria*. In general, two things have become clear from this dataset: (1) in this 'genomic' era it has become difficult to define a bacterium as a 'denitrifier' only on the basis of genetic data: are denitrifiers only bacteria that encode all enzymes of the denitrification pathway or is the definition more inclusive, and (2) functional gene sequences of Gram-positive bacteria are underrepresented and future whole genome sequencing approaches of Gram-positive denitrifiers are indispensable for an improved grasp on their denitrification potential.

In-depth research on thermophilic denitrifiers has only recently started and researchers have questioned whether supposed thermophilic geobacilli would grow and be metabolically active in non-thermophilic conditions (Rahman *et al.*, 2004, Marchant & Banat, 2010), mainly because of their high abundance in all kinds of environments all over the world (McMullan *et al.*, 2004, Zeigler, 2014). Yet – in general – studies on functional, both physiological and ecological roles, in particular on nitrogen transformations, of Gram-positive (thermophilic) organisms in both hot as cold environments are virtually nonexistent. This is due to the bias of molecular tools towards genes of Gram-negative denitrifiers and the lack of data in literature on phenotypic denitrification in Gram-positive bacteria and on denitrification in thermophilic environments as a whole.

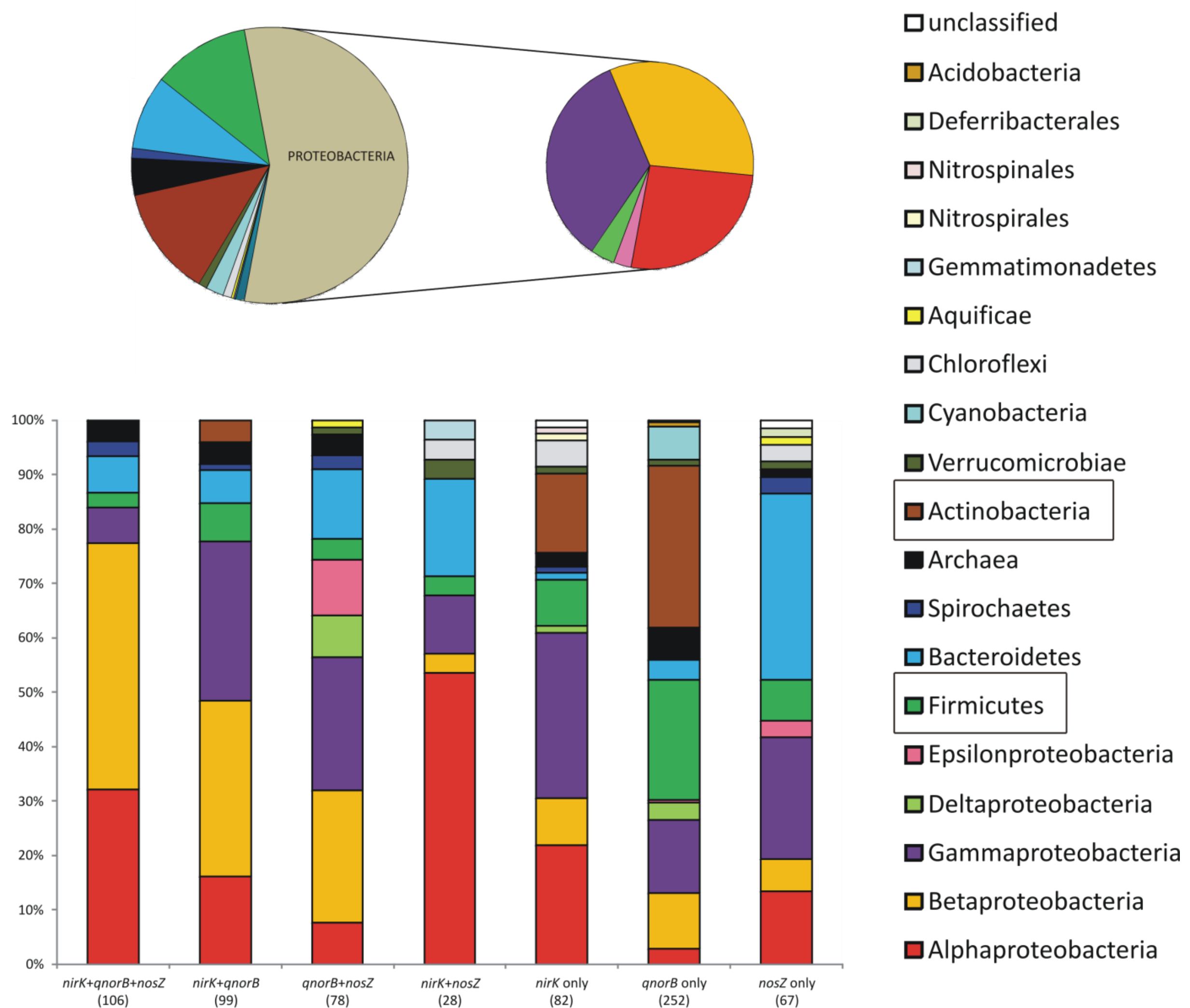


Fig. 3. 6 Phylogenetic distribution of organisms within the functional gene data set. For each bar in the diagram the total number of whole genomes with the particular gene inventory is given between parentheses.

The mentioned habitats in this chapter in which *Geobacillus* are found, e.g. composting plant materials, marine hot springs and hydrothermal vents, subterranean oil fields, waste water treatment plants,… are all hot environments with considerable anaerobic/anoxic zones or are environments that are transiently hot and anaerobic. Thermophilic denitrifying geobacilli could be relevant in these anaerobic environments. However, little physiological or molecular work has been published supporting evidence for Gram-positive denitrification within these habitats so far. Moreover, many Gram-positive bacteria have been shown to terminate denitrification with N_2O (Renner & Becker, 1970, Shoun *et al.*, 1998, Mishima *et al.*, 2009) and *Firmicutes*, a.o. geobacilli, are present in large numbers in different kinds of environments, hence they may contribute to N_2O hot-spots and the greenhouse gas effect. Thermophilic geobacilli have even been isolated from

permafrost (Vironov *et al.*, 2013). Nonetheless, it has not been proven whether geobacilli are metabolically active in thermophilic, mesophilic or even psychrophilic habitats, or how they could be in the latter two (what type of strategy they may adopt), whether they may contribute to N_2O production and which driving forces influence N_2O production by these bacteria. Or even whether they belong to the large portion of bacteria that do not directly contribute to denitrification, but remain in a 'dormant' spore-state as part of the seed bank from which different traits can be resuscitated (Krause *et al.*, 2014).

Therefore, it would be fascinating to check whether *Geobacillus* can grow and denitrify after an extended incubation at ambient temperatures (e.g. 4°C-15°C-20°C), as would occur in (agricultural) soil ecosystems in more temperate regions of Western-Europe. In addition, more elaborate phenotypic testing on environmental parameters that may influence denitrification phenotype and endpoint product stoichiometry, such as pH, the type and concentration of electron acceptor (nitrate and nitrite), the type of electron donor,… etc., with gas kinetics (Molstad *et al.*, 2007) would broaden our knowledge on the physiology of thermophilic denitrifiers, their contributions to atmospheric N_2O and maybe even Gram-positive denitrification in general.

The presence of 'atypical' or clade II *nosZ* in all *Geobacillus* strains is also intriguing. Typical and atypical *nosZ* differ in the presence of a Tat or Sec signal for transportation of N_2OR from the cytoplasm to the periplasm. There is a substantial difference between the two mechanisms in energetic cost of protein translocation: in the Sec system only 1 ATP is needed to translocate 20 amino acids across the membrane, whereas the Tat system uses an equivalent of approximately 10.000 ATP molecules (Lee *et al.*, 2006, Jones *et al.*, 2013). This would explain why atypical *nosZ* genes outnumber typical *nosZ* genes in soil metagenomes (Orellana *et al.*, 2014). However, atypical *nosZ* lacks *nosR* and *nosX*, which are accessory genes involved in the holoenzyme assembly in the periplasm (Spiro, 2012).

Several hypotheses on why the Sec system is conserved throughout microbiological communities in the environment have been proposed:

- The need for holoenzyme assembly in the cytoplasm. Because the accessory genes that are involved in assembly of the protein in the periplasm are absent in the Sec system, it was thought that the latter system assembled the complete protein in the cytoplasm. However, physiological evidence showed holoenzyme assembly occurring in the periplasm in both translocation mechanisms (Zumft & Kroneck, 2007).
- Ecological constraints on protein folding outside the cytoplasm, e.g. for microorganisms in harsh conditions such as halophiles or thermophiles. This is connected to the need for holoassembly in the cytoplasm. However, exceptions can be found within the *nosZ*

phylogeny (Fig. 3. 5)(Jones *et al.*, 2013), reducing the possibility of this kind of selective pressure as a driver for the preference of one system over the other.

- Function of atypical *nosZ* is detoxification of N_2O in non-denitrifying bacteria. N_2O can be cytotoxic to bacterial cells because it binds and inactivates VitB12, which is an essential cofactor in enzymes involved in a.o. DNA synthesis. Microorganisms that do not have VitB12-independent mechanisms to perform a.o. DNA synthesis, may prevent toxicity of exogenous N_2O by using atypical N_2OR (Sullivan *et al.*, 2013).

Did *Geobacillus* acquire this atypical *nosZ* coincidentally by HGT early in its evolution and subsequently used it in its denitrification pathway? Or have indeed constraints such as its small periplasmic space as a Gram-positive denitrifier, its thermophilic lifestyle or the absence of VitB12 independent cellular mechanisms also influenced its apparent preference for atypical *nosZ*? These are issues that still need elucidation.

Verbaendert, I., Boon, N., De Vos, P. (2014). In preparation.

Author's contributions:

IV, PDV & NB designed the experiments. IV performed the experiments, analyzed the data and wrote the manuscript. NB, PDV and IV proofread and commented on the manuscript.

Chapter 4

Genotypic exploration of the dissimilatory nitrogen metabolism in N_2O-producing *Bacillus* spp.

Summary

An initial study demonstrated the ability for dissimilatory nitrate reduction in a large set of *Bacillus* strains isolated from soil and selected from the BCCM/LMG Bacteria Collection. However, very few strains generated *nir* or *nor* amplicons when tested with a number of published primer sets frequently used in environmental monitoring studies. More recently published primers for *nirK*, *qnorB* and *nosZ* in Gram-positive geobacilli were tested and adapted to detect the genes in genomic DNA of strains from the *Bacillaceae* strain set. However, these did not result in overall high amplification rate of the denitrification genes under scrutiny, suggesting the possible presence of other mechanisms for N_2O and/or N_2 production in *Bacillaceae*. *NrfA* and *cbaA*, the latter encoding qCu_ANOR, have been implicated in DNRA and concomitant detoxification of NO to N_2O and/or N_2. Therefore, incidence of *cbaA* or qCu_ANOR– with newly developed primers – and *nrfA* – with recently improved primers – was investigated as well. Our results demonstrate that *Bacillaceae* isolated from soils may commonly have the genetic potential to use modules for both denitrification and DNRA in dissimilatory nitrate reduction.

4.1 Introduction

Nitrous oxide (N_2O) is a powerful greenhouse gas and ozone-depleting substance of which emissions have been exacerbated since intensification of agriculture by the application of synthetic nitrogen-based fertilizers. More than 50%-75% of these global N_2O emissions are hypothesized to be produced by denitrification and nitrification processes in soils, with nitrification probably being a much less potent N_2O-source because of its low ($N_2O/(NO_2^-+NO_3^-)$) product ratio at pH ≥ 5 (Richardson *et al.*, 2009, Bakken *et al.*, 2012). However, denitrification is not the only potent dissimilatory nitrate-reducing process present in soils that can cause N_2O production. DNRA is the abbreviation for 'Dissimilatory Nitrate Reduction to Ammonium', also called nitrate ammonification, but DNRA *sensu stricto* actually only comprises the reduction of nitrite to ammonium. This pathway has also been postulated to be an important N_2O producing process in fertilized or high-nitrate environments (Rütting *et al.*, 2011, Vine & Cole, 2011, Rowley *et al.*, 2012, Streminska *et al.*, 2012, Mania *et al.*, 2014). It is believed that N_2O production during DNRA occurs due to the accumulation of nitrite, which is reduced to cytotoxic NO – possibly via nitrate reductase NarG – that is successively detoxified to N_2O (Streminska *et al.*, 2012, Mania *et al.*, 2014). However, disentangling N_2O production processes such as denitrification and DNRA at a field scale is still difficult and environmental controls are not well understood (Giles *et al.*, 2012, Butterbach-Bahl *et al.*, 2013).

The potential of members of the genus *Bacillus* to perform dissimilatory reduction of nitrate and to produce N_2O during denitrification or DNRA has been demonstrated before (Smith & Zimmerman, 1981, Verbaendert *et al.*, 2011b, Streminska *et al.*, 2012, Mania *et al.*, 2014). *Bacillus vireti* LMG 21834^T even ammonifies nitrate in a denitrification-like fashion with the use of nitrous oxide reductase (N_2OR) to reduce the produced N_2O to N_2 (Mania *et al.*, 2014). Competition for nitrate between DNRA and denitrification *in situ* has been recognized for decades (Tiedje *et al.*, 1982, Tiedje, 1988), but recent whole genome analysis revealed that gene inventories for both processes can be present in one single organism, indicating that competition for nitrate may even occur at organism level. This has been observed in genomes of strains of *Opitutus terrae*, *Marivirga tractuosa* and *Shewanella loihica* (Sanford *et al.*, 2012), but also in *Bacillus azotoformans* and *Bacillus bataviensis* type strains. The latter strains also showed a significant redundancy for nitric oxide reductase (NOR) encoding genes, harboring both genes for qNOR and qCu_ANOR (Heylen & Keltjens, 2012). In addition, whole genome sequencing (WGS) has uncovered extensive sequence divergence for the responsible genes for both denitrification (*nir*, *nor*, *nos*) and DNRA (*nrfA*) in Gram-positive bacteria, but also in other bacterial groups (Verbaendert *et al.*, 2014, Welsh *et al.*, 2014). Thus sorting out N_2O production by denitrification and/or DNRA at the organismal level also still presents challenges for both molecular and physiological research.

Bacilli are significant players in natural ecosystems (Tzeneva, 2006), are part of denitrifying bacterial communities in soil (Weier & Macrae, 1992, Chèneby *et al.*, 2000, Chèneby *et al.*, 2004, Flores-Mireles *et al.*, 2007) and display a high genetic diversity, metabolic versatility and a strong adaptive capability. They lack a periplasm, which should impose specific requirements on localization and organization – and associated sequence variation (Philippot, 2002) – of enzymes involved in dissimilatory nitrate reduction, and they are often used as model organisms for the Gram-positive phylum *Firmicutes*. Yet, virtually no *Bacillus* strains are used as model organisms for processes involved in the dissimilatory reduction of nitrate, such as denitrification and DNRA. Nevertheless, an isolation campaign and an elaborate screening of the dissimilatory nitrogen metabolism of a set of 180 *Bacillus* strains retrieved from a variety of inocula and from geographically widespread origins, resulted in a set of potentially denitrifying bacilli (Heylen, 2007, Verbaendert *et al.*, 2011b). In this study, only Griess reagents were used to determine nitrate and/or nitrite removal after a two-week incubation period and only GC-measurements together with the acetylene inhibition method (AIM) to measure N_2O were performed. Hence, it was difficult to assess whether these bacilli carried out 'true' denitrification or denitrification-like nitrate ammonification under the presented conditions. Therefore, we have performed a series of experiments on a selection of these strains – including both different representatives of one specific species and representatives of different species, allowing inter- and intraspecies comparisons. Experiments were directed at revealing the presence of genes involved in denitrification and/or DNRA that may have caused the observed nitrogen removal phenotypes in this diverse set of strains. The research aimed to understand, at a genetic level, possible N_2O producing pathways within the strain set and thus to reveal the extent to which *Bacillus* – abundant in soil-bound environments – may contribute to N_2O emissions from soils. To this end, existing and newly developed degenerate oligonucleotide primer sets were tested to detect the presence of genes encoding key enzymes (*nir*, *nor*, *nos*, *cbaA*, *nrfA*) in denitrification and DNRA nitrogen transformations in these Gram-positive *Bacillaceae*.

4.2 Materials and methods

4.2.1 Bacterial cultures

Sixty-one strains belonging to the phylum *Bacillaceae* – amongst which a majority of *Bacillus* strains and two *Paenibacillus* strains – and one *Staphylococcus* strain were selected from the BBCM/LMG bacteria collection and from the LM-UGent Research collection (Table 4. 1). All strains were selected for their denitrification potential (Heylen, 2007, Behrendt *et al.*, 2010, Verbaendert *et al.*, 2011b) and were mostly derived from soil.

Table 4. 1 *Bacillus, Paenibacillus* and *Staphylococcus* strains used in this study.

Species	Strain n°	Biological origin	Growth temp	Initial isolation medium[a]
B. asahii[b]	LMG 24728^{T}	soil, Shizuoka Prefecture, Japan	28°C	n/a
B. atrophaeus	LMG 8199^{T}t$_1$	unknown	28°C	n/a
B. atrophaeus	LMG 8199^{T}t$_2$	unknown	28°C	n/a
B. azotoformans	LMG 9581^{T}	garden soil, France	28°C	n/a
B. azotoformans	LMG 15445	garden soil, France	28°C	n/a
B. azotoformans	LMG 15448	garden soil, France	28°C	n/a
B. azotoformans	LMG 15449	garden soil, France	28°C	n/a
B. bataviensis	LMG 21833^{T}	soil, hay field, Drentse A grasslands, the Netherlands	28°C	n/a
B. bataviensis	LMG 21832	soil, hay field, Drentse A grasslands, The Netherlands	28°C	n/a
B. bataviensis[c]	R-31541	luvisol soil, Melle, Belgium	28°C	TSA
B. bataviensis[c]	R-31770	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. bataviensis[c]	R-31834	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. bataviensis[c]	R-32700	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. bataviensis[c]	R-32709	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. bataviensis[c]	R-32779	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. bataviensis[c]	R-32781	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. bataviensis[c]	R-32787	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. bataviensis[c]	R-32845	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. drentensis[c]	R-31547	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. drentensis[c]	R-31550	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. drentensis[c]	R-31846	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. drentensis[c]	R-32528	luvisol soil, Melle, Belgium	28°C	G_2M^{11}
B. drentensis[c]	R-32575	luvisol soil, Melle, Belgium	28°C	TSA
B. drentensis[c]	R-32656	luvisol soil, Melle, Belgium	28°C	G_4M_3
B. drentensis[c]	R-32702	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. drentensis[c]	R-32705	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. drentensis[c]	R-32789	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. drentensis[c]	R-33773	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. indicus	LMG 22858^{T}	arsenic polluted sand, Chakdah district West Bengal, India	28°C	n/a
B. infantis	LMG 24756^{T}	blood of newborn child with sepsis, Republic of Korea	28°C	n/a
B. lehensis	LMG 24751^{T}	soil, Leh, India	28°C	n/a
B. licheniformis	LMG 17339	potato pulp for cattle feeding	37°C	n/a
B. licheniformis	LMG 17340	potato pulp for cattle feeding	37°C	n/a
B. licheniformis	LMG 6934	unknown	37°C	n/a
B. licheniformis	LMG 7559	unknown	37°C	n/a
B. licheniformis	LMG 7561	field soil	37°C	n/a
B. licheniformis	LMG 7633	chinchilla, feces	37°C	n/a
B. licheniformis[c]	R-31769	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. licheniformis[c]	R-32706	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. megaterium	LMG 7127^{T}	unknown	28°C	n/a
B. methanolicus	LMG 24730^{T}	unknown	52°C	n/a
B. mojavensis	LMG 22477	river Vélez, river mouth, Malaga, Spain	28°C	n/a
B. mycoides	LMG 7128^{T}	soil, Germany	28°C	n/a
B. plakortidis	LMG 24732^{T}	sponge (Plakortis simplex), Norway	28°C	n/a
B. pseudomycoides[c]	R-31830	luvisol soil, Melle, Belgium	28°C	TSA
B. soli[c]	R-31553	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-31841	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-32715	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-32849	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-32526	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-32694	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
B. soli[c]	R-33820	luvisol soil, Melle, Belgium	28°C	G_2M_{11}
Bacillus sp.	R-39623	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown
Bacillus sp.	R-39624	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown
Bacillus sp.	R-39625	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown
B. thuringiensis	LMG 12265	unknown	28°C	n/a
B. vietnamensis	LMG 24742^{T}	Vietnamese fish sauce, Japan	28°C	n/a
B. vireti	LMG 21834^{T}	soil, hay field, Drentse A grasslands, The Netherlands	28°C	n/a
Paenibacillus sp.	R-27048	activated sludge, Belgium	28°C	n/a
Paenibacillus uliginis	LMG 24790^{T}	fen peat soil, nitrogen fertilization long-term experiment, Paulinenaue, Germany	28°C	n/a
Staphylococcus sp.	R-34181	luvisol soil, Melle, Belgium	28°C	G_2M_{11}

[a] from Heylen (2007) and Verbaendert *et al.* (2011b)
[b] Genus name *Bacillus* is indicated with *B.*
[c] Tentative species designation on the basis of 16S rRNA gene sequence similarity analysis as described in Verbaendert *et al.* (2011b)
n/a not applicable

4.2.2 Primer design

Novel primers for the *cbaA* gene (encoding qCu_ANOR) that are used in this study are shown in Table 4. 3. Nucleotide sequences of type I and type II *cbaA* of *Bacillus azotoformans* LMG 9581^T and *Bacillus bataviensis* LMG 21833^T (Heylen & Keltjens, 2012) were used as query sequences in nBLAST and pBLAST to retrieve homologous sequences from annotated whole genome sequencing projects from the EMBL sequence database. Cut-off for query coverage of homologous genes was set at > 50% and at 1e-3 for e-values. Only sequences from Gram-positive strains were retained. Genes that did not have definite annotation in published genomes were examined by comparison of conserved positions using PSI-BLAST (Altschul *et al.*, 1997) to confirm gene identity. DNA-based alignments were made using MEGA version 6.0 (Tamura *et al.*, 2013) under amino acid settings. Localization of conserved motifs binding the Fe_B catalytic center of the protein and the asparagine (N) residue distinguishing qCu_ANOR from other heme copper oxidases (HCO) allowed for identification of potential priming sites, i.e. regions that showed distinct dissimilarity between qCu_ANOR and other related HCO (Fig 4. 1). Due to the high nucleotide sequence divergence of *cbaA*, degenerate *cbaA* type I and type II primers were designed specifically for *Bacillus* (Fig 4. 1). Primers for *Geobacillus nirK*, *qnorB* and *nosZ* (Verbaendert *et al.*, 2014) were modified to better accommodate sequence divergence of the target regions in sequences of different *Bacillus* whole genome sequences (WGS) () (Table 4. 2) and resulted in primers set given in Table 4. 3.

Table 4. 2 *Bacillus* strains of which WGS data for *nirK, qnorB* and *nosZ* genes was used for primer design.
Accession numbers of *nirK*, *qnorB* and *nosZ* genes can be found in the respective phylogenetic trees.

nirK	*qnorB*	*nosZ*
Bacillus azotoformans LMG 9581^T	*Bacillus azotoformans* LMG 9581^T (2 copies)	*Bacillus azotoformans* LMG 9581^T (3 copies)
Bacillus bataviensis LMG 21833^T	*Bacillus* sp. 1NLA3E (2 copies)	*Bacillus* sp. 17376
Bacillus sp. ZYK	*Bacillus* sp. ZYK	*Bacillus* sp. ZYK
Bacillus sp. 2_A_57_CT2	*Bacillus sonorensis* KCTC 13918	
Bacillus oceanisediminis str. 2961	*Bacillus coagulans* XZL4	
Bacillus firmus DS1	*Bacillus acidiproducens* DSM 23148	
Bacillus sp. 10403023	*Bacillus licheniformis* ATCC 14580	
	Bacillus licheniformis WX-02	

4.2.3 Genomic DNA preparation and PCR analyses

Total genomic DNA from pure cultures was extracted according to the guanidium-thiocyanate-EDTA-sarkosyl method (Pitcher *et al.*, 1989), which was adapted for Gram-positive bacteria with an additional lysozyme step, or with alkaline lysis. For the latter, a small amount of cells was lysed in 20µl alkaline lysis buffer (0.25% (w/v) SDS and 0.05M NaOH) for 15 min at 95°C. Subsequently, 180µl MilliQ-water was added and lysates were aliquoted and stored at -20°C before use. All PCRs were performed with a Veriti 96-well Thermocycler (Applied Biosystems).

This study focused on *nirK*-type and *qnorB*-type denitrification gene variants since the copper-containing type nitrite reductase and the quinol-type nitric oxide reductase seem to be the most prevalent in Gram-positive bacteria (Heylen & Keltjens, 2012). Reported *nirK*, *qnorB, nosZ* and *nrfA* PCR set-ups and programs were performed as described in literature (Braker *et al.*, 1998, Hallin & Lindgren, 1999, Kloos *et al.*, 2001, Braker & Tiedje, 2003, Mohan *et al.*, 2004, Thröback *et al.*, 2004, Jones *et al.*, 2011, Verbaendert *et al.*, 2014, Welsh *et al.*, 2014). *CbaA* and *Bacillus*-specific *nirK, qnorB* and *nosZ* PCR set-ups and programs were established (Table 4. 4). PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels followed by 30 min of staining with ethidium bromide. Bands were visualized by UV excitation.

Table 4. 3 Selected primers for amplification of *nirK*, *qnorB*, *cbaA*, *nosZ* and *nrfA* genes.

Enzyme	Gene	Primer name	Sequence 5'-3'[a]	Degeneracy	Reference
CuNiR	*nirK*	nirK1F	GGM ATG GTK CCS TGG CA	8	(Braker *et al.*, 1998)
		nirK5R	GCC TCG ATC AGR TTR TGG	4	
		F1aCu	ATC ATG GTS CTG CCG CG	2	(Hallin & Lindgren, 1999)
		R3Cu	GCC TCG ATC AGR TTG TGG TT	2	
		GnirK2F	GGK GTV TTT ATG TAC CAT TGC	6	(Verbaendert *et al.*, 2014)
		GnirK2R	SCC GCT YGC TYG CCG GAA GCA TCA C	6	
		BnirK5F	GGN GTN TTY ATG TAT CAY TGY	128	This study
		BnirK6R	RCC GCT BGC MGG DAR CAT DAC	144	
		BnirK5Fb	GGN GTN TTY ATG TAT CAY TG	64	
		BnirK6Rb	RCT BGC HGG DAR CAT DAC	324	
		BnirK6F	GGN GTN TTY ATG TAT CA	32	
		BnirK8R	RCC RCT TGC MGG RAG CAT	16	
		BnirK10R	GCC GCT BGC MGG TAA CAT	6	
qNOR	*qnorB*	qnorB2F	GGN CAY CAR GGN TAY GA	64	(Braker & Tiedje, 2003)
		qnorB7R	GGN GGR TTD ATC ADG AAN CC	288	
		GqnorB4F	GGC CAY CA AGG YTG GGA	4	(Verbaendert *et al.*, 2014)
		GqnorB6R	GGC AAR TTG ATW ARR AAV CC	48	
		BqnorB7F	GGA CAY CAR GGY TDK GA	48	This study
		BqnorB6R	GGK ARR TTG ATG AAR AAK CC	32	
		BqnorB9F	GGC CAY CAR GGA ATG GA	4	
		BqnorB8R	GGC AAG TTG ATC CAA AAA CC	0	
qCu_ANOR	*cbaA*	$cbaA_{t1}F3$	TCW GTY ATG TAY WCS TTY TAT C	64	This study
		$cbaA_{t1}F4$	TCW GTT ATG TAT ACS TTY TAT C	8	
		$cbaA_{t1}R3$	CCR CCD GTR ATR TTC AT	24	
		$cbaA_{t1}R4$	CCR CCR GTA ATR TTC AT	8	
		$cbaAt_2F1$	GSS TGG ACD TTY YTW TA	96	
		cbaA t_2F2	GMS TGG ACD TTC YTW TA	48	
		cbaA t_2R1	CGK ATA RAT MAT YAW KGT	64	
		cbaA t_2R2	CGT ATA MAT CAT YAA GGT	4	
N_2OR	*nosZ*	nosZF	CGY TGT TCM TCG ACA GCC AG	4	(Kloos *et al.*, 2001, Thröback *et al.*, 2004)
		nosZ1622R	CGC RAS GGC AAS AAG GTS CG	8	
		nosZ-F	TGG GGN GAY NTB CAY CA	192	(Jones *et al.*, 2011)
		nosZ-R	GAR CAR AAG TTI GTR CAR TA	64	
		GnosZF	TGG GGA GAT TTG CAT CA	0	(Verbaendert *et al.*, 2014)
		GnosZR	GAA CAG AAG TTC GTG CAA TA	0	
		BnosZ2F	TGG GGD GAY BTB CAY CA	108	This study
		BnosZ3F	TGG GGD GAC BTB CAC CA	27	
		BnosZ4F	TGG GGD GAC BTS CAC CA	18	
		BnosZ1R	GAR CAG AAG TTT GTR CAR TA	8	
NrfA	*nrfA*	nrfAF1	GCN TGY TGG WSN TGY AA	128	(Mohan *et al.*, 2004)
		nrfA7R1	TWN GGC ATR TGR CAR TC	64	
		nrfAF2aw	CAR TGY CAY GTB GAR TA	48	(Mohan *et al.*, 2004, Welsh *et al.*, 2014)

[a] N = A, C, G, T; Y = C or T; R = A or G; D = G, A or T

```
BAZO_06399   MQPTSSTANM SKPSFKERSN QML--GAIPQ DAIITKSYLF VAFTAVLLGG 48
BABA_00225   ------MQTA KSRDFKDKAN KIM--GISKE DALLTKSYIF VAFMAILLGG 42
BAVI_00935   ------MAIP KT--LKEKAN KEM--GISTE DARITKTYLF VAFMALLIGG 40
Baoc         -VETVIKQTG KTQAFKDKAN KVM--GISLE DAKITKSYLS VAFLAILLGG 47
Basp         -VETVIKQTG KTQALKDKAN KVM--GISLE DAKITKSYLS VAFLAILLGG 47
Bati         ---LATILNN SDLTLKEKTN KVL--GVNPQ DAKISKSYLS VAFIALLLGG 45   type I cbaA
BAZO_04705   ---------- ---------M RPITDTVNSS TRKSVVFPTL LGSVLILLMM 31   type II cbaA
Bafi         ---------- ---------- --MNEAIHPL ARKAVIWHLA VTALVIILMM 28
Baoc         ---------- ---------- --MNEAIHPL ARKTVVWHLA VTSLVLILMM 28
Alha         ---------- ---------- -----MVNAL DARLSMYHFY TAFAALLIGG 25   HCO
Anf          ---------- ------MMNG ---TWKVDAR DGKLAMAHIY VAFVALALGG 31
Ank          ---------- ------MMNG ---TWKVDAR DGKLAMAHIY VAFVALALGG 31
Brbo         ---------- -----MN-HT TIGQAQVDRP TARLSLAYVW VAYAAFGLAA 34
Brbr         ---------- MVIARKN-DD TLPHVPVQRS AATLSLAYVW VAYIAFGLAA 39
Ct           ---------- ------MTVK VKAGTEVHPK DAKLSMAYIY VAFAAFLTGA 34
GTNG_1394    ---------- ------MVQP ---LEKVDRR DAKLALAHLF VAFIALGLGG 31
GTNG_1518    ---------- ------MVQP ---LEKVDRR DAKLALAHLF VAFIALGLGG 31
Pd           DLSAESTGTP AKPADTLTQK AGLHMPYPPK DAAIVLSHII FAFAALLLGG 50
Pe           ---------- ------MVQN --LKS-FDRR DSALVLAHIL FAFFALFLGG 31
Vh           --------MG LEKIKKISNN EILPLTISKQ ESRLYMSFIY VAIISLLIGG 42
```

forward region 1 (*) — **forward region 2** (* ● *)

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BAZO_06399   LLGLLQGLNR AGLLQQMPLG LNYYQILTAH GLLLVVIFSA TFVIGYFYAG 98
BABA_00225   ILGLVQGLNR AGLL-ELPSW VNYYQVLTAH GLLLVVVLTA FFTIGYFYAG 91
BAVI_00935   LLGLLQGLNR AGLL-ELPSW LNYYQVLTAH GLLLVVVLTA FFTIGYFYAG 89
Baoc         ILGLLQGLNR AGML-ELPAW LNYYQVLTAH GLLLVVVLSA FFTIGYFYAG 96
Basp         ILGLLQGLNR AGML-ELPAW LNYYQVLTAH GLLLVVVLSA FFTIGYFYAG 96
Bati         LFGLFQGLNR AGLL-ELPSW LNYYQVLTAH GLLLVVVFSA FITIGYFYAG 94   type I cbaA
BAZO_04705   VVGLIMLLNQ GKLI-TISDG F-FYKIMTLH GTGM-IGAGA LAGTAIMYYF 78   type II cbaA
Bafi         IFGVIMLMNQ GEMI-SITPQ W-FYKIMTAH GTGM-VGIAA LGGTAILWYF 75
Baoc         IFGVIMLMNQ GEMI-SITPQ W-FYKIMTAH GTGM-VGIAA LGGTAILWYF 75
Alha         FFGLLQTLVR SEQL-TLPVD LNYYQVLTAH GVLLALVFTT FFIFGFMYAG 74   HCO
Anf          LAGLLQVLVR SGKF-ELPAG ISYYTILTTH GVLLGLVLTT FFIIGFQFAA 80
Ank          LAGLLQVLVR SGKF-ELPAG ISYYTILTTH GVLLGLVLTT FFIIGFQFAA 80
Brbo         LAGMLQGMVR GGLV-ELPSW TNYYQILTAH GVLMALIFTT YFIIGFLLSG 83
Brbr         VAGMVQGMVR GGIM-ELPSW TNYYQILTAH GVLMALIFTT YFIVGFIFSG 88
Ct           VAGVVQGLQR AGLI-DLPAG INYYQVLTAH GVLLALVFTT YFIFGFLTAG 83
GTNG_1394    FAGLLQTLVR SGKF-ELPGG ISYYTILTTH GVLLGLVLTT FFIIGFQFAA 80
GTNG_1518    FAGLLQTLVR SGKF-ELPGG ISYYTILTTH GVLLGLVLTT FFIIGFQFAA 80
Pd           VAGLLQGLVR GGMV-ELPAG IGYYQLLTAH GVLMALVFTT FFIIGFLYSG 99
Pe           IAGVMQGLVR GGML-ALPMG IGYYQLLTAH GVLMALIFTT YFIIGFLYAG 80
Vh           LMGLLQTLVR SNTI-TLPWG IGYYQILTLH GVVLALVLTT YFIIGFQFAL 91
```

forward region 3 (* *)

```
BAZO_06399   MSHTLG-GLL PKVRKIVWTG FWLKIVGTVV VVIPVLMNEA SVMYSFYPPM 157
BABA_00225   MSHTLG-GLL PKVRTMAWIG FGLKMVGFVL AVIPIIMGDA SVMYTFYPPM 140
BAVI_00935   LSHTLG-GLL PKVRKMAWIG FGLKIVGFVF AVIPILMGNA SVMYTFYPPM 138
Baoc         LSHTLG-GLL PKVRKMAWIG FWMKIFGFVL AVIPILMNEA SVMYTFYPPM 145
Basp         LSHTLG-GLL PKVRKMAWIG FWMKIFGFVL AVIPILMNEA SVMYTFYPPM 145
Bati         LSHDLG-GLL PKVRKMAWIG FWTKIVGIVL VVIPVLMNKA SVMYTFYPPM 143   type I cbaA
BAZO_04705   TSQYIK--LS KA---IFVSN IVLSILGVVM VLIGIFVFDF AAAWTFLYPL 122   type II cbaA
Bafi         LSKYVK--LN PA---ILIVN FVLFLSGVTM VLISIFVFDF SDGWTFLYPL 120
Baoc         LSKYVK--LN PS---ILIVN FVLFLTGVSM VLIAIFVFDF SDGWTFLYPL 120
Alha         VVKTTG-EFA SHRRKAAWIG FVLMLIGTIM ATTLILMNKA TVLYTFYAPL 123   HCO
Anf          VSRTAG-TLS DRTRFWGWVG FWLMTIGTAI TAFYILIGEA SVLYTFYAPL 129
Ank          VSRTAG-TLS DRVRFWGWIG FWLMTIGTAI TAFYILIGEA SVLYTFYAPL 129
Brbo         VAKTTGGSLH GTARGLGWTG WVLMTVGTAM AVVTIVTNDA SVLYTFYAPL 133
Brbr         VARTTGGSLH GAALKFGWAG WILMTIGTLM AVVTILLNEA SVLYTFYAPL 138
Ct           MSKTLG-AFP EKYRQAGWIG FYVLTLGTLL TAIMILLNEG TVLYTFYAPL 132
GTNG_1394    VSRTAG-TFT DSTRRVGWIG FWLMTIGTAM SAFFILTGQA AVLYTFYAPL 129
GTNG_1518    VSRTAG-TFT DSTRRVGWIG FWLMTIGTAM SAFFILTGQA AVLYTFYAPL 129
Pd           IANTLGGKLL PATRRFAWFG FVLMASGTVL GTIMILLNKA TVLYTFYAPM 149
Pe           VARTLGGRLL PIARNLGWLG FGLMTLGTAI ATVEILLNRA TVLYTFYAPL 130
Vh           MGKTVG--IS LKQRKVAWLS FWVMIVGTVM AAITILMGKA SVLYTFYAPL 139
```

```
BAZO_06399   KAHPM-FY-- FGLVFVVLGV WAAAIGAFIQ VAS------- ---------- 189
BABA_00225   AAAPM-FY-- IGLALIVVGV WMCAFGAFIQ VAN------- ---------- 169
BAVI_00935   AASPW-FY-- IGLALIVVGV WMCAFGAFIQ VAN------- ---------- 168
Baoc         AASPI-FY-- IGLVFIVLGV WMCAFGAFIN VAS------- ---------- 165
Basp         AASPI-FY-- IGLVFIVLGV WMCAFGAFIN VAS------- ---------- 165
Bati         AAHPV-FY-- IGLALVVVGV WILAAGAFMQ VAN------- ---------- 173   type I cbaA
BAZO_04705   PAISGGMWGA AGAVFYLGGM TVLGTGFLLF YLDTGRAIIK KYGNLGNALG 172   type II cbaA
Bafi         PAQSAKMYGA AGAVFFLSGM LILGIGFLLL YMYLAARLTA VYGGLGKALG 170
Baoc         PAQSAKMYGA AGAVFFLSGM LVLGFGFLIL YMYLAARLTA VYGGLGKALG 170
Alha         QASPF-FY-- IGLAFIIIGT YFAAYAIISQ YRS------- ---------- 153   HCO
Anf          QAHAG-FY-- IGLTLVVVGS WVSGFAMFAH YAK------- ---------- 159
Ank          QAHAG-FY-- IGLTLVVVGS WISGFAMFAH YAK------- ---------- 159
Brbo         KASPY-FY-- IGAALLVVGS WFGGFAIFSS YRK------- ---------- 163
Brbr         KASPY-FY-- IGSALLVVGS WLAGFGVFAS YRK------- ---------- 168
Ct           QAHPL-FY-- LGLTLFVVGT WIAGGALLAH YFN------- ---------- 162
GTNG_1394    QAHAG-FY-- IGLALVVVGS WVSGFAMFAH YAR------- ---------- 159
GTNG_1518    QAHAG-FY-- IGLALVVVGS WVSGFAMFAH YAR------- ---------- 159
Pd           QASVL-FY-- IALVLLVVGS WSSGFGMIYQ YRY------- ---------- 179
Pe           KASPA-FY-- IALVFVVVGS WMSGFGIFIN YRY------- ---------- 160
Vh           KAHPV-FY-- IGLALVIVGS WIAGFVNWHQ LFV------- ---------- 169
```

forward region 4

```
BAZO_06399   WRKNNPGKH- -VPIFSFFAT GVFILLVGAT LMVAVEVLFM IIPWSLGWVD 247
BABA_00225   WRKNHKGQH- -VPILSYFAT GVFVLLFFGS LPVAIEVIVM IIPWSFGWVK 217
BAVI_00935   WRKNNKGKH- -VPILSFFAT GVFILLFFGS LPVAIEVIVM IIPWSFGWVK 216
Baoc         WRKRNPGQH- -IPILSFFAT GVFVLLFFGS IPVAIEVFT- IIPWAFGWVE 118
Basp         WRKRNPGQH- -IPILSFFAT GVFVLLFFGS IPVAIEVFT- IIPWAFGWVE 118
Bati         WRKNHKGQH- -LPILSFFAT GVFILLVGAT AFVAVEVLFL ILPWTLGWVD 220   type I cbaA
BAZO_04705   WPIIFGKTMK EELPPAIVAG TMVTIVNTAA LVSGASVLIM SIINIFNPSF 222   type II cbaA
Bafi         WDIIFRGKKG YGPPAAVVAT AMVIITNSTA LLAGATVLAA SLVNIINPAI 220
Baoc         WDIIFRGKKG YGPPAAVVAT AMVIITNSTA LLAGATVLAA SLVNIINPAV 220
Alha         WRKEHKGEK- -SPLFTFMAV ATLVLWLIAT IGVVTAVTLQ YIPWSFGWTE 201   HCO
Anf          WKKAHPGQV- -SPLLTFMSV VNMVLWLVCS LGVAATVLFQ LIPWSLGYVD 207
Ank          WKKAHPGQV- -SPLLTFMSV VNMVLWLVCS LGVAATVLFQ LIPWSLGYVD 207
Brbo         WRRENRGKL- -SPLFVYMSV TTFVLWLIAT VPVAVEVLFQ LIPWSLGWTP 211
Brbr         WKRENKGKM- -SPLFVFMGV TTYVLWITAT IPVAVEVLFQ LIPWSLGLVP 216
Ct           WKRENKGQL- -TPLFGFMTV ATILLWYIAT IGVAATVLLQ LLPWSLGISE 210
GTNG_1394    WRKAHRGQA- -SPLLTFMSV TNMALWLICT LGVAATVVFQ LIPWSLGLSE 207
GTNG_1518    WRKAHRGQA- -SPLLTFMSV TNMALWLICT LGVAATVVFQ LIPWSLGLSE 227
Pd           WRRKHQGQL- -SPLFAFMSI VTMLMWLIAT IGVAATVILQ FIPWSLGWVD 207
Pe           WKKTHRGET- -TPIFTYMAV VTMLMWIIAT LGVAIEVLFQ LIPWSLGWVD 208
Vh           WKKKHPGEK- -SPLLAFMVA INMAMWFIAS LGVAVAVLVQ FIPWSLGYAE 217
```

forward region 5 (* **●) — **reverse region 1** (*)

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BAZO_06399   TINVMVARTL FWAFGHTAVN IWYLTAVSAW YVIVPKIIGG RRFSDMLTRV 297
BABA_00225   TINVMVARTL FWAFGHTLVN IWYLTAVSAW YVIVPKIISG KRWSDTLTRV 267
BAVI_00935   TINVMVARTL FWAFGHTLVN IWYLTAVSAW YVIVPKIIGG KRWSDTLTRI 266
Baoc         TINVMVARTL FWAFGHTLVN IWYLTAVSAW YVIVPKIIGG RRWNDTLTRI 168
Basp         TINVMVARTL FWAFGHTLVN IWYLTAVSAW YVIVPKIIGG RRWNDTLTRI 168
Bati         TINVLVSRTL FWAFGHTAVN IWYLTAVSAW YVIIPKIIGG KRWSDTLTRV 270   type I cbaA
BAZO_04705   TMDPLLSKNL IYAFGHIFAN SIIYMGVIAV YEIFPKYTGR PWKVYGNFLI 272   type II cbaA
Bafi         TIDPLLAKHL TYAFGHIFAN CTIYMAVIAV YEVLSEYTKR PWKANKVFLI 270
Baoc         TMDPLLAKHL TYAFGHIFAN CTIYMAVIAV YEVLSEYTKR PWKANKVFLI 270
Alha         TINVALSRTL FWFFGHPLVY FWLLPAYMAW YVTVPKIIGG KIFSDSLARL 251   HCO
Anf          RINVLVSRTL FWYFGHPLVY FWLLPAYMIW YAIIPKIIGG KMFSDSLARM 257
Ank          RINVLVSRTL FWYFGHPLVY FWLLPAYMIW YAIIPKIIGG KMFSDSLARM 257
Brbo         SINVMLSRTL FWFFGHPLVY FWLMPAYIAW YVCVPRVIGG HVFSDSLSRL 261
Brbr         TINVMLSRTL FWFFGHPLVY FWLLPAYMAW YCLVPRIIGG HVFSDSLSRL 266
Ct           RINVLLSRTL FWYFGHPLVY FWIMPAYMAW YVVIPKIIGG KLFSDALARL 260
GTNG_1394    RVNVLLSRTL FWYFGHPLVY FWLLPAYMVW YAVIPKVIGG KMFSDSLARL 267
GTNG_1518    RVNVLLSRTL FWYFGHPLVY FWLLPAYMVW YAVIPKVIGG KMFSDSLARL 277
Pd           TINVMISRTL FWYFGHPLVY FWLLPAYICW YVVIPKIIGG KIFSDALARL 257
Pe           TVNVVLSRTL FWYFGHPLVY FWLLPAYMCW YVVIPKIIGG KMFSDALARL 258
Vh           TINVLVSRTL FWYFGHPLVY FWLLPAYMAW YAIIPKIIGG KIFSDSLARL 267
```

Fig. 4.1

```
                reverse regions 2 and 3      reverse region 4
                          ••                    *
BAZO_06399   VIIALVIMNI TGGFHHQIID PGISEPIKFM HVFMSLAIGF PSLMTAYAMF 347
BABA_00225   VIIALVVMNI TGGFHHQIVD PGISPSVKFM HVFMSLAIGF PSLMTAWAMF 317
BAVI_00935   VIIALVIMNI TGGFHHQIID PGISESVKFM HVFMSLAIGF PSLMTAWAMF 316
Baoc         VIIALVIMNI TGGFHHQIID PGISEPVKYM HVFMSLAIGF PSLMTAYAMF 218
Basp         VIIALVIMNI TGGFHHQIID PGISEPVKYM HVFMSLAIGF PSLMTAYAMF 218
Bati         VIIALVIMNI TGGFHHQIID PGILPAVKFL HVFMSLAIGF PSLMTAWAMF 320     type I cbaA
BAZO_04705   AWNASTLFTM IIYPHHLLMD FAVPKWMLIL GQVLSYANGL PVLVVT--AF 320     type II cbaA
Bafi         AWNCSTLFTL MIYTHHLLMD FAVPKWMLII GQVFSYANGL PVMVVT--AY 318
Baoc         AWNCSTLFTL MIYTHHLLMD FAVPKWMLII GQVFSYANGL PVMVVT--AY 318
Alh          AFLLFILFSI PVGFHHQLLE PGISEVWKYV QVVLTFAVII PSLMTAFAMF 301     HCO
Anf          SFILFLIFSI PVGFHHQLVE PGIDPAWKYL QVVLTFMVVI PSLMTAFSMF 307
Ank          SFILFLIFSI PVGFHHQLVE PGIDPAWKYL QVVLTFMVVI PSLMTAFSMF 307
Brbo         AFISLLLFSM PVGFHHQLME PGISAGWKMI HVILTLIVVV PSLMTAFSMF 311
Brbr         AFLSLLLFSI PVGFHHQLME PGISPAWKMI HVTLTLIVVI PSLMTAFSMF 316
Ct           AFVLFLLFSI PVGFHHQLME PGISPFWKYI QTVLTFAVVV PSLMTAFTIF 310
GTNG_1394    AFILFLLFSI PVGFHHQLLE PGISPFWKYV QVVLTFMVII PSLMTAFSMF 317
GTNG_1518    AFILFLLFSI PVGFHHQLLE PGISPFWKYV QVVLTFMVII PSLMTAFSMF 327
Pd           SFILLLLFSI PVGFHHQLME PGISPLWKYI QVVLTFMVVI PSLMTAFSLF 307
Pe           SFIMFLLFSI PVGFHHQLME PGISNVWKFV QVVLTFMVVI PSLMTAFSLF 308
Vh           SFILFLFFSI PVGFHHQLTE PGIDPVWKFI QVVLTFMVII PSLMTAFSMF 317
               <------
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                                   * *          *
BAZO_06399   AVMERTGREK GGKGAFGWFK KLPWGDVRFL APFIGMAAFA PAGAGGIVQS 397
BABA_00225   AVFERTARKQ GGKGLIGWYK KMPWGDVRFL APMIAMIAFI PAGAGGIAQS 367
BAVI_00935   AVFERTARKN GGKGIVGWYK KMPWGDVRFL APFIAMLAFI PAGAGGIVQS 366
Baoc         AVFERTARKK GGRGLVGWYK KLPWGDVRFL APFIAMAAFI PAGAGGIVQS 268
Basp         AVFERTARKK GGRGLVGWYK KLPWGDVRFL APFIAMAAFI PAGAGGIVQS 268
Bati         AVFERTGRKK GGKGAFGWFK KLPWGDVRFL APFIAMAAFI PAGAGGIAQS 370     type I cbaA
BAZO_04705   GALQIIFRSG ---------- -IKW-DMPST LFFMSMFGWV MGVVPAIIDA 359     type II cbaA
Bafi         GALMIVYRSR ---------- -MKW-DFAST LFFLSMFGWV AGAIPAIIDA 357
Baoc         GALMIVYRSR ---------- -MKW-DFTST LFFLSMFGWV AGAIPAIIDA 357
Alh          ASFEIAGREK GATGLLGWFK KLPWSDVRFL APFIGMAFFI PAGAGGIINA 351     HCO
Anf          ATFEMYGRSK GATGLFGWLR KLPWGDARFF APFIGMLFFI PAGAGGLVNA 357
Ank          ATFEMYGRSK GATGLFGWLR KLPWGDARFF APFVGMLFFI PAGAGGLVNA 357
Brbo         ATFETTGRKK GGVGLFGWFK KLPWSDVRFF APFVGMLFFI PAGAGGIINA 361
BrBr         AVFETTGRKK GGVGLFGWLK KLPWTDVRFF APFVGMLFFI PGGAGGIINA 366
Ct           ATFELAGRAK GASGLFGWWK KLPWKDVRFF APFIAMVMFI PAGAGGIINA 360
GTNG_1394    ATFESYGRSQ GAKGLFGWLR KLPWGDARFF APFVGMLFFI PAGTGGIINA 367
GTNG_1518    ATFESYGRSQ GAKGLFGWLR KLPWGDARFF APFVGMLFFI PAGTGGIINA 377
Pd           ATFEISGREK GAKGLFGWLK YLPWKDVRFF APFMGMLIFI PAGAGGLINA 357
Pe           ASFEMHGRSV GAKGLFGWLK KLPWKDVRFF APFMGMLIFI PAGAGGLINA 358
Vh           ATFEITGRRK GFGGLFGWLK HLPWKDVRFL APFIGMLAFI PGGAGGIINA 367
```

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                        reverse region 5     reverse region 6
                     ** *   *•*•                   *
BAZO_06399   TNQLDQTVHN TMWIVGHFHL TLGMSVVMTF FGVSYWLIPH VSKRILTPAM 447
BABA_00225   TNQLDQVVHN TMWIVGHFHL TLGMSVVMTF FGISYWLVPY ISKRVLTPQI 417
BAVI_00935   TNQLNQVVHN TMWIAGHFHL TLGMSVVMTF FGISYWLVPY VSKRVLTPAI 416
Baoc         TNQLNQVVHN TMWIVGHFHL TLGMSVVMTF FGISYWLIPY VSKRMLTPQI 318
Basp         TNQLNQVVHN TMWIVGHFHL TLGMSVVMTF FGISYWLIPY VSKRMLTPQI 318
Bati         TNQLDQVVHN TMWIVGHFHL TLGMSVVMTF FGISYWLVPH ISGRVLTKGM 420     type I cbaA
BAZO_04705   TIAINHVMHN TKWVPGHFHM YMGIGAVAMI FGFMYYVLK- VEGLKMSSYL 408     type II cbaA
Bafi         TIVVNHVMHN TKWVPGHFHM YMGMGVVAMI IGFMYYVNK- TEGNQQHRGI 406
Baoc         TIVVNHVMHN TKWVPGHFHM YMGMGVVAMI IGFMYYVNK- TEGNQEHRGI 406
Alh          SHQMNQVVHN TIWVTGHFHI TIGATVILTF FGISYWLIPH LKGRVLTKHI 401     HCO
Anf          SHQLNQVVHN TIWVTGHFHL TLATTVVLTF FGAAYWLIPH LTGRVMTKAM 407
Ank          SHQLNQVVHN TIWVTGHFHL TLATTVVLTF FGGAYWLIPH LTGRVMTKAM 407
Brbo         SNQMNAVVHN TIIVTGHFHI TVGAAVALTF FGVSYWLIPV LTGRTLTRTA 411
Brbr         SNQINAVVHN TIWVTGHFHI TVGAAVALTF FGVSFWLIPA LTGRQLTKTA 416
Ct           SHQMNAVVHN TLWVTGHFHL TVGTTVLLTF FGISYWLIPV LTGRRLTPAI 410
GTNG_1394    SHQLNQVVHN TIWVTGHFHL TVATTVVLTF FGASYWLIPH LTGRVLTKAM 417
GTNG_1518    SHQLNQVVHN TIWVTGHFHL TVATTVVLTF FGASYWLIPH LTGRVLTKAM 427
Pd           SHQMNGVIHN TLWVTGHFHL TVATSVALTF FGITYWLIPV ITNRVLTPAM 407
Pe           SHELNAIVHN TLWVTGHFHL TVATSVALTF FGITYWLIPA MLGREMTPLM 408
Vh           SHQMNAVVHN TIWVTGHFHL TVATTVILTF FGISYWLIPH LTGRKLTPQL 417
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                         *          *     ***               *
BAZO_06399   NKLGVFQTIL WTIGMVIMSF SMHYIGLYGG SPRRTSYTTY FDHPMALSWD 497
BABA_00225   NRLGVIQTWI WTIGMIFMSG AMHAVGLLG- SPRRTSFTTY GDNATALSWN 496
BAVI_00935   NKLGVIQTII WTVGMVIMSG AMHTVGLLG- SPRRTSYTTY GDNATALGWN 466
Baoc         NKLGVIQTII WTLGMIIMSG AMHWVGLLG- SPRRTSYSTY FDNATALSWD 465
Basp         NKLGVIQTII WTLGMIIMSG AMHWVGLLG- SPRRTSYSTY FDNATALSWD 347
Bati         NKLGVVQTII WTIGMVLMSG SMHTVGLLG- SPRRTSFSDY FGSAQAAGWE 347     type I cbaA
BAZO_04705   DKLSFWVYLL FFAG---LTF SFLYSGKLS- APRR--WSQH FP-----EWV 459     type II cbaA
Bafi         DTFTIAVYFA FFTG---LVG SFLYAGGIS- APRR--WAEH LP-----QWV 447
Baoc         DTFTIAVYFV FFMG---LVG SFLYAGGIS- APRR--WSEH LP-----QWV 447
Alh          NNLGKYQIVL WTVGMFIMSA AMHFSGLSG- NPRRTQITSY NGHEAAASW- 450     HCO
Anf          NRLAIIQTIV WSIGMIFMSS AMHFAGLLG- APRRSAFSTY GDAPQALEWI 456
Ank          NRLAIIQTIV WSIGMIFMSS AMHFAGLLG- APRRSAFSTY GDAPQALEWI 456
Brbo         NRMGMVQTVF WSVGMFLMSF AMHYLGLQG- TPRRTDYTTY MDHPLALGWM 460
Brbr         NRMGMVQTVF WAVGMFLMSL AMHAMGLQG- APRRTDYTTY MDHPLALGWM 465
Ct           NRLGIIQSVL WVVGMFFMSG AMHVLGLLG- APRRTAFTTY QDHPTALAWL 459
GTNG_1394    NRLAIIQTIV WAVGMTFMSG SMHFAGLLG- APRRSAFSTY GNSPQALEWI 466
GTNG_1518    NRLAIIQTIV WAVGMTFMSG SMHFAGLLG- APRRSAFSTY GNSPQALEWI 476
Pd           NRLGIIQTVI WLVGMFFMSG SMHTAGLFG- SPRRTAYTTY NDHPDAVLWM 456
Pe           HRMGIIQTIV WMVGMFFMSG AMHTVGLLG- SPRRTAFTTY QDSADAVGWM 457
Vh           NRLGIIQTIV WTVGMTIMSG AMHIQGLLG- GPRRSAFSEY GGTEQASNWI 466
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BAZO_06399   ----PWMLAV GIGAVLLFTA VCIQVFAAFN LMFFAPKGVT EFPIGEPQSD 543
BABA_00225   ----PYLLCL AVGGTLLIIG VLLQVYAVIN MMFFAPKGDT EFPIAEEEEN 542
BAVI_00935   ----PYLLLL AVGGTLLIIG VLLQVYAVIN MMFFAPKGET EFPIAEEEEG 512
Baoc         ----PYLFFL AIGGTLLMIG VLMQVYAVFY LMFFAPKGNT EFPVAEVEED 511
Basp         ----PYLFFL AIGGTLLMIG VLMQVYAVFY LMFFAPKGNT EFPVAEVEED 393
Bati         ----PFMMVV GIGATLLLIA VIIQVYAVFN MMFFAPKGLT EFPVAEVEEN 393     type I cbaA
BAZO_04705   ----PADQLG AVAAIGIIFA TSVFIIRFAV SAKQIGTQKD EKETKEKLYA 496     type II cbaA
Bafi         ----LSDQVG AVSGIFIVLA AIIFTARFFA GLRSVGTNVP -YNKNS--AA 490
Baoc         ----LSDQVG AVSGIFIVLA AIIFTARFFS GLRCVGSQVP -YQKKS--AA 490
Alh          -G--SYEVAM SVGGTLLLFS IFLFIGLMIN LAFWAPKGSE EFPIGEEFED 506     HCO
Anf          ----PYQIAQ AVGGTILFIG IILVLIIVTN LAFFAPKGET EFPVAEVAEQ 452
Ank          ----PYQIAQ AVGGTILFIG IILVLIIVTN LAFFAPKGET EFPVAEVAEQ 452
Brbo         ----DYQRVM AFGGGLLFVA AVLLILILGY LTFYAPKGKT EYPIAET-EA 505
Brbr         ----DYQRVM AFGGGLLFVS AILLIAILLY LTFYAPKGNT EYPIAET-ET 510
Ct           EGVFSSQVTM SIGGVILFIA ALLMIYNVIN LCFLAPKEHT EFPIGEVAED 509
GTNG_1394    ----PYQIAQ AVGGTILFIG IILMLVIVIN LAFFAPKGET EFPVAEAATP 512
GTNG_1518    ----PYQIAQ AVGGTILFIG IILMLVIVIN LAFFAPKGET EFPVAEAATP 522
Pd           ----PYHVAM AIGGTILFVA VILMVYNIFA M-MRMPKGDT PYPIGEVSDQ 502
Pe           ----PYHVAM AIGGTILFIG VVMMIINIVM L-LSAPKGET EFPIAEANEA 501
Vh           ----GYQLAQ AIGGSILFIG IILMLYIFIK LAFFAPKGIE EIPIAEEEED 512
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BAZO_06399   DEKTPYWTER WGIWVVLMII TIAMAYTVPL VEMIVNGPPG SPPFKTW 593
BABA_00225   ATKTPYWTER WGMWIVVMLL VVAMAYVIPL SEFIVNAPPG SPPFKTW 592
BAVI_00935   ASKTPYWTER WGLWIVIMLI FVGMAYVIPL TEFIVNAPPG SPPFKTW 562
Baoc         EAPTPRWTER WGLWIVCMIV LVGMAYVIPL VDFIVNAPPG SPPYKTW 561
Basp         EAPTPRWTER WGLWIVCMIV LVGMAYVIPL VDFIVNAPPG SPPYKTW 443
Bati         ASKTPLWTER WGLWVVLMVL VIAMAYVLPL TDMIVNAPPG SPPFKTW 443     type I cbaA
BAZO_04705   *--------- ---------- ---------- ---------- ------- 497     type II cbaA
Bafi         G*-------- ---------- ---------- ---------- ------- 492
Baoc         G*-------- ---------- ---------- ---------- ------- 492
Alh          THQLPKLLEN WKVWIAIAIL LILIAYTIPV IDLIQDAPPG SPGYQFW 556     HCO
Anf          AERTPLVFEN WKLWIGIAVV LILIAYTVPF IDMIQNAPPG SKGFKLW 502
Ank          AERTPLVFEN WKLWIGIAVV LILIAYTVPF IDMIQNAPPG SKGFKLW 502
Brbo         EQHTPRILER WPVWVGVLLF LIVMAYGIPI ADQLTHNPPG SPPIRTW 555
Brbr         TQEVPKILER WPVWIGLVAF LIILAYGYPI LEQIQHQAPG SPPFVTW 560
Ct           ASQTPPLLEN WKLWVALAVA LILFAYTIPL MDMIQNAPPG SPPVRPW 559
GTNG_1394    QERVVLVFEN WKLWIGIVVA LILIAYTVPL IDIIQNAPPG SKGYKLW 562
GTNG_1518    QERVVLVFEN WKLWIGIVVA LILIAYTVPL IDIIQNAPPG SKGYKLW 572
Pd           AEPTPKLLEK WWIWIVLVVV LILFAYTIPV LEMLGQSGPG SPPWVTW 552
Pe           AEKAPAIFER WGVWVGVLVV LILVAYSVPV MDIISNASVG SKGFKTW 551
Vh           AEPTPMFLEN WKLWIAITLA LILFAYTIPF VDIIQNSPPG SPPFA-W 561
```

Fig. 4. 1 (continued)

Fig 4. 1 (previous two pages) Multiple alignment of *cbaA* type I and type II encoding qCu_ANOR and other non-nitric oxide heme copper oxidases (HCO). Sequences were retrieved from GenBank and aligned by ClustalW and MEGA 5.0. Denotation residue shading: yellow, conserved asparagine (N) residue distinguishing NOR from other HCO; blue and *, 100% conserved; green, conserved in *cbaA* type I; red, conserved in *cbaA* type II; pink, conserved in HCO; grey; conserved in *cbaA* type I and HCO; light green, residues in *cbaA* type I that differ in comparison to residues at the same position in either or both cbaA type II and HCO; light red; residues in *cbaA* type II that differ in comparison to residues at the same position in either or both *cbaA* type I or HCO; bold, conserved residues in either *cbaA* type I and *cbaA* type II or *cbaA* type II and HCO. Candidate regions for primer design are indicated with black rectangles, black arrows represent the regions in which designed forward and reverse primers are positioned, ● indicates histidines of the histidine sextet characteristic for HCO. BAZO, *Bacillus azotoformans* LMG 9581[T]; BABA, *Bacillus bataviensis* LMG 21833[T]; BAVI, *Bacillus vireti* LMG 21834[T]; Baoc, *Bacillus oceanisediminis* 2691 (WP_019381136 & WP_019382619); Basp, *Bacillus* sp. 2_A_57_CT2 (WP_009335537); Bati, *Bacillus timonensis* 10403023 (WP_010677984); Bafi, *Bacillus firmus* DS1 (EWG08665); Alh, *Alcalibacillus haloalkaliphilus* C5 (WP_017186287); Anf, *Anoxybacillus flavithermus* NBRC 109594 (WP_006318624); Ank, *Anoxybacillus kamchatkensis* G10 (WP_019417709); Brbo, *Brevibacillus borstelensis* AK1 (WP_003389896); Brbr, *Brevibacillus brevis* X23 (WP_017247201); Ct, *Caldalkalibacillus thermarum* TA2.A1 (WP_007502530); GTNG, *Geobacillus thermodenitrificans* NG80-2; Pd, *Paenibacillus daejeonensis* DSM 15491 (WP_020619605); Pe, *Paenibacillus elgii* B69 (WP_010501276); Vh, *Virgibacillus halodenitrificans* 1806 (WP_019378420)

Table 4. 4 Determined time-temperature profiles for *Bacillus nirK*, *cbaA, qnorB* and *nosZ* PCR primers targeting *Bacillus*.

	***cbaA* type I**			***BqnorB***		
Step	**Temp**	**Time**	**Cycle n°**	**Temp**	**Time**	**Cycle n°**
Initial denaturation	94°C	3 min		94°C	3 min	
Denaturation	94°C	30 sec	35	94°C	30 sec	35
Annealing	49°C	30 sec		52°C	30 sec	
Elongation	72°C	1 min		72°C	1 min	
Denaturation Annealing Elongation						
Elongation	72°C	10 min		72°C	10 min	
Hold	4°C	until analysis		4°C	until analysis	

	BnirK			***nosZ***		
Step	**Temp**	**Time**	**Cycle n°**	**Temp**	**Time**	**Cycle n°**
Initial denaturation	94°C	3 min		95°C	5 min	
Denaturation	94°C	30 sec		95°C	30 sec	
Annealing	51°C	30 sec	35	69°C -> 49°C	40 sec	15
Elongation	72°C	1 min		72°C	1 min	
Denaturation				95°C	30 sec	
Annealing				53°C	40 sec	25
Elongation				72°C	1 min	
Elongation	72°C	10 min		72°C	10 min	
Hold	4°C	until analysis		4°C	until analysis	

4.2.4 Sequencing of amplified *nirK*, *qnorB, cbaA*, *nosZ* and *nrfA* products.

Amplicons of the expected size were purified using the Nucleofast®96 PCR clean up membrane system (Macherey-Nagel, Germany) or the QIAquick® PCR Purification Kit (Qiagen). Sequencing reactions were performed in a total volume of 10 µl with 3 µl of purified amplicon, 0.286µl of BigDyeTM mixture (Terminator Cycle Sequencing Kit version 3.1, Applied Biosystems), 1x sequencing buffer and 1.2 µM of each of the amplification primers used as sequencing primers. The thermal program consisted of 30 cycles (96 °C for 15 s, 35 °C for 1 s, 60 °C for 4 min). Subsequently, sequencing products were purified using the BigDye XTerminator Kit (Applied Biosystems) and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). For all genes, inspection of chromatograms and assembly of sequences was performed using BioNumerics 5.1 (Applied Maths,

Sint-Martens Latem). Protein translation analyses using the bioinformatics tools TranSeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and pBLAST (Altschul *et al.*, 1997) were used to confirm that sequences encoded for (part of) the evaluated enzymes.

4.2.5 Phylogenetic analyses of retrieved genes

4.2.5.1 Datasets

For *nirK*, *qnorB* and *nosZ* downsized datasets and a similar analysis were used as described previously (Verbaendert *et al.*, 2014). nBLAST and pBLAST results of amplified *nirK*, *qnorB* and *nosZ* sequences from this study were used to update the datasets. For *nrfA* and *cbaA* novel datasets were created by retrieval from Fungene (http://fungene.cme.msu.edu/) and/or GenBank (http://www.ncbi.nlm.nih.gov/genbank/) sequence databases. nBLAST and pBLAST results of amplified *cbaA* genes resulted in a data set with mainly sequences from genome sequencing projects. For the *nrfA* sequence dataset, retrieved sequences were based on *nrfA* phylogeny from the recent publication of Welsh and co-workers (Welsh *et al.*, 2014) and was updated with nBLAST and pBLAST results of amplified *nrfA* genes.

4.2.5.2 Analysis

Inferred amino acid sequences were aligned using the MEGA 6.0 software (Tamura *et al.*, 2013) using MUSCLE (Edgar, 2004) and resulting amino acid alignments were inspected manually for errors. For each alignment, average percent amino acid identity was calculated to estimate reliability (Thompson *et al.*, 1999). Phylogenetic analysis was performed as described previously (Verbaendert *et al.*, 2014).

4.2.6 Nucleotide sequence accession numbers

NirK, *qnorB, nosZ*, *cbaA* and *nrfA* gene sequence data generated in this study will be submitted to the European Nucleotide Archive (http://www.ebi.ac.uk) upon submission of the manuscript.

4.3 Results

4.3.1 Primer design

The nitric oxide reductase qCu_ANOR belongs to the heme-copper oxidase (HCO) superfamily. All HCO enzymes contain a characteristic histidine (His) sextet in which specific histidines ligate Fe_B (NOR) or Cu_B (oxidases) of the catalytic centre, coordinate the electron-transferring heme and function as a proximal ligand to the catalytic heme. NORs can be differentiated from oxidases by the presence of a glutamate (E), glutamine (Q), aspartate (D) or asparagine (N) near the catalytic site

versus tyrosine (Y) in oxidases (Hemp & Gennis, 2008). *B. azotoformans* and *B. bataviensis* type strains were reported to contain an asparagine (N) residue in their qCu_ANOR enzymes (Heylen & Keltjens, 2012). Hence, primers designed for *cbaA* had to meet three criteria: (i) forward and reverse primer should amplify a nucleotide region containing amino acids important for catalytic function, such as the catalytically important His residues (ii) the amplified PCR product should preferentially contain the region in which the amino acid residue (N) is located distinguishing NOR from oxidases, and (iii) primers should not amplify HCO other than NORs. Five forward and six reverse candidate regions with conserved amino acid residues within NOR that also showed distinct dissimilarity between qCu_ANOR and oxidases were identified within the amino acid alignment (black rectangles) (Fig 4. 1). Closer inspection of the corresponding nucleic acid alignment indicated that forward region 3 and reverse region 2 (Fig 4. 1) were the most promising candidate regions for primer design, including at least one of the histidines characteristic for HCO and the N residue characteristic for NORs. Two forward and two reverse primers were designed for both *cbaA* type I and type II (Table 4. 3). Locations of the designed *cbaA* primers are shown in (Fig 4. 1) (black arrows). Although amino acids of *cbaA* type II were clearly different in the primer target regions, primer design for *cbaA* type II was more complicated because corresponding nucleic acid sequences showed more sequence variation, resulting in higher divergence of primer sequences. Optimum conditions for the PCR reactions were evaluated on genomic DNA of *B. azotoformans* LMG 9581^T and *B. bataviensis* LMG 21833^T which both denitrify (Verbaendert *et al.*, 2011b) and encode for qCu_ANOR (Heylen & Keltjens, 2012). Their DNA was used as template to test the performance of the primers for direct amplification of the predicted *cbaA* fragments with different primer combinations at different annealing temperatures. For *cbaA* type I, products of the expected size were obtained for primer pair $cbaA_{t1}F3$-$cbaA_{t1}R3$, but with weak amplification of only *B. azotoformans* and thus was omitted for assessment on the complete strain set. Primer pairs $cbaA_{t1}F4$-$cbaA_{t1}R3$ and $cbaA_{t1}F4$-$cbaA_{t1}R4$ delivered products of the expected size for both strains, with *B. azotoformans* yielding the most intense band with primer pair $cbaA_{t1}F4$-$cbaA_{t1}R3$ and weak amplification for *B. bataviensis* with both primer pairs. The best amplification results were obtained at an annealing temperature (Ta) of 49°C. For *cbaA* type II, primers did not yield amplification in *B. azotoformans* LMG 9581^T; when tested on the other *B. azotoformans* strains, weak amplification was observed with primer pair $cbaAt_2F1$-$cbaAt_2R2$ for LMG 15448 only at a Ta of 45°C (Table 4. 4). This unsuccessful amplification for *cbaA* type II probably was due to the high degeneracy of the primers.

Bacillus-specific *nirK*, *qnorB* and *nosZ* primers were created by modifying *nirK*, *qnorB* and *nosZ* primers for *Geobacillus* (Verbaendert *et al.*, 2014) and are listed in Table 4. 3. As for *cbaA*, optimum conditions for the PCR reactions were evaluated with genomic DNA of *B. azotoformans* LMG 9581^T and *B. bataviensis* LMG 21833^T which encode correspondingly for *nirK, qnorB* and *nosZ,*

and for *nirK* and *qnorB* (Heylen & Keltjens, 2012). Their DNA was used as template to test the performance of the primers for direct amplification of the predicted *nirK, qnorB* and *nosZ* fragments with different primer combinations at different Ta's. For *B. bataviensis* LMG 21833^T products of expected size were obtained for the primer pair combination BnirK5F-BnirK6R at a Ta of 51°C, although bands were very weak. The primer pair was further modified and products of the expected size were obtained for primer pair BnirK5Fb-BnirK6Rb and BnirK6F-BnirK8R, but only for *B. bataviensis* LMG 21833^T. The most intense bands were obtained at a Ta of 51°C and 46°C, respectively. Unexpectedly, the *B. azotoformans* type strain did never yield amplification even with primer pair BnirK6F-BnirK10R which was more specific towards *B. azotoformans*. Sequences obtained confirmed that the primers had identified the correct fragment. For *qnorB*, no products were obtained for the primer pair combination BqnorB7F-BqnorB6R, however products of the expected size were obtained for primer pair BqnorB9F-BqnorB8R, but not for the *B. bataviensis* type strain. The best amplification results were obtained at a Ta of 52°C (Table 4. 4). Obtained sequences confirmed that the primers had identified the correct fragment. For *nosZ*, products of expected size were obtained for all primer pair combinations for *B. azotoformans* only, with primer pairs BnosZ3F-BnosZ1R/BonsZ4F-BnosZ1R yielding the most intense amplification. Band intensity with primer pair BnosZ2F-BnosZ1R declined at Ta's higher than 55°C. The best amplification results were obtained at a Ta of 53°C (Table 4. 4). All sequences obtained confirmed that the primers had identified the correct fragments.

4.3.2 Detection of *nirK*, *qnorB*, *cbaA*, *nosZ* and *nrfA* in functional denitrifiers

Five priming pairs frequently used in environmental monitoring of denitrification and DNRA (Braker *et al.*, 1998, Hallin & Lindgren, 1999, Kloos *et al.*, 2001, Braker & Tiedje, 2003, Mohan *et al.*, 2004, Thröback *et al.*, 2004) were applied to amplify genes encoding nitrite reductases (*nir* and *nrfA*), nitric oxide reductases (*qnorB*) and nitrous oxide reductases (*nosZ*) (Table 4. 5). For the majority of the strains, these established PCR priming sets did not allow for amplification of the genes involved in denitrification or DNRA (Table 4. 5). This was not unexpected due to the many mismatches between available PCR primers and denitrification gene sequences in Gram-positive bacteria (Verbaendert *et al.*, 2014) and the little amount of research on *nrfA* diversity (Welsh *et al.*, 2014). Improved primer pairs for the amplification of *nirK*, *qnorB* and *nosZ* of Gram-positive bacteria (this study and Verbaendert *et al*. 2014) were evaluated as well but did not result in overall high amplification rate of the denitrification genes under investigation, except for *nosZ* (Table 4. 5). Novel primers for *cbaA* type I genes, encoding qCu_ANOR, and improved primers for *nrfA* genes (Welsh *et al.*, 2014) were succesful for the studied strain set. Table 4. 5 provides an overview of all studied strains and the results for each primer set.

Table 4. 5 Presence of genes encoding nitrite reductases (*nirK* and *nrfA*), nitric oxide reductases (*qnorB* and *cbaA*) and nitrous oxide reductase (*nosZ*). Species identification, strain number and the results of PCR-based detection of denitrification genes and *nrfA* with different PCR priming sets are given. Primer pairs frequently used for environmental monitoring are given in the columns highlighted in dark grey and primers pairs designed for the Gram-positive genus *Geobacillus* by Verbaendert *et al.* 2014 in light grey.

		nitrite reductase								nitric oxide reductase						nitrous oxide reductase					
Species	**Strain**	***nirK***						***nrfA***		***qnorB***				***cbaA***		***nosZ***					
		nirK1F-nirK5R[a]	F1aCu-R3Cu[b]	GnirK2F-GnirK2R[c]	BnirK5F-BnirK6R[d]	BnirK5Fb-BnirK6Rb[d]	BnirK6F-BnirK10R[d]	nrfAF1-nfrA7R1[e]	nrfAF2aw-nfrA-7R1[f]	qnorB2F-qnorB7R[g]	Gqnor4F-GqnorB6R[c]	BqnorB7F-BqnorB6R[d]	BqnorB9F-BqnorB8R[d]	$cbaA_{t1}F4$-$cbaA_{t1}R3$[d]	$cbaA_{t1}F4$-$cbaA_{t1}R4$[d]	nosZ-F-nosZ1622R[h]	nosZ-F-nosZ-R[i]	GnosZF-GnosZR[c]	BnosZ2F-BnosZ1R[d]	BnosZ3F-BnosZ1R[d]	BnosZ4F-BnosZ1R[d]
B. asahii	LMG 24728[T]	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. atrophaeus	LMG 8199[T]t_1	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. atrophaeus	LMG 8199[T]t_2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. azotoformans	LMG 15445	-	-	-	-	-	-	-	+	-	+A	-	-	+	+	-	-	-	+	+	+
B. azotoformans	LMG 15448	-	-	-	-	-	-	-	+	-	?	-	-	+	+	+A	-	-	+A	+	?
B. azotoformans	LMG 15449	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+
B. azotoformans	LMG 9581[T]	-	-	-	-	-	-	-	+A	-	-	-	?	+	+	-	-	-	?	+	+
B. bataviensis	LMG 21832	-	-	+	-	+	-	?	+A	-	?	-	-	-	+	-	-	-	-	-	-
B. bataviensis	LMG 21833[T]	-	-	+	?	+	-	+	-	-	?	-	-	-	+	-	-	-	-	-	-
B. bataviensis	R-31541	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+A
B. bataviensis	R-31770	-	-	-	(?)	-	-	-	+	$\underline{+}$	-	-	-	-	+	-	-	-	+	-	-
B. bataviensis	R-31834	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+A	+
B. bataviensis	R-32700	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
B. bataviensis	R-32709	-	-	-	(?)	-	-	-	+	$\underline{+}$	-	-	-	-	-	-	-	-	+	-	-
B. bataviensis	R-32779	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
B. bataviensis	R-32781	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
B. bataviensis	R-32787	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-
B. bataviensis	R-32845	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	?	+	+	-
B. drentensis	R-31547	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+
B. drentensis	R-31550	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+
B. drentensis	R-31846	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
B. drentensis	R-32528	-	-	?	(?)	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
B. drentensis	R-32575	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-
B. drentensis	R-32656	-	-	-	-	-	-	-	+	$\underline{+}$	-	-	-	-	+	-	-	-	+	?	+
B. drentensis	R-32702	-	-	-	(?)	-	-	-	+	$\underline{+}$	-	-	-	-	-	-	-	-	+	-	-
B. drentensis	R-32705	-	-	-	(?)	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+A	-
B. drentensis	R-32789	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+A	-
B. drentensis	R-33773	-	-	-	-	-	-	-	+	$\underline{+}$	+	-	-	-	+	-	-	-	+	+	-
B. indicus	LMG 22858[T]	-	-	-	-	-	-	-	+A	-	-	-	-	-	-	-	-	-	-	-	-
B. infantis	LMG 24756[T]	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. lehensis	LMG 24751[T]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	-	+	-	-	-
B. licheniformis	LMG 17339	-	-	-	-	-	-	-	+A	-	?	-	?	-	-	-	-	-	-	-	-
B. licheniformis	LMG 17340	-	-	-	-	-	-	-	+A	-	?	-	+	-	-	-	-	-	-	-	-
B. licheniformis	LMG 6934	-	-	-	-	-	-	-	+A	-	?	-	+	-	-	-	-	-	-	-	-
B. licheniformis	LMG 7559	-	-	-	-	-	-	-	+A	-	+	-	+	-	-	-	-	?	-	-	-
B. licheniformis	LMG 7561	-	-	-	-	-	-	-	-	-	?	-	+	-	-	-	-	-	-	-	-
B. licheniformis	LMG 7633	-	-	-	-	-	-	-	+A	-	?	-	+	-	-	+A	-	-	-	-	-
B. licheniformis	R-31769	-	-	-	-	-	-	-	+A	-	-	-	+	-	-	-	-	-	-	-	-
B. licheniformis	R-32706	-	-	-	-	-	-	-	+A	-	-	-	+	-	-	-	-	-	-	-	-
B. megaterium	LMG 7127[T]	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. mojavensis	LMG 22477	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-
B. mycoides	LMG 7128[T]	-	-	-	-	-	-	-	-	-	?	-	-	-	-	-	-	-	-	-	-
B. plakortidis	LMG 24732[T]	-	-	?	-	-	-	?	-	-	+	-	-	-	-	-	-	-	-	-	-
B. pseudomycoides	R-31830[u]	-	-	-	(?)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
B. soli	R-31553	-	-	-	(?)	-	-	-	+	-	-	-	-	-	+	-	-	?	+	+A	-
B. soli	R-31841	-	-	-	-	-	-	-	+	$\underline{+}$	-	-	-	-	+	-	-	-	+	+A	-
B. soli	R-32526	-	-	-	-	-	-	-	+	$\underline{+}$	-	-	-	-	+	-	-	-	+	+A	-
B. soli	R-32694[v]	-	-	-	-	-	-	-	+	$\underline{+}$	-	-	-	-	-	-	-	-	+	+A	-
B. soli	R-32715	-	-	-	-	-	-	-	-	$\underline{+}$	-	-	-	-	+	-	-	-	?	+A	-
B. soli	R-32849	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	+	+A	-
B. soli	R-33820	-	-	-	-	-	-	-	+	$\underline{+}$	-	-	-	-	-	-	-	-	+	+A	-
Bacillus sp.	R-39623	-	-	-	?	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus sp.	R-39624	-	-	-	-	-	-	-	-	-	?	-	-	-	-	-	-	-	-	-	-
Bacillus sp.	R-39625	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. thuringiensis	LMG 12265	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. vietnamensis	LMG 24742[T]	-	-	?	-	-	-	-	-	$\underline{+}$	-	-	-	-	-	-	-	-	-	-	-
B. vireti	LMG 21834[T]	-	-	-	-	-	-	?	?	-	-	-	-	-	+	-	-	-	+	+	+
Paenibacillus sp.	R-27048	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. uliginis	LMG 24790[T]	-	-	+	-	-	-	?	-	$\underline{+}$	+	-	-	-	-	-	-	-	-	-	-
S. warneri	R-34181	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, amplicon verified via sequence analysis, and, -, no amplification or aspecific amplification

$\underline{+}$, sequences obtained in Heylen (2007), and, +A, specific band accompanied by aspecific amplification

?, amplicons that could not be sequenced successfully, and, (?), very weak amplicons that could not be sequenced succesfully

[a] to [i] primer pairs described in Braker *et al*., 1998 (a); Hallin and Lindgren, 1999 (b); Verbaendert *et al.,* 2014 (c); this study (d); Mohan *et al*., 2004 (e); Welsh *et al*., 2014 (f); Braker and Tiedje, 2003 (g); Kloos *et al.* (2001) and Thröback *et al*. 2004 (h) and Jones *et al* . (2011) (i)

[u] contains gene for *cd1*-type nitrite reductase (*nirS*) (Heylen, 2007)

[v] contains gene for cNOR-type nitric oxide reductase (*cnorB*)(Heylen, 2007)

4.3.3 *nirK* – functional genotype

The presence of *nirK* was confirmed in three strains only (Table 4. 5). Primer pair combination BnirK5F-BnirK6R showed very weak amplification for fourteen other strains (e.g. Fig 4. 2), but it was not possible to confirm *nirK* presence with BnirK5F and BnirK6R as sequencing primers by direct sequencing. Repetition of PCR reactions did unfortunately not always yield detectable amplification. Pairwise comparison of *Bacillus bataviensis* LMG 21833^T and LMG 21832 and *Paenibacillus uliginis* LMG 24790^T *nirK* sequences revealed similarities between 62.7 to 99.8%. Sequencing resulted in fragments of the expected approximate 440 bp. Derived amino acid sequences displayed the characteristic SSFH amino acid motif for class II CuNiR (Jones *et al.*, 2008) and pBLAST indicated they were all similar to 'copper-containing nitrite reductase (CuNiR)'. Average percent AAI of the inferred amino acid alignments was calculated to be 42.6%, indicating reliability of the alignment. *NirK* of *Paenibacillus uliginis* strain LMG 24790^T was most closely related to nitrite reductases found in whole genome sequences of *Paenibacillus* spp., whereas *Bacillus bataviensis* LMG 21832 *nirK* clustered most closely to *nirK* of *B. bataviensis* type strain (Fig 4. 3). All three sequences belong to the previously described third type *nirK* cluster (Philippot, 2002, Verbaendert *et al.*, 2014). Surprisingly, subcluster 4 (which corresponds to subcluster 3 in Fig. 3.3 in Chapter 3) only contains *Bacillaceae nirK* sequences with *Geobacillus, Bacillus* and *Paenibacillus* sequences grouping thus far in seemingly monophyletic clades (Fig 4. 3).

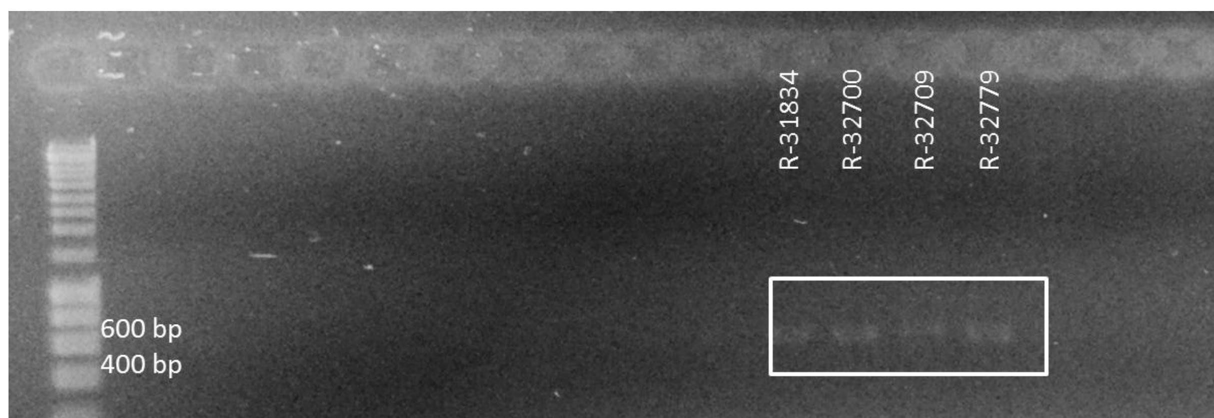


Fig 4. 2 Example of weak *nirK* amplification with primer pair BnirK5F-BnirK6R

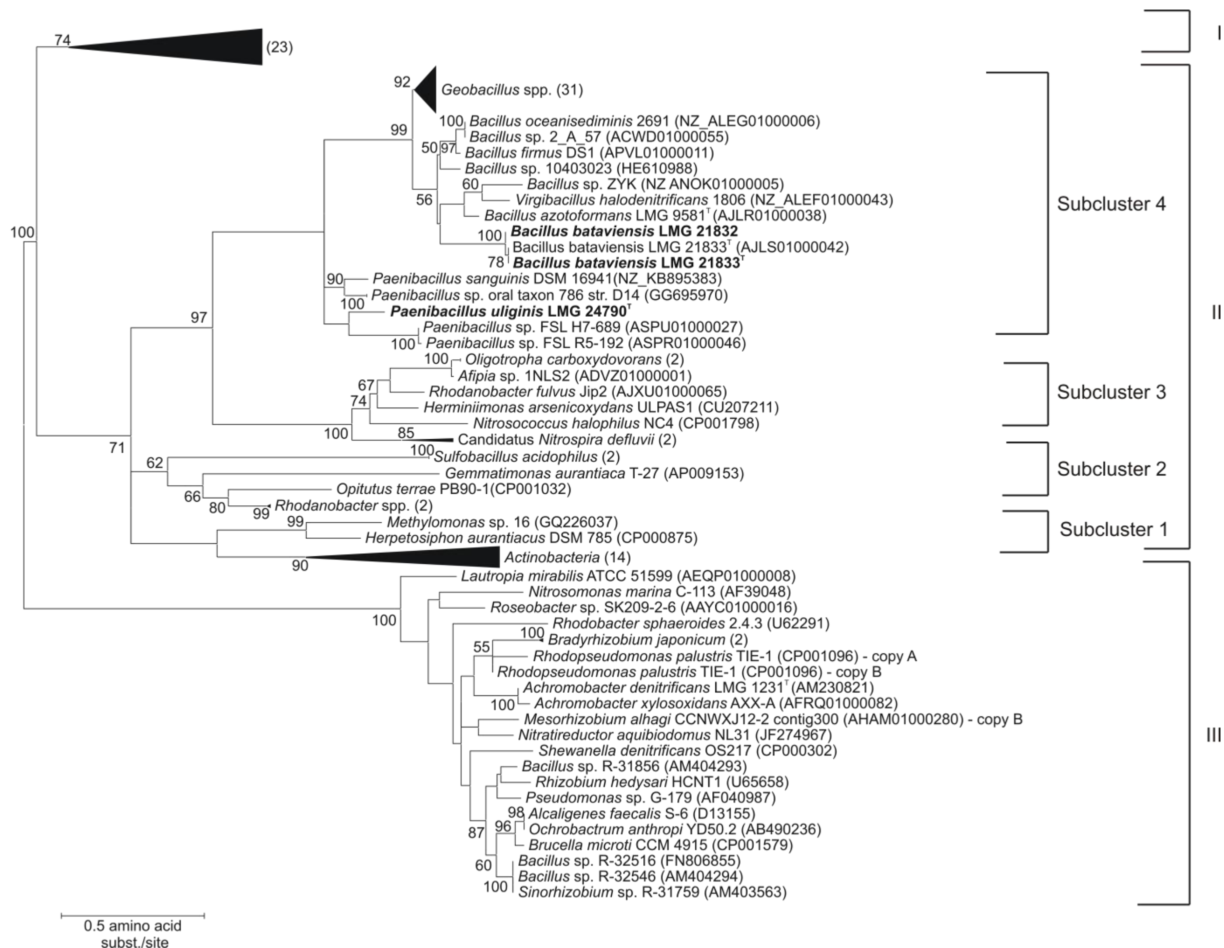


Fig 4. 3 Maximum likelihood phylogeny of *nirK* amino acid sequences. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 123 amino acid sequences and there were a total of 105 positions used in the final dataset. Sequences from this study are given in bold.

4.3.4 *qnorB* – functional genotype

Previous research (Heylen, 2007) detected the *qnorB* gene in twelve strains with primers of Braker *et al.* (2003). With *qnorB* primers of this study, the gene was detected in an extra fifteen strains (Table 4. 5), resulting in a fragment of approximately 600bp. Pairwise comparison of all retrieved sequences resulted in similarities ranging from 85.4-100%. pBLAST of derived amino acid sequences indicated they were all similar to 'nitric oxide reductase large subunit' (NOR)'. Inferred amino acid sequences also disclosed the presence of three conserved histidine residues, the highly conserved HLWVEGX$_2$E and glutamate residue, all necessary for catalytic activity. Average percent AAI of the inferred amino acid alignments was calculated to be 58%, indicating reliability of the alignment.

The *qnorB* dataset consisted of 200 genotypes and RAxML phylogenetic analysis of amino acid sequences revealed that *qnorB* amplified with *Geobacillus*-specific *qnorB* targeting primers of the strains in this dataset were most closely related to *qnorB* sequences of *Geobacillus* (Fig 4. 4), whereas *qnorB* amplified with the primers described by Braker *et al*. (2005) grouped together with

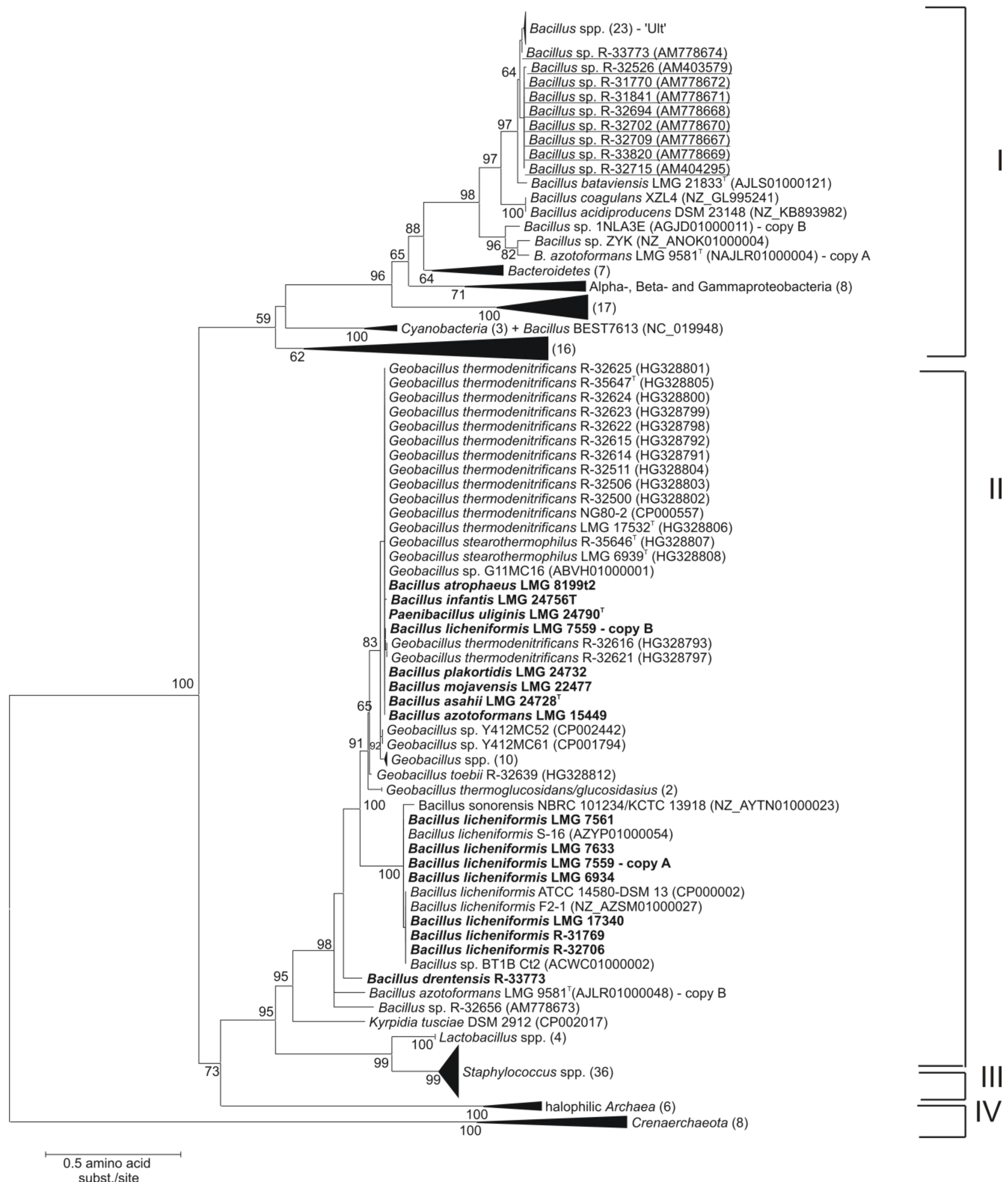


Fig 4. 4 Maximum likelihood phylogeny of *qnorB* amino acid sequences from isolates as well as available genomes in Fungene and GenBank sequence databases. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I+F) and only bootstrap support values for nodes with > 50% bootstrap probability (n=500) are given. Analysis involved 200 amino acid sequences and there were a total of 145 positions used in the final dataset. Sequences from this study are given in bold, sequences from this study for which a *qnorB* was available are underlined (Heylen, 2007).

other soil-borne *Bacillus qnorB* in cluster I (Fig 4. 4). *Bacillus licheniformis* LMG 7559 displayed the presence of two different *qnorB* types, one amplified by primer pair GqnorB4F – GqnorB6R and one by BqnorB9F – BqnorB8R grouping in different subclusters. *Bacillus licheniformis qnorB* seem to

cluster together in a monophyletic clade together with a representative of the species *Bacillus sonorensis* (Fig. 4.4). Most of the novel *qnorB* sequences grouped within cluster II, of which not many *qnorB* sequences have been amplified by PCR.

4.3.5 *cbaA* type I and type II – functional genotype

Primer pair $cbaA_{t1}F4$-$cbaA_{t1}R4$ for amplification of *cbaA* type I scored best for amplification of the gene in the total strain set. The *cbaA* gene for type I qCu_ANOR was detected in twenty-four strains (Table 4. 5), resulting in a fragment of approximately 450bp. In 2011, a bioinformatics tool for classification of heme-copper oxidases (HCO) and an HCO database was published (Sousa *et al.*, 2011). However, it could not identify the retrieved sequences as NOR because it did not include known qCu_ANOR in its database. Nevertheless, all retrieved sequences exhibited the Fe_B-binding asparagine (N) residue described for sNORs (Hemp & Gennis, 2008) and one His residue of the six histidines characteristic for HCO. Pairwise comparison of all retrieved sequences resulted in similarities ranging from 72.27% to 100%. pBLAST of derived amino acid sequences indicated they were all similar to 'cytochrome c oxidase subunit I' or 'cytochrome c'. Via pBLAST and nBLAST only a limited number of homologous sequences could be retrieved for phylogenetic analyses since not many qCu_ANOR genes have been detected in WGS and correctly or fully annotated. Average percent AAI of the inferred amino acid alignment was calculated to be 59.4%, indicating reliability of the alignment.

The *cbaA* dataset consisted of 52 genotypes and RAxML phylogenetic analysis of amino acid sequences inferred from the *cbaA* genes revealed a tree topology with three distinct clusters (Fig 4. 5): cluster I containing sequences belonging to *Firmicutes cbaA* type I sequences, cluster II comprised of *cbaA* type II sequences also associated with *Firmicutes* and cluster III with sequences encoding the sNOR enzyme in nitrifiers such as *Nitrosomonas eutropha* C91 (Fig 4. 5). *B. azotoformans cbaA* type I forms a separate clade within cluster I with *cbaA* type I sequences of *Bacillus timonensis*, *Bacillus bogoriensis* and *Caldalkalibacillus thermarum*, whereas the other retrieved *cbaA* sequences cluster together in another clade.

4.3.6 *nosZ* – functional genotype

The *nosZ* gene was detected in thirty-two strains (Table 4. 5). Amplification of the correct fragment was only successful with primer pairs developed specifically for Gram-positive bacteria belonging to the *Bacillaceae* (this study and Verbaendert *et al.*, 2014)(Table 4. 5). Amplification resulted in fragments of approximately 1500bp. Primer pair BnosZ2F-BnosZ1R did not exhibit aspecific amplification and showed the highest success rate. Amplification with BnosZ3F-BnosZ1R and BnosZ4F-BnosZ1R was more intense but more frequently showed non-specific amplification.

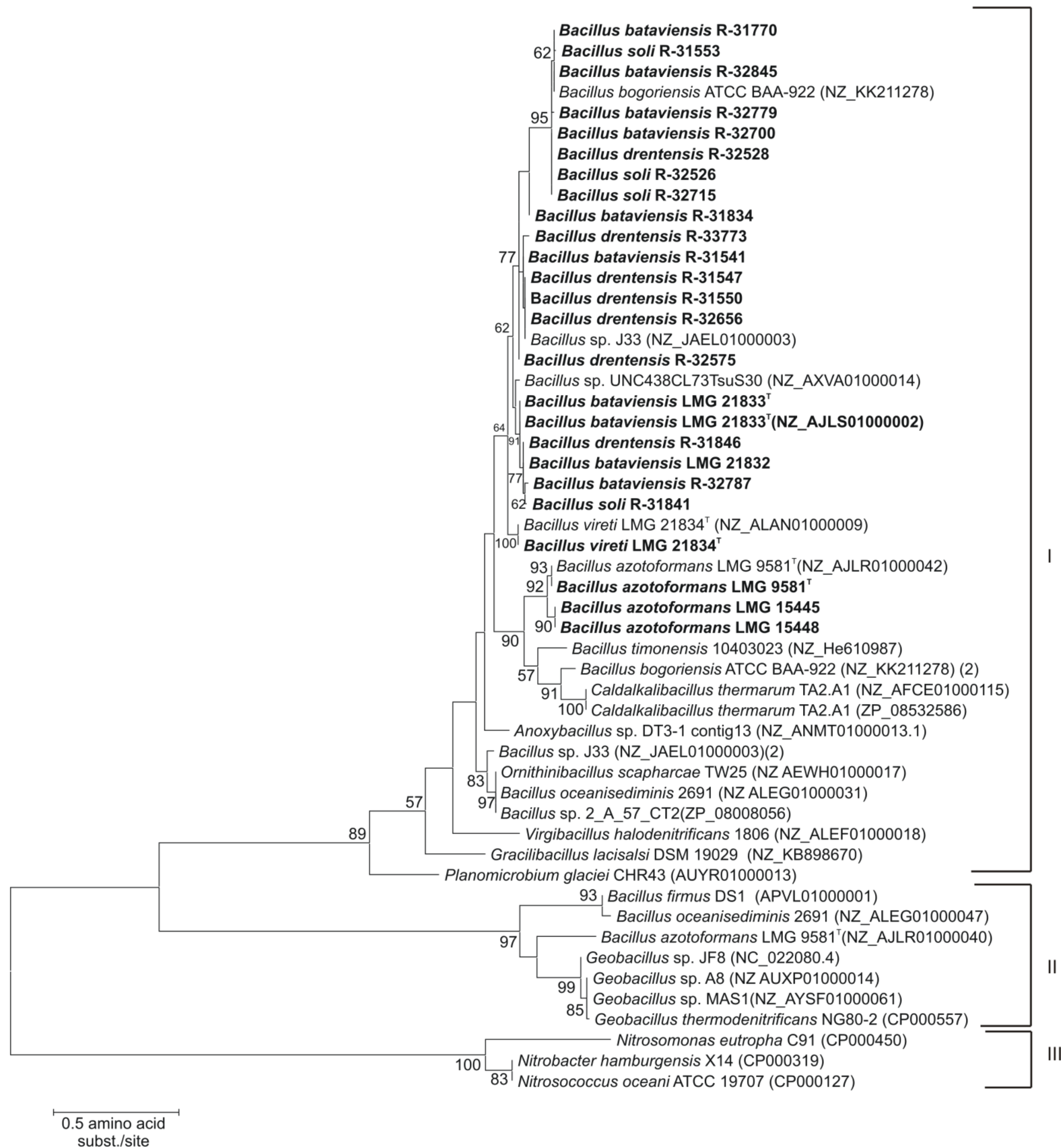


Fig 4. 5 Maximum likelihood phylogeny of *cbaA* type I and II amino acid sequences. Scales indicate corrected amino acid substitutions per site (WAG+G) and only bootstrap support values for nodes with > 50% bootstrap probability (n=500) are given. Analysis involved 52 amino acid sequences and there were a total of 166 positions used in the final dataset. Sequences from this study are given in bold.

Further optimization of the PCR conditions may eliminate non-specific products. Pairwise comparison of all retrieved sequences resulted in similarities ranging from 66.96% to 99.97%. pBLAST of derived amino acid sequences indicated they were all similar to 'Sec-dependent nitrous-oxide reductase (N_2OR)' with conservation of several His residues important for structure and functionality of N_2O-reductase (Scala & Kerkhof, 1998, Sanford *et al.*, 2012). Average percent AAI of the inferred amino acid alignments was calculated to be 53.5 %, indicating reliability of the alignment.

The *nosZ* dataset consisted of 162 genotypes and RAxML phylogenetic analysis of amino acid sequences inferred from the *nosZ* genes revealed that *nosZ* of the *Bacillaceae* belonged to the 'aty-

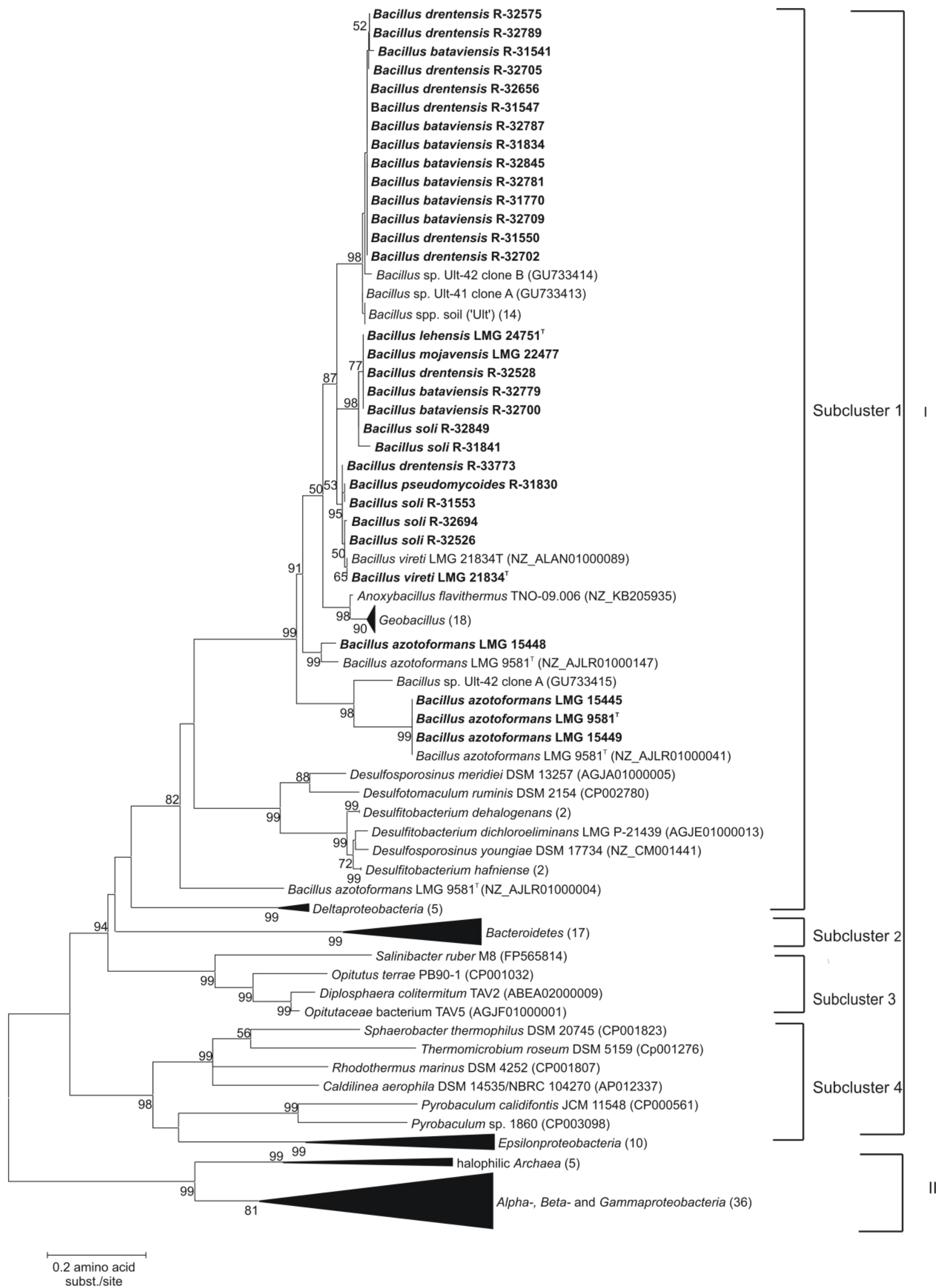


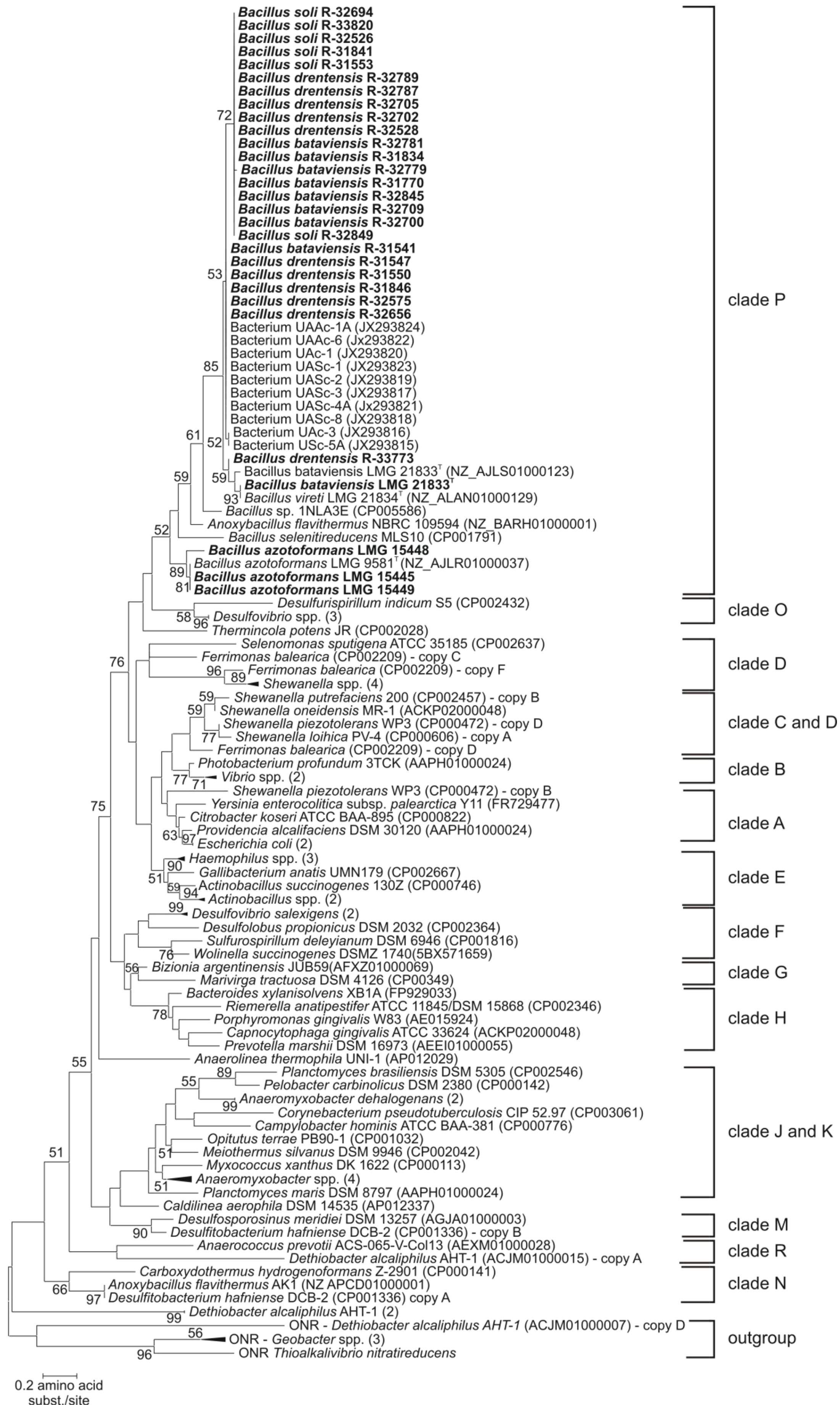
Fig 4. 6 Maximum likelihood phylogeny of *nosZ* amino acid sequences. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=500) are given. Analysis involved 162 amino acid sequences and there were a total of 198 positions used in the final dataset. Sequences from this study are given in bold.

typical' *nosZ* clade (Sanford *et al.*, 2012) (cluster I) and grouped together in a cluster with *nosZ* of other *Firmicutes* (Fig 4. 6, subcluster 1). Fourteen *nosZ* sequences were phylogenetically most closely related to *nosZ* from *Bacillus* isolated from soil fertilized with ammonium sulfate from the Ultana ('Ult') agricultural experimental site (Jones *et al.*, 2011). *NosZ* sequences of another five strains were most closely related to *nosZ* of denitrification-like nitrate ammonifying *Bacillus vireti* LMG 21834^T (Mania *et al.*, 2014). *Bacillus azotoformans nosZ* clearly clusters away from *nosZ* sequences harbored by the other *Bacillus* species that are more closely related to *nosZ* of denitrifying *Geobacillus* (Verbaendert *et al.*, 2014). Markedly, *Bacillus azotoformans nosZ* sequences also clustered together with only two types of three types of *nosZ* described in *B. azotoformans* LMG 9581^T (Heylen & Keltjens, 2012), indicating that the primers do not pick-up the different *nosZ* variants that may be present in one strain.

4.3.7 *nrfA* – functional genotype

The *nrfA* gene was detected in twenty-nine strains (Table 4. 3) resulting in a fragment of approximately 269 bp (nrfA2aw-nrfA7R1) or 500 bp (nrfAF1-nrfA7R1). With the recently published refined *nrfA* protocol (Welsh *et al.*, 2014), eighteen strains yielded an amplicon of the correct size together with amplification of non-target of 600 to 800 bp in size. Subsequent modification to a touchdown PCR protocol (5 min at 95°C; 11 cycles of 95°C for 30s, 63°C for 30 sec with a touchdown of 2°C per cycle and 72°C for 20 sec; followed by 25 cycles of 95°C for 30 sec, 53°C for 30 sec and 72°C for 20 sec and ended with 10 min at 72°C) yielded a single specific product for some of those strains and *nrfA* sequences could ultimately be obtained. *B. bataviensis* LMG 21833^T was the only strain from which *nrfA* was recovered with primers of Mohan and co-workers (2004). From *Bacillus azotoformans* LMG 9581^T and *Bacillus vireti* LMG 21834^T no *nrfA* sequences could be obtained although the strains have been reported to harbor *nrfA*. All retrieved *Bacillus nrfA* sequences displayed the characteristic *nrfA* motif KXQH between the third and fourth heme-binding motif. Pairwise comparison of all retrieved sequences resulted in similarities ranging from 72.69% to 100%. pBLAST of derived amino acid sequences indicated they were all similar to 'cytochrome c nitrite reductase'. Average percent AAI of the inferred amino acid alignments was calculated to be 52.98%, indicating reliability of the alignment.

The *nrfA* dataset consisted of 119 genotypes and RAxML phylogenetic analysis of amino acid sequences inferred from the *nrfA* genes revealed the close relatedness to *nrfA* sequences of clade P described by Welsh and colleagues (2014). (Fig 4. 7). The majority of sequences was most closely related to *nrfA* sequences of unidentified bacteria or bacterial clones (JX293815 to JX293823) originating from an agricultural soil (Urbana, Illinois, USA) (Welsh *et al.*, 2014) which roughly has a similar composition (10-20% clay, 60-70% silt, 10-20% loam) to the luvisol soil from which many of


Bacillus soli R-32694
Bacillus soli R-33820
Bacillus soli R-32526
Bacillus soli R-31841
Bacillus soli R-31553
Bacillus drentensis R-32789
Bacillus drentensis R-32787
Bacillus drentensis R-32705
Bacillus drentensis R-32702
Bacillus drentensis R-32528
Bacillus bataviensis R-32781
Bacillus bataviensis R-31834
Bacillus bataviensis R-32779
Bacillus bataviensis R-31770
Bacillus bataviensis R-32845
Bacillus bataviensis R-32709
Bacillus bataviensis R-32700
Bacillus soli R-32849
Bacillus bataviensis R-31541
Bacillus drentensis R-31547
Bacillus drentensis R-31550
Bacillus drentensis R-31846
Bacillus drentensis R-32575
Bacillus drentensis R-32656
Bacterium UAAc-1A (JX293824)
Bacterium UAAc-6 (Jx293822)
Bacterium UAc-1 (JX293820)
Bacterium UASc-1 (JX293823)
Bacterium UASc-2 (JX293819)
Bacterium UASc-3 (JX293817)
Bacterium UASc-4A (Jx293821)
Bacterium UASc-8 (JX293818)
Bacterium UAc-3 (JX293816)
Bacterium USc-5A (JX293815)
Bacillus drentensis R-33773
Bacillus bataviensis LMG 21833T (NZ_AJLS01000123)
Bacillus bataviensis LMG 21833T
Bacillus vireti LMG 21834T (NZ_ALAN01000129)
Bacillus sp. 1NLA3E (CP005586)
Anoxybacillus flavithermus NBRC 109594 (NZ_BARH01000001)
Bacillus selenitireducens MLS10 (CP001791)
Bacillus azotoformans LMG 15448
Bacillus azotoformans LMG 9581T (NZ_AJLR01000037)
Bacillus azotoformans LMG 15445
Bacillus azotoformans LMG 15449
Desulfurispirillum indicum S5 (CP002432)
Desulfovibrio spp. (3)
Thermincola potens JR (CP002028)
Selenomonas sputigena ATCC 35185 (CP002637)
Ferrimonas balearica (CP002209) - copy C
Ferrimonas balearica (CP002209) - copy F
Shewanella spp. (4)
Shewanella putrefaciens 200 (CP002457) - copy B
Shewanella oneidensis MR-1 (ACKP02000048)
Shewanella piezotolerans WP3 (CP000472) - copy D
Shewanella loihica PV-4 (CP000606) - copy A
Ferrimonas balearica (CP002209) - copy D
Photobacterium profundum 3TCK (AAPH01000024)
Vibrio spp. (2)
Shewanella piezotolerans WP3 (CP000472) - copy B
Yersinia enterocolitica subsp. palearctica Y11 (FR729477)
Citrobacter koseri ATCC BAA-895 (CP000822)
Providencia alcalifaciens DSM 30120 (AAPH01000024)
Escherichia coli (2)
Haemophilus spp. (3)
Gallibacterium anatis UMN179 (CP002667)
Actinobacillus succinogenes 130Z (CP000746)
Actinobacillus spp. (2)
Desulfovibrio salexigens (2)
Desulfolobus propionicus DSM 2032 (CP002364)
Sulfurospirillum deleyianum DSM 6946 (CP001816)
Wolinella succinogenes DSMZ 1740(5BX571659)
Bizionia argentinensis JUB59(AFXZ01000069)
Marivirga tractuosa DSM 4126 (CP00349)
Bacteroides xylanisolvens XB1A (FP929033)
Riemerella anatipestifer ATCC 11845/DSM 15868 (CP002346)
Porphyromonas gingivalis W83 (AE015924)
Capnocytophaga gingivalis ATCC 33624 (ACKP02000048)
Prevotella marshii DSM 16973 (AEEI01000055)
Anaerolinea thermophila UNI-1 (AP012029)
Planctomyces brasiliensis DSM 5305 (CP002546)
Pelobacter carbinolicus DSM 2380 (CP000142)
Anaeromyxobacter dehalogenans (2)
Corynebacterium pseudotuberculosis CIP 52.97 (CP003061)
Campylobacter hominis ATCC BAA-381 (CP000776)
Opitutus terrae PB90-1 (CP001032)
Meiothermus silvanus DSM 9946 (CP002042)
Myxococcus xanthus DK 1622 (CP000113)
Anaeromyxobacter spp. (4)
Planctomyces maris DSM 8797 (AAPH01000024)
Caldilinea aerophila DSM 14535 (AP012337)
Desulfosporosinus meridiei DSM 13257 (AGJA01000003)
Desulfitobacterium hafniense DCB-2 (CP001336) - copy B
Anaerococcus prevotii ACS-065-V-Col13 (AEXM01000028)
Dethiobacter alcaliphilus AHT-1 (ACJM01000015) - copy A
Carboxydothermus hydrogenoformans Z-2901 (CP000141)
Anoxybacillus flavithermus AK1 (NZ APCD01000001)
Desulfitobacterium hafniense DCB-2 (CP001336) copy A
Dethiobacter alcaliphilus AHT-1 (2)
ONR - Dethiobacter alcaliphilus AHT-1 (ACJM01000007) - copy D
ONR - Geobacter spp. (3)
ONR Thioalkalivibrio nitratireducens
clade P
clade O
clade D
clade C and D
clade B
clade A
clade E
clade F
clade G
clade H
clade J and K
clade M
clade R
clade N
outgroup
0.2 amino acid subst./site

the strains of this study were isolated. Although still closely related, *B. azotoformans nrfA* sequences grouped separately from this latter cluster. Although the *nrfA* gene phylogeny was based on an amino acid sequence length only spanning the region between the 3rd and 4th heme-binding motifs of the *nrfA* gene (< 270 bases), as this was the region amplified by primers nrfA2aw-nrfA7R1, *nrfA* tree topology was very similar to that of full-length *nrfA* sequence alignment of Welsh and co-workers (2014).

Fig 4. 7 Maximum likelihood phylogeny of *nrfA* amino acid sequences (previous page). Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G) and only bootstrap support values for nodes with >50% bootstrap probability (n=500) are given. Analysis involved 119 amino acid sequences and there were a total of 60 positions used in the final dataset. Clade designations according to Welsh *et* al. (2014). Sequences from this study are given in bold. ONR (Octaheme Nitrite Reductase) sequences are used as outgroups.

4.4 Discussion

We have evaluated the genetic and strain-dependent nature of the dissimilatory nitrogen metabolism within a strain set comprised by sixty-two potentially denitrifying strains mainly belonging to the *Bacillaceae* – of which many strains were isolated from soil – by evaluating the presence of genes involved in denitrification and DNRA (*nirK*, *qnorB*, *cbaA*, *nosZ* and *nrfA*). The bulk of the strain set was comprised by members of the genus *Bacillus*. *Bacillus* is a relevant genus for such comparison for several reasons: (i) *Bacillus* strains are ubiquitous in soil environments (Felske *et al.*, 1998, Janssen, 2006, Tzeneva, 2006), (ii) many *Bacillaceae* denitrifiers have been reported to have a truncated denitrification pathway producing the greenhouse gas N_2O (Denariaz *et al.*, 1989, Verbaendert *et al.*, 2011a), (iii) high nitrate concentrations (which soil microbiota can encounter e.g. after nitrogenous fertilization) have been found to stimulate members of the genus *Bacillus* to also produce the greenhouse gas N_2O, but during DNRA (Smith & Zimmerman, 1981, Bleakley & Tiedje, 1982, Streminska *et al.*, 2012), and, (iv) a diverse gene inventory for both denitrification and DNRA was recently observed in two *Bacillus* type strains (Heylen & Keltjens, 2012). These studies invoked the question to which extent potentially denitrifying bacilli previously isolated from luvisol soil and originating from a reference strain set screened for dissimilatory nitrate reduction (Verbaendert *et al.*, 2011b) would also display this diverse gene inventory and to which extent they could be important contributors to soil N_2O emissions under fluctuating environmental conditions. For this, recent primers for *nirK*, *qnorB* and *nosZ* of Gram-positive geobacilli (Verbaendert *et al.*, 2014) were tested and further modified to accommodate for the known sequence variation in *Bacillus*. In addition, amplification of *nrfA with* improved primers (Welsh *et al.*, 2014) was used as a proxy for the presence of DNRA and brand new primers detecting the genes for qCu_ANOR were developed.

B. azotoformans LMG 9581^T and *B. bataviensis* LMG 21833^T were previously reported to harbor *nirK* (Heylen & Keltjens, 2012). Yet in the studied strain set only three *nirK* sequences could be retrieved via direct sequencing, even with primers specifically designed on *Bacillus nirK* gene sequences (Table 4. 2). This underperformance could be attributed to the high sequence divergence of *nirK* in the particular target region, especially at the 5’ side of the reverse primers, hampering specific annealing and extension of the designed degenerate primers under the conditions tested. The lack of *nirK* in many strains could also be explained by the absence of denitrification and the presence of DNRA. This is a process in which nitrate is reduced to nitrite by the catalytic subunits of a nitrate reductase (NarG or NapA), followed by the further reduction of nitrite to ammonium by the nitrite reductase NrfA. Many of the strains genetically presented as nitrate ammonifying bacteria with genes for periplasmic nitrite reductase (*nrfA*), qCu_ANOR (*cbaA*) and atypical N_2OR (*nosZ*) and may use NrfA to reduce nitrite to ammonium while producing N_2O via qCu_ANOR. Nitrous oxide may

further be reduced in these strains by atypical N_2OR in a denitrification-like ammonification. This hypothesis may hold true because DNRA *sensu stricto* is the reduction of nitrite to ammonium (Simon & Klotz, 2013) and many strains were initially isolated and grown under nitrite selection on minimal medium G_2M_{11} (Heylen, 2007, Verbaendert *et al.*, 2011b) (Table 4. 1) containing 3mM nitrite as main nitrogen source.

Previous work showed that N_2O-producing bacilli included in this study could not render *qnorB* PCR-products with commonly used primer sets (positive results are included in Table 4. 5 and underlined) (Heylen, 2007). Failed attempts to amplify *qnorB* from the majority of the strains in the described *Bacillaceae* strain set with these primers (Braker & Tiedje, 2003) and recently published *qnorB* primers for Gram-positive bacteria (Verbaendert *et al.*, 2014) could be explained by the presence of qCu_ANOR (also called sNOR), a family of cytochrome c oxidases suggested to operate as NO reductases for detoxification of NOx (nitrogen oxides) formed in the periplasm (Stein *et al.*, 2007). This nitric oxide reductase was discovered in *B. azotoformans* (Suharti *et al.*, 2001), is present in *Nitrosomonas eutropha* and some other nitrifiers (Stein *et al.*, 2007) and – recently – sequences encoding this gene in type strains of *Bacillus azotoformans*, *Bacillus bataviensis* (Heylen & Keltjens, 2012) and *Bacillus vireti* (Mania *et al.*, 2014) have been published. The reported absence of a *qnorB* gene in some of the strains could indeed be explained by the presence of qCu_ANOR (Table 4. 5), although five of the *cbaA*-containing strains did also harbor *qnorB*. Molecular tools for *cbaA*-detection and assessment of its prevalence in *Bacillus* (and maybe other bacteria) were hitherto not available. As a result this is the first study to report on the successful design of primers that specifically target *cbaA* genes for members of the genus *Bacillus*. Although the successful development of primers for qCu_ANOR has not been previously described, more sequence information, e.g. through whole genome sequencing, is essential to confirm the presence of these nitric oxide reductases in other pure culture denitrifiers and bacteria producing N_2O. Tools for the detection of these and other novel nitric oxide reductases are vital, as N_2O-producing processes – such as the denitrification process and DNRA – are still often monitored cultivation-independently through the study of their functional genes and/or transcripts (Correa-Galeote *et al.*, 2013). At the moment, denitrifying and nitrate-ammonifying N_2O producing guilds harboring qCu_ANOR are completely ignored in these cultivation-independent analyses. In addition, the presence of multiple different *qnorB* genes and both *cbaA* and *qnorB* variants indicate that various soil-born members of the genus *Bacillus* have the strong potential to mitigate endogenously produced or exogenous exposure to NO.

The studied strain set included the denitrifying fen soil isolate *Paenibacillus uliginis* LMG 24790^T (Behrendt *et al.*, 2010) which was reported to not render *nirK* or *nosZ* amplicons with primer sets commonly used in environmental studies (Behrendt *et al.*, 2010). Both *nirK* and *qnorB* genes were detected in this strain with recently published *Geobacillus*-specific *nirK* and *qnorB* primers

(Verbaendert *et al.*, 2014)(Table 4. 1) and even though denitrification gene phylogenies do not correlate with organism phylogenies (Jones *et al.*, 2008), it is possible that other denitrifying paenibacilli possess these distinct *nirK* and *qnorB* variants. The primers designed for Gram-positive bacteria (Verbaendert *et al.*, 2014) for *nirK* and *qnorB* could now enable researchers to (i) explore the fen soils described by Behrendt and colleagues (2010) containing a large fraction of *Paenibacillus* denitrifiers, and (ii) study these denitrifying bacterial communities *in-situ.*

Next to the key denitrification genes encoding nitrite and nitric oxide reductases the presence of *nosZ* was investigated as well. *NosZ* was detected in thirty-one strains (50%) of the *Bacillaceae* strain set. As can be observed from the *nosZ* phylogenetic tree all retrieved *Bacillus nosZ* sequences belong to the 'atypical' *nosZ* cluster (Sanford *et al.*, 2012, Jones *et al.*, 2013)(cluster I, Fig 4. 6) together with *Geobacillus nosZ* (Verbaendert *et al.*, 2014) and *nosZ* of other soil-born *Bacillus* strains (Jones *et al.*, 2011). In contrast with typical *nosZ,* atypical *nosZ* is not only found in denitrifiers but also in bacteria with a more diverse N-metabolism, such as nitrate ammonifiers and bacteria missing *nirK*/*nirS* (Sanford *et al.*, 2012, Mania *et al.*, 2014). However, the ability to reduce nitrous oxide has so far only been detected in very few nitrate ammonifying strains (Simon *et al.*, 2004, Sanford *et al.*, 2012, Mania *et al.*, 2014) and is thought to be uncommon for organisms performing DNRA. Yet, this study revealed that multiple *nrfA*-harboring *Bacillus* strains can harbor atypical *nosZ* and may indicate that this is more common than previously thought, at least in members of the genus *Bacillus*. Recent analyses revealed that atypical *nosZ* genes outnumber typical *nosZ* genes in soil metagenomes (Orellana *et al.*, 2014). Since in the environment unused genes are swiftly purged from genomes by negative selection (Morris *et al.*, 2012), the high abundance of these genes in soils suggests an important functional or ecological role, such as detoxification of N_2O to avoid harmful effects on DNA synthesis mechanisms (Sullivan *et al.*, 2013).

PCR primer pair nrfAF1-nrfA7R1 (Mohan *et al.*, 2004) was recently reported to miss clade P genera – harboring *Bacillus* – relevant to the soil environment (Welsh *et al.*, 2014). Proposed improved primers for *nrfA* detection were reported to *in-silico* amplify *nrfA* from *Bacillus selenitireducens* MLS10 and *Bacillus* sp. 1NLA3E and thus seemed suitable to test on the *Bacillaceae* strain set. Almost half of the strains displayed the presence of *nrfA* (29 out of 62 strains – 46.7%). Ten strains displayed the presence of the correct band together with non-specific amplification, even after modification of the PCR protocol, indicating that more strains may harbor *nrfA* (Table 4. 5). However, probably primer pair nrfAF2aw- nrfA7R1 and/or corresponding PCR conditions were not sufficient for specific annealing to the *nrfA* target in those strains and further optimization may be required. *NrfA* sequences within the strain set were all related to sequences assigned to clade P (Welsh *et al.*, 2014)(Fig 4. 7), explaining the failure to amplify *nrfA* with nrfAF1-nrfA7R1 in the majority of the strains (Table 4. 5). Although nitrate ammonification by members of the genus

Bacillus has been repeatedly suggested, to date, identification of *nrfA* in any *Firmicute* genome is rare (May 2014, http://fungene.cme.msu.edu/) and very few *nrfA* gene sequences from *Bacillaceae* are available in the publicly available sequence databases. Hence, the improved *nrfA* primers of Welsh and co-workers (Welsh *et al.*, 2014) unquestionably shed light on the potential contribution of *Bacillaceae* to DNRA and possible N_2O production in certain ecosystems, such as the examined luvisol soil. *Bacillaceae* are important players of soil microbiological communities and are currently unaccounted for in environmental studies analyzing *nrfA*, *nirK, qnorB* and (typical) *nosZ* abundances and dynamics. Inclusion of their gene sequences in future primer design efforts for environmental studies may help shed light on the contribution of these bacteria to soil N_2O emissions and on the environmental conditions under which they express the involved genes.

The detection of *nirK, nrfA, cbaA*, *qnorB* and *nosZ* genes from the soil-isolated *Bacillus* strains from this study most certainly highlights the resources these strains have to potentially produce N_2O and contribute to soil N_2O emissions under the right environmental conditions, e.g. in the presence of high nitrate concentrations (Mania *et al.*, 2014) or in acidic soils (Bakken *et al.*, 2012). But although sequences for *nir*, *nor*, *cbaA, nos* and *nrfA* gene sequences were retrieved from the studied strains, they proved difficult to amplify within the strain set even after systematic optimization of PCR-protocols and with *Bacillus*-directed improvement or development of PCR primers. This observation may be explained by (1) high inter- and intra-species sequence divergence of the corresponding genes, or (2) the presence of multiple gene copies and/or on mobile elements, such as plasmids. Yet for the latter, direct evidence is scant and only available for Gram-negative bacteria not belonging to the genus *Bacillus* (Cramm *et al.*, 1997, Chan & McCormick, 2004, Coyne *et al.*, 2010, Alvarez *et al.*, 2011). Hence, whole genome sequencing (WGS) of many more Gram-positive denitrifiers and nitrate ammonifiers and detection of the involved functional gene sequences will be necessary to assess this divergence and its origin. Moreover, WGS will provide researchers with information on genetic organization, presence of regulatory genes and consequently insight in genetic evolutionary events such as horizontal gene transfer (HGT), convergent evolution of different structural types, lineage sorting, duplication, …etc. of the involved genes (Jones *et al.*, 2008). As shown for *Geobacillus nirK*, *qnorB* and *nosZ* (Verbaendert *et al.*, 2014) and other taxa and genes (Green *et al.*, 2010, Sanford *et al.*, 2012), group-specific strategies – either building on taxon assignment or gene phylogeny – for development of improved amplification protocols may be warranted as more sequence information becomes available and may solve existing problems with poor primer coverage. However, considering the difficulty with which degenerate primers designed for denitrifying *geobacilli* (Verbaendert *et al.*, 2014) amplified e.g. *nosZ* from the bacilli of this study (Table 4. 5), time and more sequencing efforts will tell how broad the target of primers designed for denitrification and DNRA genes can be. Several strains did not render any of the targeted gene sequences or showed specific

bands in combination with aspecific amplification, making direct sequencing impossible. These observations could also be attributed to the absence of the genes in the strains, but that does not explain the previous observations of N_2O production on complex or defined media (Verbaendert *et al.*, 2011b). Failure probably is due to insufficient PCR procedures – as mentioned above – and the understudied enzymatic redundancy for nitrite reduction, nitric oxide reduction and nitrous oxide reduction in Gram-positive *Bacillaceae*. As a result, to obtain the gene sequences of the enzymes involved in denitrification and DNRA of those cultivated Gram-positive representatives that did not render amplicons remains a challenge.

Agriculture management practices to mitigate N_2O emissions were suggested to possibly benefit from an approach that models N_2O production from denitrifying bacteria in response to environmental parameters (Richardson *et al.*, 2009). The same holds true for N_2O production by nitrate ammonification. To this end, detailed knowledge on the denitrifier and the nitrate-ammonifying metabolism, genes and enzymology is required. Unfortunately, although these growth mechanisms are prevalent among a wide range of prokaryotes, denitrification and DNRA and their regulatory components are almost exclusively studied in model organisms belonging to phylogenetically closely related Gram-negative *Proteobacteria*. An outstanding challenge therefore remains for the development of molecular methods to detect the diversity of nitrite and nitric oxide reductases, specifically in Gram-positive bacteria, that contribute to N_2O concentrations arising from processes in the global nitrogen cycle. Our results demonstrate that soil-isolated members of the phylum *Bacillaceae* contain distinct variants of functional genes involved in denitrification or DNRA and coordinated use of the corresponding enzymes probably depends on the environmental conditions. Hopefully, this study is a step towards further in-depth investigation of both processes in Gram-positive bacteria with the purpose of complementing current knowledge on Gram-negative model denitrifiers and nitrate-ammonifying bacteria and consequent application in modeling N_2O fluxes from soils.

4.5 Acknowledgements

The authors would like to thank Bart Hoste for help with *cbaA* primer design.

Reflection and discussion

Nitrate reducing bacteria belong to physiologically and genetically diverse functional guilds and are grouped by their ability to employ fixed nitrogen compounds as electron acceptors instead of oxygen and to reduce them to gaseous nitric oxide (NO), nitrous oxide (N_2O) and/or nitrogen gas (N_2)(denitrification) or ammonium (NH_4^+)(DNRA). At the time of phenotypic screening of the *Bacillus* reference strain set (Chapter 2), PCR priming sets for denitrification and nitrate ammonification genes targeting Gram-positive bacteria did not exist. In addition, well-known priming sets for *nor* genes failed in PCR amplification for most of the strains and there was a lack of *Bacillus* – and other Gram-positive - whole genome sequences (WGS) containing *nrfA*, *nir*, *nor* and/or *nos* genes. However, the exponential increase of genomic data has revealed the presence of a large functional diversity and redundancy in the wide range of organisms involved in denitrification and DNRA (Shapleigh, 2006, Giles *et al.*, 2012), including in a few Gram-positive denitrifiers (Ueda *et al.*, 2004, Shapleigh, 2006, Pukall *et al.*, 2009, Heylen & Keltjens, 2012). Therefore, this chapter reports on the genetic investigation of parallel pathways for nitrate/nitrite reduction in a strain set of phenotypically denitrifying *Bacillaceae* (mainly comprised of members of the genus *Bacillus*) with well-known and refined primers for *nrfA*, *nir*, *nor* and *nos* – encoding nitrite, nitric oxide and nitrous oxide reductases – and novel primers for *cbaA* – encoding qCu_ANOR. Analyses with well-known primers often used in environmental monitoring of denitrifying or nitrate-ammonifying communities were for the most part ineffective. This was not unexpected because of the high sequence divergence between existing primers and *Bacillaceae* denitrification genes (Verbaendert *et al.*, 2014). However, the unsuccessful use of degenerate primers designed for denitrification genes of *Geobacillus*, a genus closely related to *Bacillus*, suggest that broad-range primers are unlikely ever to be succesfully developed, even for specific phyla (e.g. *Bacillaceae*) or for specific groups of phylogenetically related gene sequences. In addition, many of the strains exhibited the presence of *nosZ* together with genes associated with DNRA in *Bacillus* (*nrfA* and *cbaA*). Thus results also indicate that *Bacillaceae* can be important contributors to nitrate and nitrite reduction in terrestrial and possibly other ecosystems and that even at organismal level nitrate or nitrite may have different fates, probably depending on the presented environmental conditions. In addition, they may be a potent and abundant source of nitrogen losses in soils and N_2O emissions, since both denitrification and DNRA are processes that may produce N_2O depending on the genetic content of the present microorganisms and the present abiotic environmental conditions. This research may help to close some gaps, although maybe small,

in our knowledge that currently preclude an understanding of the microbial diversity of N-cycle dynamics in soils.

We have successfully assessed the presence of denitrification and/or DNRA in a set of nitrate and/or nitrite reducing and N_2O-producing bacilli by PCR-amplification of the associated genes with existing and newly developed primers, but certain observations have only invoked more questions and/or concerns:

Firstly, primer design for the *Bacillus* denitrification genes *nirK*, *qnorB* and *nosZ* was based on primer target sites covering the same regions as described before, but in *Bacillus* WGS. Successful primers for denitrification genes seemed to be difficult to design and refined *nrfA* primers often showed aspecific amplification, a problem most likely resulting from the high sequence divergence even in gene copies of closely related bacilli and from annealing to similar non-target sequences. Switching to other conserved gene regions within the involved gene sequences, instead of using the same target regions, may have improved primer specificity and hence might have resulted in better amplification and subsequent sequencing. As mentioned before, for ten strains the refined *nrfA* primers of Welsh and co-workers (2014) yielded an amplicon of the targeted size accompanied by a larger aspecific band. On the basis of the alignment of *Bacillus nrfA* and the two available primer pairs, it was clearly needless to alter the primer sequences of Welsh *et al.* (2014) because it would not significantly improve the specificity of the primers (Fig 4. 8). However, the primer target site of the forward primer of Mohan *et al*. (2004) did show a higher number in degenerate positions of and clear mismatches with *Bacillus nrfA* gene sequences and may present an alternative target for improved *nrfA* primers for *Bacillaceae* (Fig 4. 8).

Secondly, what is detected in culture-independent microbial community analyses is ultimately determined by the use of primers. Several reports in literature have highlighted the unreliability of available primers as broad-range amplification primers because of their bias to amplification of certain genes (Behrendt *et al.*, 2010, Green *et al.*, 2010, Verbaendert *et al.*, 2011a, Sanford *et al.*, 2012). Hence, the shortcomings of available primers should always be kept in mind when used (Giles *et al.*, 2012). Of course, this also applies to the primers of the study described in this chapter (and Chapter 3). Since they have been selectively designed for members of the genus *Bacillus* – and by extension – other *Bacillaceae* in mind, these primers might result in biased PCR amplification should they be used for broad-spectrum analysis. Yet they certainly contribute to the rather limited multitude of primers available for environmental monitoring of activity and abundance of N_2O and N_2 producing Gram-positive bacteria belonging to the *Bacillaceae*.

MM, mismatch
***, 4-fold degeneracy
**, 3-fold degeneracy
*, 2-fold degeneracy

FORWARD

Organism name	Primer site nrfAF1 (Mohan *et al*. 2004)	MM	*	***	Primer site nrfAF2aw (Welsh *et al*. 2014)	MM	*	**
	G C N T G Y T G G W S N T G Y A A →				C A R T G Y C A Y G T B G A R T A →			
Bacillus selenitireducens MLS10	T C G T G C A T G A C G T G T A A	3	4	2	C A A T G T C A C G T G G A G T A	0	4	1
Bacillus sp. 1NLA3E	T C C T G T T T A A C A T G T A A	3	4	2	C A A T G T C A T G T T G A G T A	0	4	1
Bacillus azotoformans LMG 9581[T]	T C T T G T T T A A C T T G T A A	3	4	2	C A A T G T C A T G T A G A A T A	1	4	0
Bacillus *bataviensis* LMG 21833[T]	T C T T G T T A T A C A T G T A A	3	4	2	C A G T G T C A T G A T G A A T A	1	4	1
Bacillus vireti LMG 21834[T]	T C T T G C T A T A C C T G T A A	3	4	2	C A G T G C C A T G A T G A A T A	1	4	1
	5' T C T T G Y W T D A C N T G T A A				C A R T G Y C A Y G W D G A R T A			
	1[st] heme binding domain				part of 3[rd] heme binding domain			

REVERSE

Organism name	Primer site nrfAF1 (Mohan *et al*. 2004, Welsh *et al*., 2014)	MM	*
	← G A Y T G Y C A Y A T G C C N W A		
Bacillus selenitireducens MLS10	G A T T G T C A T A T G C C T T A	2	3
Bacillus sp. 1NLA3E	G A T T G T C A T A T G C C G T A	2	3
Bacillus azotoformans LMG 9581[T]	G A T T G T C A C A T G C C T T A	2	3
Bacillus *bataviensis* LMG 21833[T]	G A T T G C C A T A T G C C A T A	2	3
Bacillus vireti LMG 21834[T]	G A C T G C C A T A T G C C A T A	2	3
	G A Y T G Y C A Y A T G C C D T A 3'		
	part of 4[th] heme binding domain		

Fig 4. 8 Alignment of selected *nrfA* sequences of bacilli with published *nrfA* primers. Primer sequences are shown on the top line with specific primer names shown above the arrows. The color code indicates mismatches and 2-, 3- and 4-fold degeneracy at nucleotide positions with the respective primers. Potential updated primers are indicated in blue.

Thirdly, the *nosZ* detected in the *Bacillus* strain set strikingly only belonged to the Sec-dependent 'atypical' *nosZ* gene variant. The reasons for this preference are unclear. N_2O can be toxic to the cellular metabolism due to binding and inactivation of VitB12 which is essential for a.o. DNA synthesis (Sullivan *et al.*, 2013). As the genetic potential for nitrate ammonification seems to be quite prevalent in bacilli, they may be, as a consequence, potent N_2O producers under high nitrate concentrations, e.g. as *B. vireti* (Mania *et al.*, 2014). Presence of the atypical *nosZ* may hence prevent N_2O accumulation and damage to the cells in non-denitrifying conditions. Or members of the genus *Bacillus* may not have VitB12-independent mechanisms for DNA synthesis and thus *nosZ* may have been conserved because of a selection pressure present in the luvisol soil other than generation of a proton motive force during dissimilatory reduction of nitrate by denitrification (Sullivan *et al.*, 2013). It is remarkable however that *Geobacillus* (Chapter 3) and *Bacillus*, both belonging to the *Bacillaceae*, carry this atypical *nosZ*. Further research is needed to clarify whether this may be a reoccurring theme in denitrifying and/or nitrate ammonifying *Bacillaceae* and it raises interesting questions which bear on interpretation of *nosZ* sequences amplified directly from the environment belonging to either type.

Fourthly, because of the modularity and wide-spread horizontal gene transfer of the functional genes involved in denitrification, PCR-based detection of one or more of the genes does not necessarily imply that the bacteria under scrutiny are able of 'true' denitrification. For this, detailed phenotypic

analysis of the organisms studied is necessary. The phenotypic experiments in Chapter 2 provided us with data verifying the potential for denitrification in many strains, however, part of the strain set was isolated and functionally tested on complex medium or medium containing nitrite as main nitrogen source and it was unclear whether the high N_2O production could also have originated from DNRA. Therefore, next to the analyses of functional genes, we have performed a series of preliminary experiments on 26 of the strains studied in this chapter aiming to clarify the end-point denitrification phenotype (Table 4. 6). The majority of strains were originally isolated and tested on two defined media, G_2M_{11} (3mM KNO_2, 15 mM sodium succinate) and G_4M_3 (3mM KNO_3, 15 mM sodium succinate), or their recommended complex medium (TSB or other, supplemented with 10mM KNO_3) (Heylen *et al.*, 2006, Heylen, 2007, Verbaendert *et al.*, 2011b)(Table 4. 6). The recommended growth medium for many Bacilli is nutrient agar/broth (NA/NB) and trypticase soy agar/broth (TSA/TSB) (BCCM/LMG Bacteria Collection, http://bccm.belspo.be/about-us/bccm-lmg) and members of the genus *Bacillus* are reported to require complex growth requirements (Pichinoty *et al.*, 1976). However, complex medium – such as TSB – provides the ideal conditions for anaerobic fermentation rather than denitrification and some *Bacillus* members are also renowned for their ability to ferment. Furthermore, other than denitrifiers, bacteria that conduct DNRA are frequently capable of fermentation processes (Tiedje, 1988, Mohan *et al.*, 2004, Mohan & Cole, 2007, Kraft *et al.*, 2011). A limited experiment to assess potential fermentative growth on TSB without nitrate or nitrite in batch cultures of part of the strain set revealed that most of these were able to ferment (Table 4. 6). As a consequence, a growth medium that is less prone to anaerobic growth other than by denitrification was preferred for the preliminary experiments. Thus to avoid phenotypic plasticity within the cell-cultures and to provide the bacilli with the required growth factors, modified mineral medium (mMM) (as presented in Chapter 3 for *Geobacillus*) was used and the reduction of nitrate to gaseous products was determined by end-point measurements of N_2O and/or N_2 after 4 days (triplicate experiment). *Bacillus azotoformans* strains and *Bacillus bataviensis* strains LMG 21833^T and LMG 21832^T were all able to fully denitrify nitrate to N_2 or N_2O, as well as several other strains isolated from luvisol soil (Table 4. 6). Many other strains produced N_2O, or for *Bacillus lehensis* LMG 24751^T N_2, in small quantities ranging from 1.5 - 6.7% of the amended nitrate. These latter results contrast sharply with the previously measured N_2O concentrations in complex medium or the initial isolation medium (Chapter 2) and could potentially be explained by either (i) still restricted growth – and denitrification – on the provided mineral growth medium, or (ii) the presence of DNRA and production of N_2O as a by-product (Streminska *et al.*, 2012). The second hypothesis may explain the high N_2O concentrations in the initial experiments since TSB is reported to favor growth of nitrate ammonifiers (Smith & Zimmerman, 1981, Tiedje *et al.*, 1982) and N_2O can be a substantial by-product

Table 4. 6 Preliminary experiments on *Bacillaceae* previously reported to denitrify

Species	Strain n°	Biological origin	Growth temp (°C)	Initial isolation medium[a]	Ferm[b]	N_2O and/or N_2 production on mMM[c]
B. asahii	LMG 24728^T	soil, Shizuoka Prefecture, Japan	28°C	n/a[c]	+	n/d
B. atrophaeus	LMG 8199^{T}t$_1$	unknown	28°C	n/a	+	n/d
B. atrophaeus	LMG 8199^{T}t$_2$	unknown	28°C	n/a	+	n/d
B. azotoformans	LMG 9581^T	garden soil, France	28°C	n/a	(+)	full, N_2
B. azotoformans	LMG 15445	garden soil, France	28°C	n/a	(+)	full, N_2
B. azotoformans	LMG 15448	garden soil, France	28°C	n/a	(+)	full, N_2
B. azotoformans	LMG 15449	garden soil, France	28°C	n/a	(+)	full, N_2
B. bataviensis	LMG 21833^T	soil, unused hay field, Drentse A grasslands, the Netherlands	28°C	n/a	+	full, N_2O
B. bataviensis	LMG 21832	soil, unused hay field, Drentse A grasslands, the Netherlands	28°C	n/a	+	full, N_2O
B. bataviensis	R-31541	luvisol soil, Melle, Belgium	28°C	TSA	n/d	n/d
B. bataviensis	R-31770	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	n/d
B. bataviensis	R-31834	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. bataviensis	R-32700	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	n/d
B. bataviensis	R-32709	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	n/d
B. bataviensis	R-32779	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	n/d
B. bataviensis	R-32781	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	n/d
B. bataviensis	R-32787	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. bataviensis	R-32845	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. drentensis	R-31547	luvisol soil, Melle, Belgium	28°C	G_4M_3	-	full, N_2
B. drentensis	R-31550	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. drentensis	R-31846	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. drentensis	R-32528	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	full, N_2
B. drentensis	R-32575	luvisol soil, Melle, Belgium	28°C	TSA	-	full, N_2
B. drentensis	R-32702	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	full, N_2
B. drentensis	R-32705	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	full, N_2
B. drentensis	R-32789	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. drentensis	R-33773	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	full, N_2
B. indicus	LMG 22858^T	arsenic polluted sand, Chakdah district West Bengal, India	28°C	n/a	+	n/d
B. infantis	LMG 24756^T	blood of newborn child with sepsis, Republic of Korea	28°C	n/a	+	n/d
B. lehensis	LMG 24751^T	soil, Leh, India	28°C	n/a	+	N_2
B. licheniformis	LMG 17339	potato pulp for cattle feeding	37°C	n/a	+	N_2O
B. licheniformis	LMG 17340	potato pulp for cattle feeding	37°C	n/a	+	N_2O
B. licheniformis	LMG 6934	unknown	37°C	n/a	+	N_2O
B. licheniformis	LMG 7559	unknown	37°C	n/a	+	N_2O
B. licheniformis	LMG 7561	field soil	37°C	n/a	+	N_2O
B. licheniformis	LMG 7633	chinchilla, feces	37°C	n/a	+	N_2O
B. licheniformis	R-31769	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. licheniformis	R-32706	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. megaterium	LMG 7127^T	unknown	28°C	n/a	+	n/d
B. methanolicus	LMG 24730^T	unknown	52°C	n/a	+	n/d
B. mojavensis	LMG 22477	river Vélez, river mouth, Malaga, Spain	28°C	n/a	+	n/d
B. mycoides	LMG 7128^T	soil, Germany	28°C	n/a	+	n/d
B. plakortidis	LMG 24732^T	sponge (Plakortis simplex), Norway	28°C	n/a	+	n/d
B. pseudomycoides	R-31830	luvisol soil, Melle, Belgium	28°C	TSA	+	N_2O
B. soli	R-31553	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	+	full, N_2
B. soli	R-31841	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. soli	R-32715	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. soli	R-32849	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. soli	R-32526	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. drentensis	R-32656	luvisol soil, Melle, Belgium	28°C	G_4M_3	n/d	n/d
B. soli	R-32694	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
B. soli	R-33820	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d
Bacillus sp.	R-39623	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown	+	N_2O
Bacillus sp.	R-39624	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown	+	N_2O
Bacillus sp.	R-39625	sequencing batch reactor, Montevideo, Uruguay	28°C	unknown	+	N_2O
B. thuringiensis	LMG 12265	unknown	28°C	n/a	+	N_2O
B. vietnamensis	LMG 24742^T	Vietnamese fish sauce, Japan	28°C	n/a	+	n/d
B. vireti	LMG 21834^T	soil, unused hay field, Drentse A grasslands, the Netherlands	28°C	n/a	+	N_2O[d]
Paenibacillus sp.	R-27048	activated sludge, Belgium	28°C	n/a	n/d	n/d
Paenibacillus uliginis	LMG 24790^T	fen peat soil from a nitrogen fertilization long-term experiment in Paulinenaue, Germany	28°C	n/a	+	n/d
Staphylococcus sp.	R-34181	luvisol soil, Melle, Belgium	28°C	G_2M_{11}	n/d	n/d

[a] In (Heylen, 2007, Verbaendert *et al.*, 2011b)
[b] Anaerobic growth on TSB without added nitrate: +, after 24h, (+) after 72h, -, after 72h
[c] Experiments were performed in triplicate. mMM, modified mineral medium (Verbaendert *et al.*, 2014), full N_2/N_2O, stoichiometric reduction of nitrate to nitrogen gas (N_2) or nitrous oxide (N_2O), N_2/N_2O, reduction of nitrate to N_2 or N_2O but in non-stoichiometric quantities
[d] Reported to be a nitrate ammonifier that reduces nitrate to ammonia with potent N_2O production under high nitrate concentrations and late onset of *nosZ* transcription (Mania *et al.*, 2014)
n/a, not applicable
n/d, not determined

of detoxification (Giles *et al.*, 2012), especially when cells are incubated for longer periods of time and arrive in their stationary growth phase (Smith & Zimmerman, 1981, Smith, 1983). In addition, the majority of the strains producing N_2O were isolated from luvisol soil on G_2M_{11} medium with nitrite as main nitrogen source. Since DNRA *sensu stricto* is the respiratory reduction of nitrite to ammonium (Simon & Klotz, 2013), use of G_2M_{11} may have led to nitrite selection of nitrate ammonifiers and may be the reason for the prevalence of DNRA in our strain set. Unfortunately, this could not always be substantiated with succesful sequencing of *nrfA*. Thus further investigation of the physiological conditions under which optimal growth and denitrification and/or DNRA occurs is an absolute necessity.

Finally, the nitrate metabolism, more specifically denitrification and DNRA, are underexplored in *Bacillaceae*. Little is known, yet the genomes of denitrifying and nitrate-ammonifying *Bacillus* (Heylen & Keltjens, 2012, Mania *et al.*, 2014) were reported to contain a range of genes involved in N-cycling, such as *napAB*, *narGHIJ*, *nirK*, *qnorB*, *cbaA* and *nosZ* for denitrification, *nrfAH* for DNRA and *nasC* and *nirBD* for assimilation and this in different assortments. Hence, physiologically, the fate of nitrate in *Bacillus* can be variable. Nitrate can be reduced by both assimilatory and dissimilatory nitrate reductases to nitrite. This potentially toxic nitrite needs to be further reduced to NO. Major sources of NO in nitrate-reducing bacteria have been suggested to be: (1) the membrane-bound nitrate reductase NarG or the assimilatory nitrite reductase NirB (Smith & Zimmerman, 1981, Bleakley & Tiedje, 1982, Rowley *et al.*, 2012), (2) the NrfA nitrite reductase involved in DNRA (Vine & Cole, 2011), and (3) copper-containing dissimilatory nitrite reductase CuNiR. However, not all mechanisms are well understood. Since NO is a cytotoxin, it needs to be immediately neutralized to the more harmless nitrous oxide. This step can be performed by a multitude of nitric oxide reductase enzymes, which have either been described in detail or deduced from full genome sequences (van der Oost *et al.*, 1994, Zumft, 2005, Hino *et al.*, 2010, Stein & Klotz, 2011, Vine & Cole, 2011, Heylen & Keltjens, 2012). Additionally, the use of sec-dependent *nosZ* by soil microbiota such as Gram-positive *Bacillaceae* (Heylen & Keltjens, 2012, Mania et al., 2014, Verbaendert et al., 2014) for denitrification and detoxification of N_2O (Sullivan *et al.*, 2013) has recently been described. Hence, physiologically there is a legion of possibilities to discard toxic N-cycling intermediates in *Bacillaceae*. This was also revealed by the results of this study, suggestive of versatile N_2O-producing and -reducing abilities at least in those strains retrieved from soils. This variable and flexible N-metabolism may provide *Bacillus* and related Gram-positive denitrifiers with specific advantages over those lacking them in fluctuating environmental conditions and may modulate a more rapid response to environmental stress. In addition, some strains exhibited a

redundancy for nitric oxide reductases similar to that of *B. azotoformans* LMG 9581^T, revealing the many possibilities *Bacillus* may have for the detoxification of NO and thus the production of N_2O.

The dissimilatory N-cycling processes denitrification and DNRA both use nitrate as the initial electron acceptor and no validly confirmed physiological experiments of bacteria that have the potential to conduct both processes have been performed, nor have the conditions under which they express either process been elucidated. For *Bacillus*, only genomic information for two type strains that have gene inventories for both processes is available and only the physiological response of the nitrate-ammonifying type strain of the Gram-positive *Bacillus vireti* LMG 21834^T to high concentrations of nitrate has been investigated in detail and corroborated by sequence analysis of its whole genome (Mania *et al.*, 2014). Hence, many interesting experiments on the differential response of the two processes to environmental factors, such as pH, availability of oxygen, nitrate, nitrite and carbon, ..etc., are still indispensable to clarify the potential role of *Bacillaceae*, as important soil microbiota, in N-cycling and soil N_2O emissions. Further research may include transcriptomics, proteomics and knock-out experiments on genes of key enzymes of both pathways. Clearly, for this, selection of some model organisms from this specific phylum would enable researchers to better address the abovementioned issues without interference of the often observed taxon- and/or organism-specific variations.

Part IV

Epilogue

Chapter 5

Concluding remarks

Spore-forming bacteria of the genera *Bacillus* and *Geobacillus* are ubiquitous, their spores and vegetative cells have been obtained from virtually all parts of both the Earth's surface and subsurface represent one of the most resilient and longest-lived cells on the planet (Nicholson *et al.*, 2000, Nicholson, 2002). Bacilli have been called "Kings without crowns" (Tzeneva, 2006) because they merit more scientific attention in research aiming to unravel their eco-physiology and functionality as major players of the soil microbiota. This especially holds true for their capabilities in N-cycling, their contribution to rising global concentrations of the 'forgotten' greenhouse gas N_2O, their responses to human-induced environmental changes (e.g. the input of large amounts of reactive nitrogen) and their genetic make-up to deal with these fluctuations.

The concepts of this thesis were:

(1) to evaluate the information available on Gram-positive denitrifiers and highlight that this group of bacteria is being underexplored because of specific obstacles hampering accurate phenotypic and molecular detection of denitrification within this group of bacteria

(2) to screen soil-isolated *Bacilli* and a large set of *Bacillus* strains of the BCCM/LMG Bacteria Collection from a wide variety of origins for the potential of dissimilatory nitrate reduction with focus on denitrification and the effect of different electron donors and acceptors in the growth medium on the occurrence of the trait

(3) to screen pure cultures of Gram-positive denitrifiers belonging to *Bacillus* and *Geobacillus* for known denitrification and DNRA genes with existing molecular tools and to develop novel molecular tools for the detection of more divergent types of denitrification genes present in Gram-positive denitrifiers

(5) to test the novel molecular tools on the collected strain set of denitrifying *Bacillus* and *Geobacillus* strains.

Our research led to the isolation of surprisingly many N_2O producing – potentially denitrifying or nitrate ammonifying – strains from the genus *Bacillus* from soil and to the discovery of the

potential for denitrification and DNRA in a considerable fraction of a large *Bacillus* reference strain set comprised of many different species. Moreover, the studied strains displayed different phenotypes in nitrogen cycling physiology depending on the used isolation or growth medium with a preference for specific electron donors and/or acceptors. In addition, twenty-one *Geobacillus* strains from various origins were substantiated to have phenotypic denitrification abilities. However, screening of both *Geobacillus* and *Bacillus* strain sets with established priming pairs for denitrification genes (*nir, nor* and *nos*) was unsuccessful. Sequence comparison of priming sites of the existing molecular tools prominently exposed the general inaptness of these tools to pick up denitrification genes that are harbored by denitrifying members of the genus *Geobacillus* and other Gram-positive denitrifiers from the *Bacillaceae* phylum. Novel *nirK*, *qnorB, cbaA* and *nosZ* primers were developed on whole genome sequence information available for *Geobacillus* and *Bacillus*. Our studies showed that these were effective in retrieval of up-till-now uncharted denitrification genes from a subset of *Geobacillus* and *Bacillus* strains and can now be used in cultivation-independent research. The combination of novel primers for detection of genes involved in denitrification and in DNRA, with the successful development of primers for the genes encoding qCu_ANOR, revealed that functional modules for denitrification and DNRA occur simultaneously in soil-derived *Bacillus* strains and that development of broad-range primers for denitrification and DNRA genes will probably be rather challenging.

It can be concluded from this thesis that our knowledge on dissimilatory nitrate reduction, such as the denitrification process, in Gram-positive *Bacillaceae* is far from complete, hence many discoveries are still to be made by studying as much facets as possible of the genetic modules involved in denitrification and DNRA and of the broad diversity in physiology of Gram-positive bacteria performing these processes, regardless of whether they belong to the *Bacillaceae* or other taxa.

The last decade, many culture-independent methodologies have emerged as a means to study microbial genetic function and ecological roles of microorganisms in ecosystems. However, the cultivation of denitrifiers and nitrate ammonifiers will remain indispensable for future research because of the inherent limitations of these DNA-based high-throughput sequencing methods, such as the inability to differentiate between metabolically (in)active and dead microorganisms, poor primer coverage of primers used for amplification of functional genes and the presence of intragroup heterogeneity, the effect of DNA extraction methods (Cruaud *et al.*, 2014), the restricted associations that can be made between specific organisms and their ecological functions in a given biological context (Wang *et al.*, 2012) and the limited information that can be gained on interactions between members of the investigated ecosystem. In addition, to attempt to understand the entire complexity

of an ecosystem, including predictions on ecosystem functioning, metagenomic approaches should be accompanied by metatranscriptomics and metaproteomics, but such integrative studies will be challenging as well because of the associated data complexity. However, cultivated isolates, their genes and their associated genome sequences constitute a crucial link between these environmental observations and phenotypic capabilities because functions of environmental sequences are predicted based on homology with characterized genes in reference databases (Rappé, 2013). This type of functional inference is even relied on heavily by metagenomics, transcriptomics and proteomics (Rappé, 2013), thus, cultivation most definitely contributes to the interpretation of physiological as well as (meta) genomic data of denitrifying and ammonifying microbiota.

Although N_2O is a long-lived and potent greenhouse gas and an important contributor to the greenhouse gas effect, it has long been neglected by environmentalists and it has been referred to as 'the forgotten greenhouse gas'. The contribution, however, of microbial sources to increasing global N_2O emissions and the conditions under which this greenhouse gas is released by bacteria in different processes are often not fully considered in soil N budgets because of the lack of knowledge (Streminska *et al.*, 2012). As mentioned before, functional modules of genes typically designated to DNRA or denitrification both occurred in soil-isolated *Bacillus* strains, warranting further phenotypic experiments such as detailed gas kinetics (Molstad *et al.*, 2007), associated transcription analyses and possible knock-out experiments which may provide answers on the contribution of soil inhabitants of this Gram-positive group of bacteria to soil N_2O emissions. In general, systematic gathering of phenotypic datasets and more extensive physiological research in order to measure activities from a range of denitrifying and other nitrate respiring Gram-positive bacteria is most certainly needed. Particular attention should be paid to assessment of these traits in a standardized matter, e.g. for incubation conditions and media. This way we may reveal important trait distributions within this type of bacteria and uncover more on the regulation of the processes in these soil microbial players that until now have been disregarded. This kind of physiological research may complement the construction of biochemical models that predict responses of microbial communities and thereby greenhouse gas emissions.

Many genome sequencing projects of the past years have rendered more complete denitrification gene sequences and have uncovered a large sequence divergence, but the generation of more whole genome sequences of Gram-positive denitrifiers is still required for a better understanding of the sequence divergence of all denitrification genes and the incidence of certain gene types in this group of bacteria. This genome-derived information could address the existing and problematic poor primer coverage that is most likely rendering measures of diversity and quantification of denitrifiers *in-situ* into underestimates. In addition, the underrepresentation of

functional community components, such as for the *Bacillaceae*, through primer selection may mean that links to important abiotic variables are missed which affects which gene sequence groups – and maybe related taxa – are amplified (Giles et al 2012).

More extensive physiological experiments and whole genome sequencing should not only be performed for denitrifying and nitrate ammonifying *Bacillaceae* but also for other groups of organisms for a number of reasons. First of all, data on the presence of genes and data from whole genome sequences or even meta-omics can provide a first insight in how an organism, consortia of organisms or bacteria in certain environments may contribute to N_2O production by certain N-cycling processes and/or what environmental parameters may trigger which responses. For example, the exponential increase of genomic and data has indicated that the N-cycle processes of denitrification, DNRA and nitrification involve highly diverse functional modules, some of which are shared between these processes. The acknowledgement that N-transformation modules can be acquired and used individually are important for high throughput sequencing technologies, such as metagenomics, because many historically used molecular markers are not unambiguous indicators for particular N-cycle pathways. Secondly, functional traits have been described as the determinants of species interactions in ecosystems and thus ecosystem functioning, yet the coherence between phylogeny and the distribution of functional traits is still heavily debated in microbial ecology and the extent to which functional traits are phylogenetically conserved remains unclear. If traits are conserved at least for some microbial groups, phylogenetic diversity could serve as a proxy for functional diversity (Krause *et al.*, 2014). Thirdly, whole genome analysis has confirmed that N_2O can come from multiple processes that reduce nitrate in a dissimilatory fashion at organismal scale. These observations indicate the need for tangible and accurate physiological context. As such, it may be very informative to go back to our culture collections and check for co-occurrence of taxonomic members in functional modules and, vice versa, gather information needed on the co-occurrence of traits within taxonomic ranks. Hence, more-high throughput and more detailed surveys of phenotypic characteristics of microbial taxa may lead to a more comprehensive understanding of the identities and activities of microorganisms involved in N-cycling, particularly in soils. Researchers, however, should take into account the time-consuming factors and trial-and-error that are involved in using batch and continuous cultures to follow the fate of N, such as finding suitable concentrations of substrate to stimulate denitrification or DNRA, pinpointing the suitable growth phase for monitoring activity, controlling changes to the growth media as cells metabolize and investigation of other cultivation parameters such as pH, media composition, temperature, rate of NOx formation, shaker speed, etc… (Stein, 2011).

Many *Bacillaceae* members have been cultivated and preserved in our culture collections and have been studied for many aspects, yet researchers do not often (re-)use this wealth of already collected microbial resources to perform in-depth analysis on specific strains or strains isolated in specific conditions, in particular for the dissimilatory nitrogen metabolism. I believe that N-cycle research would surely benefit from exploration of our culture collections. There are still many missing pieces of the denitrification and DNRA inventory of genes and enzymes and their regulatory mechanisms that are yet to be discovered. Therefore, microbiologists studying denitrification and DNRA should really make an effort to archive the microbial diversity capable of these dissimilatory nitrate reduction processes and generate some kind of a microbial trait database that may in the future serve as a basis for extrapolation of physiological experiments in the laboratory to natural environments to attempt to solve problems like increasing atmospheric N_2O concentrations. In that respect we should maybe consider to study more Gram-positive bacteria as model organisms for a.o. denitrification and DNRA. Since microbes – and by extension microbial communities – are considered as key variable in how natural and anthropogenic ecosystem disruptions, such as climate change, affect ecosystem functioning, gaining better knowledge on dissimilatory nitrate and nitrite reduction in Gram-positive bacteria will provide more answers on (i) how these organisms contribute to denitrification and DNRA in the environment, and (ii) which environmental controls influence their propensity to emit greenhouse gases.

Part V

Appendices

Table S1. Summary of host organisms, protein ID, gene locus (when applicable) and accession numbers (with contig, copy and/or secondary replicon) of *nirK, norB* and *nosZ* sequences used in the phylogenetic analyses.

nirK

Species	Strain number	Gene locus tag	Protein ID	Accession number
Acaryochloris marina	MBIC11017	-	-	-
Acaryochloris sp.	CCMEE 5410	-	-	-
Achromobacter arsenitooxidans	SY8	KYC_10256	EHK66522	AGUF01000040 (Contig00041)
Achromobacter cycloclastes	unknown	-	-	-
Achromobacter cycloclastes	ACCC 03051	**n/a**	**ADK76188**	**HM060298**
Achromobacter cycloclastes	ATCC 21921 = IAM 1013	**n/a**	**CAA88564**	**Z48635**
Achromobacter denitrificans	LMG 1231^{T}	**n/a**	**CAJ76712**	**AM230821**
Achromobacter sp.	DBTN3	-	-	-
Achromobacter xylosoxidans	A8	AXYL_02390	ADP15711	CP002287
Achromobacter xylosoxidans	AXX-A	AXXA_20893	EGP44463	AFRQ01000082 (contig00092)
Achromobacter xylosoxidans	C54	HMPREF0005_05372	EFV87356	ACRC01000039 (cont 1.39)
Achromobacter xylosoxidans	GIFU1051	**n/a**	**BAA33678**	**AB013078**
Acidilobus saccharovorans	345-15	-	-	-
Acidovorax delafieldii	2AN	-	-	-
Acidovorax ebreus	TPSY	-	-	-
Acidovorax sp.	JS42	-	-	-
Acidovorax sp.	NO-1	-	-	-
Actinobacillus minor	202	AM202_01885	EEV24935	ACFT01000086 (Contig_47)
Actinobacillus minor	NM305	AM305_00399	EER46470	ACQL01000109 (contig_2)
Actinobacillus pleuropneumoniae sv. 1	str. 4074	appser1_50	EFM86442	ADOD01000001 (contig00046)
Actinobacillus pleuropneumoniae sv. 2	str. S1536	appser2_20740	EFM86596	ADOE01000048 (contig00055)
Actinobacillus pleuropneumoniae sv. 2	str. 4226	APP2_0133	EFL79427	ADXN01000001 (Contig1)
Actinobacillus pleuropneumoniae sv. 3	str. JL03	APJL_2086	ABY70631	CP000687
Actinobacillus pleuropneumoniae sv. 4	str. M62	appser4_50	EFM90771	ADOF01000001 (contig00056)
Actinobacillus pleuropneumoniae sv. 5b	L20	APL_2035	ABN75109	CP000569
Actinobacillus pleuropneumoniae sv. 6	str. Femo	APP6_0040	EFL80617	ADXO01000012 (Contig2)
Actinobacillus pleuropneumoniae sv. 7	str. AP76	APP7_2122	ACE62774	CP001091
Actinobacillus pleuropneumoniae sv. 9	str. CVJ13261	appser9_50	EFM95169	ADOI01000001 (contig00012)
Actinobacillus pleuropneumoniae sv. 10	str. D13039	appser10_160	EFM97327	ADOJ01000001 (contig00023)
Actinobacillus pleuropneumoniae sv. 12	str. 1096	appser12_160	EFN01625	ADOL01000001 (contig00075)
Actinobacillus pleuropneumoniae sv. 13	str. N273	appser13_21320	EFN01709	ADOM01000053 (contig00007)
Actinobacillus succinogenes	130Z	Asuc_0978	ABR74346	CP000746
Actinobacillus suis	H91-0380	ASU2_07220	YP_006817821	NC_018690, chromosome
Actinobacillus ureae	ATCC 25976	HMPREF0027_0689	EFX92260	AEVG01000049 (contig00049)
Actinomyces coleocanis	DSM 15436	HMPREF0044_0006	EEH64269	ACFG01000004 (contig00004)
Actinomyces odontolyticus	ATCC 17982	ACTODO_00575	EDN80136	AAYI02000004 (Actinomyces_odontolyticus-2.0_Cont201.13)
Actinomyces sp.	oral taxon 170 str. F0386	-	-	-
Actinomyces sp.	oral taxon 171 str. F0337	HMPREF9057_00320	EFW28277	AECW01000037 (A_sp_Oral_taxon_171_F0337-1.0_Cont44.1)
Actinomyces sp.	oral taxon 178 str. F0338	HMPREF9005_1248	EFW09772	AEUH01000145 (contig00145)
Actinomyces sp.	oral taxon 448 str. F0400	-	-	-
Actinomyces sp.	oral taxon 849 str. F0330	HMPREF0975_00449	EHM95490	ACTB01000030 (cont1.30)
Actinomyces urogenitalis	DSM 15434	HMPREF0058_2208	EEH64934	ACFH01000202 (contig00226)
Actinosynnema mirum	DSM 43827	Amir_4752	ACU38581	CP001630
Aequorivita sublithincola	DSM 14238	Aeqsu_0109	AFL79638	CP003280
Afipia sp.	1NLS2	AfiDRAFT_0466	EFI52480	ADVZ01000001 (ctg00009)
Aggregatibacter aphrophilus	ATCC 33389	-		-
Aggregatibacter aphrophilus	F0387	-		-
Aggregatibacter aphrophilus	NJ8700	-		-
Aggregatibacter segnis	ATCC 33393	-		-
Rhizobium radiobacter	CCNWGS0286	ATCR1_08969	EHH07078	AGSM01000004 (contig00004)
Rhizobium radiobacter	str. C58	Atu4382	AAK89058	AE007870, linear chromosome
Alcaligenes faecalis	S-6	**n/a**	**BAA02440**	**D13155**
Alcaligenes faecalis subsp. *faecalis*	LMG 1229^{T}	**-**	**-**	**n/i**
Alcaligenes faecalis subsp. *faecalis*	NCIB 8687	QWA_07574	EJC62911	AKMR01000006 (Contig_7)
Achromobacter sp.	DSM 30128	-	-	**n/i**
Alcaligenes sp.	STC1	**n/a**	**BAB21510**	**AB046603**
Achr. xylosoxidans subsp. *xylosoxidans*	NCIMB 11015	**n/a**	**AAC05831**	**AF051831**
Alcanivorax dieselolei	N1203	-	-	n/i
Alcanivorax dieselolei	B5	-	-	-
Alicycliphilus denitrificans	BC	-	-	-
Alicycliphilus denitrificans	K601	-	-	-
Alicycliphilus sp.	R-24604	-	-	-
Alicycliphilus sp.	R-24606	-	-	-
Alicycliphilus sp.	R-24611	-	-	-
Alicycliphilus sp.	R-26814	-		-
Alkalilimnicola ehrlichii	MLHE-1	-		-
Allochromatium vinosum	DSM 180	-		-
Anaeromyxobacter dehalogenans	2CP-1	-	-	-
Anaeromyxobacter dehalogenans	2CP-C	-	-	-
Anaeromyxobacter dehalogenans	DCP18	-	-	-
Anaeromyxobacter sp.	Fw109-5	-	-	-
Anaeromyxobacter sp.	K	-	-	-
Anaerophaga sp.	HS1	-	-	-
Anoxybacillus flavithermus	TNO-09.006 chrAF6	-	-	-
Arthrospira maxima	CS-328	-	-	-
Arthrospira platensis	C1	-	-	-
Arthrospira sp.	PCC 8005	-	-	-
Azoarcus aromaticum	EbN1	-	-	-
Azoarcus sp.	BH72	-	-	-
Azoarcus sp.	KH32C	AP012305	BAL27513	AP012305, plasmid pAZKH
Azospirillum amazonense	Y2	-		-

nirK

Species	Strain number	Gene locus tag	Protein ID	Accession number
Azospirillum brasilense	Sp245	**n/a**	**ABY68277**	**EU194339, plasmid p85**
		n/a	**ABY87187**	**EU221421**
		AZOBR_p310167	CCD02425	HE577330, plasmid AZOBR_p3
		-	ABY87184	EU221420
Azospirillum brasilense	SM	-		-
Azospirillum doebereinerae	GSF 71T	-	AAL73092	AY072263
Azospirillum lipoferum	4B	AZOLI_p30082	CBS89931	FQ311871, plasmid AZO_p3
Azospirillum sp.	B510	AZL_c02030	BAI75496	AP010949, plasmid pAB510c
Bacillus azotoformans	LMG 9581^{T}	BAZO_03565	WP_003329883	AJLR01000038 (contig38)
Bacillus bataviensis	LMG 21833^{T}	BABA_p06582	WP_007084344	AJLS01000042 (contig42)
Bacillus licheniformis	ATCC 14580/DSM 13	-	-	-
Bacillus sp.	1NLA3E	-	-	-
Bacillus sp.	2_A_57_CT2	HMPREF1013_03650	EFV76012	ACWD01000055 (cont1.55)
Bacillus sp.	BT1B_CT2	-	-	-
Bacillus sp.	Ult-108 clone A	-	-	-
Bacillus sp.	Ult-123 clone A	-	-	-
Bacillus sp.	Ult-130 clone A	-	-	-
Bacillus sp.	Ult-145 clone A	-	-	-
Bacillus sp.	Ult-356 clone A	-	-	-
Bacillus sp.	Ult-391 clone A	-	-	-
Bacillus sp.	Ult-41 clone A	-	-	-
Bacillus sp.	Ult-42 clone A	-	-	-
Bacillus sp.	Ult-42 clone B	-	-	-
Bacillus sp.	Ult-442 clone A	-	-	-
Bacillus sp.	Ult-46 clone A	-	-	-
Bacillus sp.	Ult-521 clone A	-	-	-
Bacillus sp.	Ult-530 clone A	-	-	-
Bacillus sp.	Ult-552 clone A	-	-	-
Bacillus sp.	Ult-640 clone A	-	-	-
Bacillus sp.	Ult-71 clone A	-	-	-
Bacillus sp.	Ult-816 clone A	-	-	-
Bacillus sp.	R-31770	-	-	-
Bacillus sp.	R-31841	-	-	-
Bacillus sp.	R-32526	-	-	-
Bacillus sp.	R-32656	-	-	-
Bacillus sp.	R-32694	-	-	-
Bacillus sp.	R-32702	-	-	-
Bacillus sp.	R-32709	-	-	-
Bacillus sp.	R-32715	-	-	-
Bacillus sp.	R-33820	-	-	-
Bacillus sp.	R-33773	-	-	-
Bacillus sp.	R-32546	**n/a**	**CAL49429**	**AM404294**
Bacillus sp.	10403023 (MM10403188)	B1040_010100019006	WP_010678487	HE610988 (genomic scaffold00004)
Bacillus sp.	SH27	-	-	-
Bacillus sp.	SH3	-	-	-
Bacillus sp.	SH11	-	-	-
Bacillus sp.	SH22	-	-	-
Bacillus sp.	SH5	-	-	-
Bacillus sp.	SH8	-	-	-
Bacillus sp.	SH10	-	-	-
Bacillus sp.	SH14	-	-	-
Bacillus sp.	SH19	-	-	-
Bacillus sp.	SH21	-	-	-
Bacillus sp.	SH25	-	-	-
Bacillus sp.	SH30	-	-	-
Bacillus sp.	SH36	-	-	-
Bacillus sp.	SH38	-	-	-
Bacillus sp.	SH41	-	-	-
Bacillus sp.	SH42	-	-	-
Bacillus sp.	SH43	-	-	-
Bacillus sp.	SH48	-	-	-
Bacillus sp.	SH51	-	-	-
Bacillus sp.	SH60	-	-	-
Bacillus sp.	SH61	-	-	-
Bacillus sp.	SH62	-	-	-
Bacillus sp.	SH63	-	-	-
Bacillus subtilis	BEST7613	-	-	-
Bdellovibrio bacteriovorus	HD100	Bd2608	CAE80401	BX842653
Beggiatoa sp.	PS	-	-	-
Belliella baltica	DSM 15883	Belba_2006	AFL84581	CP003281
Beta-proteobacterium	R1-Apr-MIB-6	**n/a**	**BAD17996**	**AB118904**
Bizionia argentinensis	JUB59	BZARG_690	EGV44369	AFXZ01000009 (contig00010)
blood disease bacterium	R229	-	-	-
Bordetella petrii	DSM 12804	-	-	-
Brachybacterium faecium	DSM 4810 strain 6-10	-	-	n/i
Bradyrhizobium japonicum	USDA 110	**n/a**	**BAC52354**	**BA000040**
Bradyrhizobium japonicum	ATCC 15067	**n/a**	**ADK76191**	**HM060301**
Bradyrhizobium japonicum	USDA 6	BJ6T_23260	BAL07605	AP012206
Bradyrhizobium sp.	BTAi1	BBta_6826	ABQ38713	CP000494
Bradyrhizobium sp.	ORS 278	BRADO1227	CAL75133	CU234118
Bradyrhizobium sp.	ORS 285	BRAO285_470012	CCD88817	CAFH01000244 (contig00049-776)
Bradyrhizobium sp.	ORS 375	BRAO375_4030011	CCD95204	CAFI01000339 (Contig00436-771)
Bradyrhizobium sp.	STM 3809	BRAS3809_3370055	CCE00380	CAFJ01000265 (Contig00348-770)
Bradyrhizobium sp.	STM 3843	BRAS3843_1900012	CCE07040	CAFK01000102 (contig 00196-775)
Bradyrhizobium sp.	S23321	S23_12320	BAL74450	AP012279

nirK				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Brucella abortus	A13334	BAA13334_II01682	AEW19619	CP003177, chromosome 2
Brucella abortus	bv. 1 str. 9-941	BruAb2_0919	AAX76305	AE017224, chromosome 2
Brucella abortus	bv. 1 str. NI010	M1G_02811	EHR17762	AGVJ01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI016	M1I_02813	EHR18604	AGVK01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI021	M1K_02814	EHR25590	AGVL01000015 (cont1.15)
Brucella abortus	bv. 1 str. NI259	M1M_02097	EHR27861	AGVM01000009 (cont1.9)
Brucella abortus	bv. 1 str. NI435a	M17_01971	EHR10282	AGVF01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI474	M19_02815	EHR08225	AGVG01000014 (cont1.14)
Brucella abortus	bv. 1 str. NI486	M1A_01383	EHR11489	AGVH01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI488	M1E_00124	EHR28066	AGVI01000002 (cont1.2)
Brucella abortus	S19	BAbS19_II08720	ACD74361	CP000888 , chromosome 2
		n/a	**ABZ79478**	**EU402949**
Brucella abortus	str. 2308 A	-	-	n/i
Brucella canis	ATCC 23365	BCAN_B0261	ABX63451	CP000873, chromosome 2
Brucella canis	HSK A52141	BCA52141_II0826	AEW15697	CP003175 , chromosome 2
Brucella ceti	str. Cudo	BCETI_6000570	EEH13611	ACJD01000006 , chromosome 2 (VBI00082_1)
Brucella inopinata	BO1	BIBO1_2258	EFM55720	ADEZ01000033 , chromosome 2 (VBI00042_6)
Brucella melitensis	ATCC 23457	BMEA_B0261	ACO02126	CP001489 , chromosome 2
Brucella melitensis	bv.1 str. 16M	BMEII0988	AAL54230	AE008918, chromosome 2
Brucella melitensis	bv. 2 str. 63/9	BASG_02325	EEZ16809	ACEM01000016 (cont1.16)
Brucella melitensis	M28	BM28_B0251	ADZ67500	CP002460 , chromosome 2
Brucella melitensis	M5-90	BM590_B0251	ADZ88368	CP001852 , chromosome 2
Brucella melitensis	NI	BMNI_II0247	AEQ09957	CP002932 , chromosome 2
Brucella microti	CCM 4915	CP001579	ACU49390	CP001579 , chromosome 2
Brucella ovis	ATCC 25840	BOV_A0236	ABQ62507	CP000709 , chromosome 2
Brucella pinnipedialis	B2/94	BPI_II255	AEK55707	CP002079, chromosome 2
Brucella sp.	BO2	BIBO2_0690	EFM60353	ADFA01000033 (VBI00229_128)
Brucella sp.	NF 2653	BROD_0684	EFM63266	ADFB01000030 (VBI00228_23)
Brucella suis	ATCC 23445	BSUIS_B0265	ABY39277	CP000912 , chromosome 2
Brucella suis	VBI22	BSVBI22_B0256	AEU07410	CP003129 , chromosome 2
Burkholderia pseudomallei	1026a	-	-	-
Burkholderia pseudomallei	1106a	BURPS1106A_A2012	ABN94946	CP000573 , chromosome 2
Burkholderia pseudomallei	1258a	-	-	-
Burkholderia pseudomallei	1258b	-	-	-
Burkholderia pseudomallei	1710b	BURPS1710b_A0520	ABA51557	CP000125, chromosome 2
Burkholderia pseudomallei	305	-	-	n/i
Burkholderia pseudomallei	354a	-	-	-
Burkholderia pseudomallei	354e	-	-	-
Burkholderia pseudomallei	576	BUC_6267	EEC31900	ACCE01000013 (BUC.Contig176)
Burkholderia pseudomallei	668	BURPS668_A2107	ABN86002	CP000571, chromosome 2
Burkholderia pseudomallei	K96243	BPSS1487	CAH38960	BX571966, chromosome 2
Burkholderia pseudomallei	MSHR346	GBP346_B1432	EEP51392	ACOJ01000001, chromosome 2 (pseudoChromo_II)
Burkholderia pseudomallei	Pakistan 9	BUH_6369	EEH27834	ACKA01000028 (BUH.Contig262)
Burkholderia thailandensis	E264	-	-	-
Burkholderia thailandensis	TXDOH	-	-	-
Caldilinea aerophila	DSM 14535 = NBRC 104270	-	-	-
Campylobacter concisus	13826	-	-	-
Campylobacter concisus	UNSWCD	-	-	-
Campylobacter curvus	525.92	-	-	-
Campylobacter fetus subsp. fetus	82-40	-	-	-
Campylobacter sp.	10_1_50	-	-	-
Cand. *Accumulibacter phosphatis* clade IIA	str. UW-1	-	-	-
Candidatus *Koribacter versatilis*	Ellin345	-	-	-
Candidatus *Nitrospira defluvii*	unknown	NIDE4252	CBK43917	FP929003, chromosome - copy A
		NIDE2534	CBK42243	FP929003, chromosome - copy B
Candidatus *Solibacter usitatus*	Ellin6076	-	-	-
Capnocytophaga gingivalis	ATCC 33624	CAPGI0001_2154	EEK14494	ACLQ01000019 (ctg1117755536039)
Capnocytophaga ochracea	F0287	-	-	-
Capnocytophaga sp.	oral taxon 338 str. F0234	HMPREF9071_1620	EGD33903	AEXX01000038 (contig00038)
Capnocytophaga sp.	oral taxon 329 str. F0087	HMPREF9074_02094	EGJ56285	AFHP01000045 (C_sporaltaxon329F0087-1.0_Cont127.2)
Capnocytophaga sp.	oral taxon 412 str. F0487	-	-	-
Capnocytophaga sp.	CM59	-	-	-
Capnocytophaga sputigena	ATCC 33612 strain Capno	CAPSP0001_1483	EEB66568	ABZV01000003 (contig00026)
Cardiobacterium hominis	ATCC 15826	HMPREF0198_0885	EEV88984	ACKY01000043 (contig00043)
Cardiobacterium valvarum	F0432	HMPREF9080_02131	EHM52825	AGCM01000121 (C_valvarumF0432-1.0_Cont630.3)
Caulobacter segnis	ATCC 21756	Cseg_3038	ADG11480	CP002008
Cellulophaga algicola	DSM 14237	-	-	-
Cellvibrio japonicus	Ueda107	-	-	-
Cellvibrio sp.	BR	-	-	-
Chelativorans sp.	BNC1	Meso_4273	ABG61243	CP000389, plasmid 1
		Meso_2243	ABG63634	CP000390
Chitinophaga pinensis	DSM 2588	-	-	-
Chloroflexus aggregans	DSM 9485	Cagg_1796	ACL24695	CP001337
Chloroflexus aurantiacus	J-10-fl	Caur_1570	ABY34789	CP000909
Chloroflexus sp.	Y-400-fl	Chy400_1706	ACM53116	CP001364
Chromobacterium violaceum	ATCC 12472	CV2007	AAQ59679	AE016825
Chroococcidiopsis thermalis	PCC 7203	-	-	-
Chryseobacterium gleum	ATCC 35910	HMPREF0204_13219	EFK34150	ACKQ02000007 (Contig314)
Chthoniobacter flavus	Ellin428	CfE428DRAFT_3240	EDY19063	ABVL01000009 (ctg71)
Citreicella sp.	357	-	-	-
Colwellia psychrerythraea	34H	-	-	-
Corynebacterium accolens	ATCC 49725	HMPREF0276_0284	EEI15669	ACGD01000001 (contig00001)
Corynebacterium accolens	ATCC 49726	HMPREF0277_1013	WP_005282173	AEED01000000 (genomic scaffold SCAFFOLD 1)
Corynebacterium aurimucosum	ATCC 700975	cauri_1056	ACP32651	CP001601
Coryn. diphtheriae bv. *intermedius*	NCTC 5011	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Corynebacterium diphtheriae gravis	NCTC13129	-	-	-
Corynebacterium diptheriae	241	-	-	-
Corynebacterium diptheriae	31A	-	-	-
Corynebacterium diptheriae	BH8	-	-	-
Corynebacterium diptheriae	C7 (beta)	-	-	-
Corynebacterium diptheriae	CDCE 8392	-	-	-
Corynebacterium diptheriae	HC01	-	-	-
Corynebacterium diptheriae	HC02	-	-	-
Corynebacterium diptheriae	HC03	-	-	-
Corynebacterium diptheriae	HC04	-	-	-
Corynebacterium diptheriae	INCA 402	-	-	-
Corynebacterium diptheriae	PW8	-	-	-
Corynebacterium diptheriae	VA01	-	-	-
Corynebacterium efficiens	YS-314	HMPREF0290_0532	EEW50838	ACLI01000031 (contig00032)
Corynebacterium pseudotuberculosis	1002	-	-	-
Corynebacterium pseudotuberculosis	267	-	-	-
Corynebacterium pseudotuberculosis	316	-	-	-
Corynebacterium pseudotuberculosis	3/99-5	-	-	-
Corynebacterium pseudotuberculosis	42/02-A	-	-	-
Corynebacterium pseudotuberculosis	C231	-	-	-
Corynebacterium pseudotuberculosis	CIP 52.97	-	-	-
Corynebacterium pseudotuberculosis	Cp162	-	-	-
Corynebacterium pseudotuberculosis	FRC41	-	-	-
Corynebacterium pseudotuberculosis	P54B96	-	-	-
Corynebacterium pseudotuberculosis	PAT10	-	-	-
Corynebacterium pseudotuberculosis	I19	-	-	-
Cupriavidus metallidurans	CH34	-	-	-
Cupriavidus necator	N-1	-	-	-
Cupriavidus necator	LMG 1201	-	-	-
Cupriavidus sp.	R-31542	-	-	-
Cupriavidus sp.	R-31543	-	-	-
Cupriavidus sp.	R-31544	-	-	-
Cupriavidus taiwanensis	LMG 19424	-	-	-
Cyanobacterium aponinum	PCC 10605	-	-	-
Cyanobacterium stanieri	PCC 7202	-	-	-
Dechloromonas aromatica	RCB	-	-	-
Dechlorosoma suillum	PS	-	-	-
Denitrovibrio acetiphilus	DSM 12809	-	-	-
Desulfitobacterium dehalogenans	ATCC 51507	-	-	-
Desulfitobacterium dichloroeliminans	LMG P-21439	-	-	-
Desulfitobacterium hafniense	DCB-2	-	-	-
Desulfitobacterium hafniense	Y51	-	-	-
Desulfomonile tiedjei	DSM 6799	-	-	-
Desulfosporosinus meridiei	DSM 13257	-	-	-
Desulfosporosinus youngiae	DSM 17734	-	-	-
Desulfotomaculum ruminis	DSM 2154	-	-	-
Desulfovibrio sp.	U5L	-	-	-
Diaphorobacter sp.	R-24610	-	-	n/i
Diaphorobacter sp.	R-24612	-	-	n/i
Diaphorobacter sp.	R-24661	-	-	-
Diaphorobacter sp.	R-25011	-	-	-
Diaphorobacter sp.	R-26815	-	-	n/i
Diaphorobacter sp.	R-26840	-	-	-
Diaphorobacter sp.	R-28417	-	-	-
Dinoroseobacter shibae	DFL 12	-	-	-
Diplosphaera colitermitum	TAV2	-	-	-
Dyadobacter fermentans	DSM 18053	-	-	-
Eikenella corrodens	ATCC 23834	-	-	-
Endosymbiont of *Riftia pachyptila*	Rifp1Symag	-	-	-
Ensifer sp.	2FB8	**n/a**	**AAL82506**	**AY078247**
Ensifer sp.	4FB6	**n/a**	**AAL82507**	**AY078248**
Enterococcus sp.	R-24626	-	-	n/i
Ferroglobus placidus	DSM 10642	-	-	-
Flavobacteriaceae bacterium	3519-10	FIC_00388	ACU06855	CP001673
Flavobacteriales bacterium	ALC-1	-	-	-
Flavobacterium columnare	ATCC 49512	FCOL_01550	AEW85159	CP003222
Flavobacterium columnare	unknown	**n/a**	**AAQ99141**	**AY387597**
Flavobacterium johnsoniae	UW101	Fjoh_2418	ABQ05445	CP000685
Flavobacterium sp.	F52	FF52_02025	EJG02931	AKZQ01000006 (Contig06)
Flavobacterium sp.	CF136 PMI10	-	-	-
Gamma-proteobacterium	HdN1	-	-	-
Gemmatimonas aurantiaca	T-27	GAU_2766	BAH39808	AP009153
Geobacillus kaustophilus	HTA426	GK0904	BAD75189	BA000043
Geobacillus kaustophilus	LMG 9819[T]	**n/a**	**CDG32545**	**HG328767**
Geobacillus sp.	C56-T3	-	-	-
Geobacillus sp.	G11MC16	G11MC16DRAFT_0524	EDY07609	ABVH01000001 (ctg12)
Geobacillus sp.	Y412MC52	-	-	-
Geobacillus sp.	Y412MC61	-	-	-
Geobacillus sp.	Y4.1MC1	GY4MC1_3069	ADP75747	CP002293
Geobacillus stearothermophilus	LMG 6939T	**n/a**	**CDG32562**	**HG328784**
Geobacillus stearothermophilus	R-35646	**n/a**	**CDG32563**	**HG328785**
Geobacillus stearothermophilus	R-32513	**n/a**	**CDG32565**	**HG328787**
Geobacillus stearothermophilus	R-32605	**n/a**	**CDG32564**	**HG328786**
Geobacillus stearothermophilus	R-32635	**n/a**	**CDG32566**	**HG328788**
Geobacillus thermodenitrificans	NG80-2	GTNG_0650	ABO66030	CP000557

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Geobacillus thermodenitrificans	LMG 17532T	**n/a**	**CDG32546**	**HG328768**
Geobacillus thermodenitrificans	R-35647	**n/a**	**CDG32561**	**HG328783**
Geobacillus thermodenitrificans	R-32614	**n/a**	**CDG32547**	**HG328769**
Geobacillus thermodenitrificans	R-32615	**n/a**	**CDG32548**	**HG328770**
Geobacillus thermodenitrificans	R-32616	**n/a**	**CDG32549**	**HG328771**
Geobacillus thermodenitrificans	R-32617	**n/a**	**CDG32550**	**HG328772**
Geobacillus thermodenitrificans	R-32618	**n/a**	**CDG32551**	**HG328773**
Geobacillus thermodenitrificans	R-32619	**n/a**	**CDG32552**	**HG328774**
Geobacillus thermodenitrificans	R-32621	**n/a**	**CDG32553**	**HG328775**
Geobacillus thermodenitrificans	R-32622	**n/a**	**CDG32554**	**HG328776**
Geobacillus thermodenitrificans	R-32623	**n/a**	**CDG32555**	**HG328777**
Geobacillus thermodenitrificans	R-32624	**n/a**	**CDG32556**	**HG328778**
Geobacillus thermodenitrificans	R-32625	**n/a**	**CDG32557**	**HG328779**
Geobacillus thermodenitrificans	R-32500	**n/a**	**CDG32558**	**HG328780**
Geobacillus thermodenitrificans	R-32506	**n/a**	**CDG32559**	**HG328781**
Geobacillus thermodenitrificans	R-32511	**n/a**	**CDG32560**	**HG328782**
Geobacillus thermoglucosidans	TNO-09.020	GT20_2711	EID43578	AJJN01000019 (contig27)
Geobacillus thermoglucosidasius	C56-YS93	Geoth_3084	AEH48959	CP002835
Geobacillus thermoleovorans	CCB_US3_UF5	-	-	-
Geobacillus toebii	R-32639	**n/a**	**CDG32567**	**HG328789**
Geobacter bemidjiensis	Bem	-	-	-
Geobacter daltonii	FRC-32	-	-	-
Geobacter metallireducens	GS-15	-	-	-
Geobacter metallireducens	RCH3	-	-	-
Geobacter sp.	M21	-	-	-
Gillisia limnaea	DSM 15749	-	-	-
Gloeocapsa sp.	PCC 7428	-	-	-
Gramella forsetii	KT0803	-	-	-
Haemophilus haemolyticus	HK386	-	-	-
Haemophilus haemolyticus	M19107	-	-	-
Haemophilus haemolyticus	M19501	-	-	-
Haemophilus haemolyticus	M21127	-	-	-
Haemophilus haemolyticus	M21621	-	-	-
Haemophilus haemolyticus	M21639	-	-	-
Haemophilus parahaemolyticus	HK385	HMPREF1050_1668	EIJ73552	AJSW01000003 (contig00021)
Haemophilus parahaemolyticus	HK411	HMPREF1054_0970	EIG27917	AJMU01000006 (contig00007)
Haemophilus parainfluenzae	ATCC 33392	HMPREF9417_1460	EGC71985	AEWU01000017 (contig00017)
Haemophilus parainfluenzae	HK262	-	-	n/i
Haemophilus parainfluenzae	HK2019	HMPREF1119_1703	EIJ29375	AJTC01000034 (contig00012)
Haemophilus parainfluenzae	T3T1	PARA_18490	CBW15949	FQ312002
Haemophilus pittmaniae	HK 85	HMPREF9952_1422	EGV05222	AFUV01000020 (ctg1129913985436)
Haemophilus sp.	oral taxon 851 str. F0397	-	-	-
Hahella chejuensis	KCTC 2396	-	-	-
Haladaptatus paucihalophilus	DX253	-	-	-
Haliscomenobacter hydrossis	DSM 1100	-	-	-
Haloarcula hispanica	ATCC 33960	HAH_1953	AEM57550	CP002921 , chromosome 1
Haloarcula marismortui	ATCC 43049	-	CAB93142	AJ278286, chromosome 1 - copy A
		rrnAC1378	AAV46307	AY596297, chromosome 1 - copy B
Halobacterium sp.	DL1	-	-	-
Haloferax denitrificans	unknown	**n/a**	**CAD89521**	**AJ557012**
Haloferax lucentense	unknown	**n/a**	**CAE46530**	**AJ582028**
Haloferax mediterranei	ATCC 33500/R4	**n/a**	**CBG76812**	**FN555205**
Haloferax volcanii	DS2	HVO_2141	ADE04765	CP001956
Halogeometricum borinquense	DSM 11551	Hbor_34420	ADQ68963	CP001692, plasmid pHBOR02
Halomicrobium mukohataei	DSM 12286	Hmuk_1967	ACV48080	CP001688
Halopiger xanaduensis	SH-6	Halxa_3282	AEH37894	CP002839 - copy A
		Halxa_2517	AEH37135	CP002839 - copy B
Halorhabdus utahensis	DSM 12940	Huta_0035	ACV10224	CP001687
Halorubrum lacusprofundi	ATCC 49239	-	-	-
Haloterrigena turkmenica	DSM 5511	Htur_3087	ADB61952	CP001860
Herminiimonas arsenicoxydans	$ULPA_{S1}$	HEAR3245	CAL63352	CU207211
Herpetosiphon aurantiacus	DSM 785	Haur_1081	ABX03729	CP000875
Hydrogenobacter thermophilus	TK-6	-	-	-
Hydrogenophaga sp.	PBC	-	-	-
Hyphomicrobium denitrificans	1NES1	HypdeDRAFT_1166	EHB77638	AGIS01000001 (ctg121)
Hyphomicrobium denitrificans	ATCC 51888	Hden_0591	ADJ22412	CP002083
Hyphomicrobium denitrificans	A3151	**n/a**	**BAC00912**	**AB076606**
Idiomarina loihiensis	L2TR	-	-	n/i
Ignavibacterium album	JCM 16511	-	-	-
Imtechella halotolerans	K1	W5A_10045	EID73898	AJJU01000017 (Contig17)
Intrasporangium calvum	DSM 43043	-	-	-
Intrasporangium sp.	4LS1	**n/a**	**ADD51574**	**GU233009**
Kangiella koreensis	DSM 16069	Kkor_2024	ACV27434	CP001707
Kingella denitrificans	ATCC 33394	HMPREF9098_0736	EGC17925	AEWV01000013 (contig00013)
Kingella kingae	ATCC 23330	HMPREF0476_1033	EGK09369	AFHS01000036 (contig00036)
Kingella kingae	PYKK081	KKB_04182	EIC13830	AJGB01000025 (Contig025)
Kingella oralis	ATCC 51147	GCWU000324_03142	EEP66738	ACJW02000008 (K_oralis-1.0.1_Cont3.1)
Kyrpidia tusciae	DSM 2912	-	-	-
Labrenzia aggregata	IAM 12614	-	-	-
Lactobac. coryniformis subsp. *coryniformis*	CECT 5711	-	-	-
Lactobac. coryniformis subsp. *coryniformis*	KCTC 3167	-	-	-
Lactobac. coryniformis subsp. *torquens*	KCTC 3535	-	-	-
Lactobacillus farciminis	KCTC 3681	-	-	-
Lactobacillus fermentum	ATCC 14931	-	-	-
Lactobacillus fermentum	CECT 5716	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Lactobacillus fermentum	IFO3956	-	-	-
Lactobacillus fermentum	28-3-CHN	-	-	-
Lactobacillus versmoldensis	KCTC 3814	-	-	-
Lautropia mirabilis	ATCC 51599	HMPREF0551_1410	EFV94993	AEQP01000008 (contig00008)
Leptonema illini	DSM 21528	-	-	-
Leptospira biflexa	serovar Patoc 'Patoc 1 (Ames)'	-	-	n/i
Leptospira biflexa	serovar Patoc 'Patoc 1 (Paris)'	LEPBI_I1063	ABZ97186	CP000786, chromosome 1
Leptospira broomii	str. 5399	-	-	-
Leptospira inadai sv. *Lyme*	str. 10	-	-	-
Leptospira licerasiae	serovar Varillal str. VAR 010	-	-	-
Leptospira meyeri	serovar Hardjo str. Went 5	LEP1GSC017_1055	WP_004786503	AKXE01000002 (ctg7180000002149)
Leptothrix cholodnii	SP-6	-	-	-
Magnetospirillum gryphiswaldense	MSR-1	-	-	-
Mannheimia succiniciproducens	MBEL55E	-	-	-
Maribacter sp.	HTCC 2170	FB2170_14923	EAR01831	CP002157
Marinilabilia sp.	AK2	-	-	-
Mariniradius saccharolyticus	AK6	-	-	-
Marinobacter aquaeolei	VT8	-	-	-
Marinobacter hydrocarbonoclasticus	617	-	-	-
Marinobacter hydrocarbonoclasticus	ATCC 49840	-	-	-
Marinobacter manganoxydans	MnI7-9	-	-	-
Marinobacter sp.	ELB17	MELB17_18054	EBA00982	AAXY01000002
Maritimibacter alkaliphilus	HTCC 2654	RB2654_12814	EAQ13954	AAMT01000003 (1099457000260)
Marivirga tractuosa	DSM 4126	Ftrac_2700	ADR22678	CP002349
Melioribacter roseus	P3M	-	-	-
Mesorhizobium alhagi	CCNWXJ12-2	MAXJ12_19213	EHK55601	AHAM01000158 (contig166) - copy A
		MAXJ12_31422	EHK53213	AHAM01000280 (contig300) - copy B
Mesorhizobium amorphae	CCNWGS0123	MEA186_34749	EHH02540	AGSN01000255 (contig00288)
Mesorhizobium australicum	WSM2073	MesauDRAFT_5725	EHB63762	AGIX01000010 (ctg00016)
Mesorhizobium ciceri biovar *biserrulae*	WSM1271	Mesci_6041	ADV15048	CP002448, plasmid pMESCI01
Mesorhizobium opportunistum	WSM2075	Mesop_6034	AEH90440	CP002279
Mesorhizobium sp.	4FB11	**n/a**	**AAL82513**	**AY078254**
Methylobacterium sp.	4-46	-	-	-
Methylocella silvestris	BL2	Msil_1519	ACK50469	CP001280
Methylocystis sp.	ATCC 49242	Met49242DRAFT_3861	EFX97847	AEVM01000035 (ctg197)
Methylocystis sp.	SC2	-	-	-
Methylomonas sp.	16a	**n/a**	**ADB12477**	**GQ226037**
Methylophaga aminisulfidivorans	MP_54_1	-	-	-
Methylotenera mobilis	JLW8	Mmol_1061	ACT47970	CP001672
Methylotenera versatilis	301	M301_1603	ADI29983	CP002056
Microlunatus phosphovorus	NM-1	-	-	-
Micromonospora aurantiaca	ATCC 27029	Micau_3956	ADL47480	CP002162
Micromonospora sp.	L5	ML5_4465	ADU09941	CP002399
Mobilicoccus pelagius	NBRC 104925	-	-	-
Moraxella catarrhalis	ETSU-9	-	-	n/i
Moraxella catarrhalis	101P30B1	E9K_02546	EGE16121	AERE01000011 (ctg00017)
Moraxella catarrhalis	103P14B1	-	-	n/i
Moraxella catarrhalis	12P80B1	-	-	n/i
Moraxella catarrhalis	46P47B1	E9M_03704	EGE13573	AERF01000022 (ctg00022)
Moraxella catarrhalis	7169	E9G_02208	EGE12206	AERC01000011 (ctg00020) - copy A
		n/a	**ACJ68085**	**EU861987 - copy B**
Moraxella catarrhalis	BBH18	MCR_0135	ADG60407	CP002005
Moraxella catarrhalis	BC1	-	-	n/i
Moraxella catarrhalis	BC7	E9S_06820	EGE19267	AERI01000023 (ctg00023)
Moraxella catarrhalis	BC8	E9U_01496	EGE21884	AERJ01000006 (ctg00006)
Moraxella catarrhalis	CO72	-	-	n/i
Moraxella catarrhalis	O35E	**n/a**	**ACJ68080**	**EU861986**
Moritella sp.	PE36	-	-	-
Muricauda ruestringensis	DSM 13258	Murru_2669	AEM71702	CP002999
Mycobacterium avium	104	-	-	-
Mycobact. avium subsp. *paratuberculosis*	k10	-	-	-
Mycobact. avium subsp. *paratuberculosis*	S397	-	-	-
Mycobacterium colombiense	CECT 3035	-	-	-
Mycobacterium intracellulare	ATCC 13950	-	-	-
Mycobacterium intracellulare	MOTT-02	-	-	-
Mycobacterium intracellulare	MOTT-64	-	-	-
Mycobacterium parascrofulaceum	ATCC BAA-614	-	-	-
Mycobacterium rhodesiae	NBB3	-	-	-
Mycobacterium sp.	JDM601	-	-	-
Mycobacterium sp.	JLS	-	-	-
Mycobacterium sp.	KMS	-	-	-
Mycobacterium sp.	MCS	-	-	-
Mycobacterium thermoresistibile	ATCC 19527	-	-	-
Mycobacterium xenopi	RIVM700367	-	-	-
Myroides injenensis	M09-0166	-	-	-
Myroides odoratimimus	CIP 101113	HMPREF9715_03084	EHO06363	AGEE01000051 (cont1.51)
Myroides odoratimimus	CIP 103059	-	-	-
Myroides odoratimimus	CCUG 10230	HMPREF9712_03423	EHO05847	AGEC01000029 (cont1.29)
Myroides odoratimimus	CCUG 3837	-	-	-
Myroides odoratimimus	CCUG 12901	HMPREF9714_03002	EHO06522	AGED01000045 (cont1.45)
Myroides odoratus	DSM 2801	-	-	-
Natrinema pellirubrum	DSM 15624	NatpeDRAFT_1672	EHA73768	AGIN01000005 (ctg251) - copy A
		NatpeDRAFT_1871	EHA73454	AGIN01000006 (ctg252) - copy B
Natronomonas pharaonis	DSM 2160	NP_1598A	CAI48890	CR936257
Neisseria bacilliformis	ATCC BAA-1200	HMPREF9123_2339	EGF08904	AFAY01000048 (contig00048)

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Neisseria cinerea	ATCC 14685	NEICINOT_05026	EEZ70840	ACDY02000017 (N_cinerea-1.0.2_Cont16.1)
Neisseria elongata subsp. *glycolytica*	ATCC 29315	NEIELOOT_00838	EFE50341	ADBF01000022 (N_elongata-1.0.1_Cont22.1)
Neisseria flavescens	NRL30031/H210	NEIFLAOT_00818	EEG34093	ACEN01000020 (N_flavescens-1.0_Cont20.1)
Neisseria flavescens	SK114	NEIFL0001_1542	EER55617	ACQV01000027 (ctg1118407793875)
Neisseria gonorrhoeae	NCCP 11945	NGK_1651	ACF30300	CP001050
Neisseria gonorrhoeae	TCDC-NG08107	NGTW08_1169	ADV08137	CP002440
Neisseria gonorrhoeae	FA 1090	NGO1276	AAW89933	AE004969
Neisseria gonorrhoeae	R10	**n/a**	**AAA25462**	**M97926**
Neisseria lactamica	020-06	NLA_6490	CBN86885	FN995097
Neisseria lactamica	ATCC 23970	NEILACOT_04256	EEZ75701	ACEQ02000013 (N_lactamica-1.0.2_Cont12.1)
Neisseria lactamica	Y92-1009	-	-	-
Neisseria macacae	ATCC 33926	HMPREF9418_0817	EGQ77692	AFQE01000037 (contig00037)
Neisseria meningitidis	M01-240355	NMBM01240355_1553	ADZ00049	CP002422
Neisseria meningitidis	M01-240149	NMBM01240149_0554	ADY97160	CP002421
Neisseria meningitidis	M01-240013	-	-	-
Neisseria meningitidis	M04-240196	-	-	-
Neisseria meningitidis	M0579	NMBM0579_0608	EGC59315	AEQH01000009 (gnmm0579c.contig.8)
Neisseria meningitidis	M13399	-	-	n/i
Neisseria meningitidis	M6190	-	-	-
Neisseria meningitidis	MC58	-	-	n/i
Neisseria meningitidis	N1568	NMXN1568_0553	EGC51538	AEQD01000024 (menX.contig.23)
Neisseria meningitidis	NM220	-	-	-
Neisseria meningitidis	NM233	-	-	-
Neisseria meningitidis	NZ-05/33	-	-	n/i
Neisseria meningitidis	CU385	-	-	n/i
Neisseria meningitidis	H44/76	-	-	n/i
Neisseria meningitidis	WUE 2594	NMAA_1353	CBY91282	FR774048
Neisseria meningitidis	alpha14	NMO_1452	CBA07163	AM889136
Neisseria meningitidis	alpha153	-	-	-
Neisseria meningitidis	alpha275	-	-	-
Neisseria meningitidis	alpha710	NMBB_1863	ADO32072	CP001561
Neisseria meningitidis	ATCC 13091	HMPREF0602_0395	EFM05097	AEEF01000022 (contig00022)
Neisseria meningitidis	OX99.30304	-	-	n/i
Neisseria meningitidis	serogroup A strain Z2491	NMA1887	CAM09006	AL157959
Neisseria meningitidis	serogroup C FAM18	NMC1549	CAM10744	AM421808
Neisseria meningitidis	053442	-	-	-
Neisseria mucosa	ATCC 25996	NEIMUCOT_06336	EFC87241	ACDX02000025 (N_mucosa-1.0.2_Cont24.1)
Neisseria mucosa	C102	HMPREF0604_00159	EFV81580	ACRG01000001 (cont1.1)
Neisseria polysaccharea	ATCC 43768	NEIPOLOT_02444	EFH21816	ADBE01000130 (N_polysaccharea-1.0.1_Cont275.1)
Neisseria sicca	ATCC 29256	NEISICOT_01651	EET44689	ACKO02000008 (N_sicca-1.0.1_Cont7.1)
Neisseria sicca	VK64	HMPREF1051_1502	EIG29850	AJMT01000042 (ctg1120005024834)
Neisseria sp.	GT4A_CT1	HMPREF1028_01611	EGY59976	ACWS01000071 (cont1.71)
Neisseria sp.	oral taxon 014 str. F0314	HMPREF9016_01005	WP_009174029	GL349411 (genomic scaffold supercont.1.1)
Neisseria subflava	NJ9703	NEISUBOT_03684	EFC52848	ACEO02000002 (N_subflava-1.0.1_Cont1.1)
Neisseria weaveri	LMG 5135	-	-	n/i
Neisseria weaveri	ATCC 51223	l13_08510	EGV36290	AFWR01000032 (NW51223.2_32)
Neisseriaceaea bacterium	NB-13	-	-	-
Niabella soli	DSM 19437	-	-	-
Niastella koreensis	GR20-10	-	-	-
Nitratifractor salsuginis	DSM 16511	-	-	-
Nitratireductor aquibiodomus	RA22	A33O_18739	EIM72710	AJXZ01000049 (Contig49)
Nitratireductor aquibiodomus	NL31	**n/a**	**AEE65031**	**JF274967**
Nitratiruptor sp.	SB155-2	-	-	-
Nitrosococcus halophilus	Nc4	Nhal_1082	ADE14254	CP001798
Nitrosococcus oceani	ATCC 19707	Noc_1090	ABA57595	CP000127
Nitrosococcus watsoni	C-113	-	-	-
Nitrosomonas marina	C-113a	**n/a**	**AAK53362**	**AF339048**
Nitrosomonas sp.	AL212	NAL212_2392	ADZ27212	CP002552
Nitrosomonas sp.	Is79A3	Nit79A3_2335	AEJ02107	CP002876
Nitrosomonas sp.	NO3W	**n/a**	**AAK53359**	**AF339045**
Nitrosomonas sp.	TA-921i-NH4	**n/a**	**AAK53363**	**AF339049**
Nitrosomonas sp.	URW	**n/a**	**AAK53360**	**AF339046**
Nitrosomonas sp.	C-56	**n/a**	**AAK53358**	**AF339044**
Nitrosomonas sp.	C-45	**n/a**	**AAK53361**	**AF339047**
Nitrosospira multiformis	ATCC 25196	Nmul_A1998	ABB75293	CP000103
Nitrosospira tenuis	Nv1	**n/a**	**ABK20188**	**EF016120**
Nitrospina gracilis	3/211	NITGR_100028	WP_005005387	CAQJ01000002 (contig 10)
Novosphingobium pentaromativorans	US6-1	-	-	-
Oceanimonas sp.	GK1	GU3_12660	AEY02287	CP003171
Ochrobactrum anthropi	LMG 2136	**n/a**	**CAJ76711**	**AM230820**
Ochrobactrum anthropi	ATCC 49188	Oant_1108	ABS13828	CP000758, chromsome 1 - copy A
		Oant_4379	ABS17079	CP000759 , chromosome 2
Ochrobactrum anthropi	FZX-1	**n/a**	**ADK76187**	**HM060297**
Ochrobactrum anthropi	YD50.2	**n/a**	**BAH28817**	**AB490236**
Ochrobactrum anthropi	YX0903	**n/a**	**ACY07615**	**GU073465**
Ochrobactrum anthropi	YX0703	**n/a**	**ADA67894**	**GU207402**
Ochrobactrum anthropi	49187	**n/a**	**AAX10149**	**AY916794**
Ochrobactrum intermedium	LMG 3301	OINT_1000886	EEQ95508	ACQA01000001, chromosome 1 (VBI00028_1)
Ochrobactrum sp.	2FB10	**n/a**	**AAL82508**	**AY078249**
Ochrobactrum sp.	3CB4	**n/a**	**AAL82509**	**AY078250**
Ochrobactrum sp.	3CB5	**n/a**	**AAL82510**	**AY078251**
Ochrobactrum sp.	4FB13	**n/a**	**AAL82511**	**AY078252**
Ochrobactrum sp.	R-24618	**n/a**	**CAJ76705**	**AM230812**
Ochrobactrum sp.	R-26825	**n/a**	**CAJ76786**	**AM230869**
Ochrobactrum sp.	R-27045	**n/a**	**CAJ76787**	**AM230870**

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Ochrobactrum sp.	R-28410	**n/a**	**CAJ76739**	**AM230887**
Oligotropha carboxidovorans	OM5	OCA5_c25200	AEI07215	CP002826
Opitutaceae bacterium	TAV-5	-	-	-
Opitutus terrae	PB90-1	Oter_1019	ACB74307	CP001032
Oscillatoria acuminata	PCC 6304	-	-	-
Oscillatoria nigro-viridis	PCC 7112	-	-	-
Owenweeksia hongkongensis	DSM 17368	-	-	-
Paenibacillus sp.	oral taxon 786 str. D14	POTG_00424	EES75193	GG695970 (genomic scaffold supercont1.1)
Parachlamydia acanthamoebae	UV-7	-	-	-
Paracoccus denitrificans	LMG 4049[T]	-	-	-
Paracoccus denitrificans	PD1222	-	-	n/i
Paracoccus denitrificans	unknown	-	-	-
Paracoccus denitrificans	NL1B8944	-	-	-
Paracoccus denitrificans	ATCC 17741	**n/a**	**ADK76189**	**HM060299**
Parvibaculum lavamentivorans	DS-1	Plav_1009	ABS62632	CP000774
Pasteurella bettyae	CCUG 2042	HMPREF1052_1571	EIJ70175	AJSX01000022 (contig00010)
Pedobacter saltans	DSM 12145	-	-	-
Persephonella marina	EX-H1	-	-	-
Phaeobacter gallaeciensis	BS107	RGBS107_00660	EDQ11847	ABIF01000008 (1102058320382)
Phaeobacter gallaeciensis	2.10	RG210_01580	EDQ08812	ABIE01000008 (1100869001550)
Phenylobacterium zuccineum	HLK1	-	-	-
Photobacterium damselae subsp. *damselae*	CIP 102761	-	-	-
Photobacterium profundum	3TCK	-	-	-
Photobacterium profundum	SS9	-	-	-
Polaromonas naphthalenivorans	CJ2	Pnap_1326	ABM36641	CP000529
Polymorphum gilvum	SL003B-26A1	-	-	-
Pontibacter sp.	BAB1700	-	-	-
Prevotella histicola	F0411	-	-	-
Prevotella multisaccharivorax	DSM 17128	-	-	-
Prevotella oulorum	F0390	-	-	-
Prevotella sp.	oral taxon 472 str. F0295	-	-	-
Prevotella sp.	oral taxon 317 str. F0108	-	-	-
Prevotella sp.	F0039	-	-	-
Propionibacterium acidiproprionici	ATCC 4875	-	-	-
Propionibacterium acnes	266	-	-	-
Propionibacterium acnes	6609	-	-	-
Propionibacterium acnes	ATCC 11828	-	-	-
Propionibacterium acnes	HL036PA1	-	-	-
Propionibacterium acnes	HL036PA2	-	-	-
Propionibacterium acnes	HL037PA2	-	-	-
Propionibacterium acnes	HL037PA3	-	-	-
Propionibacterium acnes	HL044PA1	-	-	-
Propionibacterium acnes	HL078PA1	-	-	-
Propionibacterium acnes	HL096PA2	-	-	-
Propionibacterium acnes	HL096PA3	-	-	-
Propionibacterium acnes	HL097PA1	-	-	-
Propionibacterium acnes	HL099PA1	-	-	-
Propionibacterium acnes	HL103PA1	-	-	-
Propionibacterium acnes	KPA171202	-	-	-
Propionibacterium acnes	PRP-38	-	-	-
Propionibacterium acnes	SK182	-	-	-
Propionibacterium acnes	TypeIA2 P.acn17	-	-	-
Propionibacterium acnes	TypeIA2 P.acn31	-	-	-
Propionibacterium acnes	TypeIA2 P.acn33	-	-	-
P. freudenreichii subsp. *shermanii*	CIRM-BIA1	-	-	-
Propionibacterium sp.	409-HC1	-	-	-
Propionibacterium sp.	CC003-HC2	-	-	-
Pseudoalteromonas haloplanktis	str. TAC125	PSHAa1477	CAI86552	CR954246 , chromosome 1
Pseudogulbenkiania ferrooxidans	2002	-	-	-
Pseudomonas aeruginosa	138244	-	-	-
Pseudomonas aeruginosa	152504	-	-	-
Pseudomonas aeruginosa	M18	-	-	-
Pseudomonas aeruginosa	MPAO1/P1	-	-	-
Pseudomonas aeruginosa	MPAO1/P2	-	-	-
Pseudomonas aeruginosa	NCGM2.S1	-	-	-
Pseudomonas aeruginosa	PA7	-	-	-
Pseudomonas aeruginosa	PADK2_CF510	-	-	-
Pseudomonas aeruginosa	PAO1	-	-	-
Pseudomonas aeruginosa	UCBPP-PA14	-	-	-
Pseudomonas aeruginosa	DSM 50071	-	-	-
Pseudomonas aeruginosa	19660 ExoU Island B	-	-	-
Pseudomonas aeruginosa	LESB58	-	-	-
Pseudomonas aeruginosa	DN24	**n/a**	**AAR11763**	**AY345247**
P. brassicacearum subsp. brassicacearum	NFM421	-	-	-
Pseudomonas chlororaphis	O6	PchlO6_5535	EIM17644	AHOT01000007 (Contig0027)
P. chlororaphis subsp. *aureofaciens*	ATCC 13985	-	CAA79939	Z21945
Pseudomonas denitrificans	unknown	-	-	-
Pseudomonas entomophila	str. L48	PSEEN5226	CAK17851	CT573326, chromosome
Pseudomonas fluorescens	F113	-	-	-
Pseudomonas fluorescens	Q8r1-96	-	-	-
Pseudomonas fluorescens	C7R12	-	-	-
Pseudomonas protegens	Pf-5	PFL_5501	AAY94707	CP000076
Pseudomonas mendocina	NK-01	MDS_0146	AEB56177	CP002620
Pseudomonas mendocina	CH91	-	AAL82514	AY078255
Pseudomonas sp.	2_1_26	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Pseudomonas sp.	MT-1	-	-	-
Pseudomonas sp.	G-179	**n/a**	**AAC79439**	**AF083948**
Pseudomonas sp.	R-24261	-	-	n/i
Pseudomonas sp.	S3(2012)	**n/a**	**AFP55599**	**JX082305**
Pseudomonas stutzeri	A1501	-	-	-
Pseudomonas stutzeri	ATCC 14405 = CCUG 16156	-	-	n/i
Pseudomonas stutzeri	ATCC 17588 = LMG 11199	-	-	-
Pseudomonas stutzeri	CCUG 29243	A458_10480	AFM33333	CP003677
Pseudomonas stutzeri	DSM 4166	-	-	-
Pseudomonas stutzeri	ZoBell ATCC 14405	-	-	-
Pseudomonas stutzeri	TS44	-	-	-
Pseudovibrio sp.	FO-BEG1	-	-	-
Pseudoxanthomonas suwonensis	11-1	Psesu_0353	ADV26214	CP002446
Psychrobacter sp.	1501	HMPREF9373_0600	EGK14836	AFHU01000052 (contig00052)
Psychrobacter sp.	PRwf-1	-	-	-
Psychroflexus torquis	ATCC 700755	-	-	-
Psychromonas ingrahamii	37	-	-	-
Pusilimonas sp.	T7-7	PT7_3282	AEC21822	CP002663
Pyrobaculum aerophilum	IM2	-	-	-
Pyrobaculum arsenaticum	DSM 13514	-	-	-
Pyrobaculum calidifontis	JCM 11548	-	-	-
Pyrobaculum oguniense	TE7	-	-	-
Pyrobaculum sp.	1860	-	-	-
Cupriavidus necator	H16	-	-	-
Ralstonia pickettii	12D	Rpic12D_4128	ACS65377	CP001645 , chromosome 2
Ralstonia pickettii	12J	Rpic_3912	ACD29019	CP001069, chromosome 2
Ralstonia solanacearum	str. CFBP2957	RCFBP_mp10045	CBJ52839	FP885907, plasmid RCFBPv3_mp
Ralstonia solanacearum	CMR15	CMR15_mp30147	CBJ41149	FP885896, plasmid CMR15_mp
Ralstonia solanacearum	GMI1000	RSp1503	CAD18654	AL646053, megaplasmid
Ralstonia solanacearum	IPO1609	RSIPO_03194	CAQ58776	CU914166
Ralstonia solanacearum	MolK2	RSMK05299	CAQ18424	CU694393
Ralstonia solanacearum	Po82	-	-	-
Ralstonia solanacearum	PSI07	RPSI07_3198	CBJ52550	FP885906
Ralstonia solanacearum	UW551	-	-	-
Ralstonia sp.	5_2_56FAA	HMPREF0989_00586	EGY61975	ACTT01000006 (cont1.6)
Ralstonia sp.	5_7_47FAA	HMPREF1004_00354	EFP67976	ACUF01000008 (cont1.8)
Ralstonia sp.	PBA	-	-	-
Reinekea blandensis	MED297	-	-	-
Rheinheimera nanhaiensis	E407-8	-	-	-
Rhizobium etli	CFN 42	RHE_PF00525	ABC94414	CP000138, plasmid p42f
Rhizobium hedysari	HCNT1	**n/a**	**AAB05880**	**U65658**
Rhizobium meliloti	JJ1c10	-	-	n/i
Rhizobium sp.	IAE-1	**n/a**	**ADK76190**	**HM060300**
Rhizobium sp.	PIP4	**n/a**	**AAZ06351**	**DQ096646**
Rhizobium sp.	PY13	**n/a**	**AAZ06350**	**DQ096645**
Rhizobium sp.	R-24654	**n/a**	**CAJ76707**	**AM230814**
Rhodanobacter fulvus	Jip2	UU9_14210	EIL87967	AJXU01000065 (contig065)
Rhodanobacter sp.	115	UU5_20555	EIL86839	AJXS01000480 (contig480) - copy B
		UU5_19496	EIL87082	AJXS01000454 (contig454) - copy A
Rhodanobacter denitrificans	116-2	UUC_15203	WP_007513576	AJXV01000054 (contig054)
Rhodanobacter denitrificans	2APBS1	**n/a**	**ADE28551**	**GU233006 - copy A**
		n/a	**ADE28552**	**GU233007 - copy B**
		R2APBS1DRAFT_1558	EHA65576	AGIL01000004 (ctg275) - copy C
Rhodanobacter spathiphylli	B39	-	-	-
Rhodanobacter thiooxydans	LCS2	UUA_11238	WP_008436910	AJXW01000052 (contig052)
Rhodobacter capsulatus	SB 1003	-	-	-
Rhodobacter sphaeroides	2.4.3	**n/a**	**AAB05767**	**U62291**
Rhodobacter sphaeroides	ATCC 17025	Rsph17025_1595	ABP70488	CP000661
Rhodobacter sphaeroides	KD131	RSKD131_0657	ACM00517	CP001150 , chromosome 1
Rhodobacter sphaeroides	WS8N	-	-	-
R. sphaeroides f. sp. *denitrificans*	IL106	-	-	n/i
Rhodococcus equi	103S	-	-	-
Rhodoferax ferrireducens	T118	-	-	-
Rhodopseudomonas palustris	BisA53	RPE_4071	ABJ07997	CP000463
Rhodopseudomonas palustris	CGA009	RPA3306	CAE28747	BX572603 (segment 11/16)
		RPA4145	CAE29586	BX572606 (segment 14/16)
Rhodopseudomonas palustris	DX-1	Rpdx1_4349	ADU45901	CP002418
Rhodopseudomonas palustris	TIE-1	Rpal_4623	ACF03114	CP001096 - copy A
		Rpal_3727	ACF02227	CP001096 - copy B
Rhodopseudomonas sp.	2-8	-	ADA82553	GU332847
Rhodospirillum centenum	SW	-	-	-
Rhodothermus marinus	DSM 4252	Rmar_1208	ACY48098	CP001807 + nirS (ACY47550)!!
Rhodothermus marinus	SG0.5JP17-172	SG0.5JP17-172	AEN73523	CP003029
Riemerella anatipestifer	DSM 15868	-	-	-
Riemerella anatipestifer	RA-GD	-	-	-
Riemerella anatipestifer	RA-YM	-	-	-
Riemerella anatipestifer	RA-CH-1	-	-	-
Robiginitalea biformata	HTCC 2501	-	-	-
Roseobacter denitrificans	OCh 114	-	-	-
Roseobacter litoralis	Och 149	-	-	-
Roseobacter sp.	SK209-2-6	RSK20926_01872	EBA14735	AAYC01000016
Roseovarius sp.	TM1035	-	-	-
Roseovarius sp.	217	ROS217_10017	EAQ23437	AAMV01000015 (1099463000266)
Rothia aeria	F0474	-	-	-
Rothia dentocariosa	ATCC 17931	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Rothia mucilaginosa	ATCC 25296	-	-	-
Rothia mucilaginosa	DY-18	-	-	-
Rothia mucilaginosa	M508	-	-	-
Rubrivivax gelatinosus	IL144	-	-	-
Rubrivivax gelatinosus	S1	-	-	-
Ruegeria pomeroyi	DSS-3 megaplasmid	-	-	-
Runella slithyformis	DSM 19594	-	-	-
Salinibacter ruber	M8	-	-	-
Salinisphaera shabanensis	E1L3A	SSPSH_19696	EGM25430	AFNV01000066 (Contig66)
Shewanella amazonensis	SB2B	Sama_2681	ABM00884	CP000507
Shewanella denitrificans	OS217	Sden_3482	ABE56757	CP000302
Shewanella frigidimarina	NCIMB 400	-	-	-
Shewanella halifaxensis	HAW-EB4	-	-	-
Shewanella loihica	PV-4	Shew_3335	ABO25201	CP000606
Shewanella piezotolerans	WP3	-	-	-
Shewanella putrefaciens	200	-	-	-
Shewanella putrefaciens	CN-32	-	-	-
Shewanella sediminis	HAW-EB3	-	-	-
Shewanella sp.	ANA-3	-	-	-
Shewanella sp.	MR-4	-	-	-
Shewanella sp.	MR-7	-	-	-
Shewanella sp.	W3-18-1	-	-	-
Shewanella woodyi	ATCC 51908	-	-	-
Sinorhizobium fredii	USDA 257	USDA257_c38590	AFL52404	CP003563
Sinorhizobium fredii	HH103	SFHH103_00930	CCE95429	HE616890, chromosome
Sinorhizobium fredii	NGR234	NGR_c09950	ACP24785	CP001389
Sinorhizobium medicae	WSM419	Smed_6278	ABR64876	CP000740, plasmid pSMED02
Sinorhizobium meliloti	1021	SMa1007	AAK65201	AE006469, plasmid pSymA
Sinorhizobium meliloti	BL225C	CP002741	AEG07313	CP002741, plasmid pSINMEB01
Sinorhizobium meliloti	JJ1C10	**n/a**	**AAS92898**	**AY536011, plasmid pSymA**
Sinorhizobium sp.	NP1	**n/a**	**ACM24772**	**FJ598613**
Sinorhizobium sp.	R-31759	**n/a**	**CAL48388**	**AM403563**
Sinorhizobium sp.	R-31764	**n/a**	**CAL48389**	**AM403564**
Sinorhizobium sp.	R-31816	**n/a**	**CAL48390**	**AM403565**
Sinorhizobium sp.	R-32546	**n/a**	**CAO85936**	**AM778663**
Sinorhizobium sp.	R-32549	**n/a**	**CAL48395**	**AM403570**
Solitalea canadensis	DSM 3403	SolcaDRAFT_1072	EHP05801	AGSC01000047 (Scan3403_Contig293) - copy B
		Solca_2885	AFD07908	CP003349 - copy A
Sorangium cellulosum	'So ce 56'	-	-	-
Sphaerobacter thermophilus	DSM 20745	Sthe_0152	ACZ37591	CP001823
Sphingobium yanoikuyae	XLDN2-5	-	-	-
Sphingomonas witchii	RW1	-	-	-
Staphylococcus aureus	O11	-	-	-
Staphylococcus aureus	O46	-	-	-
Staphylococcus aureus	08BA02176	-	-	-
Staphylococcus aureus	ST398/SO385	-	-	-
Staphylococcus aureus subsp. *aureus*	HO 5096 0412	-	-	-
Staphylococcus aureus subsp. *aureus*	21342	-	-	-
Staphylococcus aureus subsp. *aureus*	21310	-	-	-
Staphylococcus aureus subsp. *aureus*	21269	-	-	-
Staphylococcus aureus subsp. *aureus*	21264	-	-	-
Staphylococcus aureus subsp. *aureus*	21178	-	-	-
Staphylococcus aureus subsp. *aureus*	21195	-	-	-
Staphylococcus aureus subsp. *aureus*	21235	-	-	-
Staphylococcus aureus subsp. *aureus*	71193	-	-	-
Staphylococcus aureus subsp. *aureus*	21331	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-105	-	-	-
Staphylococcus aureus subsp. *aureus*	MRSA252	-	-	-
Staphylococcus aureus subsp. *aureus*	D139	-	-	-
Staphylococcus aureus subsp. *aureus*	JKD6159	-	-	-
Staphylococcus aureus subsp. *aureus*	CGS00	-	-	-
Staphylococcus aureus subsp. *aureus*	LGA251	-	-	-
Staphylococcus aureus subsp. *aureus*	DR10	-	-	-
Staphylococcus aureus subsp. *aureus*	ED133	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-125	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-157	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-189	-	-	-
Staphylococcus aureus subsp. *aureus*	21345	-	-	-
Staphylococcus aureus subsp. *aureus*	ATCC BAA-39	-	-	-
Staphylococcus aureus subsp. *aureus*	MN8	-	-	-
Staphylococcus aureus subsp. *aureus*	JKD6008	-	-	-
Staphylococcus aureus subsp. *aureus*	T0131	-	-	-
Staphylococcus aureus subsp. *aureus*	TCH60	-	-	-
Staphylococcus aureus subsp. *aureus*	TW20	-	-	-
Staphylococcus aureus subsp. *aureus*	H19	-	-	-
Staphylococcus aureus subsp. *aureus*	C160	-	-	-
Staphylococcus simulans	ACS-120-V-Sch1	-	-	-
Starkeya novella	DSM 506	Snov_1147	ADH88466	CP002026
Sulfobacillus acidophilus	DSM 10332	Sulac_3352	AEW06798	CP003179
Sulfobacillus acidophilus	TPY	TPY_3652	AEJ41804	CP002901
Sulfolobus islandicus	HVE10/4	-	-	-
Sulfolobus islandicus	L.S.2.15	-	-	-
Sulfolobus islandicus	REY15A	-	-	-
Sulfolobus islandicus	Y.G.57.14	-	-	-
Sulfolobus islandicus	Y.N.15.51	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Sulfolobus solfataricus	98/2	-	-	-
Sulfolobus solfataricus	P2	-	-	-
Sulfurimonas autotrophica	DSM 16294	-	-	-
Sulfurimonas denitrificans	DSM 1251	-	-	-
Sulfurimonas gotlandica	GD1 SMGD1	-	-	-
Sulfurovum sp.	NBC37-1	-	-	-
Symbiobacterium thermophilum	IAM 14863	STH741	BAD39726	AP006840
Synechocystis sp.	PCC 6803	-	-	-
Synechocystis sp.	PCC 6803 substr. GT-I	-	-	-
Synechocystis sp.	PCC 6803 substr. PCC-N	-	-	-
Synechocystis sp.	PCC 6803 substr. PCC-P	-	-	-
Syntrophobacter fumaroxidans	MPOB	-	-	-
Taylorella asinigenitalis	MCE3	TASI_1505	AEP37243	CP003059
Taylorella equigenitalis	MCE9	TEQUI_0546	ADU91488	CP002456
Thauera sp.	MZ1T	-	-	-
Thermaerobacter marianensis	DSM 12885	Tmar_1288	ADU51401	CP002344
Thermaerobacter subterraneus	DSM 13965	ThesuDRAFT_1115	EFR62310	AENY01000089 (Tsubt_Contig188)
Thermobaculum terrenum	ATCC BAA-798	Tter_2149	ACZ43050	CP001826, chromosome 2
Thermobifida fusca	YX	Tfu_2793	AAZ56827	CP000088
Thermomicrobium roseum	DSM 5159	-	-	-
Thermoproteus uzonensis	768-20	-	-	-
Thioalkalivibrio sulfidophilus	HL-EbGr7	-	-	-
Thioalkalivibrio thiocyanoxidans	ARh 4	-	-	-
Thiobacillus denitrificans	ATCC 25259	-	-	-
Thiocapsa marina	5811	-	-	-
Thiocystis violascens	DSM 198	-	-	-
Turneriella parva	DSM 21527	Turpa_2099	AFM12745	CP002959
Veillonella parvula	ATCC 17745	-	-	-
Veillonella parvula	DSM 2008	-	-	-
Veillonella sp.	oral taxon 158 str. F0412	-	-	-
Veilonella dispar	ATCC 17748	-	-	-
Veilonella parvula	ACS-068-V-Sch12	-	-	-
Vibrio orientalis	CIP 102891 = ATCC 33934	-	-	-
Vibrio tubiashii	ATCC 19109	-	-	-
Vibrio tubiashii	NCIMB 1337 = ATCC 19106	-	-	-
Vulcanisaeta distributa	DSM 14429	-	-	-
Waddlia chondrophila	WSU 86-1044	-	-	-
Waddlia chondrophila	2032/99	-	-	-
Xanthobacter autotrophicus	Py2	-	-	-
Zobellia galactanivorans	DsiJT	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Acaryochloris marina	MBIC11017	AM1_0481	ABW25535	CP000828, genome - copy A
		AM1_A0139	ABW31648	CP000838, plasmid pREB1 - copy B
Acaryochloris sp.	CCMEE 5410	ACCM5_010100035995	WP_010481731	AFEJ01000420 (contig00578)
Achromobacter arsenitooxidans	SY8	KYC_10271	EHK66525	AGUF01000040 (Contig00041)
Achromobacter cycloclastes	unknown	-	-	-
Achromobacter cycloclastes	ACCC 03051	-	-	-
Achromobacter cycloclastes	ATCC 21921 = IAM 1013	-	-	-
Achromobacter denitrificans	LMG 1231[T]	**n/a**	**CAK95692**	**AM284322**
Achromobacter sp.	DBTN3	**n/a**	**ACZ64697**	**GU181420**
Achromobacter xylosoxidans	A8	AXYL_02387	ADP15708	CP002287
Achromobacter xylosoxidans	AXX-A	AXXA_20873	EGP44459	AFRQ01000082 (contig00092)
Achromobacter xylosoxidans	C54	HMPREF0005_05369	EFV87353	ACRC01000039 (cont 1.39)
Achromobacter xylosoxidans	GIFU1051	-	-	-
Acidilobus saccharovorans	345-15	ASAC_0647	ADL19053	CP001742
Acidovorax delafieldii	2AN	AcdelDRAFT_2688	EER59746	ACQT01000100 (ctg00827)
Acidovorax ebreus	TPSY	Dtpsy_1000	ACM32478	CP001392
Acidovorax sp.	JS42	Ajs_1084	ABM41319	CP00539
Acidovorax sp.	NO-1	KYG_09275	EHL23016	AGTS01000075 (contig79)
Actinobacillus minor	202	-	-	-
Actinobacillus minor	NM305	-	-	-
Actinobacillus pleuropneumoniae sv. 1	str. 4074	-	-	-
Actinobacillus pleuropneumoniae sv. 2	str. S1536	-	-	-
Actinobacillus pleuropneumoniae sv. 2	str. 4226	-	-	-
Actinobacillus pleuropneumoniae sv. 3	str. JL03	-	-	-
Actinobacillus pleuropneumoniae sv. 4	str. M62	-	-	-
Actinobacillus pleuropneumoniae sv. 5b	L20	-	-	-
Actinobacillus pleuropneumoniae sv. 6	str. Femo	-	-	-
Actinobacillus pleuropneumoniae sv. 7	str. AP76	-	-	-
Actinobacillus pleuropneumoniae sv. 9	str. CVJ13261	-	-	-
Actinobacillus pleuropneumoniae sv. 10	str. D13039	-	-	-
Actinobacillus pleuropneumoniae sv. 12	str. 1096	-	-	-
Actinobacillus pleuropneumoniae sv. 13	str. N273	-	-	-
Actinobacillus succinogenes	130Z	Asuc_1602	ABR74956	CP000746
Actinobacillus suis	H91-0380	-	-	-
Actinobacillus ureae	ATCC 25976	-	-	-
Actinomyces coleocanis	DSM 15436	-	-	-
Actinomyces odontolyticus	ATCC 17982	-	-	-
Actinomyces sp.	oral taxon 170 str. F0386	HMPREF9056_00645	EGF57061	AFBL01000014 (A_spOraltaxon170F0386-1.0_Cont17.2)
Actinomyces sp.	oral taxon 171 str. F0337	HMPREF9057_01868	EFW26754	AECW01000290 (A_sp_Oral_taxon_171_F0337-1.0_Cont318.6)
Actinomyces sp.	oral taxon 178 str. F0338	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Actinomyces sp.	oral taxon 448 str. F0400	HMPREF9062_1327	EGQ73949	AFQC01000047 (contig00047)
Actinomyces sp.	oral taxon 849 str. F0330	HMPREF0975_00125	EHM95821	ACTB01000007 (cont1.7)
Actinomyces urogenitalis	DSM 15434	-	-	-
Actinosynnema mirum	DSM 43827	-	-	-
Aequorivita sublithincola	DSM 14238	-	-	-
Afipia sp.	1NLS2	-	-	n/i
Aggregatibacter aphrophilus	ATCC 33389	ATCC33389_1825	EGY30498	AEWB01000019 (contig00020)
Aggregatibacter aphrophilus	F0387	HMPREF9335_00871	EHB90309	ACZJ01000005 (cont1.5)
Aggregatibacter aphrophilus	NJ8700	NT05HA_1475	ACS97816	CP001607
Aggregatibacter segnis	ATCC 33393	HMPREF9064_0493	EFU67830	AEPS01000003 (contig00003)
Rhizobium radiobacter	CCNWGS0286	-	-	n/i
Rhizobium radiobacter	str. C58	-	-	-
Alcaligenes faecalis	S-6	-	-	-
Alcaligenes faecalis subsp. *faecalis*	LMG 1229^T	**n/a**	**CAK95709**	**AM284323**
Alcaligenes faecalis subsp. *faecalis*	NCIB 8687	QWA_07579	EJC62912	AKMR01000006 (Contig_7)
Achromobacter sp.	DSM 30128	**-**	**CAD45383**	**AJ507330**
Alcaligenes sp.	STC1	-	-	-
Achrom. xylosoxidans subsp. *xylosoxidans*	NCIMB 11015	**n/a**	**CAD45384**	**AJ507331**
Alcanivorax dieselolei	N1203	**n/a**	**BAH56661**	**AB453735**
Alcanivorax dieselolei	B5	B5T_03073	AFT71341	CP003466
Alicycliphilus denitrificans	BC	Alide_0128	ADU97914	CP002449
Alicycliphilus denitrificans	K601	Alide2_0119	AEB82557	CP002657
Alicycliphilus sp.	R-24604	**n/a**	**CAK95711**	**AM284325**
Alicycliphilus sp.	R-24606	**n/a**	**CAK95712**	**AM284326**
Alicycliphilus sp.	R-24611	**n/a**	**CAK95713**	**AM284328**
Alicycliphilus sp.	R-26814	**n/a**	**CAK95718**	**AM284335**
Alkalilimnicola ehrlichii	MLHE-1	-	-	n/i
Allochromatium vinosum	DSM 180	Alvin_0930	ADC61875	CP001896
Anaeromyxobacter dehalogenans	2CP-1	A2cp1_3860	ACL67183	CP001359 - copy A
		A2cp1_2550	ACL65887	CP001359 - copy B
Anaeromyxobacter dehalogenans	2CP-C	Adeh_3720	ABC83486	CP000251 - copy A
		Adeh_1403	ABC81177	CP000251 - copy B
		Adeh_3178	ABC82946	CP000251- copy C
Anaeromyxobacter dehalogenans	DCP18	-	-	n/i
Anaeromyxobacter sp.	Fw109-5	Anae109_2758	ABS26958	CP000769
Anaeromyxobacter sp.	K	AnaeK_3777	ACG74988	CP001131 - copy A
		AnaeK_2454	ACG73680	CP001131 - copy B
Anaerophaga sp.	HS1	AnHS1_010100003178	WP_010526654	AFSL01000014 (Contig14)
Anoxybacillus flavithermus	TNO-09.006 chrAF6	-	-	-
Arthrospira maxima	CS-328	AmaxDRAFT_1830	EDZ95560	ABYK01000010 (ctg10)
Arthrospira platensis	C1	SPLC1_S101990	EKD10169	AFXD01000010, chromosome 2 (scaffold 10)
Arthrospira sp.	PCC 8005	ARTHRO_790069	CCE20201	CAFN01000670 (Contig4774-2130)
Azoarcus aromaticum	EbN1	-	-	-
Azoarcus sp.	BH72	-	-	n/i
Azoarcus sp.	KH32C	-	-	n/i
Azospirillum amazonense	Y2	-	-	-
Azospirillum brasilense	Sp245	-	-	n/i
Azospirillum brasilense	SM	-	-	-
Azospirillum doebereinerae	GSF 71T	-	-	-
Azospirillum lipoferum	4B	-	-	n/i
Azospirillum sp.	B510	-	-	n/i
Bacillus azotoformans	LMG 9581^T	BAZO_00190	EKN71191	AJLR01000004 (contig04) - qnorB1
		BAZO_08916	WP_003331062	AJLR01000048 (contig47) - qnorB2
Bacillus bataviensis	LMG 21833^T	BABA_17212	WP_007086436	AJLS01000121 (contig121) - qnorB1
Bacillus licheniformis	ATCC 14580/DSM 13	BL01473	AAU23610	CP000002
Bacillus sp.	1NLA3E	B1NLA3EDRAFT_1367	EHP27021	AGJD01000003 (ctg163) - copy A
		B1NLA3EDRAFT_3340	EHP25171	AGJD01000011 (ctg155) - copy B
Bacillus sp.	2_A_57_CT2	-	-	-
Bacillus sp.	BT1B_CT2	HMPREF1012_00847	EFV73263	ACWC01000002 (cont1.2)
Bacillus sp.	Ult-108 clone A	-	-	-
Bacillus sp.	Ult-123 clone A	-	-	-
Bacillus sp.	Ult-130 clone A	-	-	-
Bacillus sp.	Ult-145 clone A	-	-	-
Bacillus sp.	Ult-356 clone A	-	-	-
Bacillus sp.	Ult-391 clone A	-	-	-
Bacillus sp.	Ult-41 clone A	-	-	-
Bacillus sp.	Ult-42 clone A	-	-	-
Bacillus sp.	Ult-42 clone B	-	-	-
Bacillus sp.	Ult-442 clone A	-	-	-
Bacillus sp.	Ult-46 clone A	-	-	-
Bacillus sp.	Ult-521 clone A	-	-	-
Bacillus sp.	Ult-530 clone A	-	-	-
Bacillus sp.	Ult-552 clone A	-	-	-
Bacillus sp.	Ult-640 clone A	-	-	-
Bacillus sp.	Ult-71 clone A	-	-	-
Bacillus sp.	Ult-816 clone A	-	-	-
Bacillus sp.	R-31770	**n/a**	**CAO85945**	**AM778672**
Bacillus sp.	R-31841	**n/a**	**CAO85944**	**AM778671**
Bacillus sp.	R-32526	**n/a**	**CAL48404**	**AM403579**
Bacillus sp.	R-32656	**n/a**	**CAO85946**	**AM778673**
Bacillus sp.	R-32694	**n/a**	**CAO85941**	**AM778668**
Bacillus sp.	R-32702	**n/a**	**CAO85943**	**AM778670**
Bacillus sp.	R-32709	**n/a**	**CAO85940**	**AM778667**
Bacillus sp.	R-32715	**n/a**	**CAL49430**	**AM404295**

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Bacillus sp.	R-33820	**n/a**	**CAO85942**	**AM778669**
Bacillus sp.	R-33773	**n/a**	**CAO85947**	**AM778674**
Bacillus sp.	R-32546	-	-	-
Bacillus sp.	10403023 (MM10403188)	-	-	-
Bacillus sp.	SH27	**n/a**	**ACB12224**	**EU374123**
Bacillus sp.	SH3	**n/a**	**ACB12214**	**EU374113**
Bacillus sp.	SH11	**n/a**	**ACB12218**	**EU374117**
Bacillus sp.	SH22	**n/a**	**ACB12222**	**EU374121**
Bacillus sp.	SH5	**n/a**	**ACB12215**	**EU374114**
Bacillus sp.	SH8	**n/a**	**ACB12216**	**EU374115**
Bacillus sp.	SH10	**n/a**	**ACB12217**	**EU374116**
Bacillus sp.	SH14	**n/a**	**ACB12219**	**EU374118**
Bacillus sp.	SH19	**n/a**	**ACB12220**	**EU374119**
Bacillus sp.	SH21	**n/a**	**ACB12221**	**EU374120**
Bacillus sp.	SH25	**n/a**	**ACB12223**	**EU374122**
Bacillus sp.	SH30	**n/a**	**ACB12225**	**EU374124**
Bacillus sp.	SH36	**n/a**	**ACB12226**	**EU374125**
Bacillus sp.	SH38	**n/a**	**ACB12227**	**EU374126**
Bacillus sp.	SH41	**n/a**	**ACB12228**	**EU374127**
Bacillus sp.	SH42	**n/a**	**ACB12229**	**EU374128**
Bacillus sp.	SH43	**n/a**	**ACB12230**	**EU374129**
Bacillus sp.	SH48	**n/a**	**ACB12231**	**EU374130**
Bacillus sp.	SH51	**n/a**	**ACB12232**	**EU374131**
Bacillus sp.	SH60	**n/a**	**ACB12233**	**EU374132**
Bacillus sp.	SH61	**n/a**	**ACB12234**	**EU374133**
Bacillus sp.	SH62	**n/a**	**ACB12235**	**EU374134**
Bacillus sp.	SH63	**n/a**	**ACB12236**	**EU374135**
Bacillus subtilis	BEST7613	BEST7613_4400	YP_007254964	NC_019948
Bdellovibrio bacteriovorus	HD100	-	-	-
Beggiatoa sp.	PS	BGP_3622	EDN69502	ABBZ01000429 (genomic contig22609_3622-3624)
		BDB_100018	CCA80339	ABBZ01000717 (genomic contig24680_5178-5180)
Belliella baltica	DSM 15883	-	-	n/i
Beta-proteobacterium	R1-Apr-MIB-6	-	-	-
Bizionia argentinensis	JUB59	-	-	-
blood disease bacterium	R229	BDB_100018	CCA80339	FR854066
Bordetella petrii	DSM 12804	Bpet3560	CAP43903	AM902716
Brachybacterium faecium	DSM 4810 strain 6-10	Bfae_28580	ACU86624	CP001643
Bradyrhizobium japonicum	USDA 110	-	-	n/i
Bradyrhizobium japonicum	ATCC 15067	-	-	-
Bradyrhizobium japonicum	USDA 6	-	-	n/i
Bradyrhizobium sp.	BTAi1	-	-	n/i
Bradyrhizobium sp.	ORS 278	-	-	n/i
Bradyrhizobium sp.	ORS 285	-	-	n/i
Bradyrhizobium sp.	ORS 375	-	-	n/i
Bradyrhizobium sp.	STM 3809	-	-	n/i
Bradyrhizobium sp.	STM 3843	-	-	n/i
Bradyrhizobium sp.	S23321	-	-	n/i
Brucella abortus	A13334	-	-	n/i
Brucella abortus	bv. 1 str. 9-941	-	-	-
Brucella abortus	bv. 1 str. NI010	-	-	n/i
Brucella abortus	bv. 1 str. NI016	-	-	n/i
Brucella abortus	bv. 1 str. NI021	-	-	n/i
Brucella abortus	bv. 1 str. NI259	-	-	n/i
Brucella abortus	bv. 1 str. NI435a	-	-	n/i
Brucella abortus	bv. 1 str. NI474	-	-	n/i
Brucella abortus	bv. 1 str. NI486	-	-	n/i
Brucella abortus	bv. 1 str. NI488	-	-	n/i
Brucella abortus	S19	-	-	-
Brucella abortus	str. 2308 A	-	-	n/i
Brucella canis	ATCC 23365	-	-	n/i
Brucella canis	HSK A52141	-	-	n/i
Brucella ceti	str. Cudo	-	-	-
Brucella inopinata	BO1	-	-	-
Brucella melitensis	ATCC 23457	-	-	n/i
Brucella melitensis	bv.1 str. 16M	-	-	-
Brucella melitensis	bv. 2 str. 63/9	-	-	-
Brucella melitensis	M28	-	-	n/i
Brucella melitensis	M5-90	-	-	n/i
Brucella melitensis	NI	-	-	n/i
Brucella microti	CCM 4915	-	-	n/i
Brucella ovis	ATCC 25840	-	-	-
Brucella pinnipedialis	B2/94	-	-	n/i
Brucella sp.	BO2	-	-	-
Brucella sp.	NF 2653	-	-	-
Brucella suis	ATCC 23445	-	-	n/i
Brucella suis	VBI22	-	-	n/i
Brucella suis	1330	-	-	-
Burkholderia mallei	ATCC 23344	BMA0633	AAU49689	CP000010, chromosome 1
Burkholderia mallei	GB8 horse 4	BMAGB8_0672	EEP85023	AAHO01000047 (contig_368)
Burkholderia mallei	PRL-20	-	-	n/i
		BMAPRL20_A0645	EES42422	AAZP01000166 (gcontig_1105338590729)
Burkholderia mallei	NCTC 10229	BMA10229_A2908	ABN01863	CP000546, chromosome 1
Burkholderia mallei	NCTC 10247	BMA10247_1694	ABO05816	CP000548, chromosome 1
Burkholderia mallei	SAVP1	BMASAVP1_0547	ABM48792	CP000525, chromosome 2
Burkholderia oklahomensis	C6786	BokIC_010100012193	WP_010116299	ABBG01000230

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Burkholderia oklahomensis	EO147	BoklE_010100012790	WP_010105151	ABBF01000339
Burkholderia pseudomallei	1026a	BP1026A_4020	EIF56798	AHJA01000245 (Contig0245)
Burkholderia pseudomallei	1106a	BURPS1106A_A1965	ABN94583	CP000573, chromosome 2
Burkholderia pseudomallei	1258a	BP1258A_0811	EIF67972	AHJB01000045 (Contig0045)
Burkholderia pseudomallei	1258b	BP1258B_0904	EIF69805	AHJC01000044 (Contig0044)
Burkholderia pseudomallei	1710b	BURPS1710b_2802	ABA50825	CP000124 , chromosome 1
Burkholderia pseudomallei	305	BURPS305_7410	EBA51041	AAYX01000001
		BP354A_0894	EIF81918	AGVS01000121 (Contig0121)
Burkholderia pseudomallei	354a	-	-	n/i
Burkholderia pseudomallei	354e	BP354E_0707	EIF77624	AHJD01000047 (Contig0047)
Burkholderia pseudomallei	576	BUC_3000	EEC36018	ACCE01000003 (BUC.Contig186)
Burkholderia pseudomallei	668	BURPS668_2680	ABN82161	CP000570, chromosome 1
Burkholderia pseudomallei	K96243	BPSL2351	CAH36354	BX571965, chromosome 1
Burkholderia pseudomallei	MSHR346	GBP346_A2859	ACQ96401	CP001408, chromosome 1
Burkholderia pseudomallei	Pakistan 9	BUH_2790	EEH24033	ACKA01000064 (BUH.Contig251)
Burkholderia thailandensis	E264	BTH_I1813	ABC38039	CP000086, chromosome 1
Burkholderia thailandensis	TXDOH	BthaT_010100024070	WP_009904577	ABBD01000554
Caldilinea aerophila	DSM 14535 = NBRC 104270	-	-	-
Campylobacter concisus	13826	CCC13826_1535	EAT98940	CP000792
Campylobacter concisus	UNSWCD	UNSWCD_247	EIF07271	AENQ01000014 (Contig_14)
Campylobacter curvus	525.92	CCV52592_1608	EAU00686	CP000767
Campylobacter fetus subsp. fetus	82-40	-	-	-
Campylobacter sp.	10_1_50	HMPREF1019_00956	EHL90154	ACWJ01000012
Cand. *Accumulibacter phosphatis* clade IIA	str. UW-1	CAP2UW1_2326	ACV35617	CP001715
Candidatus *Koribacter versatilis*	Ellin345	Acid345_0364	ABF39369	CP000360
Candidatus *Nitrospira defluvii*	unknown	-	-	-
Candidatus *Solibacter usitatus*	Ellin6076	Acid_2936	ABJ83921	CP000473
Capnocytophaga gingivalis	ATCC 33624	-	-	-
Capnocytophaga ochracea	F0287	HMPREF1977_0085	EFS98578	AEOH01000003 (contig00003)
Capnocytophaga sp.	oral taxon 338 str. F0234	-	-	-
Capnocytophaga sp.	oral taxon 329 str. F0087	HMPREF9074_03499	EGJ54958	AFHP01000080 (C_sporaltaxon329F0087-1.0_Cont297.1)
Capnocytophaga sp.	oral taxon 412 str. F0487	HMPREF1321_1370	EIW91935	AJZR01000040 (ctg1120005442970)
Capnocytophaga sp.	CM59	-	-	n/i
Capnocytophaga sputigena	ATCC 33612 strain Capno	CAPSP0001_0314	EEB65097	ABZV01000015 (Capno contig00009)
Cardiobacterium hominis	ATCC 15826	HMPREF0198_2276	EEV87596	ACKY01000119 (contig00187)
Cardiobacterium valvarum	F0432	HMPREF9080_00108	EHM56084	AGCM01000006 (C_valvarumF0432-1.0_Cont5.1)
Caulobacter segnis	ATCC 21756	Cseg_3031	ADG11473	CP002008
Cellulophaga algicola	DSM 14237	Celal_3930	ADV51174	CP002453
Cellvibrio japonicus	Ueda107	CJA_1546	ACE84886	CP000934
Cellvibrio sp.	BR	O59_004021	EIK43228	AICM01000010 (O59_Contig10)
Chelativorans sp.	BNC1	-	-	-
Chitinophaga pinensis	DSM 2588	Cpin_5786	ACU63206	CP001699
Chloroflexus aggregans	DSM 9485	-	-	-
Chloroflexus aurantiacus	J-10-fl	-	-	-
Chloroflexus sp.	Y-400-fl	-	-	-
Chromobacterium violaceum	ATCC 12472	CV_3494	AAQ61155	AE016825
Chroococcidiopsis thermalis	PCC 7203	Chro_3365	YP_007092694	NC_019695, chromosome
Chryseobacterium gleum	ATCC 35910	EFK34155	HMPREF0204_13224	ACKQ02000007 (Contig314)
Chthoniobacter flavus	Ellin428	-	-	-
Citreicella sp.	357	-	-	-
Colwellia psychrerythraea	34H	-	-	-
Corynebacterium accolens	ATCC 49725	HMPREF0276_1372	EEI14462	ACGD01000026 (contig00027)
Corynebacterium accolens	ATCC 49726	-	-	n/i
Corynebacterium aurimucosum	ATCC 700975	-	-	-
Corynebact. diphtheriae bv. *intermedius*	NCTC 5011	W5M_10471	EIK55371	AJVH01000028 (contig28)
Corynebacterium diphtheriae gravis	NCTC13129	DIP2249	CAE50773	BX248360 (segment 7/8)
Corynebacterium diptheriae	241	CD241_2139	AEX45191	CP003207
Corynebacterium diptheriae	31A	CD31A_2275	AEX42940	CP003206
Corynebacterium diptheriae	BH8	CDBH8_2232	AEX49746	CP003209
Corynebacterium diptheriae	C7 (beta)	CDC7B_2223	AEX68408	CP003210
Corynebacterium diptheriae	CDCE 8392	CDCE8392_2140	AEX73123	CP003211
Corynebacterium diptheriae	HC01	CDHC01_2139	AEX75380	CP003212
Corynebacterium diptheriae	HC02	CDHC02_2110	AEX77597	CP003213
Corynebacterium diptheriae	HC03	CDHC03_2131	AEX79856	CP003214
Corynebacterium diptheriae	HC04	CDHC04_2159	AEX82148	CP003215
Corynebacterium diptheriae	INCA 402	CDB402_2096	AEX47385	CP003208
Corynebacterium diptheriae	PW8	CDPW8_2214	AEX70857	CP003216
Corynebacterium diptheriae	VA01	CDVA01_2055	AEX84319	CP003217
Corynebacterium efficiens	YS-314	-	-	-
Corynebacterium pseudotuberculosis	1002	Cp1002_0125	ADL20031	CP001809
Corynebacterium pseudotuberculosis	267	Cp267_0135	AFH51043	CP003407
Corynebacterium pseudotuberculosis	316	Cp316_0140	AFB71474	CP003077
Corynebacterium pseudotuberculosis	3/99-5	Cp3995_0128	AEX38604	CP003152
Corynebacterium pseudotuberculosis	42/02-A	Cp4202_0124	AEP69400	CP003062
Corynebacterium pseudotuberculosis	C231	CpC231_0127	ADL09624	CP001829
Corynebacterium pseudotuberculosis	CIP 52.97	CpCIP5297_0136	AEQ05701	CP003061
Corynebacterium pseudotuberculosis	Cp162	Cp162_0131	AFM06508	CP003652
Corynebacterium pseudotuberculosis	FRC41	cpfrc_00128	ADK27921	CP002097
Corynebacterium pseudotuberculosis	P54B96	CpP54B96_0131	AFF21294	CP003385
Corynebacterium pseudotuberculosis	PAT10	CpPAT10_0127	AEK91471	CP002924
Corynebacterium pseudotuberculosis	I19	CpI19_0128	ADO25423	CP002251
Cupriavidus metallidurans	CH34	-	-	n/i
Cupriavidus necator	N-1	CNE_2c22880	AEI81236	CP002878, chromosome 2
Cupriavidus necator	LMG 1201	-	CAK95706	AM284317

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Cupriavidus sp.	R-31542	-	CAO85949	AM778676
Cupriavidus sp.	R-31543	-	CAO85950	AM778677
Cupriavidus sp.	R-31544	-	CAO85948	AM778675
Cupriavidus taiwanensis	LMG 19424	RALTA_B2087	CAQ72664	CU633750, chromosome 2
Cyanobacterium aponinum	PCC 10605	Cyan10605_3228	YP_007163318	NC_019776, chromosome
Cyanobacterium stanieri	PCC 7202	Cyast_2435	YP_007166030	NC_019778
Dechloromonas aromatica	RCB	-	-	-
Dechlorosoma suillum	PS	-	-	n/i
Denitrovibrio acetiphilus	DSM 12809	-	-	-
Desulfitobacterium dehalogenans	ATCC 51507	-	-	n/i
Desulfitobacterium dichloroeliminans	LMG P-21439	-	-	-
Desulfitobacterium hafniense	DCB-2	-	-	n/i
Desulfitobacterium hafniense	Y51	-	-	-
Desulfomonile tiedjei	DSM 6799	Desti_2522	AFM25202	CP003360
Desulfosporosinus meridiei	DSM 13257	-	-	-
Desulfosporosinus youngiae	DSM 17734	-	-	n/i
Desulfotomaculum ruminis	DSM 2154	-	-	-
Desulfovibrio sp.	U5L	DesU5LDRAFT_0749	EIG52452	JH600068 (genomic scaffold DesU5Lscaffold_2)
Diaphorobacter sp.	R-24610	**n/a**	**CAK95693**	**AM284327**
Diaphorobacter sp.	R-24612	**n/a**	**CAK95714**	**AM284329**
Diaphorobacter sp.	R-24661	**n/a**	**CAK95710**	**AM284324**
Diaphorobacter sp.	R-25011	**n/a**	**CAK95717**	**AM284334**
Diaphorobacter sp.	R-26815	**n/a**	**CAK95719**	**AM284336**
Diaphorobacter sp.	R-26840	**n/a**	**CAK95707**	**AM284320**
Diaphorobacter sp.	R-28417	**n/a**	**CAK95708**	**AM284321**
Dinoroseobacter shibae	DFL 12	-	-	-
Diplosphaera colitermitum	TAV2	-	-	n/i
Dyadobacter fermentans	DSM 18053	-	-	-
Eikenella corrodens	ATCC 23834	EIKCOROL_00933	EEG24299	ACEA01000017 (E_corrodens-1.0_Cont1.4)
Endosymbiont of *Riftia pachyptila*	Rifp1Symag	-	-	-
Ensifer sp.	2FB8	-	-	-
Ensifer sp.	4FB6	-	-	-
Enterococcus sp.	R-24626	**n/a**	**CAK95715**	**AM284330**
Ferroglobus placidus	DSM 10642	-	-	n/i
Flavobacteriaceae bacterium	3519-10	-	-	-
Flavobacteriales bacterium	ALC-1	-	-	-
Flavobacterium columnare	ATCC 49512	-	-	n/i
Flavobacterium columnare	unknown	-	-	-
Flavobacterium johnsoniae	UW101	Fjoh_2413	ABQ05440	CP000685
Flavobacterium sp.	F52	FF52_02000	WP_008462514	AKZQ01000006 (Contig06)
Flavobacterium sp.	CF136 PMI10	PMI10_02384	WP_007808030	AKJZ01000033 (PMI10_contig42.42)
Gamma-proteobacterium	HdN1	HDN1F_20450	CBL45628	FP929140
Gemmatimonas aurantiaca	T-27	-	-	-
Geobacillus kaustophilus	HTA426	GK0758	BAD75043	BA000043
Geobacillus kaustophilus	LMG 9819^T	**n/a**	**CDG32568**	**HG328790**
Geobacillus sp.	C56-T3	GC56T3_2788	ADI27733	CP002050
Geobacillus sp.	G11MC16	G11MC16DRAFT_0532	EDY07617	ABVH01000001 (ctg12)
Geobacillus sp.	Y412MC52	GYMC52_0678	ADU93167	CP002442
Geobacillus sp.	Y412MC61	GYMC61_1556	ACX78193	CP001794
Geobacillus sp.	Y4.1MC1	GY4MC1_3075	ADP75753	CP002293
Geobacillus stearothermophilus	LMG 6939T	**n/a**	**CDG32586**	**HG328808**
Geobacillus stearothermophilus	R-35646	**n/a**	**CDG32585**	**HG328807**
Geobacillus stearothermophilus	R-32513	**n/a**	**CDG32587**	**HG328809**
Geobacillus stearothermophilus	R-32605	**n/a**	**CDG32588**	**HG328810**
Geobacillus stearothermophilus	R-32635	**n/a**	**CDG32589**	**HG328811**
Geobacillus thermodenitrificans	NG80-2	GTNG_0643	ABO66023	CP000557
Geobacillus thermodenitrificans	LMG 17532T	**n/a**	**CDG32584**	**HG328806**
Geobacillus thermodenitrificans	R-35647	**n/a**	**CDG32583**	**HG328805**
Geobacillus thermodenitrificans	R-32614	**n/a**	**CDG32569**	**HG328791**
Geobacillus thermodenitrificans	R-32615	**n/a**	**CDG32570**	**HG328792**
Geobacillus thermodenitrificans	R-32616	**n/a**	**CDG32571**	**HG328793**
Geobacillus thermodenitrificans	R-32617	**n/a**	**CDG32572**	**HG328794**
Geobacillus thermodenitrificans	R-32618	**n/a**	**CDG32573**	**HG328795**
Geobacillus thermodenitrificans	R-32619	**n/a**	**CDG32574**	**HG328796**
Geobacillus thermodenitrificans	R-32621	**n/a**	**CDG32575**	**HG328797**
Geobacillus thermodenitrificans	R-32622	**n/a**	**CDG32576**	**HG328798**
Geobacillus thermodenitrificans	R-32623	**n/a**	**CDG32577**	**HG328799**
Geobacillus thermodenitrificans	R-32624	**n/a**	**CDG32578**	**HG328800**
Geobacillus thermodenitrificans	R-32625	**n/a**	**CDG32579**	**HG328801**
Geobacillus thermodenitrificans	R-32500	**n/a**	**CDG32580**	**HG328802**
Geobacillus thermodenitrificans	R-32506	**n/a**	**CDG32581**	**HG328803**
Geobacillus thermodenitrificans	R-32511	**n/a**	**CDG32582**	**HG328804**
Geobacillus thermoglucosidans	TNO-09.020	GT20_2715	EID43582	AJJN01000019 (contig27)
Geobacillus thermoglucosidasius	C56-YS93	Geoth_3090	AEH48965	CP002835
Geobacillus thermoleovorans	CCB_US3_UF5	GTCCBUS3UF5_8530	AEV18176	CP003125
Geobacillus toebii	R-32639	**n/a**	**CDG32590**	**HG328812**
Geobacter bemidjiensis	Bem	Gbem_3901	ACH40893	CP001124
Geobacter daltonii	FRC-32	Geob_0018	ACM18395	CP001390
Geobacter metallireducens	GS-15	Gmet_3493	ABB33698	CP000148
Geobacter metallireducens	RCH3	GeomeDRAFT_2270	EHP85799	AGJM01000024 (ctg00045)
Geobacter sp.	M21	GM21_3986	ACT20002	CP001661
Gillisia limnaea	DSM 15749	-	-	-
Gloeocapsa sp.	PCC 7428	Glo7428_4622	YP_007130218	NC_019745
Gramella forsetii	KT0803	-	-	-
Haemophilus haemolyticus	HK386	HMPREF1053_0482	EIJ74638	AJSV01000006 (contig00002)

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Haemophilus haemolyticus	M19107	GG7_1502	EGT75234	AFQN01000097
Haemophilus haemolyticus	M19501	GG9_1557	EGT74070	AFQO01000016
Haemophilus haemolyticus	M21127	GGA_1449	EGT74967	AFQP01000022 (M21127_022)
Haemophilus haemolyticus	M21621	GGC_0047	EGT81894	AFQQ01000001 (M21621_001)
Haemophilus haemolyticus	M21639	GGE_0248	EGT83120	AFQR01000006 (M21639_006)
Haemophilus parahaemolyticus	HK385	-	-	-
Haemophilus parahaemolyticus	HK411	-	-	-
Haemophilus parainfluenzae	ATCC 33392	HMPREF9417_0833	EGC72688	AEWU01000013 (contig00013)
Haemophilus parainfluenzae	HK262	HMPREF1118_1478	EIF37739	AJMW01000055 (contig00013)
Haemophilus parainfluenzae	HK2019	HMPREF1119_1398	EIJ29196	AJTC01000037 (contig00008)
Haemophilus parainfluenzae	T3T1	PARA_00150	CBW14123	FQ312002
Haemophilus pittmaniae	HK 85	-	-	-
Haemophilus sp.	oral taxon 851 str. F0397	HMPREF9096_00051	EHO49301	AGRK01000002 (H_sporaltaxon851F0397-1.0_Cont0.2)
Hahella chejuensis	KCTC 2396	HCH_04410	ABC31114	CP000155
Haladaptatus paucihalophilus	DX253	ZOD2009_20962	EFW90104	AEMG01000029 (contig00031)
Haliscomenobacter hydrossis	DSM 1100	Halhy_2238	AEE50119	CP002691
Haloarcula hispanica	ATCC 33960	HAH_2739	AEM58324	CP002921, chromosome 1
Haloarcula marismortui	ATCC 43049	rrnAC2272	AAV47100	AY596297, chromosome 1
Halobacterium sp.	DL1	-	-	-
Haloferax denitrificans	unknown	-	-	-
Haloferax lucentense	unknown	-	-	-
Haloferax mediterranei	ATCC 33500/R4	-	-	-
Haloferax volcanii	DS2	HVO_2147	ADE03879	CP001956
Halogeometricum borinquense	DSM 11551	Hbor_34300	ADQ68951	CP001692, plasmid pHBOR02
Halomicrobium mukohataei	DSM 12286	Hmuk_1980	ACV48093	CP001688
Halopiger xanaduensis	SH-6	Halxa_1516	AEH36148	CP002839 - copy A
		Halxa_4018	AEH38623	CP002839 - copy B
Halorhabdus utahensis	DSM 12940	-	-	-
Halorubrum lacusprofundi	ATCC 49239	Hlac_1902	ACM57480	CP001365, chromosome 1
Haloterrigena turkmenica	DSM 5511	Htur_0182	ADB59083	CP001860 - copy A
		Htur_0873	ADB59768	CP001860 - copy B
Herminiimonas arsenicoxydans	$ULPA_{S1}$	HEAR0048	CAL60284	CU207211
Herpetosiphon aurantiacus	DSM 785	-	-	-
Hydrogenobacter thermophilus	TK-6	-	-	-
Hydrogenophaga sp.	PBC	Q5W_0292	EIK92794	AJWL01000008 (contig9)
Hyphomicrobium denitrificans	1NES1	-	-	n/i
Hyphomicrobium denitrificans	ATCC 51888	-	-	n/i
Hyphomicrobium denitrificans	A3151	-	-	-
Idiomarina loihiensis	L2TR	IL0186	AAV81029	AE017340
Ignavibacterium album	JCM 16511	-	-	-
Imtechella halotolerans	K1	-	-	n/i
Intrasporangium calvum	DSM 43043	Intca_0298	ADU46853	CP002343
Intrasporangium sp.	4LS1	-	-	-
Kangiella koreensis	DSM 16069	Kkor_2038	ACV27448	CP001707
Kingella denitrificans	ATCC 33394	HMPREF9098_0738	EGC17927	AEWV01000013 (contig00013)
Kingella kingae	ATCC 23330	EGK09370	HMPREF0476_1034	AFHS01000036 (contig00036)
Kingella kingae	PYKK081	-	-	-
Kingella oralis	ATCC 51147	GCWU000324_03144	EEP66740	ACJW02000008 (K_oralis-1.0.1_Cont3.1)
Kyrpidia tusciae	DSM 2912	Btus_2112	ADG06795	CP002017
Labrenzia aggregata	IAM 12614	-	-	-
Lactobac. coryniformis subsp. *coryniformis*	CECT 5711	A11Y_60269	WP_003679986	AKFP01000079 (COR000079)
Lactobac. coryniformis subsp. *coryniformis*	KCTC 3167	LcorcK3_010100012707	WP_010011804	GL544636 (scaffold00050)
Lactobac. coryniformis subsp. *torquens*	KCTC 3535	LcortK3_010100002862	WP_010012682	AEOS01000077(contig00086)
Lactobacillus farciminis	KCTC 3681	LfarK3_010100006566	WP_010019570	GL575018 (genomic scaffold scaffold00003)
Lactobacillus fermentum	ATCC 14931	HMPREF0511_1469	EEI21575	ACGI01000109 (contig00134)
Lactobacillus fermentum	CECT 5716	LC40_1039	ADJ41603	CP002033
Lactobacillus fermentum	IFO3956	LAF_1643	BAG27979	AP008937
Lactobacillus fermentum	28-3-CHN	HMPREF0513_00350	WP_003685301	GG704700 (genomic scaffold supercont1.2)
Lactobacillus versmoldensis	KCTC 3814	LverK3_010100008579	WP_010625032	BACR01000037
Lautropia mirabilis	ATCC 51599	HMPREF0551_1739	EFV94483	AEQP01000017 (contig00017)
Leptonema illini	DSM 21528	-	-	n/i
Leptospira biflexa	serovar Patoc 'Patoc 1 (Ames)'	-	-	n/i
Leptospira biflexa	serovar Patoc 'Patoc 1 (Paris)'	-	-	n/i
Leptospira broomii	str. 5399	-	-	-
Leptospira inadai sv. *Lyme*	str. 10	-	-	-
Leptospira licerasiae	serovar Varillal str. VAR 010	-	-	n/i
Leptospira meyeri	serovar Hardjo str. Went 5	-	-	n/i
Leptothrix cholodnii	SP-6	-	-	-
Magnetospirillum gryphiswaldense	MSR-1	-	-	n/i
Mannheimia succiniciproducens	MBEL55E	MS0294	AAU36901	AE016827
Maribacter sp.	HTCC 2170	-	-	-
Marinilabilia sp.	AK2	-	-	-
Mariniradius saccharolyticus	AK6	-	-	-
Marinobacter aquaeolei	VT8	-	-	-
Marinobacter hydrocarbonoclasticus	617	-	-	-
Marinobacter hydrocarbonoclasticus	ATCC 49840	MARHY3054	CCG96517	FO203363
Marinobacter manganoxydans	MnI7-9	-	-	-
Marinobacter sp.	ELB17	-	-	-
Maritimibacter alkaliphilus	HTCC 2654	-	-	-
Marivirga tractuosa	DSM 4126	-	-	n/i
Melioribacter roseus	P3M	-	-	-
Mesorhizobium alhagi	CCNWXJ12-2	-	-	-
Mesorhizobium amorphae	CCNWGS0123	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Mesorhizobium australicum	WSM2073	-	-	-
Mesorhizobium ciceri biovar *biserrulae*	WSM1271	-	-	-
Mesorhizobium opportunistum	WSM2075	-	-	-
Mesorhizobium sp.	4FB11	-	-	-
Methylobacterium sp.	4-46	-	-	-
Methylocella silvestris	BL2	-	-	n/i
Methylocystis sp.	ATCC 49242	-	-	-
Methylocystis sp.	SC2	SC2p1_00450	CCD31960	FO000001, plasmid 1
		SC2p2_01300	CCD32286	FO000002, plasmid 2
Methylomonas sp.	16a	-	-	-
Methylophaga aminisulfidivorans	MP_54_1	MAMP_02723	EGL55729	AFIG01000001
Methylotenera mobilis	JLW8	-	-	n/i
Methylotenera versatilis	301	-	-	-
Microlunatus phosphovorus	NM-1	MLP_45230	BAK37537	AP012204
Micromonospora aurantiaca	ATCC 27029	-	-	-
Micromonospora sp.	L5	-	-	-
Mobilicoccus pelagius	NBRC 104925	MOPEL_041_00110	GAB48068	BAFE01000037 (contig: MOPEL041)
Moraxella catarrhalis	ETSU-9	ETSU-9	ACJ68087	EU861988
Moraxella catarrhalis	101P30B1	E9Y_02956	EGE25320	AEPC01000014 (ctg00014)
Moraxella catarrhalis	103P14B1	E9K_02526	EGE16117	AERE01000011 (ctg00017)
Moraxella catarrhalis	12P80B1	E9O_08814	EGE13682	AERG01000048 (ctg00062)
Moraxella catarrhalis	46P47B1	E9M_03684	EGE13569	AERF01000022 (ctg00022)
Moraxella catarrhalis	7169	E9G_02188	EGE12202	AERC01000011 (ctg00020)
		n/a	**ACJ68083**	**EU861987**
Moraxella catarrhalis	BBH18	MCR_0131	ADG60403	CP002005
Moraxella catarrhalis	BC1	E9Q_02703	EGE18978	AERH01000017 (ctg00016)
Moraxella catarrhalis	BC7	E9S_06840	EGE19271	AERI01000023 (ctg00023)
Moraxella catarrhalis	BC8	E9U_01516	EGE21888	AERJ01000006 (ctg00006)
Moraxella catarrhalis	CO72	E9W_00440	EGE26426	AERK01000001 (ctg00001)
Moraxella catarrhalis	O35E	EA1_00670	EGE27851	AERL01000003 (ctg00003)
		n/a	**ACJ68079**	**EU861986**
Moritella sp.	PE36	-	-	-
Muricauda ruestringensis	DSM 13258	-	-	n/i
Mycobacterium avium	104	MAV_4011	ABK65825	CP000479
Mycobact. avium subsp. *paratuberculosis*	k10	MAP_3181	AAS05729	AE016958
Mycobact. avium subsp. *paratuberculosis*	S397	MAPs_04650	EGO38309	AFIF01000102 (MavS397DRAFT__contig00221)
Mycobacterium colombiense	CECT 3035	MCOL_19202	EGT84744	AFVW01000008 (contig00008)
Mycobacterium intracellulare	ATCC 13950	OCU_38590	AFC45078	CP003322 - copy A
		OCU_19850	AFC43204	CP003322 - copy B
Mycobacterium intracellulare	MOTT-02	OCO_38620	AFC50225	CP003323 - copy A
		OCO_19730	AFC48336	CP003323 - copy B
Mycobacterium intracellulare	MOTT-64	OCQ_39780	AFC55490	CP003324 - copy A
		OCQ_18400	AFC53352	CP003324 - copy B
Mycobacterium parascrofulaceum	ATCC BAA-614	HMPREF0591_4306	EFG75777	ADNV01000299 (contig00386)
Mycobacterium rhodesiae	NBB3	MycrhN_0866	AEV71497	CP003169
Mycobacterium sp.	JDM601	JDM601_2974	AEF36974	CP002329
Mycobacterium sp.	JLS	Mjls_1539	ABN97337	CP000580
Mycobacterium sp.	KMS	Mkms_1592	ABL90801	CP000518
Mycobacterium sp.	MCS	Mmcs_1569	ABG07680	CP000384
Mycobacterium thermoresistibile	ATCC 19527	KEK_12388	EHI11695	AGVE01000046 (contig46)
Mycobacterium xenopi	RIVM700367	MXEN_08492	EID14647	AJFI01000042 (contig42)
Myroides injenensis	M09-0166	MinjM_010100004000	WP_010250761	BAEX01000015
Myroides odoratimimus	CIP 101113	HMPREF9715_03090	EHO06369	AGEE01000051 (cont1.51)
Myroides odoratimimus	CIP 103059	-	-	n/i
Myroides odoratimimus	CCUG 10230	HMPREF9712_03439	EHO05853	AGEC01000029 (cont1.29)
Myroides odoratimimus	CCUG 3837	HMPREF9711_03441	EKB02235	AGZK01000045.1 (cont1.45)
Myroides odoratimimus	CCUG 12901	HMPREF9714_03008	EHO06528	AGED01000045 (cont1.45)
Myroides odoratus	DSM 2801	Myrod_2504	EHQ43325	CM001437
Natrinema pellirubrum	DSM 15624	NatpeDRAFT_2115	EHA73199	AGIN01000007 (ctg250)
Natronomonas pharaonis	DSM 2160	-	-	-
Neisseria bacilliformis	ATCC BAA-1200	HMPREF9123_2340	EGF08905	AFAY01000048 (contig00048)
Neisseria cinerea	ATCC 14685	NEICINOT_05025	EEZ70839	ACDY02000017 (N_cinerea-1.0.2_Cont16.1)
Neisseria elongata subsp. *glycolytica*	ATCC 29315	NEIELOOT_00840	EFE50343	ADBF01000022 (N_elongata-1.0.1_Cont22.1)
Neisseria flavescens	NRL30031/H210	NEIFLAOT_00819	EEG34094	ACEN01000020 (N_flavescens-1.0_Cont20.1)
Neisseria flavescens	SK114	NEIFL0001_1541	EER55675	ACQV01000027 (ctg1118407793875)
Neisseria gonorrhoeae	NCCP 11945	NGK_1489	ACF30147	CP001050
Neisseria gonorrhoeae	TCDC-NG08107	NGTW08_1168	ADV08136	CP002440
Neisseria gonorrhoeae	FA 1090	NGO1275	AAW89932	AE004969
Neisseria gonorrhoeae	R10	-	-	-
Neisseria lactamica	020-06	NLA_6500	CBN86886	FN995097
Neisseria lactamica	ATCC 23970	-	-	n/i
Neisseria lactamica	Y92-1009	-	-	-
Neisseria macacae	ATCC 33926	HMPREF9418_0816	EGQ77691	AFQE01000037 (contig00037)
Neisseria meningitidis	M01-240355	NMBM01240355_1552	ADZ00048	CP002422
Neisseria meningitidis	M01-240149	NMBM01240149_0555	ADY97161	CP002421
Neisseria meningitidis	M01-240013	NMBM01240013_0671	EGC67109	AEQL01000019 (gnmgb013c.contig.18)
Neisseria meningitidis	M04-240196	NMBM04240196_0602	ADZ01099	CP002423
Neisseria meningitidis	M0579	NMBM0579_0609	EGC59316	AEQH01000009 (gnmm0579c.contig.8)
Neisseria meningitidis	M13399	NMBM13399_0607	EGC57340	AEQG01000017 (gnmm13399.contig.16)
Neisseria meningitidis	M6190	NMBM6190_0497	EGC55375	AEQF01000016 (gnmm6190c.contig.15)
Neisseria meningitidis	MC58	NMB1622	AAF41974	AE002098
Neisseria meningitidis	N1568	NMXN1568_0554	EGC51539	AEQD01000024 (menX.contig.23)
Neisseria meningitidis	NM220	NMY220_1522	EHP14602	AGRR01000253 (ntnm06c.contig.300)

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Neisseria meningitidis	NM233	NMY233_1502	EHP14994	AGRQ01000199 (ntnm05c.contig.198)
Neisseria meningitidis	NZ-05/33	NMBNZ0533_1599	ADZ04001	CP002424
Neisseria meningitidis	CU385	NMBCU385_0568	EGC63246	AEQJ01000019 (gnmcu385c.contig.18)
Neisseria meningitidis	H44/76	NMBH4476_0604	ADY95238	CP002420
		NMH_2252	EFV62859	AEQZ01000044 (contig7)
Neisseria meningitidis	WUE 2594	NMAA_1352	CBY91281	FR774048
Neisseria meningitidis	alpha14	NMO_1451	CBA07160	AM889136
Neisseria meningitidis	alpha153	NME_0084	CBA03469	AM889137
Neisseria meningitidis	alpha275	NMW_0421	CBA05109	AM889138
Neisseria meningitidis	alpha710	NMBB_1862	ADO32071	CP001561
Neisseria meningitidis	ATCC 13091	HMPREF0602_0396	EFM05098	AEEF01000022 (contig00022)
Neisseria meningitidis	OX99.30304	NMBOX9930304_0551	EGC53382	AEQE01000027 (gnmox.contig.26)
Neisseria meningitidis	serogroup A strain Z2491	NMA1886	CAM09005	AL157959
Neisseria meningitidis	serogroup C FAM18	NMC1548	CAM10743	AM421808
Neisseria meningitidis	053442	NMCC_1531	ABX73688	CP000381
Neisseria mucosa	ATCC 25996	NEIMUCOT_06335	EFC87240	ACDX02000025 (N_mucosa-1.0.2_Cont24.1)
Neisseria mucosa	C102	HMPREF0604_00160	EFV81581	ACRG01000001 (Cont1.1)
Neisseria polysaccharea	ATCC 43768	NEIPOLOT_02445	EFH21817	ADBE01000130 (N_polysacchareae-1.0.1_Cont275.1)
Neisseria sicca	ATCC 29256	NEISICOT_01650	EET44688	ACKO02000008 (N_sicca-1.0.1_Cont7.1)
Neisseria sicca	VK64	HMPREF1051_1503	EIG29849	AJMT01000042 (ctg120005024834)
Neisseria sp.	GT4A_CT1	HMPREF1028_01612	EGY59977	ACWS01000071 (Cont1.71)
Neisseria sp.	oral taxon 014 str. F0314	-	-	n/i
Neisseria subflava	NJ9703	NEISUBOT_03686	EFC52850	ACEO02000002 (N_subflava-1.0.1_Cont1.1)
Neisseria weaveri	LMG 5135	l11_03480	EGV38901	AFWQ01000003 (NW5135.2_3)
Neisseria weaveri	ATCC 51223	l13_08520	EGV36291	AFWR01000032 (NW51223.2_32)
Neisseriaceaea bacterium	NB-13	-	-	GU066897
Niabella soli	DSM 19437	NiasoDRAFT_2853	EHP53274	AGSA01000027 (Nsol19437_Contig173)
Niastella koreensis	GR20-10	-	-	-
Nitratifractor salsuginis	DSM 16511	-	-	n/i
Nitratireductor aquibiodomus	RA22	-	-	-
Nitratireductor aquibiodomus	NL31	-	-	-
Nitratiruptor sp.	SB155-2	-	-	n/i
Nitrosococcus halophilus	Nc4	-	-	n/i
Nitrosococcus oceani	ATCC 19707	-	-	-
Nitrosococcus watsoni	C-113	Nwat_0420	ADJ27385	CP002086
Nitrosomonas marina	C-113a	-	-	n/i
Nitrosomonas sp.	AL212	-	-	n/i
Nitrosomonas sp.	Is79A3	-	-	-
Nitrosomonas sp.	NO3W	-	-	n/i
Nitrosomonas sp.	TA-921i-NH4	-	-	n/i
Nitrosomonas sp.	URW	-	-	n/i
Nitrosomonas sp.	C-56	-	-	-
Nitrosomonas sp.	C-45	-	-	-
Nitrosospira multiformis	ATCC 25196	-	-	-
Nitrosospira tenuis	Nv1	-	-	-
Nitrospina gracilis	3/211	-	-	-
Novosphingobium pentaromativorans	US6-1	NSU_4542	EHJ58490	AGFM01000077 (contig00077)
Oceanimonas sp.	GK1	GU3_00450	AEX99846	CP003171
Ochrobactrum anthropi	LMG 2136	-	-	-
Ochrobactrum anthropi	ATCC 49188	-	-	n/i
Ochrobactrum anthropi	FZX-1	-	-	-
Ochrobactrum anthropi	YD50.2	-	-	n/i
Ochrobactrum anthropi	YX0903	-	-	-
Ochrobactrum anthropi	YX0703	-	-	-
Ochrobactrum anthropi	49187	-	-	-
Ochrobactrum intermedium	LMG 3301	-	-	-
Ochrobactrum sp.	2FB10	-	-	-
Ochrobactrum sp.	3CB4	-	-	-
Ochrobactrum sp.	3CB5	-	-	-
Ochrobactrum sp.	4FB13	-	-	-
Ochrobactrum sp.	R-24618	-	-	-
Ochrobactrum sp.	R-26825	-	-	-
Ochrobactrum sp.	R-27045	-	-	-
Ochrobactrum sp.	R-28410	-	-	-
Oligotropha carboxidovorans	OM5	-	-	-
Opitutaceae bacterium	TAV-5	-	-	-
Opitutus terrae	PB90-1			-
Oscillatoria acuminata	PCC 6304	Oscil6304_4731	YP_007088162	NC_019693
Oscillatoria nigro-viridis	PCC 7112	Osc7112_0369	YP_007113400	NC_019729
Owenweeksia hongkongensis	DSM 17368	-	-	-
Paenibacillus sp.	oral taxon 786 str. D14	-	-	-
Parachlamydia acanthamoebae	UV-7	PUV_24980	CCB87448	FR872580
Paracoccus denitrificans	LMG 4049^T	-	CAK95689	AM284316
Paracoccus denitrificans	PD1222	-	-	n/i
Paracoccus denitrificans	unknown	-	-	-
Paracoccus denitrificans	NL1B8944	-	-	-
Paracoccus denitrificans	ATCC 17741	-	-	-
Parvibaculum lavamentivorans	DS-1	Plav_1004	ABS62627	CP000774
Pasteurella bettyae	CCUG 2042	-	-	-
Pedobacter saltans	DSM 12145	-	-	-
Persephonella marina	EX-H1	-	-	n/i
Phaeobacter gallaeciensis	BS107	-	-	n/i
Phaeobacter gallaeciensis	2.10	-	-	n/i
Phenylobacterium zuccineum	HLK1	PHZ_c0619	ACG77033	CP000747
Photobact. damselae subsp. *damselae*	CIP 102761	VDA_001352	EEZ40327	ADBS01000001 (Contig58)

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Photobacterium profundum	3TCK	-	-	-
Photobacterium profundum	SS9	-	-	-
Polaromonas naphthalenivorans	CJ2	Pnap_1328	ABM36643	CP000529
Polymorphum gilvum	SL003B-26A1	-	-	CP002568
Pontibacter sp.	BAB1700	O71_22439	WP_007660492	AKIS01000202 (contig202)
Prevotella histicola	F0411	-	-	-
Prevotella multisaccharivorax	DSM 17128	-	-	-
Prevotella oulorum	F0390	-	-	-
Prevotella sp.	oral taxon 472 str. F0295	HMPREF6745_2817	EEX51751	ACZS01000166 (contig00231)
Prevotella sp.	oral taxon 317 str. F0108	HMPREF0670_01137	EEX51751	GG740072
Prevotella sp.	F0039	HMPREF0669_01421	WP_009228594	GG740059
Propionibacterium acidiproprionici	ATCC 4875	PACID_27170	YP_006981839	NC_019395
Propionibacterium acnes	266	PAZ_c20630	AEE73187	CP002409
Propionibacterium acnes	6609	TIB1ST10_10055	AEH30283	CP002815
Propionibacterium acnes	ATCC 11828	TIIST44_02655	AER05050	CP003084
Propionibacterium acnes	HL036PA1	HMPREF9604_01023	EFS58915	ADYL01000004 (P_acnesHL036PA1-1.0_Cont2.1)
Propionibacterium acnes	HL036PA2	HMPREF9605_02551	EFS60059	ADYU01000024 (P_acnesHL036PA2-1.0_Cont16.1)
Propionibacterium acnes	HL037PA2	HMPREF9621_01713	EFS73878	ADYH01000037 (P_acnesHL037PA2-1.0_Cont53.3)
Propionibacterium acnes	HL037PA3	HMPREF9622_01501	EFT15500	ADXV01000031 (P_acnesHL037PA3-1.0_Cont25.1)
Propionibacterium acnes	HL044PA1	HMPREF9607_02337	EFS91432	ADZU01000040 (P_acnesHL044PA1-1.0_Cont33.2)
Propionibacterium acnes	HL078PA1	HMPREF9569_01456	EFT52941	ADZG01000049 (P_acnesHL078PA1-1.0_Cont89.1)
Propionibacterium acnes	HL096PA2	HMPREF9338_01136	EGE72632	ADWC01000005 (P_acnesHL096PA2-1.0_Cont2.1)
Propionibacterium acnes	HL096PA3	HMPREF9337_02401	EGE67462	ADWB01000033 (P_acnesHL096PA3-1.0_Cont108.1)
Propionibacterium acnes	HL097PA1	HMPREF9344_01376	EGE74809	ADWI01000012 (P_acnesHL097PA1-1.0_Cont32.1)
Propionibacterium acnes	HL099PA1	HMPREF9343_00705	EGF75096	ADWH01000031 (P_acnesHL099PA1-1.0_Cont61.1)
Propionibacterium acnes	HL103PA1	HMPREF9341_02314	EGE67618	ADWF01000008 (P_acnesHL103PA1-1.0_Cont9.3)
Propionibacterium acnes	KPA171202	PPA1975	AAT83691	AE017283
Propionibacterium acnes	PRP-38	TICEST70_01450	EIA12893	AIJP01000001 (contig001)
Propionibacterium acnes	SK182	HMPREF9205_1241	EGR94721	AFUM01000009 (ctg1123499743119)
Propionibacterium acnes	TypeIA2 P.acn17	TIA2EST22_09670	AEW82232	CP003196
Propionibacterium acnes	TypeIA2 P.acn31	TIA2EST36_09650	AEW84498	CP003197
Propionibacterium acnes	TypeIA2 P.acn33	TIA2EST2_09610	AEW79990	CP003195
P. freudenreichii subsp. *shermanii*	CIRM-BIA1	PFREUD_00980	CBL55602	FN806773
Propionibacterium sp.	409-HC1	HMPREF9947_1083	EGL46329	AFIK01000017 (ctg1128491765063)
Propionibacterium sp.	CC003-HC2	HMPREF9949_0105	EGR91283	AFUK01000001
Pseudoalteromonas haloplanktis	str. TAC125	PSHAa2417	CAI87466	CR954246, chromosome 1
Pseudogulbenkiania ferrooxidans	2002	-	-	n/i
Pseudomonas aeruginosa	138244	-	-	n/i
Pseudomonas aeruginosa	152504	-	-	n/i
Pseudomonas aeruginosa	M18	-	-	n/i
Pseudomonas aeruginosa	MPAO1/P1	-	-	n/i
Pseudomonas aeruginosa	MPAO1/P2	-	-	n/i
Pseudomonas aeruginosa	NCGM2.S1	-	-	n/i
Pseudomonas aeruginosa	PA7	-	-	n/i
Pseudomonas aeruginosa	PADK2_CF510	-	-	n/i
Pseudomonas aeruginosa	PAO1	-	-	-
Pseudomonas aeruginosa	UCBPP-PA14	-	-	-
Pseudomonas aeruginosa	DSM 50071	-	-	-
Pseudomonas aeruginosa	19660 ExoU Island B	EXB34	ABD94720	DQ437743
Pseudomonas aeruginosa	LESB58	-	-	n/i
Pseudomonas aeruginosa	DN24	-	-	-
P. brassicacearum subsp. brassicacearum	NFM421	-	-	n/i
Pseudomonas chlororaphis	O6	-	-	n/i
P. chlororaphis subsp. *aureofaciens*	ATCC 13985	-	-	-
Pseudomonas denitrificans	unknown	-	-	-
Pseudomonas entomophila	str. L48	-	-	-
Pseudomonas fluorescens	F113	-	-	n/i
Pseudomonas fluorescens	Q8r1-96	-	-	n/i
Pseudomonas fluorescens	C7R12	-	-	-
Pseudomonas protegens	Pf-5	-	-	-
Pseudomonas mendocina	NK-01	-	-	n/i
Pseudomonas mendocina	CH91	-	-	-
Pseudomonas sp.	2_1_26	-	-	n/i
Pseudomonas sp.	MT-1	-	-	-
Pseudomonas sp.	G-179	-	-	-
Pseudomonas sp.	R-24261	**n/a**	**CAK95690**	**AM284318**
Pseudomonas sp.	S3(2012)	-	-	-
Pseudomonas stutzeri	A1501	-	-	-
Pseudomonas stutzeri	ATCC 14405 = CCUG 16156	-	-	n/i
Pseudomonas stutzeri	ATCC 17588 = LMG 11199	-	-	n/i
Pseudomonas stutzeri	CCUG 29243	-	-	n/i
Pseudomonas stutzeri	DSM 4166	-	-	n/i
Pseudomonas stutzeri	ZoBell ATCC 14405	-	-	-
Pseudomonas stutzeri	TS44	-	-	n/i
Pseudovibrio sp.	FO-BEG1	-	-	n/i
Pseudoxanthomonas suwonensis	11-1	Psesu_0363	ADV26224	CP002446
Psychrobacter sp.	1501	HMPREF9373_1777	EGK11392	AFHU01000147 (contig00147)
Psychrobacter sp.	PRwf-1	PsycPRwf_1520	ABQ94463	CP000713
Psychroflexus torquis	ATCC 700755	-	-	-
Psychromonas ingrahamii	37	-	-	-
Pusilimonas sp.	T7-7	PT7_0538	AEC19078	CP002663 - copy A
		PT7_2183	AEC20723	CP002663 - copy B
Pyrobaculum aerophilum	IM2	PAE3603	AAL65035	AE009441
Pyrobaculum arsenaticum	DSM 13514	Pars_0502	ABP50097	CP000660

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Pyrobaculum calidifontis	JCM 11548	Pcal_1908	ABO09324	CP000561
Pyrobaculum oguniense	TE7	Pogu_1845	AFA39872	CP003316
Pyrobaculum sp.	1860	P186_0748	AET32196	CP003098
Cupriavidus necator	H16	**n/a**	**AAC45803**	**AF002661**
		n/a	**AAC45801**	**AF002217, megaplasmid pHG1**
		H16_B2323	CAJ97105	AM260480 , chromosome 2
Ralstonia pickettii	12D	Rpic12D_4126	ACS65375	CP001645, chromosome 2
Ralstonia pickettii	12J	Rpic_4013	ACD29117	CP001069, chromosome 2
Ralstonia solanacearum	str. CFBP2957	RCFBP_mp10043	CBJ52837	FP885907, plasmid RCFBPv3_mp
Ralstonia solanacearum	CMR15	-	-	n/i
Ralstonia solanacearum	GMI1000	RSp1505	CAD18656	AL646053 (megaplasmid)
Ralstonia solanacearum	IPO1609	RSIPO_03196	CAQ58778	CU914166
Ralstonia solanacearum	MolK2	RSMK05297	CAQ18422	CU694393
Ralstonia solanacearum	Po82	RSPO_m00042	AEG70684	CP002820, megaplasmid
Ralstonia solanacearum	PSI07	RPSI07_3196	CBJ52548	FP885906, chromosome
Ralstonia solanacearum	UW551	RRSL_00759	EAP71019	AAKL01000070 (Cont0518)
Ralstonia sp.	5_2_56FAA	HMPREF0989_00584	EGY61973	ACTT01000006 (cont1.6)
Ralstonia sp.	5_7_47FAA	HMPREF1004_00356	EFP67978	ACUF01000008 (cont1.8)
Ralstonia sp.	PBA	MW7_1769	EIZ03729	AKCV01000022 (contig24)
Reinekea blandensis	MED297	-	-	-
Rheinheimera nanhaiensis	E407-8	RNAN_0925	GAB57954	BAFK01000004 (contig: CTG004)
Rhizobium etli	CFN 42	-	-	-
Rhizobium hedysari	HCNT1	-	-	-
Rhizobium meliloti	JJ1c10	-	-	n/i
Rhizobium sp.	IAE-1	-	-	-
Rhizobium sp.	PIP4	-	-	-
Rhizobium sp.	PY13	-	-	-
Rhizobium sp.	R-24654	-	-	-
Rhodanobacter fulvus	Jip2	UU9_06139	EIL90362	AJXU01000028 (contig028)
Rhodanobacter sp.	115	UU5_06228	EIL96808	AJXS01000157 (contig157)
Rhodanobacter denitrificans	116-2	UUC_01627	EIM04569	AJXV01000003 (contig003) - copy B
		UUC_09598	EIM02309	AJXV01000027 (contig027) - copy A
Rhodanobacter denitrificans	2APBS1	R2APBS1DRAFT_2393	EHA64725	AGIL01000007 (ctg272)
Rhodanobacter spathiphylli	B39	UU7_08950	EIL93273	AJXT01000021 (contig021) - copy A
		UU7_09730	EIL93040	AJXT01000024 (contig024) - copy B
Rhodanobacter thiooxydans	LCS2	UUA_00495	EIM02931	AJXW01000003 (contig003) - copy B
		UUA_04773	EIM01356	AJXW01000020 (contig020) - copy A
Rhodobacter capsulatus	SB 1003	-	-	-
Rhodobacter sphaeroides	2.4.3	-	-	-
Rhodobacter sphaeroides	ATCC 17025	-	-	-
Rhodobacter sphaeroides	KD131	-	-	n/i
Rhodobacter sphaeroides	WS8N	-	-	n/i
R. sphaeroides f. sp. *denitrificans*	IL106	-	-	n/i
Rhodococcus equi	103S	REQ_03280	CBH46468	FN563149, chromosome
Rhodoferax ferrireducens	T118	Rfer_1886	ABD69612	CP000267
Rhodopseudomonas palustris	BisA53	-	-	-
Rhodopseudomonas palustris	CGA009	-	-	-
Rhodopseudomonas palustris	DX-1	-	-	n/i
Rhodopseudomonas palustris	TIE-1	-	-	n/i
Rhodopseudomonas sp.	2-8	-	-	-
Rhodospirillum centenum	SW	RC1_3976	ACJ01317	CP000613
Rhodothermus marinus	DSM 4252	-	-	-
Rhodothermus marinus	SG0.5JP17-172	-	-	-
Riemerella anatipestifer	DSM 15868	-	-	-
				-
Riemerella anatipestifer	RA-GD	-	-	-
Riemerella anatipestifer	RA-YM	-	-	-
Riemerella anatipestifer	RA-CH-1	-	-	-
Robiginitalea biformata	HTCC 2501	-	-	-
Roseobacter denitrificans	OCh 114	-	-	-
Roseobacter litoralis	Och 149	-	-	n/i
Roseobacter sp.	SK209-2-6	-	-	-
Roseovarius sp.	TM1035	-	-	-
Roseovarius sp.	217	-	-	-
Rothia aeria	F0474	HMPREF1324_0747	EID51972	AJJQ01000004 (contig00007)
Rothia dentocariosa	ATCC 17931	HMPREF0733_11863	ADP41320	CP002280
Rothia mucilaginosa	ATCC 25296	ROTMU0001_1629	EET74784	ACVO01000024 (contig00032)
Rothia mucilaginosa	DY-18	RMDY18_11300	BAI64962	AP011540
Rothia mucilaginosa	M508	HMPREF0737_01045	EHB87922	ACSB01000008 (cont1.8)
Rubrivivax gelatinosus	IL144	-	-	n/i
Rubrivivax gelatinosus	S1	-	-	-
Ruegeria pomeroyi	DSS-3 megaplasmid	-	-	n/i
Runella slithyformis	DSM 19594	-	-	-
Salinibacter ruber	M8	-	-	-
Salinisphaera shabanensis	E1L3A	-	-	-
Shewanella amazonensis	SB2B	Sama_0728	ABL98936	CP000507
Shewanella denitrificans	OS217	-	-	-
Shewanella frigidimarina	NCIMB 400	Sfri_3253	ABI73089	CP000447
Shewanella halifaxensis	HAW-EB4	Shal_3554	ABZ78097	CP000931
Shewanella loihica	PV-4	Shew_0657	ABO22529	CP000606
Shewanella piezotolerans	WP3	swp_0833	ACJ27644	CP000472
Shewanella putrefaciens	200	Sput200_3181	ADV55577	CP002457
Shewanella putrefaciens	CN-32	Sputcn32_3072	ABP76785	CP000681
Shewanella sediminis	HAW-EB3	Ssed_3822	ABV38426	CP000821
Shewanella sp.	ANA-3	Shewana3_0846	ABK47084	CP000469, chromosome 1

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Shewanella sp.	MR-4	Shewmr4_3089	ABI40160	CP000446
Shewanella sp.	MR-7	Shewmr7_0883	ABI41882	CP000444
Shewanella sp.	W3-18-1	Sputw3181_0873	ABM23723	CP000503
Shewanella woodyi	ATCC 51908	Swoo_0761	ACA85056	CP000961
Sinorhizobium fredii	USDA 257	-	-	n/i
Sinorhizobium fredii	HH103	-	-	-
Sinorhizobium fredii	NGR234	-	-	n/i
Sinorhizobium medicae	WSM419	-	-	n/i
Sinorhizobium meliloti	1021	-	-	-
Sinorhizobium meliloti	BL225C	-	-	n/i
Sinorhizobium meliloti	JJ1C10	-	-	n/i
Sinorhizobium sp.	NP1	-	-	-
Sinorhizobium sp.	R-31759	-	-	-
Sinorhizobium sp.	R-31764	-	-	-
Sinorhizobium sp.	R-31816	-	-	-
Sinorhizobium sp.	R-32546	-	-	-
Sinorhizobium sp.	R-32549	-	-	-
Solitalea canadensis	DSM 3403	Solca_2916	AFD07938	CP003349 - copy A
		SolcaDRAFT_1103	EHP05832	AGSC01000047 (Scan3403_Contig293) - copy B
Sorangium cellulosum	'So ce 56'	sce8584	CAN98754	AM746676 - copy A
		sce3281	CAN93440	AM746676 - copy B
Sphaerobacter thermophilus	DSM 20745	-	-	-
Sphingobium yanoikuyae	XLDN2-5	SyanX_010100002072	WP_010335701	AFXE01000019 (contig000019)
Sphingomonas witchii	RW1	Swit_4614	ABQ70952	CP000699
		Swit_5200	ABQ71311	CP000700, plasmid pSWIT01
Staphylococcus aureus	O11	SAO11_0983	EGA97960	AEUQ01000008 (contig008)
Staphylococcus aureus	O46	SAO46_0640	EGB01031	AEUR01000006 (contig006)
Staphylococcus aureus	08BA02176	C248_0252	AFR72266	CP003808
Staphylococcus aureus	ST398/SO385	SAPIG0277	CAQ48715	AM990992
Staphylococcus aureus subsp. *aureus*	HO 5096 0412	SAEMRSA15_02230	CCG14941	HE681097
Staphylococcus aureus subsp. *aureus*	21342	SA21342_0505	EHQ64570	AHKU01000108 (contig00005)
Staphylococcus aureus subsp. *aureus*	21310	SA21310_0750	EGL94648	AFNP01000014 (contig00025)
Staphylococcus aureus subsp. *aureus*	21269	SA21269_1284	EGS91557	AFTU01000005 (contig00029)
Staphylococcus aureus subsp. *aureus*	21264	SA21264_1668	EHO88427	AHJX01000047 (contig00008)
Staphylococcus aureus subsp. *aureus*	21178	SA21178_1913	EHM63862	AGRN01000019 (contig00027)
Staphylococcus aureus subsp. *aureus*	21195	SA21195_1295	EGS98783	AFTM01000014 (contig00031)
Staphylococcus aureus subsp. *aureus*	21235	SA21235_1287	EGS83663	AFTQ01000002 (contig00035)
Staphylococcus aureus subsp. *aureus*	71193	ST398NM01_0277	AFH68581	CP003045
Staphylococcus aureus subsp. *aureus*	21331	SA21331_0938	EHM74662	AGTV01000036 (contig00004)
Staphylococcus aureus subsp. *aureus*	IS-105	IS105_0116	EHS26870	AHLR01000077 (contig00001)
Staphylococcus aureus subsp. *aureus*	MRSA252	SAR0261	CAG39287	BX571856
Staphylococcus aureus subsp. *aureus*	D139	WP_000062637	SATG_01500	GG730161 (genomic scaffold supercont1.3)
Staphylococcus aureus subsp. *aureus*	JKD6159	SAA6159_00241	ADL22197	CP002114
Staphylococcus aureus subsp. *aureus*	CGS00	CGSSa00_01086	EFU25108	ABWS01000003 (ctg90)
Staphylococcus aureus subsp. *aureus*	LGA251	SARLGA251_02280	CCC86997	FR821779
Staphylococcus aureus subsp. *aureus*	DR10	ST398NM02_0277	EIA14585	AIDT01000004 (Contig0018)
Staphylococcus aureus subsp. *aureus*	ED133	SAOV_0204	ADI96742	CP001996
Staphylococcus aureus subsp. *aureus*	IS-125	IS125_1761	EHS72147	AHVC01000034 (contig00108)
Staphylococcus aureus subsp. *aureus*	IS-157	IS157_0747	EHS76816	AICH01000107 (contig00010)
Staphylococcus aureus subsp. *aureus*	IS-189	IS189_2165	EHS77188	AICJ01000041 (contig00064)
Staphylococcus aureus subsp. *aureus*	21345	SA21345_2591	EHQ69720	AHKW01000023 (contig00039)
Staphylococcus aureus subsp. *aureus*	ATCC BAA-39	HMPREF0783_1232	EFM07122	AEEK01000046 (contig00076)
Staphylococcus aureus subsp. *aureus*	MN8	HMPREF0769_10220	EFH96218	ACJA02000001 (Contig000001)
Staphylococcus aureus subsp. *aureus*	JKD6008	SAA6008_00238	ADL64313	CP002120
Staphylococcus aureus subsp. *aureus*	T0131	SAT0131_00257	AEB87369	CP002643
Staphylococcus aureus subsp. *aureus*	TCH60	HMPREF0772_10238	ADQ75700	CP002110
Staphylococcus aureus subsp. *aureus*	TW20	SATW20_02660	CBI48150	FN433596
Staphylococcus aureus subsp. *aureus*	H19	SAUG_02006	WP_000062633	GG730336 (genomic scaffold supercont1.2)
Staphylococcus aureus subsp. *aureus*	C160	SFAG_00775	WP_000062635	GG730274 (genomic scaffold supercont1.2)
Staphylococcus simulans	ACS-120-V-Sch1	HMPREF9310_02008	EKS24181	AGZX01000027 (ACS-120-V-Sch1_cont1.27)
Starkeya novella	DSM 506	-	-	n/i
Sulfobacillus acidophilus	DSM 10332	-	-	-
Sulfobacillus acidophilus	TPY	-	-	-
Sulfolobus islandicus	HVE10/4	SiH_0816	ADX82169	CP002426
Sulfolobus islandicus	L.S.2.15	LS215_1118	ACP35138	CP001399
Sulfolobus islandicus	REY15A	SiRe_0538	ADX84624	CP002425
Sulfolobus islandicus	Y.G.57.14	YG5714_0509	ACP44803	CP001403
Sulfolobus islandicus	Y.N.15.51	YN1551_2203	ACP49203	CP001404
Sulfolobus solfataricus	98/2	Ssol_2401	ACX92559	CP001800
Sulfolobus solfataricus	P2	SSO1571	AAK41784	AE006641
Sulfurimonas autotrophica	DSM 16294	-	-	n/i
Sulfurimonas denitrificans	DSM 1251	-	-	-
Sulfurimonas gotlandica	GD1 SMGD1	-	-	n/i
Sulfurovum sp.	NBC37-1	-	-	n/i
Symbiobacterium thermophilum	IAM 14863	-	-	-
Synechocystis sp.	PCC 6803	SYNGTS_3089	BAK51837	AP012205
Synechocystis sp.	PCC 6803 substr. GT-I	SYNGTI_3088	BAL30835	AP012276
Synechocystis sp.	PCC 6803 substr. PCC-N	SYNPCCN_3087	BAL34004	AP012277
Synechocystis sp.	PCC 6803 substr. PCC-P	SYNPCCP_3087	BAL37173	AP012278
Syntrophobacter fumaroxidans	MPOB	Sfum_2932	ABK18607	CP000478
Taylorella asinigenitalis	MCE3	-	-	-
Taylorella equigenitalis	MCE9	-	-	-
Thauera sp.	MZ1T	-	-	n/i
Thermaerobacter marianensis	DSM 12885	-	-	-
Thermaerobacter subterraneus	DSM 13965	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Thermobaculum terrenum	ATCC BAA-798	-	-	-
Thermobifida fusca	YX	-	-	-
Thermomicrobium roseum	DSM 5159	-	-	-
Thermoproteus uzonensis	768-20	TUZN_1529	AEA12999	CP002590
Thioalkalivibrio sulfidophilus	HL-EbGr7	-	-	-
Thioalkalivibrio thiocyanoxidans	ARh 4	-	-	n/i
Thiobacillus denitrificans	ATCC 25259	-	-	-
Thiocapsa marina	5811	-	-	n/i
Thiocystis violascens	DSM 198	Thivi_3685	AFL75535	CP003154
Turneriella parva	DSM 21527	-	-	-
Veillonella parvula	ATCC 17745	HMPREF1035_0889	EFB86588	ADFU01000008 (contig00017)
Veillonella parvula	DSM 2008	Vpar_1513	ACZ25189	CP001820
Veillonella sp.	oral taxon 158 str. F0412	HMPREF9199_1587	EFR60983	AENU01000007 (ctg1126953305818)
Veilonella dispar	ATCC 17748	VEIDISOL_00439	EEP66365	ACIK02000004 (V_dispar-1.0.1_Cont0.2)
Veilonella parvula	ACS-068-V-Sch12	HMPREF9323_1047	EGL78360	AEXI01000001 (contig00017)
Vibrio orientalis	CIP 102891 = ATCC 33934	-	-	-
Vibrio tubiashii	ATCC 19109	-	-	-
Vibrio tubiashii	NCIMB 1337 = ATCC 19106	-	-	-
Vulcanisaeta distributa	DSM 14429	Vdis_1451	ADN50837	CP002100
Waddlia chondrophila	WSU 86-1044	wcw_0480	ADI37851	CP001928
Waddlia chondrophila	2032/99	WCH_BJ09000	CCB92015	FR872660
Xanthobacter autotrophicus	Py2	Xaut_3044	ABS68274	CP000781
Zobellia galactanivorans	DsiJT	zobellia_4311	CAZ98446	FP476056, chromosome

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Acaryochloris marina	MBIC11017	-	-	-
Acaryochloris sp.	CCMEE 5410	-	-	-
Achromobacter arsenitooxidans	SY8	KYC_21059	EHK64322	AGUF01000065 (Contig00066)
Achromobacter cycloclastes	unknown	**n/a**	**CAA64426**	**X94977**
Achromobacter cycloclastes	ACCC 03051	-	-	-
Achromobacter cycloclastes	ATCC 21921 = IAM 1013	**n/a**	**AAD09157**	**AF047429 - copy A**
		n/a	**CAA75425**	**Y15161 - copy B**
Achromobacter denitrificans	LMG 1231[T]	-	-	-
Achromobacter sp.	DBTN3	-	-	n/i
Achromobacter xylosoxidans	A8	AXYL_04548	ADP17863	CP002287
Achromobacter xylosoxidans	AXX-A	AXXA_01591	EGP48295	AFRQ01000013 (contig00023)
Achromobacter xylosoxidans	C54	HMPREF0005_03872	EFV85183	ACRC01000453 (cont1.453)
Achromobacter xylosoxidans	GIFU1051	-	-	-
Acidilobus saccharovorans	345-15	-	-	-
Acidovorax delafieldii	2AN	AcdelDRAFT_2697	EER59730	ACQT01000101 (ctg00228)
Acidovorax ebreus	TPSY	Dtpsy_1060	ACM32537	CP001392
Acidovorax sp.	JS42	Ajs_1139	ABM41372	CP000539
Acidovorax sp.	NO-1	KYG_23550	EHL20417	AGTS01000159 (contig165)
Actinobacillus minor	202	-	-	-
Actinobacillus minor	NM305	-	-	-
Actinobacillus pleuropneumoniae sv. 1	str. 4074	-	-	-
Actinobacillus pleuropneumoniae sv. 2	str. S1536	-	-	-
Actinobacillus pleuropneumoniae sv. 2	str. 4226	-	-	-
Actinobacillus pleuropneumoniae sv. 3	str. JL03	-	-	-
Actinobacillus pleuropneumoniae sv. 4	str. M62	-	-	-
Actinobacillus pleuropneumoniae sv. 5b	L20	-	-	-
Actinobacillus pleuropneumoniae sv. 6	str. Femo	-	-	-
Actinobacillus pleuropneumoniae sv. 7	str. AP76	-	-	-
Actinobacillus pleuropneumoniae sv. 9	str. CVJ13261	-	-	-
Actinobacillus pleuropneumoniae sv. 10	str. D13039	-	-	-
Actinobacillus pleuropneumoniae sv. 12	str. 1096	-	-	-
Actinobacillus pleuropneumoniae sv. 13	str. N273	-	-	-
Actinobacillus succinogenes	130Z	-	-	-
Actinobacillus suis	H91-0380	-	-	-
Actinobacillus ureae	ATCC 25976	-	-	-
Actinomyces coleocanis	DSM 15436	-	-	-
Actinomyces odontolyticus	ATCC 17982	-	-	-
Actinomyces sp.	oral taxon 170 str. F0386	-	-	-
Actinomyces sp.	oral taxon 171 str. F0337	-	-	-
Actinomyces sp.	oral taxon 178 str. F0338	-	-	-
Actinomyces sp.	oral taxon 448 str. F0400	-	-	-
Actinomyces sp.	oral taxon 849 str. F0330	-	-	-
Actinomyces urogenitalis	DSM 15434	-	-	-
Actinosynnema mirum	DSM 43827	-	-	-
Aequorivita sublithincola	DSM 14238	KYG_23550	EHL20417	CP003280
Afipia sp.	1NLS2	AfiDRAFT_3697	EFI49990	ADVZ01000006 (ctg00010)
Aggregatibacter aphrophilus	ATCC 33389	-	-	-
Aggregatibacter aphrophilus	F0387	-	-	-
Aggregatibacter aphrophilus	NJ8700	-	-	-
Aggregatibacter segnis	ATCC 33393	-	-	-
Rhizobium radiobacter	CCNWGS0286	-	-	-
Rhizobium radiobacter	str. C58	-	-	-
Alcaligenes faecalis	S-6	-	-	-
Alcaligenes faecalis subsp. *faecalis*	LMG 1229[T]	-	-	-
Alcaligenes faecalis subsp. *faecalis*	NCIB 8687	QWA_15677	EJC61301	AKMR01000017 (Contig_18)

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Achromobacter sp.	DSM 30128	-	-	-
Alcaligenes sp.	STC1	-	-	-
Achrom. xylosoxidans subsp. *xylosoxidans*	NCIMB 11015	-	-	n/i
Alcanivorax dieselolei	N1203	-	-	-
Alcanivorax dieselolei	B5	-	-	-
Alicycliphilus denitrificans	BC	Alide_3077	ADV00801	CP002449
Alicycliphilus denitrificans	K601	Alide2_1370	AEB83772	CP002657
Alicycliphilus sp.	R-24604	-	-	n/i
Alicycliphilus sp.	R-24606	-	-	-
Alicycliphilus sp.	R-24611	-	-	n/i
Alicycliphilus sp.	R-26814	-	-	-
Alkalilimnicola ehrlichii	MLHE-1	Mlg_1074	ABI56426	CP000453
Allochromatium vinosum	DSM 180	-	-	-
Anaeromyxobacter dehalogenans	2CP-1	A2cp1_1556	ACL64900	CP001359
Anaeromyxobacter dehalogenans	2CP-C	A2cp1_1556	ACL64900	CP000251
Anaeromyxobacter dehalogenans	DCP18	-	AFB35544	JN882602
Anaeromyxobacter sp.	Fw109-5	Anae109_0244	ABS24462	CP000769
Anaeromyxobacter sp.	K	AnaeK_1461	ACG72692	CP001131
Anaerophaga sp.	HS1	-	-	-
Anoxybacillus flavithermus	TNO-09.006 chrAF6	AF6_1580	WP_004890845	KB205935 (genomic scaffold chrAF6)
Arthrospira maxima	CS-328	-	-	-
Arthrospira platensis	C1	-	-	-
Arthrospira sp.	PCC 8005	-	-	-
Azoarcus aromaticum	EbN1	CAI09713	CAI09713	CR555306
Azoarcus sp.	BH72	azo3113	CAL95730	AM406670
Azoarcus sp.	KH32C	AZKH_2415	BAL24721	AP012304
Azospirillum amazonense	Y2	AZA_78631	EGY01621	AFBX01000297 (contig00354)
Azospirillum brasilense	Sp245	AZOBR_p340004	CCD02766	HE577330, plasmid AZOBR_p3
Azospirillum brasilense	SM	**n/a**	**ACJ06409**	**FJ358638**
Azospirillum doebereinerae	GSF 71T	-	-	-
Azospirillum lipoferum	4B	AZOLI_p20731	CBS89841	FQ311870 (plasmid AZO_p2)
Azospirillum sp.	B510	-	-	-
Bacillus azotoformans	LMG 9581[T]	BAZO_00120	WP_003329179	AJLR01000004 (contig 04) - nosZ1
		BAZO_05340	WP_003330276	AJLR01000041 (contig41) - nosZ2
		BAZO_18226	WP_003332806	AJLR01000147 (contig147) - nosZ3
Bacillus bataviensis	LMG 21833[T]	-	-	-
Bacillus licheniformis	ATCC 14580/DSM 13	-	-	-
Bacillus sp.	1NLA3E	-	-	-
Bacillus sp.	2_A_57_CT2	HMPREF1013_04202	EFV75425	ACWD01000061 (cont1.61)
Bacillus sp.	BT1B_CT2	-	-	-
Bacillus sp.	Ult-108 clone A	**n/a**	**ADX01156**	**GU733403**
Bacillus sp.	Ult-123 clone A	**n/a**	**ADX01157**	**GU733404**
Bacillus sp.	Ult-130 clone A	**n/a**	**ADX01165**	**GU733412**
Bacillus sp.	Ult-145 clone A	**n/a**	**ADX01162**	**GU733409**
Bacillus sp.	Ult-356 clone A	**n/a**	**ADX01152**	**GU733399**
Bacillus sp.	Ult-391 clone A	**n/a**	**ADX01160**	**GU733407**
Bacillus sp.	Ult-41 clone A	**n/a**	**ADX01166**	**GU733413**
Bacillus sp.	Ult-42 clone A	**n/a**	**ADX01168**	**GU733415**
Bacillus sp.	Ult-42 clone B	**n/a**	**ADX01167**	**GU733414**
Bacillus sp.	Ult-442 clone A	**n/a**	**ADX01164**	**GU733411**
Bacillus sp.	Ult-46 clone A	**n/a**	**ADX01153**	**GU733400**
Bacillus sp.	Ult-521 clone A	**n/a**	**ADX01154**	**GU733401**
Bacillus sp.	Ult-530 clone A	**n/a**	**ADX01155**	**GU733402**
Bacillus sp.	Ult-552 clone A	**n/a**	**ADX01158**	**GU733405**
Bacillus sp.	Ult-640 clone A	**n/a**	**ADX01163**	**GU733410**
Bacillus sp.	Ult-71 clone A	**n/a**	**ADX01161**	**GU733408**
Bacillus sp.	Ult-816 clone A	**n/a**	**ADX01159**	**GU733406**
Bacillus sp.	R-31770	-	-	-
Bacillus sp.	R-31841	-	-	-
Bacillus sp.	R-32526	-	-	-
Bacillus sp.	R-32656	-	-	-
Bacillus sp.	R-32694	-	-	-
Bacillus sp.	R-32702	-	-	-
Bacillus sp.	R-32709	-	-	-
Bacillus sp.	R-32715	-	-	-
Bacillus sp.	R-33820	-	-	-
Bacillus sp.	R-33773	-	-	-
Bacillus sp.	R-32546	-	-	-
Bacillus sp.	10403023 (MM10403188)	-	-	-
Bacillus sp.	SH27	-	-	-
Bacillus sp.	SH3	-	-	-
Bacillus sp.	SH11	-	-	-
Bacillus sp.	SH22	-	-	-
Bacillus sp.	SH5	-	-	-
Bacillus sp.	SH8	-	-	-
Bacillus sp.	SH10	-	-	-
Bacillus sp.	SH14	-	-	-
Bacillus sp.	SH19	-	-	-
Bacillus sp.	SH21	-	-	-
Bacillus sp.	SH25	-	-	-
Bacillus sp.	SH30	-	-	-
Bacillus sp.	SH36	-	-	-
Bacillus sp.	SH38	-	-	-
Bacillus sp.	SH41	-	-	-
Bacillus sp.	SH42	-	-	-

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Bacillus sp.	SH43	-	-	-
Bacillus sp.	SH48	-	-	-
Bacillus sp.	SH51	-	-	-
Bacillus sp.	SH60	-	-	-
Bacillus sp.	SH61	-	-	-
Bacillus sp.	SH62	-	-	-
Bacillus sp.	SH63	-	-	-
Bacillus subtilis	BEST7613	-	-	-
Bdellovibrio bacteriovorus	HD100	-	-	-
Beggiatoa sp.	PS	-	-	-
Belliella baltica	DSM 15883	Belba_1979	AFL84554	CP003281
Beta-proteobacterium	R1-Apr-MIB-6	-	-	-
Bizionia argentinensis	JUB59	-	-	-
blood disease bacterium	R229	-	-	-
Bordetella petrii	DSM 12804	Bpet4339	CAP44690	AM902716
Brachybacterium faecium	DSM 4810 strain 6-10	-	-	-
Bradyrhizobium japonicum	USDA 110	**n/a**	**BAC45580**	**BA000040**
		n/a	**CAA05521**	**AJ002531**
Bradyrhizobium japonicum	ATCC 15067	-	-	-
Bradyrhizobium japonicum	USDA 6	-	-	-
Bradyrhizobium sp.	BTAi1	BBta_6008	ABQ37945	CP000494
Bradyrhizobium sp.	ORS 278	-	-	-
Bradyrhizobium sp.	ORS 285	BRAO285_180041	CCD86231	CAFH01000090 (contig00020-776)
Bradyrhizobium sp.	ORS 375	BRAO375_10006	CCD90352	CAFI01000001 (Contig00001-771)
Bradyrhizobium sp.	STM 3809	BRAS3809_6700012	CCE02902	CAFJ01000635 (Contig00741-770)
Bradyrhizobium sp.	STM 3843	BRAS3843_1560030	CCE06052	CAFK01000064 (contig 00162-775)
Bradyrhizobium sp.	S23321	-	-	-
Brucella abortus	A13334	-	-	n/i
Brucella abortus	bv. 1 str. 9-941	BruAb2_0905	AAX76292	AE017224 , chromosome 2
Brucella abortus	bv. 1 str. NI010	M1G_02798	EHR17749	AGVJ01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI016	M1I_02800	EHR18591	AGVK01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI021	M1K_02801	EHR25577	AGVL01000015 (cont1.15)
Brucella abortus	bv. 1 str. NI259	M1M_02084	EHR27848	AGVM01000009 (cont1.9)
Brucella abortus	bv. 1 str. NI435a	M17_01984	EHR10295	AGVF01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI474	M19_02802	EHR08212	AGVG01000014 (cont1.14)
Brucella abortus	bv. 1 str. NI486	M1A_01396	EHR11502	AGVH01000011 (cont1.11)
Brucella abortus	bv. 1 str. NI488	M1E_00137	EHR28079	AGVI01000002 (cont1.2)
Brucella abortus	S19	-	-	-
Brucella abortus	str. 2308 A	BAAA_7000920	EEP62369	ACOR01000007, chromosome 2 (VBI00023_1)
Brucella canis	ATCC 23365	BCAN_B0277	ABX63466	CP000873 , chromosome 2
Brucella canis	HSK A52141	BCA52141_II0802	AEW15681	CP003175 , chromosome 2
Brucella ceti	str. Cudo	BCETI_6000588	EEH13627	ACJD01000006 , chromosome 2 (VBI0082_1)
Brucella inopinata	BO1	BIBO1_2266	EFM55728	ADEZ01000033 , chromosome 2 (VBI00042_6)
Brucella melitensis	ATCC 23457	-	-	-
Brucella melitensis	bv.1 str. 16M	-	-	n/i
Brucella melitensis	bv. 2 str. 63/9	-	-	n/i
Brucella melitensis	M28	-	-	n/i
Brucella melitensis	M5-90	-	-	n/i
Brucella melitensis	NI	-	-	n/i
Brucella microti	CCM 4915	BMI_II270	ACU49406	CP001579, chromosome 2
Brucella ovis	ATCC 25840	BOV_A0251	ABQ62504	CP000709, chromosome 2
Brucella pinnipedialis	B2/94	BPI_II273	AEK55724	CP002079, chromosome 2
Brucella sp.	BO2	BIBO2_0683	EFM60346	ADFA01000033 (VBI00229_128)
Brucella sp.	NF 2653	BROD_0675	EFM63257	ADFB01000030 (VBI00228_23)
Brucella suis	ATCC 23445	BSUIS_B0281	ABY39292	CP000912, chromosome 2
Brucella suis	VBI22	BSVBI22_B0271	AEU07424	CP003129, chromosome 2
Brucella suis	1330	BRA0275	AAN33476	AE014292, chromosome 2
Burkholderia mallei	ATCC 23344	BMA0995	AAU49344	CP000010, chromosome 1
Burkholderia mallei	GB8 horse 4	BMAGB8_1059	EEP87368	AAHO01000011 (contig_378)
Burkholderia mallei	PRL-20	BMAPRL20_A1393	EES47342	AAZP01000003 (contig_1105338605131)
Burkholderia mallei	NCTC 10229	-	-	-
Burkholderia mallei	NCTC 10247	-	-	-
Burkholderia mallei	SAVP1	-	-	-
Burkholderia oklahomensis	C6786	-	-	-
Burkholderia oklahomensis	EO147	-	-	-
Burkholderia pseudomallei	1026a	BP1026A_6258	EIF52799	AHJA01000290 (Contig0290)
Burkholderia pseudomallei	1106a	BURPS1106A_2115	ABN92010	CP000572, chromosome 1
Burkholderia pseudomallei	1258a	BP1258A_1221	EIF66468	AHJB01000076 (Contig0076)
Burkholderia pseudomallei	1258b	BP1258B_1314	EIF68141	AHJC01000071 (Contig0071)
Burkholderia pseudomallei	1710b	BURPS1710b_2255	ABA47928	CP000124, chromosome 1
Burkholderia pseudomallei	305	BURPS305_6720	EBA51672	AAYX01000001
Burkholderia pseudomallei	354a	BP354A_1468	EIF81342	AGVS01000181 (Contig0181)
Burkholderia pseudomallei	354e	BP354E_1123	EIF77060	AHJD01000062 (Contig0063)
Burkholderia pseudomallei	576	BUC_1969	EEC35055	ACCE01000004 (BUC.Contig184)
Burkholderia pseudomallei	668	BURPS668_2060	ABN81842	CP000570, chromosome 1
Burkholderia pseudomallei	K96243	BPSL1607	CAH35607	BX571965, chromosome 1
Burkholderia pseudomallei	MSHR346	GBP346_A2185	ACQ98966	CP001408, chromosome 2
Burkholderia pseudomallei	Pakistan 9	BUH_1778	EEH26874	ACKA01000037 (BUH.Contig274)
Burkholderia thailandensis	E264	BTH_I2325	ABC36800	CP000086, chromosome 1
Burkholderia thailandensis	TXDOH	-	-	-
Caldilinea aerophila	DSM 14535 = NBRC 104270	CLDAP_07150	BAL98754	AP012337
Campylobacter concisus	13826	CCC13826_1729	EAT98130	CP000792
Campylobacter concisus	UNSWCD	UNSWCD_777	EIF06641	AENQ01000027 (Contig_27)
Campylobacter curvus	525.92	-	-	-
Campylobacter fetus subsp. fetus	82-40	CFF8240_0440	ABK83015	CP000487

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Campylobacter sp.	10_1_50	-	-	n/i
Cand. *Accumulibacter phosphatis* clade IIA	str. UW-1	-	-	n/i
Candidatus *Koribacter versatilis*	Ellin345	-	-	-
Candidatus *Nitrospira defluvii*	unknown	-	-	-
Candidatus *Solibacter usitatus*	Ellin6076	-	-	-
Capnocytophaga gingivalis	ATCC 33624	CAPGI0001_1095	EEK13943	ACLQ01000023 (ctg1117755536027)
Capnocytophaga ochracea	F0287	-	-	-
Capnocytophaga sp.	oral taxon 338 str. F0234	HMPREF9071_0694	EGD34772	AEXX01000015 (contig00015)
Capnocytophaga sp.	oral taxon 329 str. F0087	-	-	-
Capnocytophaga sp.	oral taxon 412 str. F0487	-	-	-
Capnocytophaga sp.	CM59	HMPREF1154_2417	WP_009640866	ALNN01000005 (ctg120006916608)
Capnocytophaga sputigena	ATCC 33612 strain Capno	-	-	-
Cardiobacterium hominis	ATCC 15826	HMPREF0198_2357	EEV87546	ACKY01000128 (contig00206)
Cardiobacterium valvarum	F0432	-	-	-
Caulobacter segnis	ATCC 21756	-	-	-
Cellulophaga algicola	DSM 14237	Celal_3154	Celal_3154	CP002453
Cellvibrio japonicus	Ueda107	-	-	-
Cellvibrio sp.	BR	-	-	-
Chelativorans sp.	BNC1	-	-	-
Chitinophaga pinensis	DSM 2588	-	-	-
Chloroflexus aggregans	DSM 9485	-	-	-
Chloroflexus aurantiacus	J-10-fl	-	-	-
Chloroflexus sp.	Y-400-fl	-	-	-
Chromobacterium violaceum	ATCC 12472	-	-	-
Chroococcidiopsis thermalis	PCC 7203	-	-	-
Chryseobacterium gleum	ATCC 35910	-	-	-
Chthoniobacter flavus	Ellin428	-	-	-
Citreicella sp.	357	C357_07946	EIE51575	AJKJ01000063 (C357_074)
Colwellia psychrerythraea	34H	CPS_4732	AAZ27785	CP000083
Corynebacterium accolens	ATCC 49725	-	-	-
Corynebacterium accolens	ATCC 49726	-	-	-
Corynebacterium aurimucosum	ATCC 700975	-	-	-
Corynebact. diphtheriae bv. *intermedius*	NCTC 5011	-	-	-
Corynebact. diphtheriae gravis	NCTC13129	-	-	-
Corynebacterium diptheriae	241	-	-	-
Corynebacterium diptheriae	31A	-	-	-
Corynebacterium diptheriae	BH8	-	-	-
Corynebacterium diptheriae	C7 (beta)	-	-	-
Corynebacterium diptheriae	CDCE 8392	-	-	-
Corynebacterium diptheriae	HC01	-	-	-
Corynebacterium diptheriae	HC02	-	-	-
Corynebacterium diptheriae	HC03	-	-	-
Corynebacterium diptheriae	HC04	-	-	-
Corynebacterium diptheriae	INCA 402	-	-	-
Corynebacterium diptheriae	PW8	-	-	-
Corynebacterium diptheriae	VA01	-	-	-
Corynebacterium efficiens	YS-314	-	-	-
Corynebacterium pseudotuberculosis	1002	-	-	-
Corynebacterium pseudotuberculosis	267	-	-	-
Corynebacterium pseudotuberculosis	316	-	-	-
Corynebacterium pseudotuberculosis	3/99-5	-	-	-
Corynebacterium pseudotuberculosis	42/02-A	-	-	-
Corynebacterium pseudotuberculosis	C231	-	-	-
Corynebacterium pseudotuberculosis	CIP 52.97	-	-	-
Corynebacterium pseudotuberculosis	Cp162	-	-	-
Corynebacterium pseudotuberculosis	FRC41	-	-	-
Corynebacterium pseudotuberculosis	P54B96	-	-	-
Corynebacterium pseudotuberculosis	PAT10	-	-	-
Corynebacterium pseudotuberculosis	I19	-	-	-
Cupriavidus metallidurans	CH34	Rmet_4917	ABF11779	CP000353, megaplasmid
Cupriavidus necator	N-1	-	-	-
Cupriavidus necator	LMG 1201	-	-	-
Cupriavidus sp.	R-31542	-	-	-
Cupriavidus sp.	R-31543	-	-	-
Cupriavidus sp.	R-31544	-	-	-
Cupriavidus taiwanensis	LMG 19424	-	-	-
Cyanobacterium aponinum	PCC 10605	-	-	-
Cyanobacterium stanieri	PCC 7202	-	-	-
Dechloromonas aromatica	RCB	Daro_1571	AAZ46320	CP000089 - copy A
		Daro_1575	AAZ46324	CP000089 - copy B
Dechlorosoma suillum	PS	Dsui_0882	AEV25288	CP003153 - copy A
		Dsui_3309	AEV27640	CP003153 - copy B
Denitrovibrio acetiphilus	DSM 12809	Dacet_0941	ADD67719	CP001968
Desulfitobacterium dehalogenans	ATCC 51507	DesdeDRAFT_2873	EHP57149	AGJH01000017 (ctg121)
		Desde_0186	AFL98667	CP003348
Desulfitobacterium dichloroeliminans	LMG P-21439	Desdi_0167	AGA67727	CP003344
Desulfitobacterium hafniense	DCB-2	Dhaf_0209	ACL18277	CP001336
Desulfitobacterium hafniense	Y51	DSY0263	BAE82052	AP008230
Desulfomonile tiedjei	DSM 6799	Desti_0657	AFM23383	CP003360
Desulfosporosinus meridiei	DSM 13257	Desmer_3457	AFQ45307	CP003629
Desulfosporosinus youngiae	DSM 17734	DesyoDRAFT_0219	WP_007778287	CM001441
Desulfotomaculum ruminis	DSM 2154	Desru_1528	AEG59793	CP002780
Desulfovibrio sp.	U5L	-	-	-
Diaphorobacter sp.	R-24610	-	-	-
Diaphorobacter sp.	R-24612	-	-	-

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Diaphorobacter sp.	R-24661	-	-	-
Diaphorobacter sp.	R-25011	-	-	n/i
Diaphorobacter sp.	R-26815	-	-	n/i
Diaphorobacter sp.	R-26840	-	-	-
Diaphorobacter sp.	R-28417	-	-	n/i
Dinoroseobacter shibae	DFL 12	Dshi_3194	ABV94927	CP000830
Diplosphaera colitermitum	TAV2	ObacDRAFT_8860	EEG21941	ABEA02000009 (ctg793)
Dyadobacter fermentans	DSM 18053	Dfer_0693	ACT91955	CP001619
Eikenella corrodens	ATCC 23834	-	-	-
Endosymbiont of *Riftia pachyptila*	Rifp1Symag	Rifp1Sym_ag00460	EGV52541	AFOC01000007
Ensifer sp.	2FB8	-	-	-
Ensifer sp.	4FB6	-	-	-
Enterococcus sp.	R-24626	-	-	-
Ferroglobus placidus	DSM 10642	Ferp_0128	ADC64316	CP001899
Flavobacteriaceae bacterium	3519-10	FIC_02107	ACU08544	CP001673
Flavobacteriales bacterium	ALC-1	FBALC1_12122	EDP71844	ABHI01000001 (1103813602123)
Flavobacterium columnare	ATCC 49512	FCOL_10605	AEW86926	CP003222
Flavobacterium columnare	unknown	-	-	-
Flavobacterium johnsoniae	UW101	-	-	-
Flavobacterium sp.	F52	FF52_21889	WP_008469074	AKZQ01000042 (Contig42)
Flavobacterium sp.	CF136 PMI10	-	-	-
Gamma-proteobacterium	HdN1	HDN1F_37580	CBL47341	FP929140
Gemmatimonas aurantiaca	T-27	GAU_1385	BAH38427	AP009153
Geobacillus kaustophilus	HTA426	-	-	-
Geobacillus kaustophilus	LMG 9819^T	**n/a**	**CDG32591**	**HG328813**
Geobacillus sp.	C56-T3	-	-	-
Geobacillus sp.	G11MC16	G11MC16DRAFT_1616	EDY06149	ABVH01000004 (ctg44)
Geobacillus sp.	Y412MC52	-	-	-
Geobacillus sp.	Y412MC61	-	-	-
Geobacillus sp.	Y4.1MC1	-	-	-
Geobacillus stearothermophilus	LMG 6939T	**n/a**	**n/a**	--
Geobacillus stearothermophilus	R-35646	**n/a**	**n/a**	--
Geobacillus stearothermophilus	R-32513	**n/a**	**n/a**	--
Geobacillus stearothermophilus	R-32605	**n/a**	**n/a**	--
Geobacillus stearothermophilus	R-32635	**n/a**	**n/a**	--
Geobacillus thermodenitrificans	NG80-2	GTNG_1734	ABO67098	**CP000557**
Geobacillus thermodenitrificans	LMG 17532T	**n/a**	**CDG32592**	**HG328814**
Geobacillus thermodenitrificans	R-35647	**n/a**	**CDG32606**	**HG328828**
Geobacillus thermodenitrificans	R-32614	**n/a**	**CDG32593**	**HG328815**
Geobacillus thermodenitrificans	R-32615	**n/a**	**CDG32594**	**HG328816**
Geobacillus thermodenitrificans	R-32616	**n/a**	**CDG32595**	**HG328817**
Geobacillus thermodenitrificans	R-32617	**n/a**	**CDG32596**	**HG328818**
Geobacillus thermodenitrificans	R-32618	**n/a**	**CDG32597**	**HG328819**
Geobacillus thermodenitrificans	R-32619	**n/a**	**R-32619**	**HG328820**
Geobacillus thermodenitrificans	R-32621	**n/a**	**CDG32599**	**HG328821**
Geobacillus thermodenitrificans	R-32622	**n/a**	**CDG32600**	**HG328822**
Geobacillus thermodenitrificans	R-32623	**n/a**	**CDG32601**	**HG328823**
Geobacillus thermodenitrificans	R-32624	**n/a**	**CDG32602**	**HG328824**
Geobacillus thermodenitrificans	R-32625	**n/a**	**n/a**	--
Geobacillus thermodenitrificans	R-32500	**n/a**	**CDG32603**	**HG328825**
Geobacillus thermodenitrificans	R-32506	**n/a**	**CDG32604**	**HG328826**
Geobacillus thermodenitrificans	R-32511	**n/a**	**CDG32605**	**HG328827**
Geobacillus thermoglucosidans	TNO-09.020	-	-	-
Geobacillus thermoglucosidasius	C56-YS93	-	-	-
Geobacillus thermoleovorans	CCB_US3_UF5	-	-	-
Geobacillus toebii	R-32639	**n/a**	**n/a**	--
Geobacter bemidjiensis	Bem	-	-	-
Geobacter daltonii	FRC-32	-	-	-
Geobacter metallireducens	GS-15	-	-	-
Geobacter metallireducens	RCH3	-	-	-
Geobacter sp.	M21	-	-	-
Gillisia limnaea	DSM 15749	WP_006989513	Gilli_2588	JH594606 (genomic scaffold Gilliscaffold_2)
Gloeocapsa sp.	PCC 7428	-	-	-
Gramella forsetii	KT0803	GFO_1411	CAL66385	CU207366
Haemophilus haemolyticus	HK386	-	-	-
Haemophilus haemolyticus	M19107	-	-	-
Haemophilus haemolyticus	M19501	-	-	-
Haemophilus haemolyticus	M21127	-	-	-
Haemophilus haemolyticus	M21621	-	-	-
Haemophilus haemolyticus	M21639	-	-	-
Haemophilus parahaemolyticus	HK385	-	-	-
Haemophilus parahaemolyticus	HK411	-	-	-
Haemophilus parainfluenzae	ATCC 33392	-	-	-
Haemophilus parainfluenzae	HK262	-	-	-
Haemophilus parainfluenzae	HK2019	-	-	-
Haemophilus parainfluenzae	T3T1	-	-	-
Haemophilus pittmaniae	HK 85	-	-	-
Haemophilus sp.	oral taxon 851 str. F0397	-	-	-
Hahella chejuensis	KCTC 2396	HCH_03388	ABC30138	CP000155
Haladaptatus paucihalophilus	DX253	-	-	-
Haliscomenobacter hydrossis	DSM 1100	Halhy_5487	AEE53312	CP002691
Haloarcula hispanica	ATCC 33960	HAH_1235	AEM56850	CP002921
Haloarcula marismortui	ATCC 43049	rrnAC0390	AAV45430	AY596297, chromosome 1
Halobacterium sp.	DL1	HalDL1DRAFT_0282	EHB59940	AGIR01000001 (ctg46)
Haloferax denitrificans	unknown	-	-	-

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Haloferax lucentense	unknown	-	-	-
Haloferax mediterranei	ATCC 33500/R4	-	-	-
Haloferax volcanii	DS2	-	-	-
Halogeometricum borinquense	DSM 11551	Hbor_30430	ADQ68580	CP001691, plasmid pHBOR01
Halomicrobium mukohataei	DSM 12286	-	-	-
Halopiger xanaduensis	SH-6	Halxa_1765	AEH36393	CP002839
Halorhabdus utahensis	DSM 12940	-	-	-
Halorubrum lacusprofundi	ATCC 49239	-	-	-
Haloterrigena turkmenica	DSM 5511	-	-	-
Herminiimonas arsenicoxydans	ULPA$_{S1}$	-	-	-
Herpetosiphon aurantiacus	DSM 785	-	-	-
Hydrogenobacter thermophilus	TK-6	Hydth_0164	ADO44574	CP002221
		HTH_0163	BAI68630	AP011112
Hydrogenophaga sp.	PBC	-	-	-
Hyphomicrobium denitrificans	1NES1	HypdeDRAFT_2067	EHB75193	AGIS01000003 (ctg119)
Hyphomicrobium denitrificans	ATCC 51888	Hden_1882	ADJ23685	CP002083
Hyphomicrobium denitrificans	A3151	-	-	-
Idiomarina loihiensis	L2TR	-	-	-
Ignavibacterium album	JCM 16511	IALB_0848	AFH48560	CP003418
Imtechella halotolerans	K1	W5A_02370	EID76831	AJJU01000002 (Contig2)
Intrasporangium calvum	DSM 43043	-	-	-
Intrasporangium sp.	4LS1	-	-	-
Kangiella koreensis	DSM 16069	-	-	-
Kingella denitrificans	ATCC 33394	HMPREF9098_2364	EGC16195	AEWV01000045 (contig00045)
Kingella kingae	ATCC 23330	HMPREF0476_1276	EGK08585	AFHS01000044 (contig00044)
Kingella kingae	PYKK081	KKB_00412	EIC14568	AJGB01000003 (Contig003)
Kingella oralis	ATCC 51147	EEP66765	EEP66765	ACJW02000008 (Cont3.1)
Kyrpidia tusciae	DSM 2912	-	-	-
Labrenzia aggregata	IAM 12614	SIAM614_31426	EAV41876	AAUW01000018 (1101096003805)
Lactobac. coryniformis subsp. *coryniformis*	CECT 5711	-	-	-
Lactobac. coryniformis subsp. *coryniformis*	KCTC 3167	-	-	-
Lactobac. coryniformis subsp. *torquens*	KCTC 3535	-	-	-
Lactobacillus farciminis	KCTC 3681	-	-	-
Lactobacillus fermentum	ATCC 14931	-	-	-
Lactobacillus fermentum	CECT 5716	-	-	-
Lactobacillus fermentum	IFO3956	-	-	-
Lactobacillus fermentum	28-3-CHN	-	-	-
Lactobacillus versmoldensis	KCTC 3814	-	-	-
Lautropia mirabilis	ATCC 51599	HMPREF0551_2644	EFV93748	AEQP01000024 (contig00024)
Leptonema illini	DSM 21528	Lepil_2345	WP_002772740	JH597773 (Lepilscaffold_1)
Leptospira biflexa	serovar Patoc 'Patoc 1 (Ames)'	LBF_0371	ABZ92917	CP000777, chromosome 1
Leptospira biflexa	serovar Patoc 'Patoc 1 (Paris)'	LEPBI_I0383	ABZ96525	CP000786
Leptospira broomii	str. 5399	Lbro5_010100005839	WP_010569222	AHMO01000010 (ctg1130121829115)
Leptospira inadai sv. *Lyme*	str. 10	LinasL1_010100017112	WP_010418013	AHMM01000030 (ctg1130156687200)
Leptospira licerasiae	serovar Varillal str. VAR 010	LEP1GSC185_2202	EIE02269	AHOO02000005 (7180000002868)
Leptospira meyeri	serovar Hardjo str. Went 5	-	-	-
Leptothrix cholodnii	SP-6	Lcho_1483	ACB33751	CP001013
Magnetospirillum gryphiswaldense	MSR-1	MGR_2761	CAM74903	CU459003
Mannheimia succiniciproducens	MBEL55E	-	-	-
Maribacter sp.	HTCC 2170	FB2170_03800	EAR02377	CP002157
Marinilabilia sp.	AK2	MaAK2_010100016341	WP_010611132	JH636570 (genomic scaffold contig103)
Mariniradius saccharolyticus	AK6	C943_1045	ZP_20928481	AMZY01000014 (contig14)
Marinobacter aquaeolei	VT8	Maqu_3078	ABM20152	CP000514
Marinobacter hydrocarbonoclasticus	617	**n/a**	**ABF83472**	**DQ504302**
Marinobacter hydrocarbonoclasticus	ATCC 49840	MARHY3020	CCG96484	FO203363
Marinobacter manganoxydans	MnI7-9	KYE_16308	EHJ03394	AGTR01000080 (contig00005)
Marinobacter sp.	ELB17	MELB17_04642	EBA00378	AAXY01000004 (1101232001196)
Maritimibacter alkaliphilus	HTCC 2654	RB2654_12844	EAQ13960	AAMT01000003 (1099457000260)
Marivirga tractuosa	DSM 4126	Ftrac_2708	ADR22686	CP002349
Melioribacter roseus	P3M	YP_006527356	MROS_1104	NC_018178
Mesorhizobium alhagi	CCNWXJ12-2	-	-	-
Mesorhizobium amorphae	CCNWGS0123	-	-	-
Mesorhizobium australicum	WSM2073	-	-	-
Mesorhizobium ciceri biovar *biserrulae*	WSM1271	-	-	-
Mesorhizobium opportunistum	WSM2075	-	-	-
Mesorhizobium sp.	4FB11	-	-	n/i
Methylobacterium sp.	4-46	M446_2861	ACA17284	CP000943
Methylocella silvestris	BL2	-	-	-
Methylocystis sp.	ATCC 49242	-	-	-
Methylocystis sp.	SC2	-	-	
Methylomonas sp.	16a	-		-
Methylophaga aminisulfidivorans	MP_54_1	-	-	-
Methylotenera mobilis	JLW8	-	-	-
Methylotenera versatilis	301	-	-	-
Microlunatus phosphovorus	NM-1	-	-	-
Micromonospora aurantiaca	ATCC 27029	-	-	-
Micromonospora sp.	L5	-	-	-
Mobilicoccus pelagius	NBRC 104925	-	-	-
Moraxella catarrhalis	ETSU-9	-	-	-
Moraxella catarrhalis	101P30B1	-	-	-
Moraxella catarrhalis	103P14B1	-	-	-
Moraxella catarrhalis	12P80B1	-	-	-
Moraxella catarrhalis	46P47B1	-	-	-
Moraxella catarrhalis	7169	-	-	-
Moraxella catarrhalis	BBH18	-	-	-

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Moraxella catarrhalis	BC1	-	-	-
Moraxella catarrhalis	BC7	-	-	-
Moraxella catarrhalis	BC8	-	-	-
Moraxella catarrhalis	CO72	-	-	-
Moraxella catarrhalis	O35E	-	-	-
Moritella sp.	PE36	PE36_02157	EDM68746	ABCQ01000003 (1099400000729)
Muricauda ruestringensis	DSM 13258	Murru_2821	AEM71845	CP002999
Mycobacterium avium	104	-	-	-
Mycobact. avium subsp. *paratuberculosis*	k10	-	-	-
Mycobact. avium subsp. *paratuberculosis*	S397	-	-	-
Mycobacterium colombiense	CECT 3035	-	-	-
Mycobacterium intracellulare	ATCC 13950	-	-	-
Mycobacterium intracellulare	MOTT-02	-	-	-
Mycobacterium intracellulare	MOTT-64	-	-	-
Mycobacterium parascrofulaceum	ATCC BAA-614	-	-	-
Mycobacterium rhodesiae	NBB3	-	-	-
Mycobacterium sp.	JDM601	-	-	-
Mycobacterium sp.	JLS	-	-	-
Mycobacterium sp.	KMS	-	-	-
Mycobacterium sp.	MCS	-	-	-
Mycobacterium thermoresistibile	ATCC 19527	-	-	-
Mycobacterium xenopi	RIVM700367	-	-	-
Myroides injenensis	M09-0166	MinjM_010100014177	WP_010256415	BAEX01000118
Myroides odoratimimus	CIP 101113	HMPREF9715_02175	EHO10957	AGEE01000024.1 (cont1.24)
Myroides odoratimimus	CIP 103059	HMPREF9716_02789	EKB05496	AGZJ01000060.1 (cont1.60)
Myroides odoratimimus	CCUG 10230	-	-	-
Myroides odoratimimus	CCUG 3837	-	-	-
Myroides odoratimimus	CCUG 12901	-	-	-
Myroides odoratus	DSM 2801	EHQ44117	Myrod_3304	CM001437
Natrinema pellirubrum	DSM 15624	-	-	-
Natronomonas pharaonis	DSM 2160	-	-	-
Neisseria bacilliformis	ATCC BAA-1200	-	-	-
Neisseria cinerea	ATCC 14685	NEICINOT_03693	EEZ72291	ACDY02000003 (N_cinerea-1.0.2_Cont2.1)
Neisseria elongata subsp. *glycolytica*	ATCC 29315	-	-	-
Neisseria flavescens	NRL30031/H210	NEIFLAOT_00428	EEG34450	ACEN01000010 (N_flavescens-1.0_Cont9.1)
Neisseria flavescens	SK114	NEIFL0001_1752	EER56771	ACQV01000016 (ctg1118407793878)
Neisseria gonorrhoeae	NCCP 11945	-	-	n/i
Neisseria gonorrhoeae	TCDC-NG08107	-	-	n/i
Neisseria gonorrhoeae	FA 1090	-	-	-
Neisseria gonorrhoeae	R10	-	-	-
Neisseria lactamica	020-06	NLA_16550	CBN87860	FN995097
Neisseria lactamica	ATCC 23970	NEILACOT_04489	EEZ75519	ACEQ02000016 (N_lactamica-1.0.2_Cont15.1)
Neisseria lactamica	Y92-1009	NLY_33640	CBX21744	CACL01000004 (contig4)
Neisseria macacae	ATCC 33926	HMPREF9418_1081	EGQ77397	AFQE01000049 (contig00049)
Neisseria meningitidis	M01-240355	-	-	-
Neisseria meningitidis	M01-240149	-	-	-
Neisseria meningitidis	M01-240013	-	-	-
Neisseria meningitidis	M04-240196	-	-	-
Neisseria meningitidis	M0579	-	-	-
Neisseria meningitidis	M13399	-	-	-
Neisseria meningitidis	M6190	-	-	-
Neisseria meningitidis	MC58	-	-	-
Neisseria meningitidis	N1568	-	-	-
Neisseria meningitidis	NM220	-	-	-
Neisseria meningitidis	NM233	-	-	-
Neisseria meningitidis	NZ-05/33	-	-	-
Neisseria meningitidis	CU385	-	-	-
Neisseria meningitidis	H44/76	-	-	-
Neisseria meningitidis	WUE 2594	-	-	-
Neisseria meningitidis	alpha14	-	-	-
Neisseria meningitidis	alpha153	-	-	-
Neisseria meningitidis	alpha275	-	-	-
Neisseria meningitidis	alpha710	-	-	-
Neisseria meningitidis	ATCC 13091	-	-	-
Neisseria meningitidis	OX99.30304	-	-	-
Neisseria meningitidis	serogroup A strain Z2491	-	-	-
Neisseria meningitidis	serogroup C FAM18	-	-	-
Neisseria meningitidis	053442	-	-	-
Neisseria mucosa	ATCC 25996	NEIMUCOT_03818	EFC90015	ACDX02000001 (N_mucosa-1.0.2_Cont0.1)
Neisseria mucosa	C102	HMPREF0604_01580	EFV80059	ACRG01000016 (cont1.16)
Neisseria polysaccharea	ATCC 43768	NEIPOLOT_02085	EFH22084	ADBE01000112 (N_polysaccharea-1.0.1_Cont223.1)
Neisseria sicca	ATCC 29256	NEISICOT_02084	EET44131	ACKO02000012 (N_sicca-1.0.1_Cont11.1)
Neisseria sicca	VK64	HMPREF1051_0982	EIG25451	AJMT01000176 (ctg120005024803)
Neisseria sp.	GT4A_CT1	-	-	n/i
Neisseria sp.	oral taxon 014 str. F0314	-	-	-
Neisseria subflava	NJ9703	NEISUBOT_04005	EFC52650	ACEO02000003 (N_subflava-1.0.1_Cont2.1)
Neisseria weaveri	LMG 5135	-	-	-
Neisseria weaveri	ATCC 51223	-	-	-
Neisseriaceaea bacterium	NB-13	-	-	-
Niabella soli	DSM 19437	NiasoDRAFT_2870	EHP53291	AGSA01000027 (Nsol19437_Contig173)
Niastella koreensis	GR20-10	Niako_5286	AEW01523	CP003178
Nitratifractor salsuginis	DSM 16511	Nitsa_0495	ADV45765	CP002452
Nitratireductor aquibiodomus	RA22	-	-	-
Nitratireductor aquibiodomus	NL31	-	-	-
Nitratiruptor sp.	SB155-2	NIS_1789	BAF70894	AP009178

nosZ				
Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Nitrosococcus halophilus	Nc4	-	-	-
Nitrosococcus oceani	ATCC 19707	-	-	-
Nitrosococcus watsoni	C-113	-	-	-
Nitrosomonas marina	C-113a	-	-	-
Nitrosomonas sp.	AL212	-	-	-
Nitrosomonas sp.	Is79A3	-	-	-
Nitrosomonas sp.	NO3W	-	-	-
Nitrosomonas sp.	TA-921i-NH4	-	-	-
Nitrosomonas sp.	URW	-	-	-
Nitrosomonas sp.	C-56	-	-	-
Nitrosomonas sp.	C-45	-	-	-
Nitrosospira multiformis	ATCC 25196	-	-	-
Nitrosospira tenuis	Nv1	-	-	-
Nitrospina gracilis	3/211	-	-	-
Novosphingobium pentaromativorans	US6-1	-	-	-
Oceanimonas sp.	GK1	-	-	-
Ochrobactrum anthropi	LMG 2136	-	-	-
Ochrobactrum anthropi	ATCC 49188	Oant_4346	ABS17046	CP000759, chromosome 2
Ochrobactrum anthropi	FZX-1	-	-	-
Ochrobactrum anthropi	YD50.2	**n/a**	**BAH28827**	**AB490237**
Ochrobactrum anthropi	YX0903	-	-	-
Ochrobactrum anthropi	YX0703	-	-	-
Ochrobactrum anthropi	49187	-	-	-
Ochrobactrum intermedium	LMG 3301	-	-	-
Ochrobactrum sp.	2FB10	-	-	-
Ochrobactrum sp.	3CB4	-	-	-
Ochrobactrum sp.	3CB5	-	-	-
Ochrobactrum sp.	4FB13	-	-	-
Ochrobactrum sp.	R-24618	-	-	-
Ochrobactrum sp.	R-26825	-	-	-
Ochrobactrum sp.	R-27045	-	-	-
Ochrobactrum sp.	R-28410	-	-	-
Oligotropha carboxidovorans	OM5	OCA5_c29290	AEI07620	CP002826
		OCAR_5030	ACI92165	CP001196
Opitutaceae bacterium	TAV-5	Opit5DRAFT_0181	EHP35500	AGJF01000001 (ctg159)
Opitutus terrae	PB90-1	Oter_1803	ACB75087	CP001032
Oscillatoria acuminata	PCC 6304	-	-	-
Oscillatoria nigro-viridis	PCC 7112	-	-	-
Owenweeksia hongkongensis	DSM 17368	Oweho_3463	AEV34412	CP003156
Paenibacillus sp.	oral taxon 786 str. D14			
Parachlamydia acanthamoebae	UV-7	-	-	-
Paracoccus denitrificans	LMG 4049^T	-	-	-
Paracoccus denitrificans	PD1222	Pden_4219	ABL72283	CP000490, chromosome 2
		n/a	**CAB53351**	**AJ010260**
Paracoccus denitrificans	unknown	**n/a**	**AAC38342**	**AF016058**
Paracoccus denitrificans	NL1B8944	**n/a**	**CAA52798**	**X74792**
Paracoccus denitrificans	ATCC 17741	-	-	-
Parvibaculum lavamentivorans	DS-1	-	-	-
Pasteurella bettyae	CCUG 2042	-	-	-
Pedobacter saltans	DSM 12145	Pedsa_3647	ADY54176	CP002545
Persephonella marina	EX-H1	PERMA_1257	ACO03623	CP001230
Phaeobacter gallaeciensis	BS107	-	-	-
Phaeobacter gallaeciensis	2.10	-	-	-
Phenylobacterium zuccineum	HLK1	-	-	-
Photobact. damselae subsp. *damselae*	CIP 102761	-	-	-
Photobacterium profundum	3TCK	P3TCK_09998	EAS41064	AAPH01000041 (1099451005207)
Photobacterium profundum	SS9	PBPRB0847	CAG22719	CR378677, chromosome 2 (segment 3/7)
Polaromonas naphthalenivorans	CJ2	-	-	-
Polymorphum gilvum	SL003B-26A1	SL003B_0564	ADZ68997	CP002568
Pontibacter sp.	BAB1700	-	-	-
Prevotella histicola	F0411	HMPREF9138_01448	EHG15872	AFXP01000014 (cont1.14)
Prevotella multisaccharivorax	DSM 17128	Premu_2180	WP_007575219	GL945017 (genomic scaffold_3)
Prevotella oulorum	F0390	HMPREF9431_01063	EGV32295	ADGI01000036 (cont1.36)
Prevotella sp.	oral taxon 472 str. F0295	-	-	-
Prevotella sp.	oral taxon 317 str. F0108	-	-	-
Prevotella sp.	F0039	-	-	-
Propionibacterium acidiproprionici	ATCC 4875	-	-	-
Propionibacterium acnes	266	-	-	-
Propionibacterium acnes	6609	-	-	-
Propionibacterium acnes	ATCC 11828	-	-	-
Propionibacterium acnes	HL036PA1	-	-	-
Propionibacterium acnes	HL036PA2	-	-	-
Propionibacterium acnes	HL037PA2	-	-	-
Propionibacterium acnes	HL037PA3	-	-	-
Propionibacterium acnes	HL044PA1	-	-	-
Propionibacterium acnes	HL078PA1	-	-	-
Propionibacterium acnes	HL096PA2	-	-	-
Propionibacterium acnes	HL096PA3	-	-	-
Propionibacterium acnes	HL097PA1	-	-	-
Propionibacterium acnes	HL099PA1	-	-	-
Propionibacterium acnes	HL103PA1	-	-	-
Propionibacterium acnes	KPA171202	-	-	-
Propionibacterium acnes	PRP-38	-	-	-
Propionibacterium acnes	SK182	-	-	-
Propionibacterium acnes	TypeIA2 P.acn17	-	-	-

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Species	**Strain number**	**Gene locus tag**	**Protein ID**	**Accession number**
Propionibacterium acnes	TypeIA2 P.acn31	-	-	-
Propionibacterium acnes	TypeIA2 P.acn33	-	-	-
P. freudenreichii subsp. *shermanii*	CIRM-BIA1	-	-	-
Propionibacterium sp.	409-HC1	-	-	-
Propionibacterium sp.	CC003-HC2	-	-	-
Pseudoalteromonas haloplanktis	str. TAC125	-	-	-
Pseudogulbenkiania ferrooxidans	2002	FuraDRAFT_0338	EEG10356	ACIS01000001 (ctg1)
Pseudomonas aeruginosa	138244	PA13_05074	EGM22012	AEVV01000011 (PA13contig011)
Pseudomonas aeruginosa	152504	PA15_32324	EGM12139	AEVW01000342 (PA15contig342)
Pseudomonas aeruginosa	M18	PAM18_1574	AEO74062	CP002496
Pseudomonas aeruginosa	MPAO1/P1	O1O_08318	EHS37950	AHKM01000014 (P1_contig_c14)
Pseudomonas aeruginosa	MPAO1/P2	O1Q_21986	EHS40357	AHKN01000081 (P2_contig_c81)
Pseudomonas aeruginosa	NCGM2.S1	NCGM2_4528	BAK91351	AP012280
Pseudomonas aeruginosa	PA7	PSPA7_1737	ABR85628	CP000744
Pseudomonas aeruginosa	PADK2_CF510	CF510_18463	EIE45224	AJHI01000055 (contig_055)
Pseudomonas aeruginosa	PAO1	PA3392	AAG06780	AE004091
Pseudomonas aeruginosa	UCBPP-PA14	PA14_20190	ABJ12646	CP000438
Pseudomonas aeruginosa	DSM 50071	**n/a**	**CAA46381**	**X65277**
Pseudomonas aeruginosa	19660 ExoU Island B	-	-	-
Pseudomonas aeruginosa	LESB58	PLES_16671	CAW26395	FM209186
Pseudomonas aeruginosa	DN24	-	-	-
P. brassicacearum subsp. brassicacearum	NFM421	PSEBR_a2644	AEA68936	CP002585 - copy A
		PSEBR_a2099	AEA68359	CP002585 - copy B
Pseudomonas chlororaphis	O6	-	-	-
P. chlororaphis subsp. *aureofaciens*	ATCC 13985	-	-	-
Pseudomonas denitrificans	unknown	**n/a**	**AAC38343**	**AF016059**
Pseudomonas entomophila	str. L48	-	-	-
Pseudomonas fluorescens	F113	PSF113_2997	AEV63001	CP003150 - copy A
		PSF113_3423	AEV63425	CP003150 - copy B
Pseudomonas fluorescens	Q8r1-96	PflQ8_2674	EIK64386	AHPO01000004 (ctg1124377998473)
Pseudomonas fluorescens	C7R12	**n/a**	**AAD22389**	**AF056319 - copy A**
		n/a	**AAG34386**	**AF197468 - copy B**
Pseudomonas protegens	Pf-5	-	-	-
Pseudomonas mendocina	NK-01	MDS_0139	AEB56170	CP002620
Pseudomonas mendocina	CH91	-	-	-
Pseudomonas sp.	2_1_26	HMPREF1030_00829	EHF15316	ACWU01000020 (cont1.20)
Pseudomonas sp.	MT-1	**n/a**	**BAC00874**	**AB054991**
Pseudomonas sp.	G-179	-	-	-
Pseudomonas sp.	R-24261	-	-	-
Pseudomonas sp.	S3(2012)	-	-	-
Pseudomonas stutzeri	A1501	**n/a**	**AAZ43126**	**AY957390 - copy A**
		PST_3550	ABP81178	CP000304 - copy B
Pseudomonas stutzeri	ATCC 14405 = CCUG 16156	PstZobell_01047	EHY76008	AGSL01000016 (contig00018)
Pseudomonas stutzeri	ATCC 17588 = LMG 11199	PSTAB_3522	AEJ06803	CP002881
Pseudomonas stutzeri	CCUG 29243	A458_03705	AFM31993	CP003677
Pseudomonas stutzeri	DSM 4166	PSTAA_3647	AEA85507	CP002622
Pseudomonas stutzeri	ZoBell ATCC 14405	**n/a**	**CAA37714**	**X53676**
Pseudomonas stutzeri	TS44	YO5_06707	EIK53500	AJXE01000006 (contig00006) - copy A
		YO5_13846	EIK51269	AJXE01000042 (contig00042) - copy B
Pseudovibrio sp.	FO-BEG1	PSE_3128	AEV37636	CP003147
Pseudoxanthomonas suwonensis	11-1	-	-	-
Psychrobacter sp.	1501	-	-	-
Psychrobacter sp.	PRwf-1	-	-	-
Psychroflexus torquis	ATCC 700755	P700755_000691	AFU67703	CP003879
Psychromonas ingrahamii	37	Ping_1428	ABM03245	CP000510
Pusilimonas sp.	T7-7	-	-	-
Pyrobaculum aerophilum	IM2	-	-	-
Pyrobaculum arsenaticum	DSM 13514	-	-	-
Pyrobaculum calidifontis	JCM 11548	ABO09344	Pcal_1928	CP000561
Pyrobaculum oguniense	TE7			-
Pyrobaculum sp.	1860	P186_0766	AET32212	CP003098
Cupriavidus necator	H16	PHG252	AAP86001	AY305378, megaplasmid pHG1
		n/a	**CAA46383**	**X65278**
Ralstonia pickettii	12D	Rpic12D_4171	ACS65419	CP001645, chromosome 2
Ralstonia pickettii	12J	Rpic_4059	ACD29162	CP001069, chromosome 2
Ralstonia solanacearum	str. CFBP2957	-	-	-
Ralstonia solanacearum	CMR15	-	-	n/i
Ralstonia solanacearum	GMI1000	RSp1368	CAD18519	AL646053, megaplasmid
Ralstonia solanacearum	IPO1609	-	-	-
Ralstonia solanacearum	MolK2	-	-	-
Ralstonia solanacearum	Po82	-	-	-
Ralstonia solanacearum	PSI07	-	-	-
Ralstonia solanacearum	UW551	-	-	-
Ralstonia sp.	5_2_56FAA	**n/a**	**EGY62020**	**ACTT01000006 (cont1.6)**
Ralstonia sp.	5_7_47FAA	-	-	n/i
Ralstonia sp.	PBA	-	-	-
Reinekea blandensis	MED297	MED297_07556	EAR08081	AAOE01000026 (1099646006996)
Rheinheimera nanhaiensis	E407-8	-	-	-
Rhizobium etli	CFN 42	-	-	-
Rhizobium hedysari	HCNT1	-	-	-
Rhizobium meliloti	JJ1c10	**n/a**	**AAC44023**	**RMU47133**
Rhizobium sp.	IAE-1	-	-	-
Rhizobium sp.	PIP4	-	-	-
Rhizobium sp.	PY13	-	-	-
Rhizobium sp.	R-24654	-	-	-

nosZ				
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Rhodanobacter fulvus	Jip2	UU9_01404	EIL92558	AJXU01000007 (contig007)
Rhodanobacter sp.	115	UU5_20685	EIL86864	AJXS01000480 (contig480)
Rhodanobacter denitrificans	116-2	UUC_01812	EIM04606	AJXV01000003 (contig003)
Rhodanobacter denitrificans	2APBS1	R2APBS1DRAFT_1707	EHA65725	AGIL01000004 (ctg275)
		n/a	**ADD51573**	**GU233008**
Rhodanobacter spathiphylli	B39	UU7_06253	EIL94026	AJXT01000009 (contig009)
Rhodanobacter thiooxydans	LCS2	UUA_00745	EIM02979	AJXW01000003 (contig003)
Rhodobacter capsulatus	SB 1003	RCAP_rcp00075	ADE87331	CP001313, plasmid pRCB133
Rhodobacter sphaeroides	2.4.3	-	-	-
Rhodobacter sphaeroides	ATCC 17025	-	-	-
Rhodobacter sphaeroides	KD131	-	-	-
Rhodobacter sphaeroides	WS8N	RSWS8N_21059	EGJ19390	AFER01000004, plasmid pWS8N_B PlasmidB
R. sphaeroides f. sp. *denitrificans*	IL106	**n/a**	**AAD43473**	**AF125260**
Rhodococcus equi	103S	-	-	-
Rhodoferax ferrireducens	T118	-	-	-
Rhodopseudomonas palustris	BisA53	RPE_3096	ABJ07032	CP000463
Rhodopseudomonas palustris	CGA009	RPA2061	CAE27502	BX572599
Rhodopseudomonas palustris	DX-1	Rpdx1_3443	ADU45013	CP002418
Rhodopseudomonas palustris	TIE-1	Rpal_2351	ACF00866	CP001096
Rhodopseudomonas sp.	2-8	-	-	-
Rhodospirillum centenum	SW	RC1_3912	ACJ01254	CP000613
Rhodothermus marinus	DSM 4252	Rmar_2012	ACY48893	CP001807
Rhodothermus marinus	SG0.5JP17-172	Rhom172_0852	AEN72785	CP003029
Riemerella anatipestifer	DSM 15868	RA0C_1969	AFD56840	CP003388 - copy A
		Riean_1674	ADQ82830	CP002346 - copy B
Riemerella anatipestifer	RA-GD	RIA_0512	ADZ11677	CP002562
Riemerella anatipestifer	RA-YM	RAYM_04561	EFT35470	AENH01000026 (scaffold6)
Riemerella anatipestifer	RA-CH-1	HYPO	HYPO	NC_018609.1
Robiginitalea biformata	HTCC 2501	RB2501_05465	EAR16321	CP001712
Roseobacter denitrificans	OCh 114	RD1_1547	ABG31176	CP000362
Roseobacter litoralis	Och 149	RLO149_c031550	AEI95111	CP002623
Roseobacter sp.	SK209-2-6	RSK20926_03559	EBA16850	AAYC01000005 (1101232001808)
Roseovarius sp.	TM1035	RTM1035_04615	EDM32870	ABCL01000002 (1101493006647)
Roseovarius sp.	217	ROS217_15191	EAQ26533	AAMV01000002 (1099463000286)
Rothia aeria	F0474	-	-	-
Rothia dentocariosa	ATCC 17931	-	-	-
Rothia mucilaginosa	ATCC 25296	-	-	-
Rothia mucilaginosa	DY-18	-	-	-
Rothia mucilaginosa	M508	-	-	-
Rubrivivax gelatinosus	IL144	RGE_19210	BAL95262	AP012320
Rubrivivax gelatinosus	S1	**n/a**	**ACX46124**	**GQ900543**
Ruegeria pomeroyi	DSS-3 megaplasmid	SPOA0050	AAV97190	CP000032, megaplasmid
Runella slithyformis	DSM 19594	Runsl_0111	AEI46569	CP002859
Salinibacter ruber	M8	SRM_00384	CBH23305	FP565814
Salinisphaera shabanensis	E1L3A	SSPSH_18537	EGM26302	AFNV01000057 (Contig57)
Shewanella amazonensis	SB2B	-	-	-
Shewanella denitrificans	OS217	Sden_2219	ABE55499	CP000302
Shewanella frigidimarina	NCIMB 400	-	-	-
Shewanella halifaxensis	HAW-EB4	-	-	-
Shewanella loihica	PV-4	Shew_3400	ABO25266	CP000606
Shewanella piezotolerans	WP3	-	-	-
Shewanella putrefaciens	200	-	-	-
Shewanella putrefaciens	CN-32	-	-	-
Shewanella sediminis	HAW-EB3	-	-	-
Shewanella sp.	ANA-3	-	-	-
Shewanella sp.	MR-4	-	-	-
Shewanella sp.	MR-7	-	-	-
Shewanella sp.	W3-18-1	-	-	-
Shewanella woodyi	ATCC 51908	-	-	-
Sinorhizobium fredii	USDA 257	USDA257_c39060	AFL52451	CP003563
Sinorhizobium fredii	HH103	-	-	-
Sinorhizobium fredii	NGR234	-	-	-
Sinorhizobium medicae	WSM419	-	-	-
Sinorhizobium meliloti	1021	SMa1182	AAK65301	AE006469, plasmid pSymA
Sinorhizobium meliloti	BL225C	SinmeB_6148	AEG07276	CP002741, plasmid pSINMEB01
Sinorhizobium meliloti	JJ1C10	-	-	-
Sinorhizobium sp.	NP1	-	-	-
Sinorhizobium sp.	R-31759	-	-	-
Sinorhizobium sp.	R-31764	-	-	-
Sinorhizobium sp.	R-31816	-	-	-
Sinorhizobium sp.	R-32546	-	-	-
Sinorhizobium sp.	R-32549	-	-	-
Solitalea canadensis	DSM 3403	Solca_1787	AFD06851	CP003349
		SolcaDRAFT_0417	EHP07940	AGSC01000045 (Scan3403_Contig291)
Sorangium cellulosum	'So ce 56'	-	-	-
Sphaerobacter thermophilus	DSM 20745	Sthe_0735	ACZ38172	CP001823, chromosome 1
Sphingobium yanoikuyae	XLDN2-5	-	-	-
Sphingomonas witchii	RW1	-	-	-
Staphylococcus aureus	O11	-	-	-
Staphylococcus aureus	O46	-	-	-
Staphylococcus aureus	08BA02176	-	-	-
Staphylococcus aureus	ST398/SO385	-	-	-
Staphylococcus aureus subsp. *aureus*	HO 5096 0412	-	-	-
Staphylococcus aureus subsp. *aureus*	21342	-	-	-
Staphylococcus aureus subsp. *aureus*	21310	-	-	-

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Staphylococcus aureus subsp. *aureus*	21269	-	-	-
Staphylococcus aureus subsp. *aureus*	21264	-	-	-
Staphylococcus aureus subsp. *aureus*	21178	-	-	-
Staphylococcus aureus subsp. *aureus*	21195	-	-	-
Staphylococcus aureus subsp. *aureus*	21235	-	-	-
Staphylococcus aureus subsp. *aureus*	71193	-	-	-
Staphylococcus aureus subsp. *aureus*	21331	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-105	-	-	-
Staphylococcus aureus subsp. *aureus*	MRSA252	-	-	-
Staphylococcus aureus subsp. *aureus*	D139	-	-	-
Staphylococcus aureus subsp. *aureus*	JKD6159	-	-	-
Staphylococcus aureus subsp. *aureus*	CGS00	-	-	-
Staphylococcus aureus subsp. *aureus*	LGA251	-	-	-
Staphylococcus aureus subsp. *aureus*	DR10	-	-	-
Staphylococcus aureus subsp. *aureus*	ED133	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-125	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-157	-	-	-
Staphylococcus aureus subsp. *aureus*	IS-189	-	-	-
Staphylococcus aureus subsp. *aureus*	21345	-	-	-
Staphylococcus aureus subsp. *aureus*	ATCC BAA-39	-	-	-
Staphylococcus aureus subsp. *aureus*	MN8	-	-	-
Staphylococcus aureus subsp. *aureus*	JKD6008	-	-	-
Staphylococcus aureus subsp. *aureus*	T0131	-	-	-
Staphylococcus aureus subsp. *aureus*	TCH60	-	-	-
Staphylococcus aureus subsp. *aureus*	TW20	-	-	-
Staphylococcus aureus subsp. *aureus*	H19	-	-	-
Staphylococcus aureus subsp. *aureus*	C160	-	-	-
Staphylococcus simulans	ACS-120-V-Sch1	-	-	-
Starkeya novella	DSM 506	-	-	-
Sulfobacillus acidophilus	DSM 10332	-	-	-
Sulfobacillus acidophilus	TPY	-	-	-
Sulfolobus islandicus	HVE10/4	-	-	-
Sulfolobus islandicus	L.S.2.15	-	-	-
Sulfolobus islandicus	REY15A	-	-	-
Sulfolobus islandicus	Y.G.57.14	-	-	-
Sulfolobus islandicus	Y.N.15.51	-	-	-
Sulfolobus solfataricus	98/2	-	-	-
Sulfolobus solfataricus	P2	-	-	-
Sulfurimonas autotrophica	DSM 16294	Saut_1988	ADN10031	CP002205
Sulfurimonas denitrificans	DSM 1251	Tmden_1770	ABB45044	CP000153 - copy A
		Tmden_1298	ABB44576	CP000153 - copy B
Sulfurimonas gotlandica	GD1 SMGD1	SMGD1_2343	EHP30866	AFRZ01000001
Sulfurovum sp.	NBC37-1	SUN_2179	BAF73119	AP009179
Symbiobacterium thermophilum	IAM 14863	-	-	-
Synechocystis sp.	PCC 6803	-	-	-
Synechocystis sp.	PCC 6803 substr. GT-I	-	-	-
Synechocystis sp.	PCC 6803 substr. PCC-N	-	-	-
Synechocystis sp.	PCC 6803 substr. PCC-P	-	-	-
Syntrophobacter fumaroxidans	MPOB	-	-	-
Taylorella asinigenitalis	MCE3	-	-	-
Taylorella equigenitalis	MCE9	-	-	-
Thauera sp.	MZ1T	Tmz1t_0664	ACK53436	CP001281
Thermaerobacter marianensis	DSM 12885	-	-	-
Thermaerobacter subterraneus	DSM 13965	-	-	-
Thermobaculum terrenum	ATCC BAA-798	-	-	-
Thermobifida fusca	YX	-	-	-
Thermomicrobium roseum	DSM 5159	trd_A0453	ACM06733	CP001276, plasmid
Thermoproteus uzonensis	768-20	-	-	-
Thioalkalivibrio sulfidophilus	HL-EbGr7	Tgr7_2564	ACL73640	CP001339
Thioalkalivibrio thiocyanoxidans	ARh 4	ThithDRAFT_1801	EGZ35502	AGFB01000002 (ctg51)
Thiobacillus denitrificans	ATCC 25259	Tbd_1389	AAZ97342	CP000116
Thiocapsa marina	5811	ThimaDRAFT_0916	EGV19470	AFWV01000003 (ctg473)
Thiocystis violascens	DSM 198	-	-	-
Turneriella parva	DSM 21527	-	-	-
Veillonella parvula	ATCC 17745	-	-	-
Veillonella parvula	DSM 2008	-	-	-
Veillonella sp.	oral taxon 158 str. F0412	-	-	-
Veilonella dispar	ATCC 17748	-	-	-
Veilonella parvula	ACS-068-V-Sch12	-	-	-
Vibrio orientalis	CIP 102891 = ATCC 33934	VIA_003610	EEX92965	ACZV01000005 (Contig80) - copy A
		VIOR3934_07838	EGU46648	AFWH01000062 (VIOR3934_6) - copy B
Vibrio tubiashii	ATCC 19109	VITU9109_23699	EGU50109	AFWI01000182 (VITU9109_59)
Vibrio tubiashii	NCIMB 1337 = ATCC 19106	VT1337_14057	EIF03334	AHHF01000072
Vulcanisaeta distributa	DSM 14429	-	-	-
Waddlia chondrophila	WSU 86-1044	-	-	-
Waddlia chondrophila	2032/99	-	-	-
Xanthobacter autotrophicus	Py2	-	-	-
Zobellia galactanivorans	DsiJT	CAZ96392	CAZ96392	FP476056

[a] Retrieval of sequences from Fungene (http://fungene.cme.msu.edu) and GenBank sequence databases in November-December 2012; entries in bold represent PCR-derived sequences
-, not found in Fungene or Genbank databases; -- no amplification with novel primers
n/a, not applicable since not part of a full genome sequence
n/I, not included in analyses (due to length, lack of necessary conserved residues, presence of multiple stopcodons in all ORFs or of multiple N-residues)

Fig. S1 Comprehensive maximum likelihood phylogeny of *nirK* amino acid sequences. From isolates as well as available genomes in Fungene and GenBank sequence databases. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 394 amino acid sequences and there were a total of 94 positions used in the final dataset. Sequences from this study are given in bold.

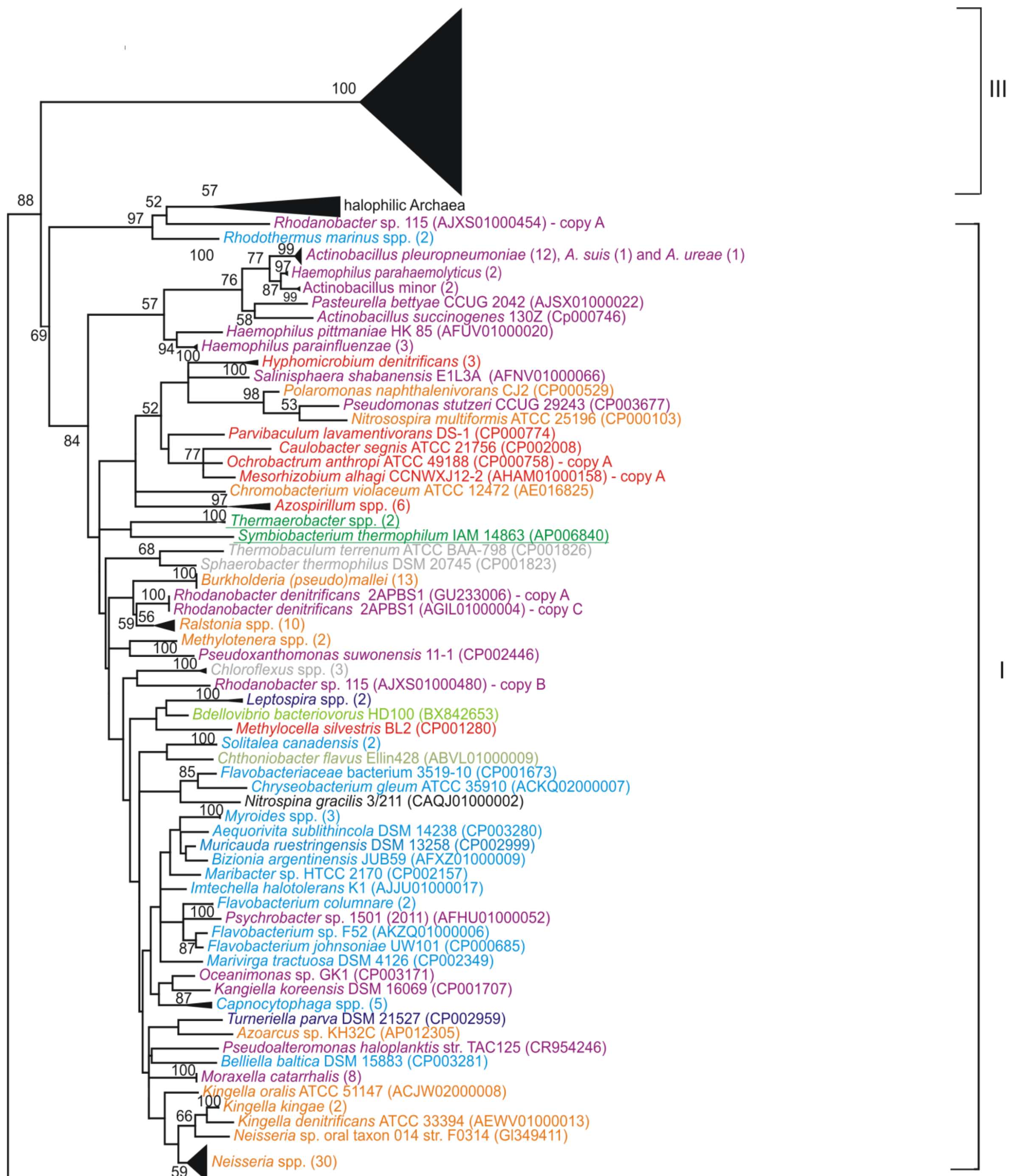


Fig. S1 (continued)

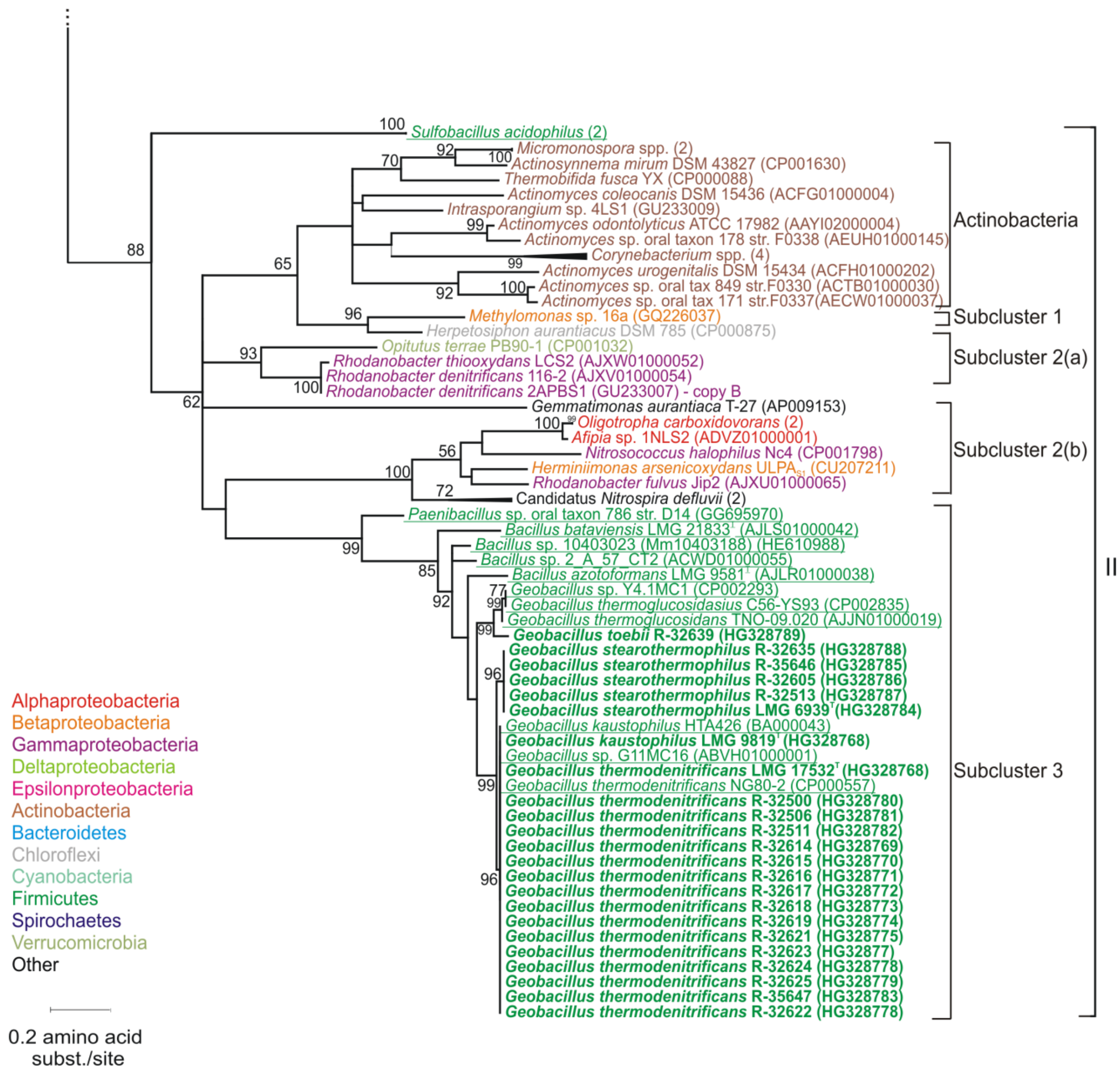


Fig. S2 Comprehensive maximum likelihood phylogeny of *qnorB* amino acid sequences. From isolates as well as available genomes in Fungene and GenBank sequence databases. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I+F) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 539 amino acid sequences and there were a total of 145 positions used in the final dataset. Sequences from this study are given in bold.

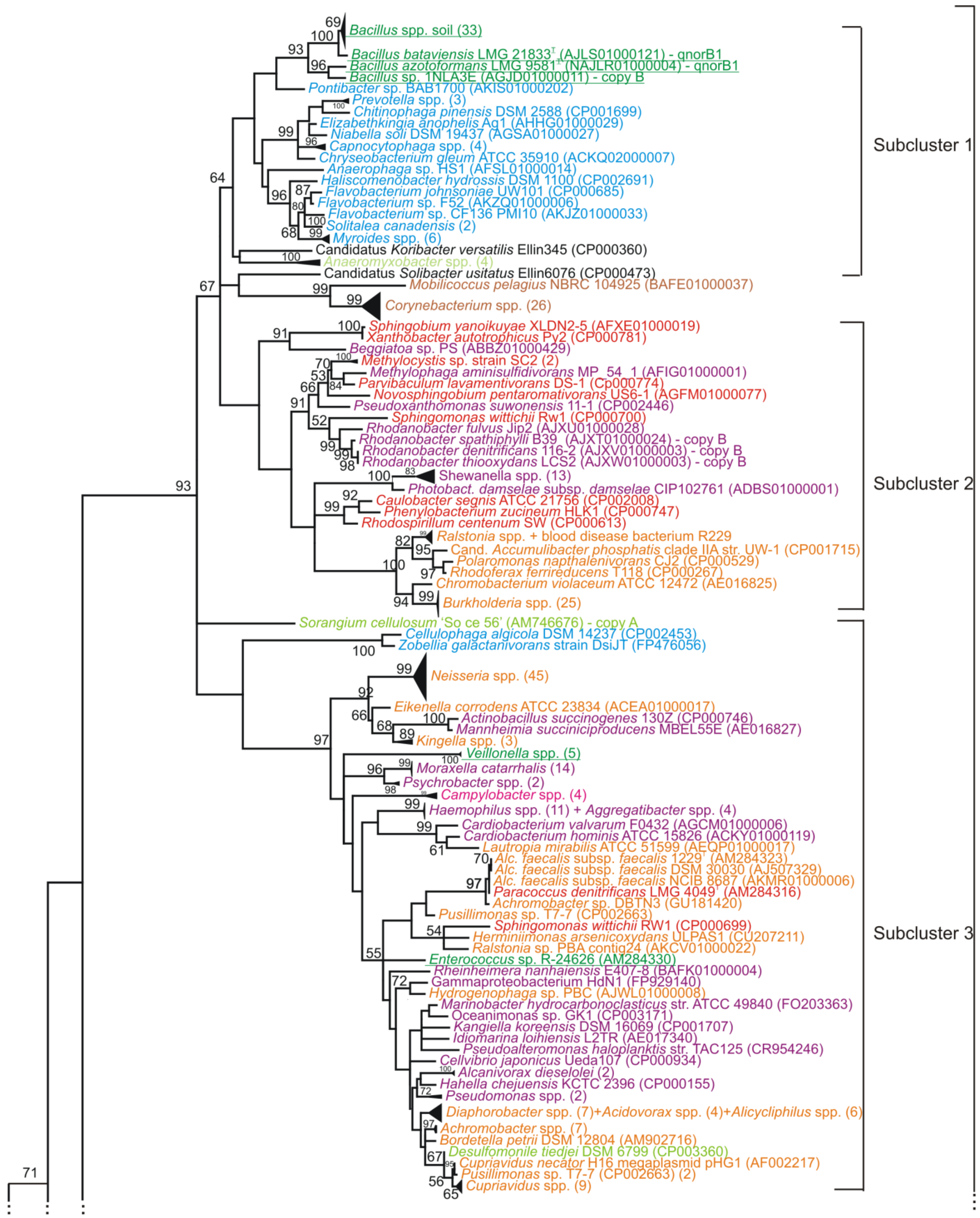


Fig. S2 (continued)

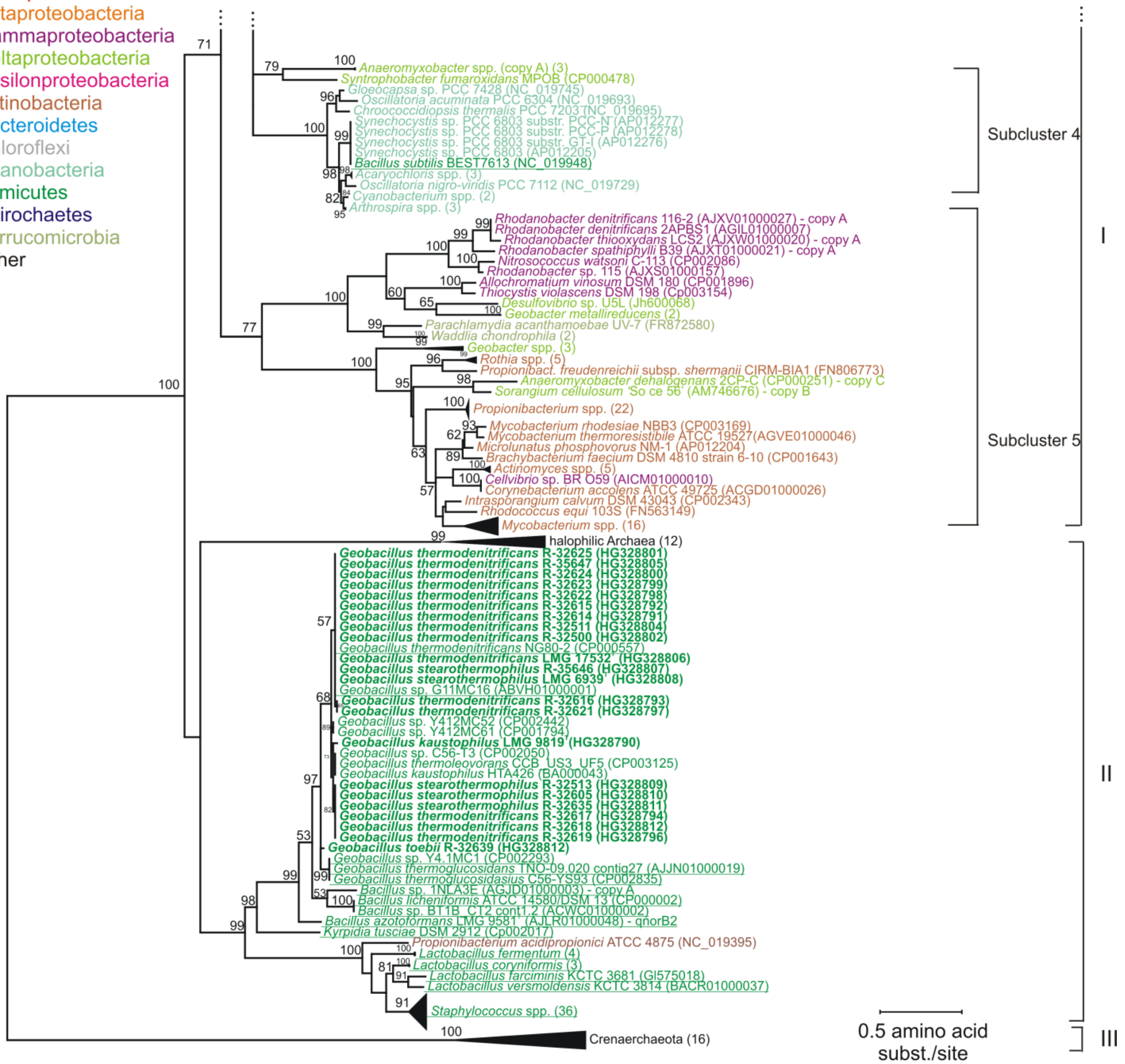


Fig. S3 Comprehensive maximum likelihood phylogeny of *nosZ* amino acid sequences. From isolates as well as available genomes in Fungene and GenBank. Collapsed clades (black triangles), with the number of sequences per clade in parenthesis, are used to simplify the figure. Scales indicate corrected amino acid substitutions per site (WAG+G+I) and only bootstrap support values for nodes with > 50% bootstrap probability (n=1000) are given. Analysis involved 264 amino acid sequences and there were a total of 338 positions used in the final dataset. Sequences from this study are given in bold.

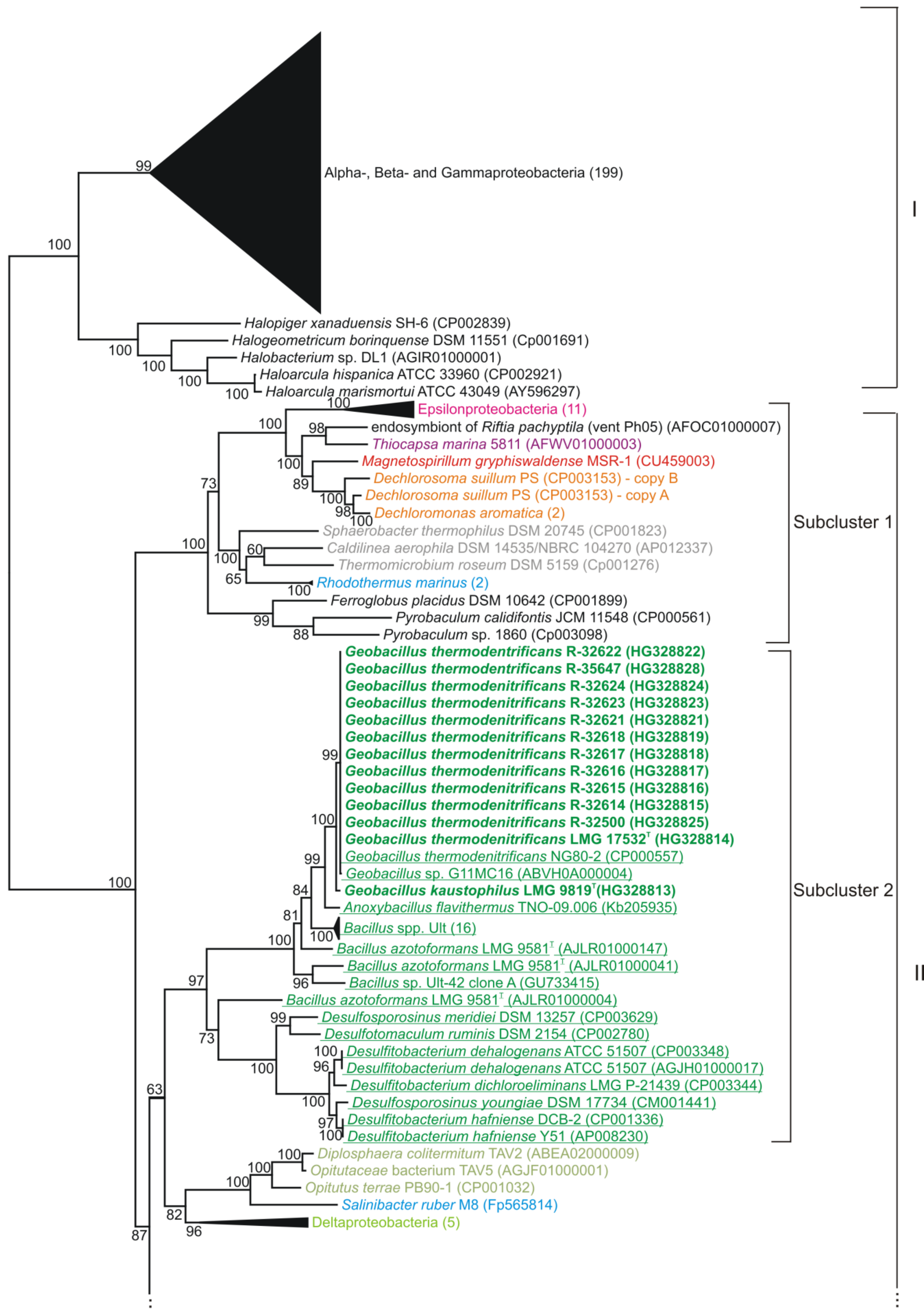
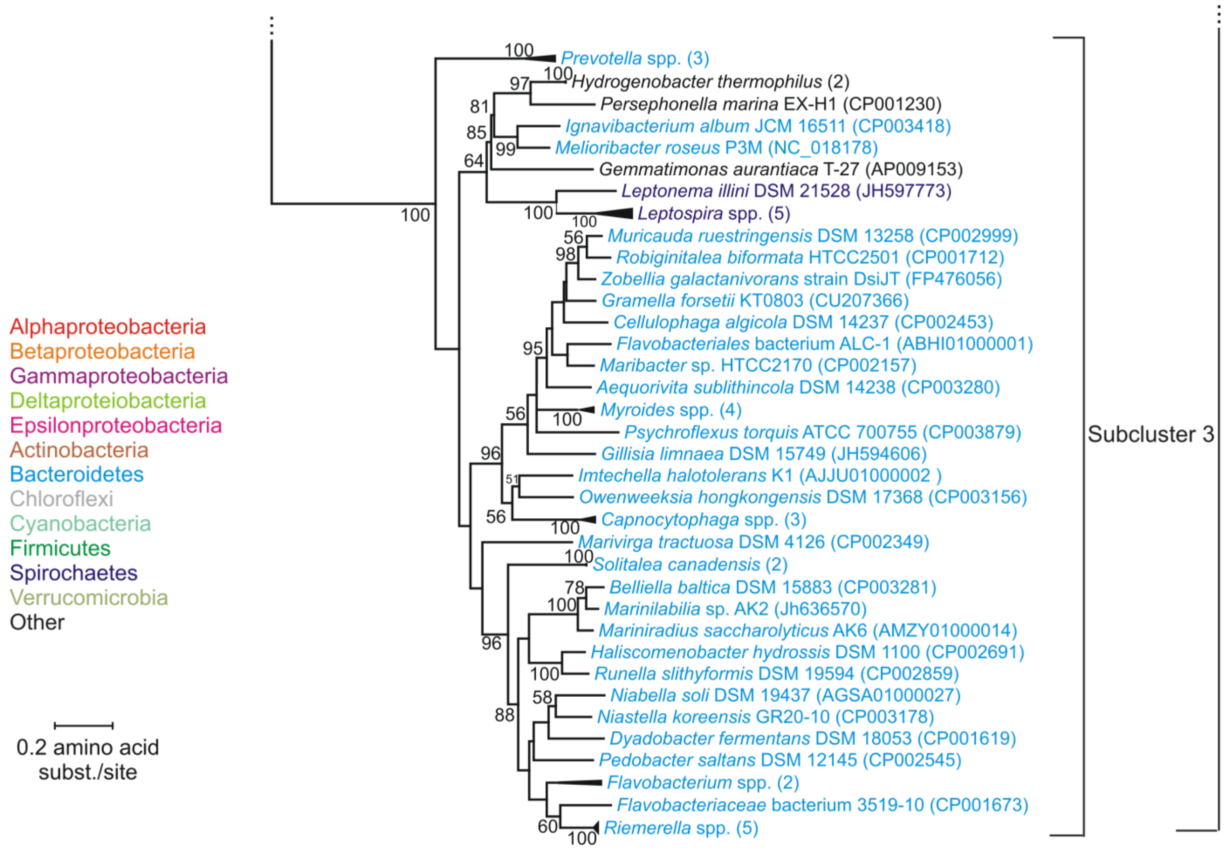


Fig. S3 (continued)



Part VI

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Part VI

Curriculum vitae

PERSONAL INFORMATION

Ines Verbaendert

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Born on January 24th 1983 (Antwerp, Belgium)
Married

WORK EXPERIENCE

2007-2014 DOCTORAL RESEARCHER, Department Biochemistry and Microbiology (WE10), Ghent University, Ghent, Belgium

EDUCATION

2007-2014 PhD Biotechnology, Ghent University
Dissertation: 'Denitrification in Gram-positive bacteria, with focus on members of the *Bacillaceae*'
Promotors: Prof. Dr. Paul De Vos, Prof. Dr. Ir. Nico Boon

2006-2007 MSc 'Applied Microbial Systematics', Ghent University (graduated magna cum laude)
Dissertation: 'Real-time PCR detection of *Pseudomonas cichorii*, the causal agent of midrib rot in lettuce'

2001-2006 Licentiate Biomedical Sciences, Ghent University (graduated cum laude)
Dissertation: 'Presence of multiresistant Gram-negative rods in the environment of patients infected or colonized with these micro-organisms'

COURSES

2012 Veilig gebruik van gassen – Seminar 'Welzijn en Milieu'
2010 Advanced Academic English – conference skills, Ghent University
2009 5th Follow-up Training Workshop on BioNumerics and GelCompar II – Applied Maths
Basisassistententraining en feedbackgesprek – Ghent University
2008 Advanced Academic English – writing skills, Ghent University
55th Int. Basic Training Workshop on BioNumerics & GelCompar II – Applied Maths
Time Management – personal effectiveness, Doctoral Schools, Ghent University
Quality Research Skills – research and validation, Doctoral Schools, Ghent University
2007 Introduction to EndNote

SCIENTIFIC ACTIVITIES

Invited lectures:

2013 **Verbaendert, I.**, Boon, N., De Vos, P. Novel primers target divergent *nirK, qnorB* and *nosZ* genes in denitrifying geobacilli. 18th European Nitrogen Cycle Meeting, Darmstadt, Germany.

2011 **Verbaendert, I.**, Boeckx, P., Boon, N., De Vos, P., Heylen, K. Denitrification in closely related *Geobacillus* strains. 2nd International Conference on Nitrification (ICoN2) and the 16th European Nitrogen Cycle Meeting, Nijmegen, the Netherlands.

2010 **Verbaendert, I.**, Boon, N., De Vos, P., Heylen, K. Denitrification in *Bacillus*. Conference Biochemical Society – Enzymology and Ecology of the Nitrogen Cycle, Birmingham, UK.

Poster presentations:

2013 **Verbaendert, I.**, Hoefman, S., Boeckx, P, Boon, N., De Vos, P. Primers for divergent *nirK, qnorB* and *nosZ* of thermophilic denitrifying geobacilli. Symposium Belgian Society Microbiology – Microbial Diversity for Science and Industry, Brussels, Belgium

2011 **Verbaendert, I.**, Boeckx, P., De Vos, P., Heylen, K. Closely related *Geobacillus* strains show genotypic and phenotypic differences in denitrification. 100th Anniversary NVvM & Scientific Spring Meeting, Arnhem, the Netherlands.

Verbaendert, I., Boeckx, P., De Vos, P., Heylen, K. Denitrification in closely related *Geobacillus* strains. 2nd International Conference on Nitrification (ICoN2) and the 16th European Nitrogen Cycle Meeting, Nijmegen, the Netherlands.

Verbaendert, I., Boeckx, P., De Vos, P., Heylen, K. Symposium Belgian Society Microbiology – Life, Death and Survival of Micro-organisms, Brussels, Belgium.

2010 **Verbaendert, I.**, Boon, N., De Vos, P., Heylen, K. Denitrification in *Bacillus*. Conference Biochemical Society – Enzymology and Ecology of the Nitrogen Cycle, Birmingham, UK.

Verbaendert, I., Boon, N., De Vos, P., Heylen, K. Denitrification: a common feature in members of *Bacillus*, Symposium Belgian Society Microbiology - Molecular dialogue in host-parasite interaction, Brussels, Belgium.

2009 **Verbaendert, I.**, Lebbe, L., Boon, N., De Vos, P. *Bacillus*: to denitrify or not to denitrify? Poster presentation, FEMS 3rd Congress of European Microbiologists – Microbes and Man, interdependence and future challenges, Gothenburg, Sweden

Verbaendert, I., Lebbe, L., Boon, N., De Vos, P. Denitrification in *Bacillus* by yet unknown genes? Symposium Belgian Society Microbiology – Analyzing complex microbial communities and their host microbe interactions, Brussels, Belgium.

2008 **Verbaendert, I.**, Heylen, K., Boon, N., De Vos, P. To denitrify or not to denitrify – an issue for Gram-positive bacteria? – Symposium Belgian Society Microbiology – Stress responses in the Microbial World, Brussels, Belgium.

De Vos, P., **Verbaendert, I.**, Boon, N., Heylen, K. To denitrify or not to denitrify? The question for Gram-positive bacteria – American Society for Microbiology 108th General Meeting, Boston, Massachusetts, USA.

Conferences

2012 Mini-symposium on metaproteogenomics (Belgian Society Microbiology) – Functional analysis of microbial communities and consortia without cultivation, Brussels, Belgium.

Symposium Belgian Society Microbiology – Posttranscriptional regulation and epigenetics in microorganisms, Brussels, Belgium

2007 Denitrification and related aspects – COST Action meeting 856, Uppsala, Sweden

Publications

A1 publications

Cottyn, B., Baeyen, S., Pauwelyn, E., **Verbaendert, I.**, De Vos, P., Bleyaert, P., Höfte, M., and Maes, M. (2011). Development of a real-time PCR assay for *Pseudomonas cichorii*, the causal agent of midrib-rot in greenhouse-grown lettuce, and its detection in irrigating water. Plant Pathology 60 (3): 453-461.

Verbaendert, I., Boon, N., De Vos, P., and Heylen, K. (2011). Denitrification is a common feature among members of the genus *Bacillus*. Systematic and Applied Microbiology 34, 385-391.

Verbaendert, I., De Vos, P., Boon, N., and Heylen K. (2011). Denitrification in Gram-positive bacteria: an underexplored trait. Biochemical Society Transactions 39 (1): 254-258

Verbaendert, I., Hoefman, S., Boeckx, P., Boon, N., De Vos, P. (2014). Primers targeting divergent *nirK, qnorB* and *nosZ* genes in denitrifying *Geobacillus*. FEMS Microbiology Ecology 89: 162-180.

B2 publication

Verbaendert, I., and De Vos, P. (2011) Studying denitrification by aerobic endospore-forming bacteria in soil. In Soil Biology: Aerobic Endospore-forming Soil Bacteria (Logan, N. & De Vos, P., eds.), Springer, pp. 271-285.2011

International mobility & grants

2011 FEMS Research Fellowship for 1 month at UMB (Norwegian University of Life Sciences) on the effect of suboptimal pH and nitrogen source on denitrification kinetics in *Bacillus* strains.

TEACHING AND OTHER ACADEMIC ACTIVITIES

Practical courses

2007 – 2014 Supervisor of 2nd year practical course "General Microbiology", Bachelor Biochemistry and Biotechnology
Supervisor of 3rd year practical course "General Microbiology", Bachelor Biology
Organiser & supervisor (2007-present) of 3rd year practical course "Microbial Diversity and Evolution", Bachelor Biochemistry and Biotechnology

Master projects

2008 – 2009 Aaron Plovie, Sarah Devriese
2009 – 2010 Anneleen Decloedt
2010 – 2011 Jan Van der Roost
2011 – 2012 Annelies Torfs
2012 – 2013 Jasmien Vercruysse

Thesis students

2008 – 2009 Malgorzata Lewandowska

Other

2002 – 2006 student representative in Study Program Commission Biomedical Sciences
2006 Prize Prof. Dr. L. De Ridder for social commitment and dedication for fellow Biomedical Sciences students
2007 – 2009 Secretary of BAG (Biomedical Alumni Ghent)
2008 – 2014 Member First Aid Team Ledeganck building
2010 – 2014 Member of Department Council Biochemistry and Microbiology