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**CHARACTERISATION OF METAL-RESISTANT AND
CHLOROAROMATICS-DEGRADING BACTERIA**

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**KARAKTERISERING VAN METAAL-RESISTENTE EN
CHLOORAROMAAT-DEGRADERENDE BACTERIËN**

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CHAPTER 1
LITERATURE OVERVIEW

“According to classical aerodynamics, it is impossible for a bumblebee to fly”
Doctor Who

CHAPTER 1 LITERATURE OVERVIEW

1.1 INTRODUCTION

This study deals with the classification and identification of bacteria that either show resistance to toxic metals or that are capable of degrading chlorinated aromatic compounds. Both characteristics have in common that they can possibly be applied for bioremediation. In this first and introducing chapter, an outline of the available scientific literature is given. The different paragraphs introduce various aspects of this work.

Some general aspects of bacterial systematics are discussed (1.2), with a focus on the current species definition. Contiguous, a brief overview of the methods that are presently used in bacterial systematics is given.

The bacteria of primary importance to this study belong to the genera *Burkholderia* and *Ralstonia*. Therefore, these two genera are discussed and their taxonomical and ecological background is presented in 1.3.

Bacterial aspects of bioremediation processes are discussed (1.4). The first section focuses on bacterial resistance mechanisms against toxic metals, and some bioremediation approaches derived from them. Emphasis in this section is placed on *Ralstonia metallidurans* CH34. The second section deals with degradation of chlorinated aromatic compounds. The general degradation pathway is outlined and the metabolism of 2,4-dichlorophenoxyacetic acid and chloroanilines are discussed in more detail. Finally, the third section discusses the different approaches useful for bioaugmentation, still in the context of chloroaromatics biodegradation. Special emphasis is placed on bioaugmentation by plasmid-mediated dissemination of catabolic genes.

1.2 BACTERIAL SYSTEMATICS

1.2.1 General definitions

The term systematics, generally taken as synonym for taxonomy, can be defined as “the scientific study of the kinds and diversity of organisms and of any and all relationships among them” [252]. In bacterial systematics, a polyphasic approach is the preferable basis for classification [104, 309]. Colwell [57] introduced the term “polyphasic taxonomy” to refer to classifications based on a consensus of all available methods, combining phenotypic, genotypic, and phylogenetic data. It is also generally accepted that bacterial classification should reflect as close as possible the phylogenetic or ancestral relationships between bacteria [325]. A central outcome of phylogenetic classification is that taxa should be monophyletic, meaning that all members of any taxon under consideration share the same common ancestor. A further requirement is that taxa sharing more recent common ancestry in time are considered to be more closely related to each other than they are to other taxa [166]. In the absence of real time units, relationships are expressed in terms of evolutionary changes.

The species is the basic unit in bacterial taxonomy. Since the “biological” species concept, stating that species are population of actually or potentially interbreeding populations of organisms [186], obviously does not apply to asexually reproducing organisms like bacteria, an alternative species definition is required. A bacterial species is defined as a group of strains, sharing 70% or greater DNA-DNA relatedness with 5°C or less ΔT_m (the difference in melting temperature between the heterologous and homologous DNA hybrids) [316]. Phenotypic and chemotaxonomic features should agree with this definition. A type strain is designated that serves as name bearer and as reference specimen of the species [264]. Vandamme and co-authors, in their review on polyphasic taxonomy, consider bacterial species to be “condensed nodes in an otherwise cloudy, confluent taxonomic space” [309].

The bacterial species concept based on DNA hybridisation has been criticised for arbitrarily imposing divisions upon a continuum [257] and DNA hybridisation has been designated a “primitive form of genetic typing... that only superficially scans for genome identity” [295]. Despite this criticism, the current species definition [316] was re-confirmed in a recent meeting of the ad hoc committee of the International Committee for the Systematics of Prokaryotes stating that “The current species definition is pragmatic, operational and universally applicable, and serves the community well” [262].

Interesting in this respect is the view of population geneticists, who propose four different types of population structures within bacterial species [255, 256]. At one extreme there are clonal species, in which genetic exchange is not frequent enough to prevent the individualization of discrete evolutionary lines. This involves a lack of gene segregation and non-random association of genotypes occurring at different loci. At the opposite end of the spectrum there are panmictic species, in which no lineages can be identified and, through basically sexual multiplication, fully randomised reassortment of genetic loci occurs continuously and blurs the vertical lineages. An intermediate

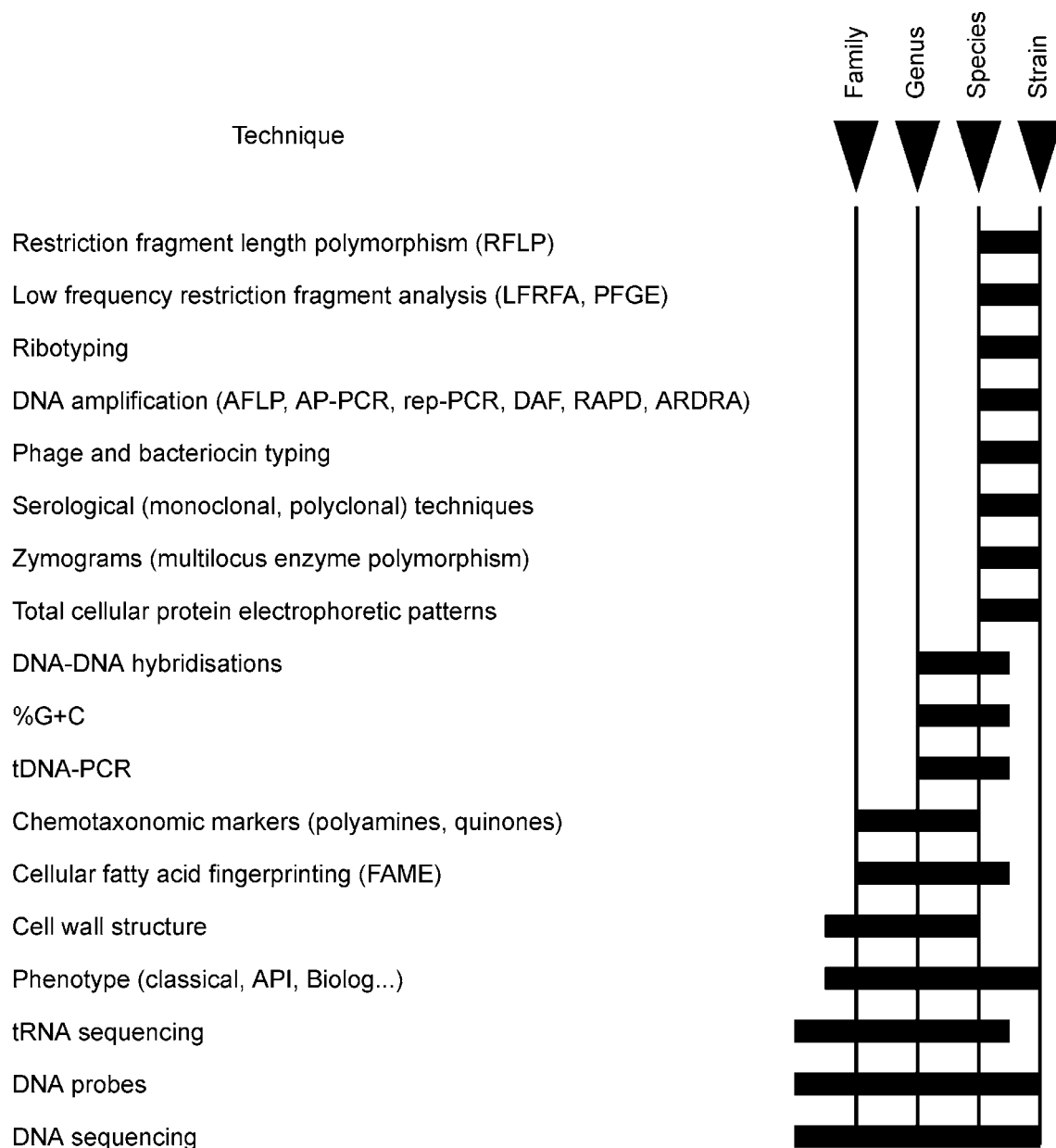


Fig. 1.2: Taxonomic resolution of currently applied techniques (reprinted from Vandamme et al., 1996 [309]).

1.2.2.1 “Classical” phenotypic characterisation

The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. The morphology of a bacterium can be described at a cellular level (cell shape, endospore, flagella, inclusion bodies, and staining characteristics) and at the colony level (colony colour, dimensions, and form). The physiological and biochemical features are determined via growth tests at different temperatures, pH values, salt concentrations, or atmospheric conditions, growth tests in the presence of various substances such as antibiotics, enzyme assays, tests for the metabolism of compounds, etc. Miniaturized phenotypic fingerprinting systems have been developed to facilitate and standardise a number of these classical tests. Mostly,

these test kits contain a battery of dehydrated reagents to which a standardized inoculum is added. Examples include BIOLOG plates (Biolog, Inc., Hayward, CA, USA) for the oxidation of carbon sources, several available API strips (bioMérieux SA, La Balme-les-Grottes, France) containing a whole array of substrate utilization tests, enzyme assays, etc. that are discriminative for the specific group of organisms under investigation, and VITEK (bioMérieux Vitek, Inc., Hazelwood, MI, USA), a more sophisticated and fully automated system for phenotypic identification and antimicrobial susceptibility testing of bacteria.

1.2.2.2 Chemotaxonomic methods

The term chemotaxonomy refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria [309]. Various cell components have been evaluated for their use as chemotaxonomic markers in bacteria e.g. polyamines, peptidoglycans, polar lipids, lipopolysaccharides, fatty acids, quinones, and proteins. Although the function of polyamines is not yet entirely clear, these molecules are universally present in cells and their quantitative and qualitative variability makes them a suitable taxonomic marker. Polyamines can be analysed by gas- or high-pressure liquid chromatography [115]. Analysis of peptidoglycans, components of the cell wall, is mainly important in gram-positive bacteria [244]. Polar lipids are the main constituent of bacterial membranes and display variation in the polar group as well as in the substituting fatty acids. Polar lipids can be determined by thin layer chromatography [158]. Lipopolysaccharides from Gram-negative bacteria, analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), form ladder-like patterns that contain taxonomic information at the species level [72]. Fatty acids, components from lipids and lipopolysaccharides, vary in chain length, double-bond position, and substituent groups. This fatty acid variability has been proven to be very valuable for the characterisation of bacterial taxa [271]. Isoprenoid quinones are part of the electron transport chain and can also be used to characterise bacteria at different taxonomic levels [56]. In whole-cell protein analysis, cell-free extracts of bacteria grown in standardised conditions are analysed with SDS-PAGE. The technique is very useful for comparing and grouping of large numbers of closely related strains [228].

Several sophisticated analytical methods, providing information about the total chemical composition of the cell, have been applied for the characterisation and/or identification of bacteria. Examples are Fourier-Transformed Infrared Spectroscopy (FT-IR) [121], RAMAN spectroscopy [147], pyrolysis mass spectrometry [103], and Matrix-assisted Laser Desorption/Ionisation with Time-of-Flight (MALDI-TOF) [45, 58] or spray-ionisation mass spectrometry [290].

1.2.2.3 Phenotypic typing methods

The term “typing method” generally refers to a technique that allows the allocation of isolates to distinct intraspecific (lower than species level) groups. Serotyping is based on the presence of variability in the antigenic constituents of the cells [122]. Antibodies can be either monoclonal or polyclonal and can target cell envelopes, flagella, toxins, enzymes, etc. Multi locus enzyme electrophoresis (MLEE) is

a very powerful phenotypic typing method that has been extensively used in population genetics [248]. In this technique, the electrophoretic mobility in non-denaturing conditions of a set of enzymes is compared. Differences in mobility indicate the existence of polymorphisms in the encoding gene.

1.2.2.4 Whole-genome DNA hybridisation and nucleotide composition

Since the bacterial species concept is based on DNA hybridisation values, whole-genome DNA hybridisation is the reference method (or “gold standard” [309]) to establish relationships within and between species. The percent DNA binding is considered to be an indirect parameter of the sequence similarity between two entire genomes. This technique will be discussed in more detail in Chapter 4.

The nucleotide composition of a bacterial genome, usually expressed as mole% guanine + cytosine (%G+C), is an important descriptive parameter for bacterial species that will be extensively dealt with in Chapter 3.

1.2.2.5 DNA-based typing methods

During the last two decades, a whole array of DNA-based typing methods has been developed. DNA can be analysed by digestion with restriction enzymes, followed by separation of the resulting DNA fragments by gel electrophoresis. This general technique is referred to as restriction fragment length polymorphisms (RFLP). Digestion of whole-genomic DNA with restriction enzymes usually generates a very large number of DNA fragments. This results in complex patterns, that are very difficult or impossible to analyse. Therefore, the complexity of the patterns has to be reduced and this can be achieved in several ways.

Rare-cutting restriction enzymes, recognising a specific sequence of six to eight bases, can be selected to digest genomic DNA. This procedure, referred to as low-frequency restriction fragment analysis, yields very large fragments that can only be separated by a specialized electrophoretic technique, generally known as pulsed field gel electrophoresis (PFGE; for a review see [184]).

Complex patterns resulting from restriction analysis with frequent cutting enzymes can be transferred to a membrane (blotting) and only a subset of the fragments revealed via hybridisation with a labelled probe. When rRNA is used as probe, this technique is called ribotyping [111]. Different ribotyping protocols exist and the technique has been automated and commercialised as RiboPrinter (DuPont, Wilmington, DE, USA).

A combination of restriction enzyme digestion and PCR yielded the amplified fragment length polymorphism (AFLP) technique [313]. PCR is used to selectively amplify particular DNA fragments from the pool of restriction fragments. The restriction is done with two restriction enzymes, yielding DNA fragments with two different types of sticky ends, combined randomly. To these ends, short oligonucleotides (adapters) are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers, containing the same sequence as the adapters but extended to include one or more selective bases. Only fragments which completely match the primer sequence are amplified.

RFLP can also be performed on isolated plasmid DNA, this technique is known as plasmid typing. In comparison with RFLP of genomic DNA, a simpler banding pattern is obtained because the DNA fragment analysed is much smaller. This is the method of choice to establish the identity of plasmids with equal molecular weight. However, its applicability for strain typing is obviously confined to strains that contain plasmids. Since plasmids are mobile genetic elements, it can be argued whether the technique is suitable for strain characterisation.

Banding patterns of low complexity can be generated by restriction endonuclease analysis of PCR-amplified genes or gene fragments. The technique is designated amplified ribosomal DNA restriction analysis (ARDRA) when 16S or 23S rDNA with or without spacer regions is amplified and digested [123]. Other genes e.g. gyrases, RNA polymerase subunits, and genes coding for steps in the intermediary metabolism can be targeted as well. Restriction analysis of several genes can be combined as multi locus restriction analysis (MLRT) to increase the specificity of the technique [52].

A different approach in DNA-based typing methods, not involving restriction enzymes, is represented by the PCR-generated banding patterns. Various protocols in which short arbitrary sequences were used as primers in the PCR assay were described and designated arbitrarily primed PCR (AP-PCR; [318]), randomly amplified polymorphic DNA analysis (RAPD; [323]), or DNA-amplified fingerprinting (DAF; [37]). Alternatively, PCR primers can be directed against repetitive elements dispersed throughout bacterial genomes. Examples of such repetitive elements are REP- (repetitive extragenic palindromic; [267]), ERIC- (enterobacterial repetitive intergenic consensus; [132]), and BOX-elements [183], but also tRNA gene fragments [187].

1.2.2.6 Sequencing of selected genes

Direct 16S rDNA sequencing has replaced the older methods of 16S rRNA cataloguing and reverse-transcriptase sequencing of 16S rRNA. The obtained high-quality 16S rDNA gene sequences provide a phylogenetic framework that serves as the backbone for modern microbial taxonomy [309]. Stackebrandt and Goebel concluded, based on a literature overview, that organisms sharing less than 97% 16S rDNA sequence similarity will not give a DNA reassociation value higher than 70%. Therefore, rRNA sequence analysis may exclude the need for DNA hybridisation studies for the description of new species, provided that the rDNA similarity level is below 97% and that rDNA sequence data of all relevant taxa are available for comparison [263]. Currently, all species descriptions should include an almost complete 16S rRNA gene sequence derived from the type strain of the proposed species [262]. An international electronic database (<http://rdp.cme.msu.edu/html/>) has been constructed that provides a large set of cured and aligned 16S rDNA sequences along with analysis services through an internet-based protocol [178].

Since a growing number of reports suggest that horizontal gene transfer may be more common than previously thought [67, 134, 174, 232], concern has been raised about founding bacterial phylogeny on the sequence of a single molecule. Furthermore, ribosomal RNA genes have been proposed to serve as sites for homologous recombination between unrelated micro-organisms [268] and at least one instance of gene transfer of rRNA has been observed [330]. It is therefore increasingly recognised

that analyses based on 16S rDNA should be supported by sequence data on other phylogenetic markers. Several other macromolecules have been examined for this purpose. Among others, 23S rDNA, the beta subunit of ATP-ase, elongation factor Tu, chaperonin, HSP60 heat shock protein, various ribosomal proteins, RNA polymerases, RecA protein and tRNA's were shown to be valuable molecular chronometers in support of bacterial systematics. These alternative macromolecules should be universally present in bacteria, their genes should not (or only at a very low frequency) transmit horizontally, and their molecular evolution rate should be comparable or somewhat higher than that of 16S rDNA, making them more suitable for differentiation of closely related organisms [309].

By comparative analysis, signature sequences can often be identified within phylogenetic marker sequences. In such cases, oligonucleotides can be designed against these signature sequences and used in hybridisation- or PCR-assays. The latter rapid tests can conveniently replace the more laborious and costly sequence determination for identification purposes. Diagnostic hybridisation probes, specific PCR primers or a combination of both have been used to identify pathogenic, environmental, and food-borne bacteria in numerous studies (e.g. [167, 222, 258, 317]).

In multi locus sequence typing (MLST) [179], sequences of multiple housekeeping genes are determined and used to assign an isolate to a specific type. Because of the unambiguous nature and electronic portability of nucleotide sequence data, results are highly reproducible and can easily be exchanged between laboratories. Internet-based MLST databases of several, mainly clinically important species are already constructed (see <http://www.mlst.net/new/index.htm>).

1.2.2.7 Whole-genome sequencing

Ultimately, all taxonomical information about a bacterium is encrypted in its genome sequence. However, whole-genome analysis is not trivial and there is no consensus yet on what is the best way to analyse and compare genomes. Some intriguing results have already been obtained, revealing a far more complex taxonomic situation than suggested by classifications based upon single gene sequences. Bacterial genomes appear to be mosaic structures of ancestral parts interspersed with genes and gene clusters recently acquired by horizontal transfer, as evidenced by the presence of mobile genetic elements, differences in codon usage, and %G+C bias [24, 157, 241]. For example, it was estimated that in *Escherichia coli*, about 18% of the genome resulted from the stable integration of transferred genes [157]. Bansal and Meyer [14] compared 37 bacterial, archaeal, and eukaryote genomes for the number of orthologs (protein domains from a common ancestor and presumably having the same function) present. Their results suggest that Eukaryotes clearly differ in gene content from Bacteria and Archaea, as can be expected from their placement in a separate domain. However, Archaea were not significantly different in terms of gene content from the Bacteria as a whole, which is inconsistent with their division in two separate domains [14]. As whole-genome analysis techniques will be refined and with increasingly more complete genome sequences becoming available (for the current status see: <http://wit.integratedgenomics.com/GOLD/>), a wealth of new insights is to be expected from this recent development. Of special interest to the study presented here, are the

complete genome sequencing projects of *Ralstonia metallidurans* CH34 and *Burkholderia* sp. LB400, that are currently in their final closing and annotation steps.

1.2.2.8 Uncultivable organisms

At present, only 0.001 – 15% of the bacteria in a natural bacterial community can be cultured, in comparison to total counts [3]. In contrast, most of the techniques described above rely on a pure culture as starting material. To study organisms that are refractory to cultivation, specific (usually genomic) methods were developed (Fig. 1.3).

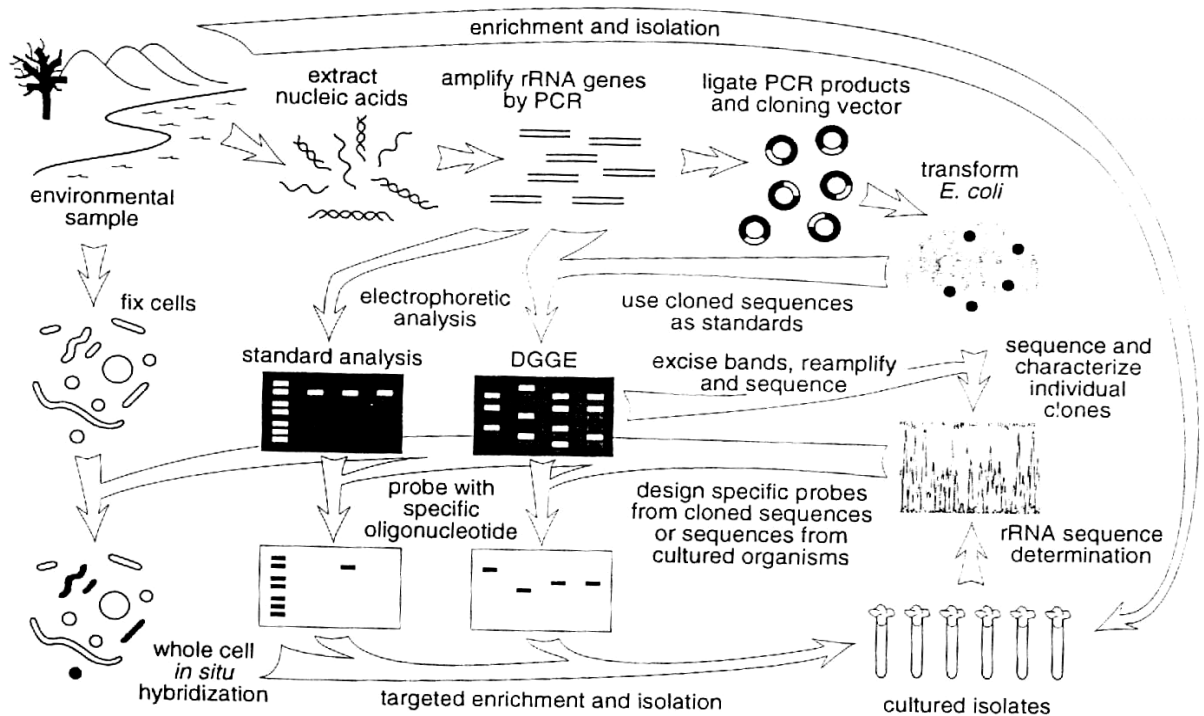


FIG. 1.3: Overview of molecular methods used in molecular microbial ecology (reprinted from Head et al., 1998 [120]).

Ribosomal RNA or protein-encoding genes from natural samples can be PCR-amplified with universal primers, cloned and sequenced. PCR-assisted 16S rRNA sequence retrieval was pioneered by Giovannoni and co-workers, who revealed unexpected biodiversity in bacterioplankton of the Atlantic Sargasso Sea [101]. However, in complex natural communities, no quantitative assumptions about relative abundances should be made based on frequency of clone recovery.

rRNA-directed fluorescent in situ hybridisation (FISH) has proven to be a powerful tool in the study of cultivable and uncultivable bacteria in their natural environments (for a review see [2]). By comparative analysis, sequence idiosyncrasies are localised within rRNA that may serve as target sites for fluorescently labelled hybridisation probes. These probes are applied on immobilised environmental samples and allow for rapid identification and quantitative monitoring (by direct counts) of micro-organisms.

Microbial communities can be studied by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of 16S rRNA genes [202]. The PCR reaction is performed using conserved primers, with one primer additionally containing an about 40 bases long GC-rich stretch (“GC clamp”). Depending on its melting temperature (hence on its base composition), each DNA fragment halts at a specific position in the gel that contains a gradient of denaturing agent. For a given microbial community composition, a specific banding pattern is obtained and changes in the microbial community can often be observed as changes in the DGGE banding pattern. DGGE has also been applied for the 16S-23S intergenic spacer region, allowing discrimination at a finer level compared to 16S rRNA gene fragments [34]. Temperature gradient gel electrophoresis (TGGE) has also been applied in microbial ecology studies and is based on a similar principle as DGGE, but relies on a temperature gradient for denaturation [203]. Another technique that has been used to study bacterial communities is terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes [170]. Using a 5'-labelled primer, 16S rRNA genes from total community DNA are PCR-amplified. The PCR amplicons are subsequently digested with restriction enzymes and analysed using an automated DNA sequencer. A specific pattern of fluorescently labelled terminal restriction fragments is obtained for a given microbial community.

1.2.3 General conclusions and future perspectives

16S rDNA sequencing contributed tremendously to our understanding of bacterial phylogeny. In many cases however, the 16S rRNA gene does not contain sufficient sequence divergence for reliable species designation. For this reason and because of recent evidence for horizontal transfer of the rRNA operon, more and more researchers agree that phylogenies based on 16S rDNA sequence data should be supported by sequence data on additional phylogenetic markers.

As the bacterial species definition remains based on DNA-DNA hybridisation data, efforts towards improvement and facilitation of this technique are still required. New DNA-DNA hybridisation protocols should be carefully calibrated against older, more established methods. A problem inherent to DNA-DNA hybridisations is the generation of non-cumulative data. Therefore, from a practical point of view, a species definition based on other markers (e.g. the sequences of multiple housekeeping genes) would be desirable, provided that these markers show sufficient congruence with DNA-DNA hybridisation data.

With an increasing number of whole-genome sequences from related organisms (representing several strains within one genus or species) made available, the relationship between DNA-DNA hybridisation data and overall sequence similarity will start to unravel.

A whole array of genotypic typing methods has proven to be useful for the determination of inter- and intraspecies bacterial relatedness. Further efforts in this field should not only focus on the development of new methods, but also on the standardisation of the generated data and on efficient database construction. Reproducible techniques, generating data that can be easily put into electronic form (such as MLST), deserve special attention because they allow development of “virtual” global databases, e.g. for clinically important organisms.

The development of sophisticated analytical methods holds a promise for fast and reliable phenotypic typing based on whole-cell content.

With the advent of molecular techniques, the existence of a tremendous diversity of yet non-cultivable micro-organisms became apparent. The present challenge consists of linking the phylogeny of these organisms to their function in a given ecosystem. Developments of techniques (either molecular tools or cultivation approaches) that can contribute to this understanding should therefore be encouraged.

1.3 ORGANISMS OF INTEREST IN THIS STUDY

1.3.1 Taxonomy and ecology of the genus *Burkholderia*

The genus *Burkholderia* is a phylogenetically well defined (Fig. 1.4), but functionally extremely diverse group of organisms. It contains primary plant and human pathogens, human opportunistic pathogens (e.g. for cystic fibrosis patients) as well as strains useful for biocontrol, bioremediation, and plant growth promotion. Since its creation in 1992 by Yabuuchi et al. [327], the genus *Burkholderia* underwent several taxonomic revisions and numerous new *Burkholderia* species have been described (Fig. 1.5).



FIG. 1.4: 16S rDNA based phylogenetic tree showing the positions of all currently recognized *Burkholderia* species and representatives of related genera. The scale bar indicates 5% sequence dissimilarity (reprinted from Coenye et al., submitted [54]).

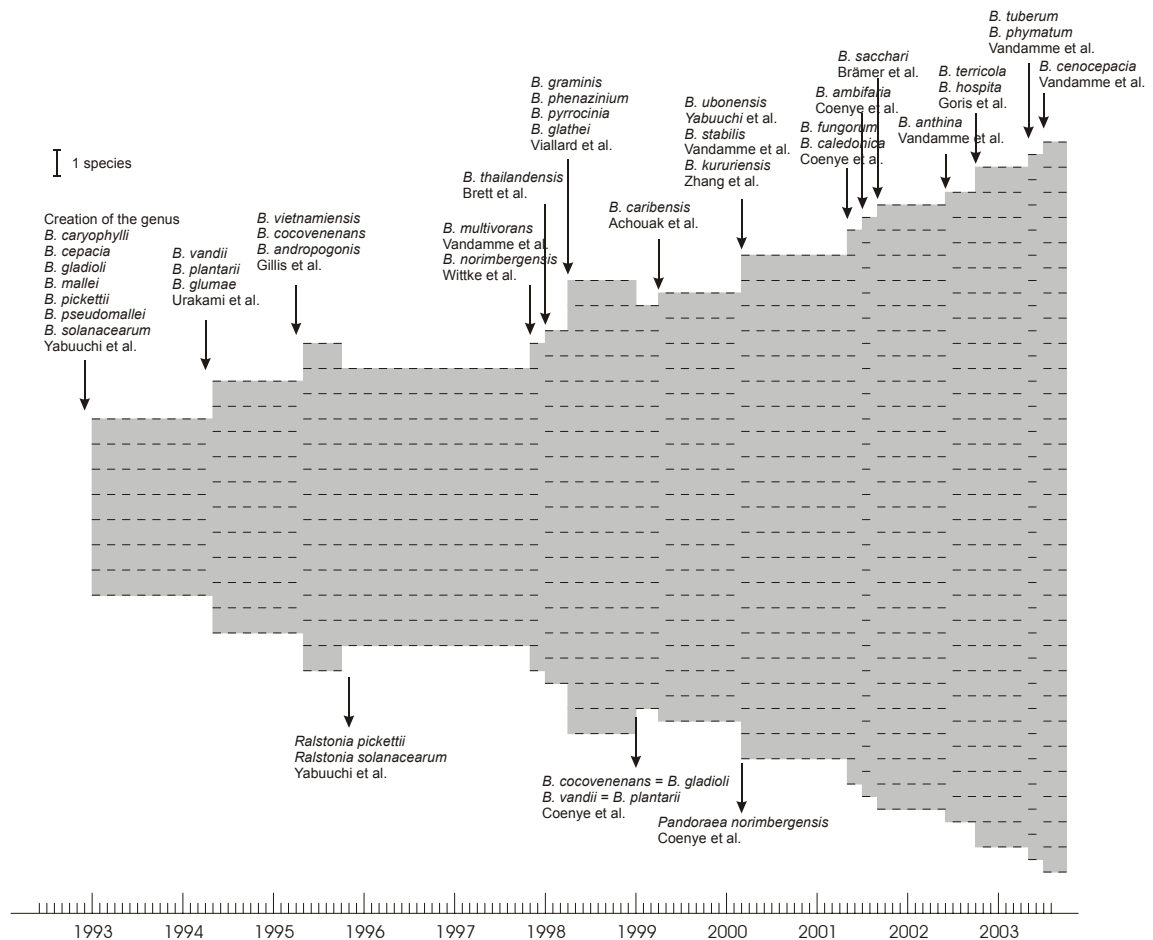


FIG. 1.5: Taxonomic revisions and description of new species in the genus *Burkholderia*.

1.3.1.1 Soil inhabitants

Burkholderia strains are frequently isolated from soil, especially in the vicinity of plant roots. Several *Burkholderia* species contain only soil or rhizosphere inhabitants without a known ecological function. *B. glathei* [335], *B. graminis* [311], *B. caribensis* [1], and *B. caledonica* [51] were isolated from fossil lateritic soil, soils and rhizospheres in France and South Australia, vertisol microaggregates in Martinique, and rhizosphere soil in Scotland, respectively. In the framework of the present study, soil organisms that picked up the 2,4-D catabolic plasmids pJP4 or pEMT1 were classified as *B. terricola* and *B. hospita* [106]. Later on, additional isolates from rhizosphere soil of garden flowers in Scotland were also identified as *B. terricola* [107].

1.3.1.2 Nitrogen-fixing and nodulating strains

B. vietnamiensis, isolated from the rhizosphere of rice plants in Vietnam, was the first *Burkholderia* species reported able to fix molecular nitrogen [100]. Later studies showed that nitrogen fixation is in fact a widespread feature in this genus, with N₂-fixing isolates found in many geographically distinct locations and in association with different host plants [59, 87, 88]. Nitrogen-fixing *Burkholderia*, nodulating the roots of

Leguminosae were described by Moulin and co-workers [199] and subsequently classified as *B. tuberum* and *B. phymatum* [303]. This finding was particularly interesting since *Leguminosae* root nodulation was long believed to be an exclusive feature of α -*Proteobacteria*.

1.3.1.3 Plant endosymbionts

An even more pronounced association with plants is found in plant endosymbiotic *Burkholderia* sp. These as yet uncultivable bacterial endophytes were detected in leaf galls of *Psychotria* (*Rubiaceae*, angiosperms) and provisionally named ‘*Candidatus Burkholderia kirkii*’ [300]. Intracellular and presently uncultivable bacteria in arbuscular mycorrhizal fungi are phylogenetically related to *Burkholderia* [23] and were recently named ‘*Candidatus Glomeribacter gigasporarum*’ [22]. These bacteria contain nitrogen fixation genes and were therefore hypothesised to perform nitrogen fixation in symbiosis with the fungus [198].

1.3.1.4 Plant pathogens

The genus *Burkholderia* also contains several plant pathogens, among them are several former *Pseudomonas* species that were reclassified as *Burkholderia* [100, 289, 327]. *B. caryophylli* causes bacterial wilt in carnation (*Dianthus caryophyllus* L.) and other ornamental plants [13]. *B. gladioli* is a plant pathogen frequently isolated from *Gladiolus* and *Iris* spp. [221]. *B. andropogonis* causes leaf spots, streaks and stripes on a wide variety of host plants, such as the economically important corn, coffee, chick pea and velvet bean [117]. *B. glumae* [152] and *B. plantarii* [10] are rice pathogens, causing sheath necrosis and blight of seedlings, respectively. *B. vandii*, the species name proposed for a group of plant-associated, antibiotic-producing strains [289], was later shown to be a junior synonym of *B. plantarii* [50].

1.3.1.5 The *Burkholderia cepacia* complex

Pseudomonas cepacia was originally described as a plant pathogen, causing bacterial rot of onions bulbs [36]. In the last two decades, a group of organisms that is now referred to as the “*B. cepacia* complex” has emerged as opportunistic pathogens of humans with cystic fibrosis. Vandamme et al. (1997) showed that presumed *B. cepacia* isolates from cystic fibrosis patients consisted of several phenotypically similar but genomic distinct species, or genomovars. All *B. cepacia*-like genomovars are collectively referred to as the “*B. cepacia* complex”. Currently, nine genomovars are known within the *B. cepacia* complex: genomovar I contains the original onion pathogenic strains and retained the name *B. cepacia*; genomovar II was named *B. multivorans* [307]; genomovar III recently received the binominal name *B. cenocepacia* [306]; *B. cepacia* genomovar IV became *B. stabilis* in 2000 [308]; genomovar V was identified as *B. vietnamiensis* [307]; a proposal to name genomovar VI *B. dolosa* is currently underway [310]; genomovar VII was classified as *B. ambifaria* [53]; for genomovar VIII the name *B. anthina* was proposed [305]; and genomovar IX was identified as *B. pyrrocinia* [305]. The clinical importance of *B. cepacia* complex is reflected in the observation that about 20% of all infected cystic fibrosis patients develop “*cepacia* syndrome”, which is characterized by a rapid, necrotising pneumonia

and death. Furthermore, the pathogen is relatively unresponsive to antibiotic therapy and is transmissible between patients [108]. Not all genomovars contribute evenly to infections in cystic fibrosis patients; *B. multivorans* (genomovar II) and especially *B. cenocepacia* (genomovar III) infections are predominant and only a small proportion of human infections is caused by strains belonging to other genomovars [169, 259].

B. cepacia complex bacteria have several potential biotechnological applications, as antagonists of soil-borne pathogens (biocontrol) or plant growth promoting agents and for bioremediation purposes. The mechanism of biocontrol is generally unknown but has been attributed to the production of antibiotics and siderophores [189]. Plant-growth promoting characteristics are attributed to production of plant hormones, suppression of deleterious rhizosphere bacteria and, in case of nitrogen-fixing strains, increased nitrogen availability to the hosts. [149, 301]. Isolates belonging to the *B. cepacia* complex have been found to degrade xenobiotics like trichloroethylene, chlorophenol, toluene, 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) [21, 65, 92, 279]. Concern has risen about the health risks for cystic fibrosis patients, resulting from large-scale release of *B. cepacia* complex bacteria in the environment for agricultural or bioremediation purposes [130, 168]. Risk assessment is extremely difficult, since no clear set of “virulence genes” has been identified in strains that have been associated with human infection and because estimations for the background level exposure of cystic fibrosis patients to *B. cepacia* complex bacteria vary widely [223]. Recent studies suggest that *B. cepacia* complex bacteria are common inhabitants of soil, plant rhizosphere, and water [11, 91, 197].

1.3.1.6 Biotechnological interest

The genus *Burkholderia* contains several strains, distinct from *B. cepacia* complex bacteria, that could be useful for bioremediation purposes. These include trichloroethylene, aniline, toluene, PAH's, PCB's, and biphenyl degrading organisms. The name *B. kururiensis* was proposed for a trichloroethylene-degrading bacterium (strain KP23) isolated from a Japanese aquifer contaminated with this compound [333]. However, the taxonomic status of many xenobiotic-degrading *Burkholderia* strains is uncertain and these strains are referred to in the literature as ‘*Burkholderia* sp.’ e.g. strains JS150, HY1, and RP007 [140, 141, 156]. In the current study, the PCB and biphenyl degrading strain *Burkholderia* sp. LB400 [28] was shown to be related at the species level to a coffee plant rhizosphere isolate and strain obtained from a blood culture.

Other possible biotechnological applications include the industrial production of the antibiotics precursor phenazine by strains of *B. phenazinium* [195], an organism originally described as *Pseudomonas phenazinium* [19], but later reclassified as *Burkholderia* [327]. The name *B. sacchari* was proposed for strain IPT101, isolated from soil of a sugar-cane plantation [30]. Strain IPT101 accumulates large amounts of polyhydroxyalkanoates when grown on carbohydrates and propionic acid [102] and could be valuable for the industrial production of these natural thermoplastics from renewable carbon sources.

1.3.1.7 *Human pathogens*

B. mallei and *B. pseudomallei* are primary human pathogens. *B. mallei* causes glanders, an infectious disease primarily affecting horses and other domestic animals. Human infection has occurred rarely and sporadically among laboratory workers and those in direct and prolonged contact with infected domestic animals. Depending upon the route of entry, *B. mallei* can cause localized, pus-forming cutaneous infections, pulmonary infections, bloodstream infections, and chronic suppurative infections of the skin. Pulmonary and septicaemic forms of the disease are associated with a high mortality. *B. pseudomallei* is the aetiologic agent of melioidosis. This is predominately a disease of tropical climates, especially in South-East Asia and Northern Australia where it is endemic. Melioidosis is clinically and pathologically similar to glanders disease, but the ecology and epidemiology of melioidosis are different from glanders. *B. pseudomallei* is found in contaminated water and soil and can spread to humans and animals through direct contact with the contaminated source. Both *B. mallei* and *B. pseudomallei* were studied as potential biological warfare weapons [319]. An avirulent environmental organism that antigenically cross-reacts with *B. pseudomallei* was described in 1998 as *B. thailandensis* [31]. *B. ubonensis* is the name proposed for a *B. thailandensis*-like roadside soil isolate [326].

1.3.1.8 *B. gladioli and B. fungorum*

B. gladioli and *B. fungorum* are two species that excellently illustrate the versatility of *Burkholderia* strains and their ability to colonize various ecological niches. As mentioned before, *B. gladioli* is known as a plant pathogen, invading several different host plants. It is also associated with infections in cystic fibrosis and other immunocompromised patients [16, 44, 110, 127, 235]. *B. cocovenenans* and *Pseudomonas antimicrobica* are junior synonyms of *B. gladioli* [47, 50]. The former two species contained toxoflavin-producing strains and strains isolated from cases of food poisoning (*B. cocovenenans*) [297, 334] and an antagonistic strain for plant pathogens, isolated from the mealy bug (*P. antimicrobica*) [8]. The name *B. fungorum* was proposed for isolates from very diverse sources as the white-rot fungus *Phanerochaete chrysosporium*, mouse nose, haemoglobin solution, vaginal secretion of a pregnant woman, human cerebrospinal fluid, and sputum of cystic fibrosis patients [51]. A symbiotic relationship between *B. fungorum* and the white-rot fungus has been suggested, in which the bacterium metabolises the aromatic compounds resulting from lignin degradation by the fungus [247]. Remarkably, *Phanerochaete chrysosporium* has been reported to degrade a variety of xenobiotics like chlorophenols, herbicides, pesticides, trinitrotoluene, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons [9, 82, 329], a capacity also present in various *Burkholderia* strains. Furthermore, strain N2P5 from PAH-contaminated soil enriched with phenanthrene [200] and a nitrogen-recycling *Tetraponera* ant endosymbiont [296] are both phylogenetically very closely related to *B. fungorum*.

1.3.1.9 *Genome flexibility*

As shown above, members of the genus *Burkholderia* are nutritionally and ecologically highly versatile, which is also reflected in their genome complexity and

plasticity [159]. The overall genome size of *Burkholderia* is relatively large and varies substantially, in the range of 4.5 to 9 Mb. Their genome consists of multiple, megabase-sized DNA molecules that replicate from their own origin or replication (replicons) [42, 240]. These replicons can be called “chromosomes” if they encode genes essential for cell growth and viability. Alternatively, when the replicon is dispensable for the cell, it is designated “megaplasmid”. The number of replicons, as well as the total genome size, varies among strains even within a species (Table 1.1). Although the existence of multiple replicons is best documented in the *B. cepacia* complex, the phenomenon is not confined to this group. Strains of *B. glumae*, *B. glathei*, *B. gladioli*, and several *Ralstonia* species also harbour more than one Megabase-size replicon [240, 321]. Numerous insertion elements are present in the genomes of *Burkholderia* strains and contribute to genome plasticity [42, 159]. Insertion elements flanking genes can facilitate their movement either within the organism or between organisms if they insert, for example, into transmissible plasmids. Furthermore, linkage disequilibrium analysis of allelic variation in environmental *B. cepacia* complex strains indicated a non-clonal population structure with associated widespread gene transfer across the population [324]. The demonstrated high rates of recombination also mirrors the variable nature of the natural environments of these organisms.

TABLE 1.1: Genome sizes and numbers of replicons in *B. cepacia* complex strains (after Parke et al., 2001 [223]).

| Strain | Large replicons | Genome size (Mb) |
|--|-----------------|------------------|
| Genomovar I (<i>B. cepacia</i>) | | |
| ATCC 25416 (LMG 1222c, GEP031) | 3.5, 3.1, 1.1 | 7.7 |
| ATCC 17759 (LMG 2161, CEP080) | 3.5, 3.3, 1.1 | 7.9 |
| CEP521 | 3.7, 2.0 | 5.7 |
| Genomovar II (<i>B. multivorans</i>) | | |
| C3430 | 3.4, 2.1, 1.1 | 6.6 |
| C5274 | 3.3, 2.4, 1.3 | 7.0 |
| LMG 18822 (C5393) | 3.1, 2.0, 0.6 | 5.7 |
| C5568 | 3.4, 2.2, 0.6 | 6.2 |
| ATCC 17616 (LMG 17588, CEP144) | 3.4, 2.5, 0.9 | 6.8 |
| LMG 14280 (FC365) | 3.3, 2.2, 0.8 | 6.3 |
| LMG 14293 (FC366) | 3.5, 2.2, 0.6 | 6.3 |
| LMG 13010 (FC445) | 3.6, 2.7, 0.6 | 6.9 |
| Genomovar III (<i>B. cenocepacia</i>) | | |
| C4455 | 3.9, 3.0, 1.0 | 7.9 |
| LMG 12614 (FC363) | 3.8, 3.0, 1.1 | 7.9 |
| LMG 12615 (FC364) | 3.7, 2.9, 1.1 | 7.7 |
| LMG 16656 (J2315) | 3.7, 3.1, 0.8 | 7.6 |
| LMG 18827 (C5424) | 3.6, 3.0, 0.8 | 7.4 |
| LMG 18828 (C6433) | 3.4, 3.0, 1.4 | 7.8 |
| LMG 18830 (CEP511) | 3.3, 2.7, 1.3 | 7.3 |
| CEP024 | 3.4, 2.7, 0.9 | 7.0 |
| CEP565 | 3.6, 3.1, 0.6 | 7.3 |
| Genomovar IV (<i>B. stabilis</i>) | | |
| LMG 14294 (FC367) | 3.7, 3.2, 1.3 | 8.2 |
| LMG 7000 (FC362) | 3.9, 3.3, 1.4 | 8.6 |
| Genomovar V (<i>B. vietnamiensis</i>) | | |
| CEP040 | 3.3, 2.3, 1.1 | 6.7 |
| ATCC 29424 (DB01, LMG 17830) | 3.4, 2.4, 1.3 | 7.1 |

| Strain | Large replicons | Genome size (Mb) |
|---------------------------------------|--------------------|------------------|
| ATCC 53617 (G4) | 3.4, 3.2, 1.2, 1.1 | 8.9 |
| LMG 6998 (FC370) | 3.1, 2.1, 1.2 | 6.4 |
| LMG 10929 (FC369) | 3.7, 2.6, 1.3 | 7.6 |
| LMG 18836 (FC441) | 3.0, 1.8, 1.2 | 6.0 |
| ATCC 39277 (FC433) | 3.6, 3.1, 1.1 | 7.8 |
| Genomovar VII (<i>B. ambifaria</i>) | | |
| ATCC 17760 (383) | 3.4, 1.3 | 4.7 |
| BcF | 3.5, 2.6, 1.3 | 7.4 |
| AMMDR1 | 3.4, 2.8, 1.0 | 7.2 |

Although the exact molecular and physiological background remains unknown, the observed genome flexibility could be one of the key factors governing the remarkable functional diversity within the genus *Burkholderia*. Whole-genome sequence information of strains of several *Burkholderia* species (*B. cenocepacia*, *B. pseudomallei*, and *B. mallei*) are available or will be available in the near future (*Burkholderia* sp. LB400, *B. vietnamiensis* G4). It can be expected that thorough analysis of this sequence information will provide further insights into mechanisms determining functional diversity and evolution.

1.3.2 Taxonomy and ecology of the genus *Ralstonia*

The genus *Ralstonia* was created by Yabuuchi [328] to accommodate two generically misclassified *Burkholderia* species (*B. pickettii* and *B. solanacearum* and one *Alcaligenes eutrophus*. Since its creation, the number of *Ralstonia* species has rapidly increased, as illustrated in Fig. 1.6. A 16S rDNA based phylogenetic tree, showing the current taxonomical situation of the genus *Ralstonia*, is presented in Fig. 1.7.

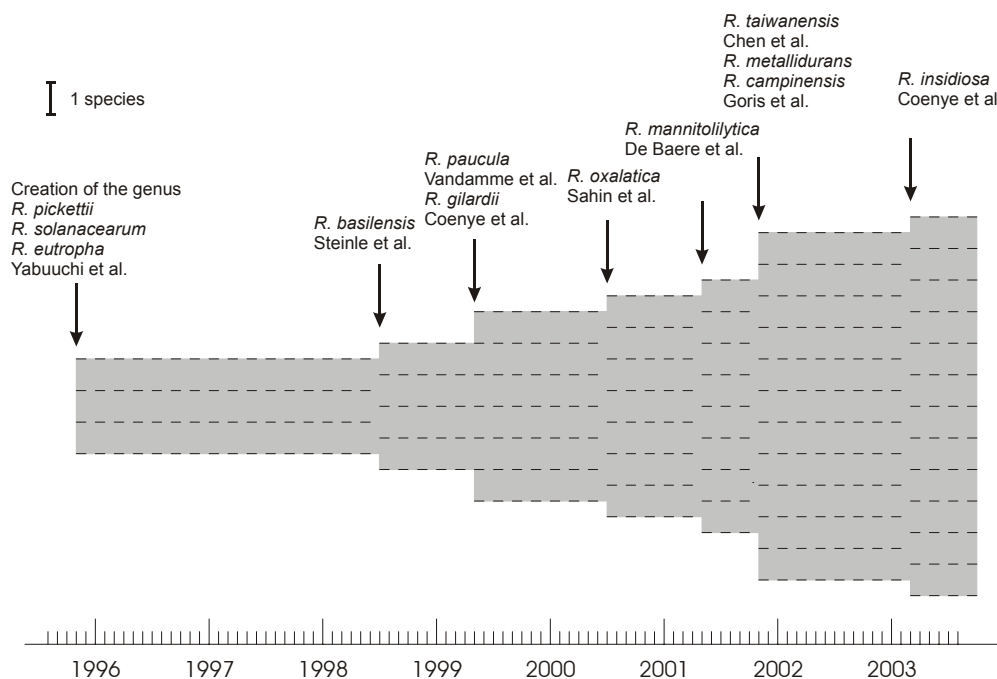


FIG. 1.6: Taxonomic revisions and description of new species in the genus *Ralstonia*.

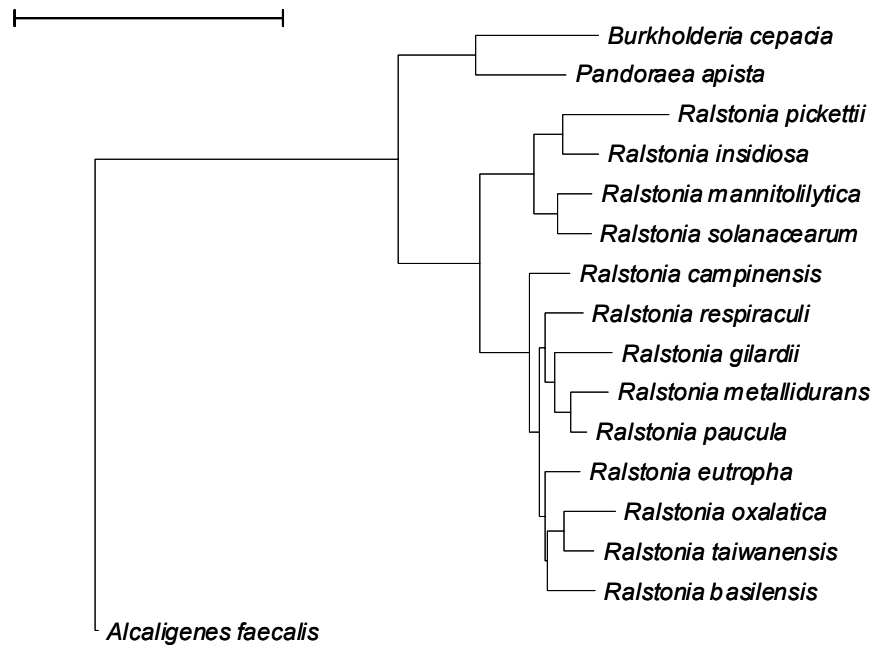


FIG. 1.7: 16S rDNA based phylogenetic tree showing the positions of all currently recognized *Ralstonia* species and some reference species of related genera. The scale bar indicates 5% sequence dissimilarity.

1.3.2.1 Plant pathogens

Ralstonia solanacearum is one of the most devastating plant pathogens in the tropics, subtropics and warm temperate regions of the world. The species exhibits a great degree of phenotypic and genotypic diversity and has been divided in five races based on host affinity [35] and six biovars based on biochemical properties [118]. The only agreement between these two schemes is that biovar 2 strains most often belong to race 3. The pathogen is soil-borne and causes bacterial wilt in several hundreds of plant species, representing more than 50 families [119]. The host range includes not only solanaceous plants (e.g. tomato, potato, tobacco, and eggplant) but also some leguminous plants (such as groundnut and French bean), a few mono-cotyledons (mainly banana and ginger) and several trees and shrubs (e.g. mulberry, olive, cassava, and eucalyptus) [98]. Long distance spread of the disease mainly occurs by means of propagation organs in trade, where infection may be latent or evident [118].

Phylogenetic analysis gave evidence that *Pseudomonas syzygii* [238] and the ‘blood disease bacterium’ also belong to the genus *Ralstonia* [273]. *Pseudomonas syzygii*, a xylem-limited bacterium that grows poorly or not at all on common bacteriological media, is the causal agent of the Sumatra disease of cloves (*Syzygium aromaticum*) [20, 238]. The ‘blood disease bacterium’ causes a vascular wilt of banana (*Musa* spp.) in Indonesia, termed blood disease because of the reddish exudates formed [85, 96].

1.3.2.2 Clinical importance

Some *Ralstonia* species, such as *R. pickettii*, *R. mannitolilytica*, *R. insidiosa*, *R. paucula*, and *R. gilardii*, are known as opportunistic human pathogens. Besides from

environmental sources, representatives of these species were also recovered from samples taken from infected patients. In nearly all cases, patients suffered from an underlying disease, affection immunocompetence. The name *Pseudomonas pickettii* was originally proposed by Ralston and co-workers for a group of clinically related strains [229]. Since then, the organism has been found responsible for several nosocomial infections that were often associated with contaminated solutions, like antibiotics [139], disinfectants [218], saline [153], and “sterile” water [237]. These observations can be partly explained by the ability of *R. pickettii* strains to pass through a 0.2 µm filter and to proliferate in low-mineral aqueous solutions over a wide range of temperatures [4]. Some environmental isolates of *R. pickettii* may be useful for the degradation of xenobiotics [133, 138] and bioremediation of selenium [234].

Some *R. pickettii*-like strains, isolated from blood, sputum, and patients suffering from a recurrent meningitis and an abdominal haematoma were classified as *R. mannitolilytica* [68]. This opportunistic pathogen was later also detected in sputum of cystic fibrosis patients [49, 90].

Recently, the name *R. insidiosa* was proposed for a group of organisms recovered from environmental (soil, water, and activated sludge) and human clinical samples (sputum of leukaemia and CF patients) [48].

The former Centers for Disease Control group IVc-2 was classified as *R. paucula* by Vandamme and co-workers [304]. As for *R. pickettii*, *R. paucula* infections are often associated with contaminated water sources and indwelling devices [181, 201]. Recognition of *R. paucula* in the clinical laboratory is important, since it can cause serious infections and may be refractory to standard antibiotic regimens [219].

R. gilardii also contains both environmental and human clinical isolates (from cerebrospinal fluid, furuncle, and bone marrow) [46] and is sporadically recovered from sputum of cystic fibrosis patients [49].

1.3.2.3 Xenobiotics degraders

R. eutropha is an environmental organism that originally attracted attention because of the ability of some strains (e.g. H16) to grow chemolithotrophically with oxidation of molecular hydrogen as energy source. Numerous papers have focussed on the polyhydroxyalkanoate metabolism of this organism, with the industrial production of bioplastics as the final objective [176]. Several *R. eutropha* strains have been reported to degrade xenobiotics, especially aromatic and chloroaromatic compounds. However, not all strains described in the literature as *R. eutropha* are true representatives of this species. A well-studied example is strain JMP134, the natural host of plasmid pJP4. This organism is designated *R. eutropha* in numerous studies concerning its 2,4-dichlorophenoxyacetate metabolism, but low DNA pairing values with the *R. eutropha* type strain indicate that this strain represents a species distinct from *R. eutropha* [135]. Another example is the PCB-degrading strain *R. eutropha* H850, which is in fact a representative of *Pandoraea pnomenus* (P. Vandamme, personal communication).

The oxalate-oxidising strain Ox1 resembles *R. eutropha* phenotypically and was reclassified as *R. oxalatica* [336].

The name *R. basilensis* was proposed for a 2,6-dichlorophenol-mineralizing isolate from a freshwater pond sediment [266]. Recently, an atrazine-mineralising soil isolate was also identified as *R. basilensis* [265].

1.3.2.4 Metal resistance

Some *Ralstonia* strains show resistance against toxic metals, with the best-studied example undoubtedly being *R. metallidurans* CH34. Strain CH34 is a facultative chemolithotroph that was isolated from a decantation tank of a zinc factory [191, 192] and shows plasmid-mediated resistance to cadmium, cobalt, chromate, copper, mercury, nickel, lead, thallium, and zinc [190]. The taxonomy of this remarkable organism underwent several changes, possibly leading to confusion for researchers in the field. Originally it was described as *Alcaligenes eutrophus* and after the proposal of the genus *Ralstonia* [328], the strain was designated *Ralstonia* sp. or *Ralstonia eutropha* in the scientific literature. However, Jenni and co-workers showed already in 1988 that CH34 is in fact not a true *R. eutropha* representative [135]. In the context of the present work, the valid binominal name *Ralstonia metallidurans* was assigned to CH34 and related organisms [105]. Other *Ralstonia* species known to contain metal-resistant strains are *R. campinensis* and *R. basilensis* [105]. Heavy-metal resistant *Ralstonia* strains have several potential biotechnological applications, like biosensors for the presence of metal ions or bioreactors designed to target removal of heavy metals from polluted effluents [55, 76].

1.3.2.5 Symbiotic nitrogen fixation

R. taiwanensis was the first validly described β -proteobacterium for which root nodule formation and symbiotic nitrogen fixation with leguminous plants (*Mimosa* sp.) was observed [41]. Remarkably, the species contains also one strain isolated from sputum of a cystic fibrosis patient.

1.3.2.6 Diversity of the genus *Ralstonia*

Concerning the ecology and diversity of *Ralstonia* species, largely similar conclusions can be drawn as for the related genus *Burkholderia*. Both genera comprise extremely versatile organisms, occupying very diverse ecological niches. As for *Burkholderia* genomes, the genomes of *Ralstonia* species have been shown to consist of multiple replicons [240] and to contain several insertion elements [162, 261, 276], indicating genome plasticity. Likewise, full-genome sequence comparisons could assist in revealing the mechanisms that make this group of organisms successful colonisers of plants and humans and that determine their competitiveness in diverse ecosystems as soil and water.

1.4 MICROBIOLOGICAL ASPECTS OF BIOREMEDIATION

1.4.1 Toxic metals: resistance mechanisms and bioremediation approaches

1.4.1.1 General bacterial resistance mechanisms against metals

Metals can be divided in three main categories, based on their interactions with living organisms: (i) essential and principally non-toxic metals (e.g. Ca and Mg), (ii) metals that are essential, but become toxic in high doses (such as Fe, Mn, Zn, Cu, Co, Ni, and Mo), and (iii) toxic metals without a biologically relevant function (e.g. Hg and Cd). For toxicity, not only the particular element is important, but also its chemical speciation (e.g. Hg^{2+} is much more toxic than metallic Hg). The toxic effect of metals is mostly due to their reactivity against the sulfhydryl groups of proteins, which they block and inactivate.

Resistance mechanisms against toxic metals probably emerged shortly after prokaryote life started [250]. However, the recent anthropogenic metal mobilisation from ores has created novel, metal-loaded niches with a strong selective pressure for metal resistance.

Eukaryotes are more sensitive to metal toxicity than bacteria and their general resistance mechanism is the expression of metallothioneins: short (approx. 60 amino acids) cysteine-rich metal-chelating proteins. Bacterial metallothioneins are rare and have only been reported in the cyanobacterium *Synechococcus* [239]. Instead, the majority of bacterial resistance mechanisms is based on energy-dependent efflux of toxic ions. Some of these efflux systems are ATPases and others are chemiosmotic cation/proton antiporters. Systems employing metal ion sequestration or enzymatic detoxification were also described. All systems are specific to certain metal ions, hence there is no general mechanism for resistance to all metals [250]. These metal resistance determinants were often found to be located on plasmids, but frequently, related chromosomally encoded systems were detected later on [250]. A well-studied example of heavy metal resistance is that of *Ralstonia metallidurans* CH34 [274]. Therefore, and because of the relevance of this organism in the present study, its metal resistance mechanisms will be discussed below.

1.4.1.2 Metal resistance determinants of *R. metallidurans* CH34

R. metallidurans CH34 contains numerous determinants encoding resistances to toxic heavy metals, located either on the bacterial chromosome or on one of the two indigenous megaplasmids pMOL28 (180 kb [275]) and pMOL30 (238 kb [194]).

The gene cluster *czc* is located on plasmid pMOL30 and confers resistance against Cd^{2+} , Zn^{2+} , and Co^{2+} . It consists of an operon encoding the structural genes *czcCBA*, flanked by regulatory genes, *czcNI* upstream and *czcDRS* downstream of *czcCBA* [209, 298]. Trans-envelope metal ion efflux is mediated by the CzcCB₂A protein complex, that functions as a chemiosmotic divalent cation/proton antiporter [210], as illustrated in Fig. 1.8. In this model [233], the CzcA protein is the actual cation-proton antiporter that transports divalent cations of cobalt, zinc, and cadmium across the cytoplasmic

membrane. CzcB funnels the cations through the periplasmic space, while CzcC drags a hypothetical outer membrane protein (OmpY) adjacent to the Czc complex. OmpY then releases the cations through the outer membrane into the extracellular space. The transcriptional regulation of *czc* is still under active study. Currently, it is hypothesized that transcription starts at four promoters (*czcNp*, *czcIp*, *czcCp*, and *czcDp*) and leads to a variety of transcripts in the *czcNICBA* region and to a tricistronic *czcDRS* message [112]. CzcN and CzcI may regulate the activity of a hypothetical extracellular function sigma factor while the two-component regulatory system, made up of CzcR (response regulator) and CzcS (histidine kinase sensor) [298], regulates the expression of CzcN. CzcD is a heavy metal ion transporter and is also involved in regulation of the *czc* system [5].

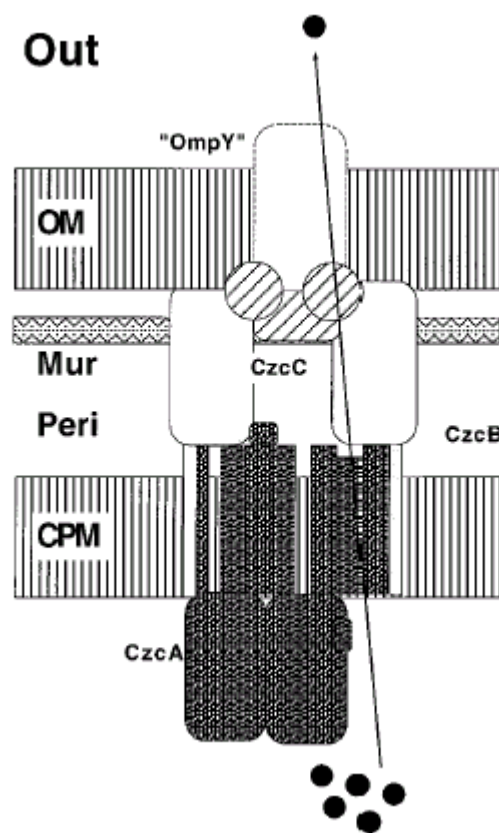


FIG. 1.8: Model for the function of the Czc efflux complex (reprinted from Rensing et al., 1997 [233]).

The *cnr* determinant of plasmid pMOL28 mediates inducible resistance to Co^{2+} and Ni^{2+} and consists of a structural locus, *cnrCBA*, located downstream of a regulatory locus, *cnrYXH* [165]. The structural genes encode a three-component cation/proton antiporter [81], whose topological orientation in the membrane has been elucidated largely by comparison with the *czc* system, with which it shares high homology at the protein level [210, 224]. From the regulatory genes, *cnrH* encodes an extracellular function sigma factor [173]. The gene products of *cnrX* and *cnrY* are periplasmic proteins, with CnrY possibly functioning as a repressor or anti-sigma factor and CnrX as a periplasmic Ni^{2+} sensor [109, 276].

Two gene clusters conferring chromate resistance, designated *chr*, are present on plasmid pMOL28 [208]. These efflux systems probably encode chromate/sulphate antiporters [212]. Recently, possible regulators for chromate resistance and an additional detoxification system, exerting superoxide dismutase activity, were identified [136].

A lead resistance determinant (designated *pbr*), encoding a Pb efflux ATPase, was recently detected on pMOL30 [29].

The megaplastids of *R. metallidurans* CH34 furthermore harbour two transposons conferring mercury resistance to its host [77].

1.4.1.3 Bioremediation strategies for metal pollution

It has been estimated that metal pollution has far more consequences than all other types of pollution, of any origin, put together [214]. The availability of remediation tools to protect affected sites from noxious effects is therefore highly desirable. However, unlike organic contaminants that can be degraded to harmless inorganic substances, toxic metals cannot be destroyed. Remediating the pollution they cause can therefore only be envisioned as their immobilisation in a non-bioavailable form, or their re-speciation into less toxic forms [293]. Chemical approaches are available for metal remediation, but often lack the specificity required to treat target metals against a background of competing ions. Biological systems, by their nature, are more specific and bioremediation approaches have been developed that offer the potential for highly selective removal of toxic metals. Additionally, some of these biological methods can be applied for *in situ* remediation, an approach that is not feasible with chemical methods.

Bioremediation approaches can be divided based on the different (and sometimes overlapping) phenomena that are responsible for metal removal or immobilisation: enzymatic transformation, metal precipitation, and biosorption [293]. Examples of the different systems will be discussed briefly below, with special emphasis on the biotechnological use of *R. metallidurans* CH34.

Enzymatic transformation

The best known example of bacterial metal detoxification by enzymatic transformation is the reduction of Hg^{2+} by mercuric reductase to Hg^0 (for a review, see [128]). This mechanism has been applied for Hg^{2+} removal from chloralkali electrolysis water in a pilot-scale bioreactor, inoculated with seven mercury-resistant *Pseudomonas* sp. Good retention efficiency was observed and metallic mercury was retained in the reactor at very high loadings [314].

Metal precipitation

Precipitation of toxic metals can occur through dissimilatory reduction or via the formation of insoluble salts such as metal sulphides, phosphates, and carbonates. Dissimilatory reduction is independent from metal intake by the cells and therefore, the reaction product generally ends up in the extracellular medium. Sometimes both phenomena overlap, as in the case of some sulphate-reducing bacteria. These bacteria

can mediate reductive precipitation of toxic metals by a periplasmic hydrogenase [171] and form insoluble sulphide complexes under anaerobic conditions [320]. One of the few commercial metal biotreatment processes, operated at the Budelco Zinc Refinery in the Netherlands, is based on the use of sulphate-reducing bacteria. *R. metallidurans* CH34 has the ability to precipitate toxic cationic metals within an extracellular coat of insoluble metal carbonates. This is formed by the generation of a high concentration of metal ions at the cell surface in a zone of high local pH, caused by the uptake of protons by metabolically active cells [211].

Biosorption

Biosorption is defined as the metabolism-independent sorption of heavy metals to biomass. Plant, algal or microbial biomass can be used to remove metals from aqueous solutions. Biosorbents may be viewed as natural ion-exchange materials that primarily contain weakly acidic and basic groups [151]. As for synthetic resins, the biosorbent can be stripped and regenerated for reuse. Biosorption of toxic metals can be enhanced in a recombinant *R. metallidurans* CH34 derivative, expressing mouse metallothioneins on its surface [150]. In this soil experiment, a degree of metal precipitation was observed around the bacterial cells, decreasing overall toxic metal bioavailability. The approach could therefore be a valuable instrument for the *in situ* protection of micro- and macrobiota from metal toxicity. Another bioremediation procedure is based on the ability of *R. metallidurans* CH34 to solubilise metals in soil via the production of siderophores and to adsorb them into their biomass. Addition of the strain to a sandy soil slurry contaminated with metals, followed by removal of the bacterium by a water flotation and flocculation process, reduced the cadmium, lead and zinc concentrations 6 to 7-fold [75].

1.4.2 The degradation of chlorinated aromatics

1.4.2.1 Introduction

Mineralization of organic compounds primarily results from microbial metabolism and is an essential element of the global carbon cycle. Most biogenic compounds have been present for millions of years, which has allowed micro-organisms to develop efficient enzymatic pathways for their degradation. Man-made organic chemicals often have structures not generally found in nature, which present a real challenge to the microbial enzymes that catalyse the degradation of naturally occurring organics. These xenobiotics are therefore often poorly degradable and may persist in the environment. However, even though most xenobiotics have only been around for a few decades, some microbial enzymes effecting their degradation have already evolved, and bacterial and fungal cultures capable of utilizing various xenobiotics as a sole source of carbon and energy have been isolated.

An important class of xenobiotics are the chlorinated aromatics, which have been produced in vast quantities over the last 60 years for use as biocides (herbicides, fungicides, insecticides), as intermediates in the synthesis of fine chemicals, and for numerous other applications. They enter the environment either in the form of point source pollution (e.g. from land fills or at production sites) or dispersed pollution (by

volatilisation or agricultural use). Since several years, public concern has risen about the health and ecological risks derived from the presence of chloroaromatics in the environment.

1.4.2.2 General degradation pathways

The biodegradation of a chlorinated aromatic compound can only be considered complete when its carbon skeleton is converted into intermediary metabolites and its organic chlorine is returned to the mineral state. The critical step is the cleavage of the chlorine-carbon bond. Two main strategies can be differentiated: (i) the chlorine substituent is removed as an initial step in degradation, via reductive, hydrolytic or oxygenolytic mechanisms, or (ii) dechlorination occurs after cleavage of the aromatic ring from an aliphatic intermediate. Although degradation of chloroaromatics is possible in the absence of molecular oxygen, much more data are available about degradation in aerobic conditions. Hence, the discussion below will focus only on aerobic biodegradation pathways. For reviews on this subject, see [114, 230, 231].

The general aerobic biodegradation pathway for chlorinated aromatic compounds can be divided into three pathway segments (Fig. 1.9): peripheral pathways, the modified *ortho*-cleavage pathway, and the late 3-oxoadipate pathway.

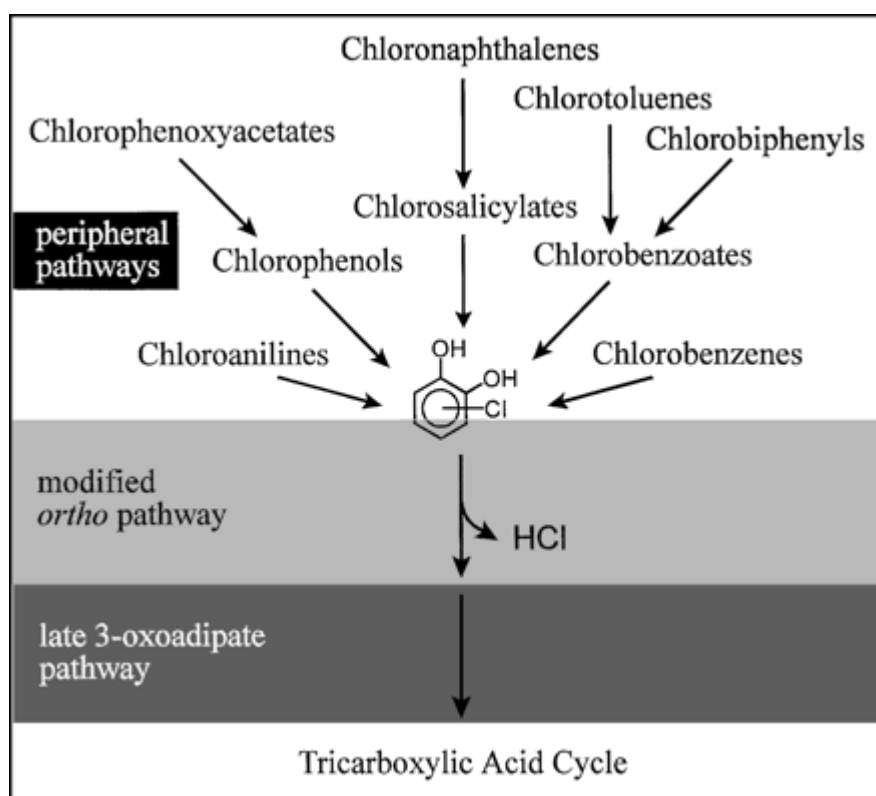


FIG. 1.9: Chloroaromatics are funnelled via peripheral pathways into chlorocatechols, which are further degraded by a few central pathways (reprinted from Reineke, 1998 [230])

Peripheral or funnelling pathways consist of various enzymes that convert a multitude of chlorinated aromatics into chlorocatechols, the central intermediates in the degradation of chloroaromatics (Fig. 1.10). The activation of the aromatic ring structure

by incorporation of two adjacent hydroxyl groups through a multicomponent dioxygenase is hereby essential.

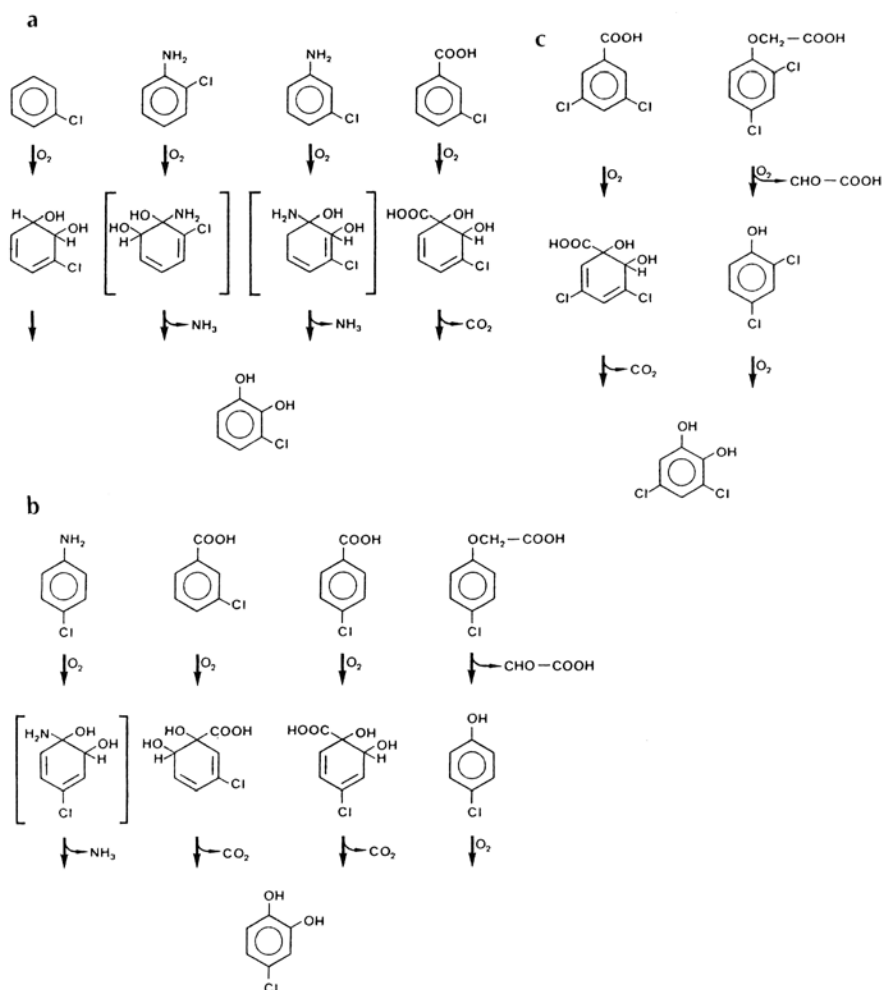


FIG. 1.10: Peripheral pathways generating chlorocatechols; (a) 3-chlorocatechol, (b) 4-chlorocatechol, (c) 3,5-dichlorocatechol. (reprinted from Reineke and Knackmuss, 1988 [231])

Chlorocatechols are generally degraded through the modified *ortho*-cleavage pathway (Fig. 1.11). Intradiol ring cleavage by chlorocatechol 1,2-dioxygenase with consumption of molecular oxygen results in the formation of the corresponding chloro-*cis,cis*-muconate. Further degradation by a chloromuconate cycloisomerase leads to a lactone, which eliminates chlorine as HCl from position 4 or 5, bringing about the formation of an exocyclic double bond. In this way, a (chloro)dienelactone is formed. It is transformed to the corresponding (chloro)maleylacetate via hydrolytic cleavage by (chloro)dienelactone hydrolase. Maleylacetate reductase reduces the carbon-carbon double bond and eliminates a possible chlorine substituent from position 2. In this way, 3-oxoadipate results from 2-chloromaleylacetate or 3-chloro-4-oxoadipate results from 2,3-dichloromaleylacetate.

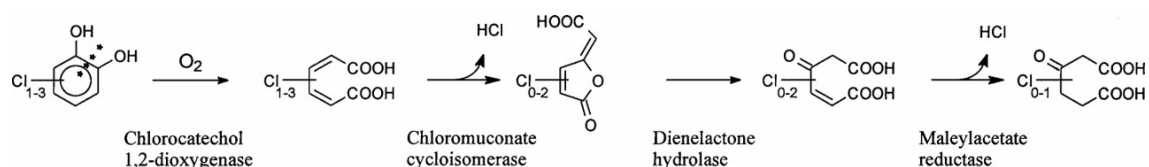


FIG. 1.11: Principle of the modified *ortho*-pathway. The degradation of di- and trichlorocatechols involves two dechlorinating steps. (reprinted from Reineke, 1998 [230])

The last two enzymes of the well-known 3-oxoadipate pathway (3-oxoadipate:succinyl-CoA transferase and 3-oxoadipyl-CoA thiolase) are required for chlorinated 3-oxoadipates to be converted into Krebs cycle intermediates (Fig. 1.12).

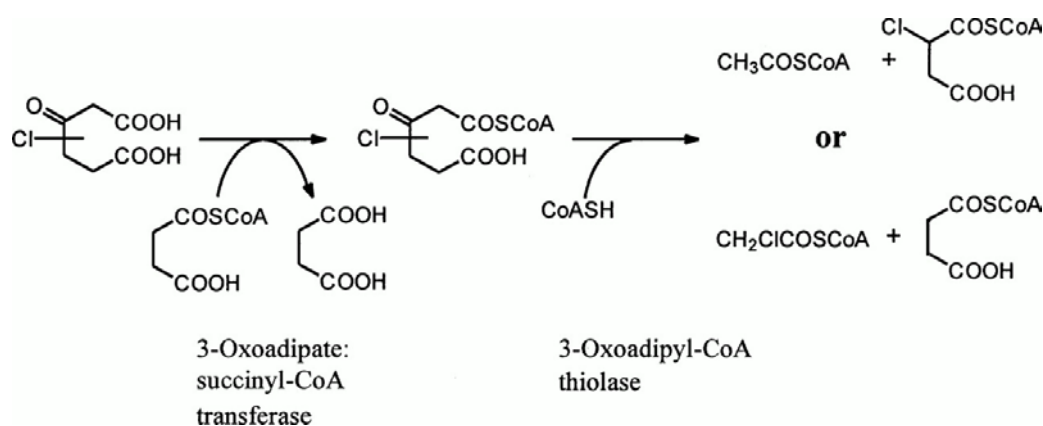


FIG. 1.12: Metabolism of chlorinated 3-oxoadipates in the late 3-oxoadipate pathway. (reprinted from Reineke, 1998 [230])

In the biodegradation of polychlorinated aromatic compounds, chlorinated para-hydroquinones seem to be the central metabolites instead of chlorocatechols. In these cases the aromatic ring is cleaved only after removal of all or most of the chlorine substituents [114].

Disappearance of a compound in a degradation test does not necessarily mean that it has been degraded. Various biotransformation reactions have been observed, such as O-methylation, acetylation, oxidative coupling and condensation, leading to dead-end products that are sometimes even more toxic than the original compound. This is often the case for O-methylation reactions, which make the compound more lipophilic and therefore increase bioaccumulation. Furthermore, chlorophenols may cross-couple with other phenolics, such as humic acids. This reaction explains the formation of humus-bound pesticide residues in soil.

1.4.2.3 Degradation of 2,4-dichlorophenoxyacetic acid

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was introduced in 1946, and rapidly became the most widely used herbicide in the world. It is a selective herbicide, mainly used in agriculture for broadleaf weed control in wheat and small grains, sorghum, corn, rice, sugar cane, soybeans, and pasture. It is also applied on roadsides, non-crop areas, in forestry, in lawn and turf care, and on aquatic weeds. 2,4-

D is relatively low in toxicity to humans and animals and is biodegraded rather easily, with a half-life of about one week in soil and two to four weeks in natural waters.

The best-known 2,4-D degrader is strain JMP134, isolated from Australian soil by selection for its ability to use 2,4-D as sole source of carbon and energy [79]. JMP134 was originally assigned to *Alcaligenes eutrophus* (now *Ralstonia eutropha*) and is referred to accordingly in nearly all later studies, although DNA-DNA hybridisation data indicate that it should be assigned to a different *Ralstonia* species [135]. Awaiting a formal taxonomical study reclassifying this strain, JMP134 will be referred to as *Ralstonia* sp. in the present study.

Ralstonia sp. JMP134, with its large catabolic plasmid pJP4, has been studied extensively for more than two decades and has become a model system for the enzymatic pathways and the genetic regulation of bacterial metabolism of halogenated aromatic compounds. The degradation pathways for 2,4-D and 3-chlorobenzoate (which partly overlap), as encoded on a 22 kb segment of plasmid pJP4, are shown in Fig. 1.13. The genes for peripheral enzymes in the 2,4-D degradative pathway are *tfdA* and *tfdB*, encoding a 2,4-D/ α -ketoglutarate dioxygenase and a 2,4-dichlorophenol hydroxylase, respectively [89, 93]. Two chlorocatechol catabolic gene clusters are present on plasmid pJP4 (Fig. 1.13). The existence of a first chlorocatechol gene cluster was shown by transposon mutagenesis [80]. This cluster comprises besides *tfdB*, the genes *tfdC* for a chlorocatechol dioxygenase, *tfdD* for a chloromuconate cycloisomerase, *tfdE* for a dienelactone hydrolase, and *tfdF* for an enzyme later suggested to be a (chloro)maleylacetate reductase [246]. Later on, the presence of a second gene cluster, *tfd_{II}*, was reported [163]. In addition to genes homologous to those of the *tfd_I* cluster, *tfd_{II}* contains the *tfdK* gene. TfdK facilitates 2,4-D uptake [163] and is essential for the chemotaxis of *Ralstonia* sp. JMP134 towards this chloroaromatic compound [116]. The *tfd_{II}* cluster, together with the identical regulatory genes *tfdS* and *tfdR*, is located on a composite transposon flanked by ISJP4 and a remnant of a second copy of it between *tfdA* and *tfdS* [162]. A third putative regulator gene, *tfdT*, localized directly upstream of *tfdC*, is interrupted by the insertion sequence ISJP4, which renders it inactive [161]. Messenger RNA and functional enzymes are produced from both chlorocatechol catabolic gene clusters [154, 160]. Several possible reasons for the maintenance of gene cluster *tfd_{II}*, containing almost isofunctional genes to *tfd_I*, on plasmid pJP4 were suggested: (i) uptake of 2,4-D is facilitated by the permease TfdK, encoded on *tfd_{II}* [163], (ii) *tfd_{II}* supplies a functional transcriptional activator, i.e. TfdR [154], and (iii) maintaining a second *tfd* gene cluster increases catechol degradation by a gene dosage effect [227].

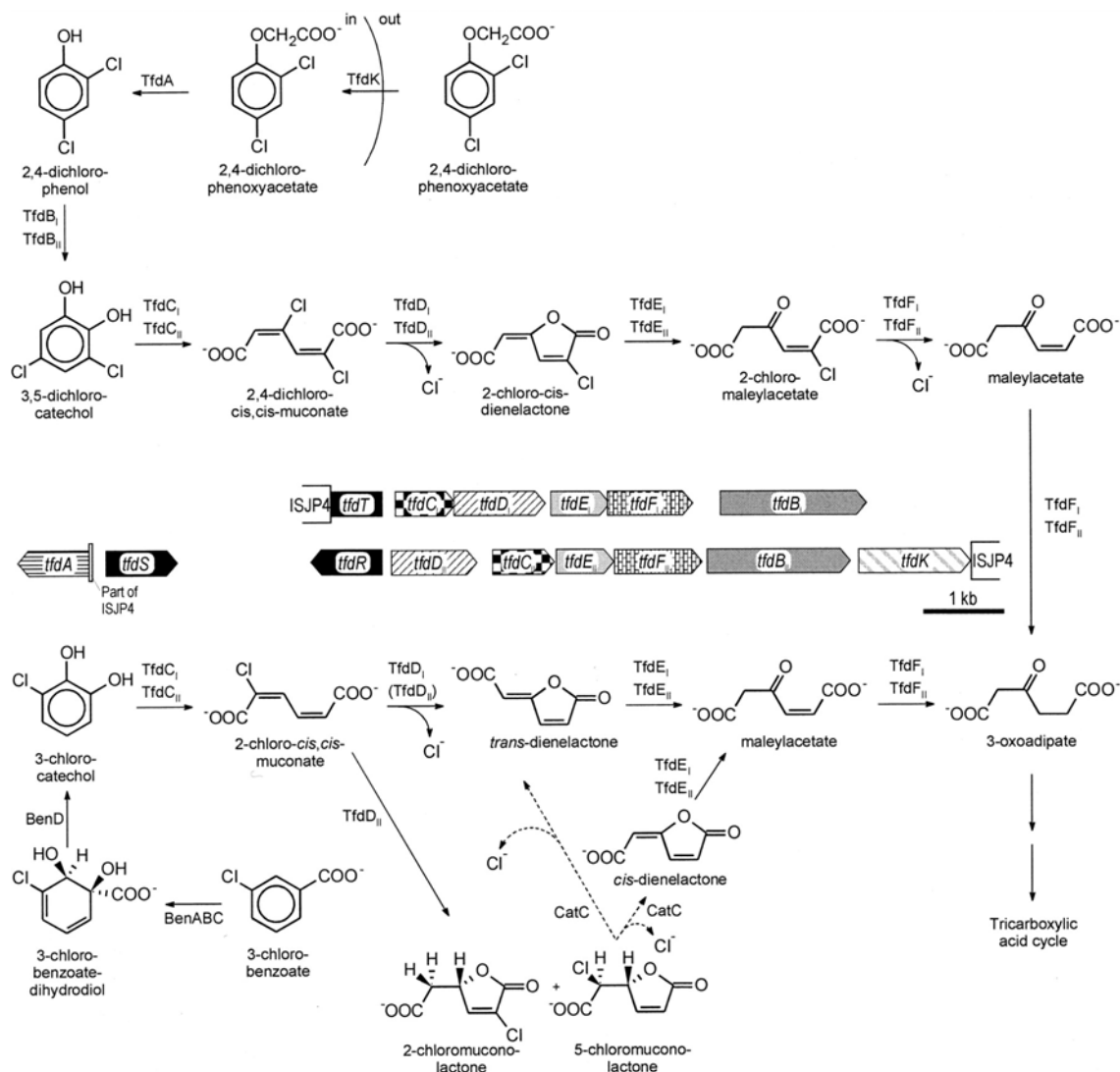


FIG. 1.13: Degradative pathways of 2,4-D (top) and 3-chlorobenzoate (bottom) by *Ralstonia* sp. JMP134 (pJP4) and the two chlorocatechol gene clusters of pJP4. The two clusters, which are contiguous on pJP4, are shown separately to facilitate comparison. (reprinted from Schlömann, 2002 [245])

Other 2,4-D degrading bacteria have been shown to carry *tfd* genes with various degrees of similarity [21, 39, 137, 177, 291] or no similarity at all [142, 148] to the *tfd* genes from *Ralstonia* sp. JMP134. In nearly all cases, these genes were found to be located on transmissible plasmids. Overall, available data suggest that an extensive interspecies transfer of a variety of degradative genes has occurred in the evolution of 2,4-D-degrading bacteria [94, 188, 292].

1.4.2.4 Degradation of chloroanilines

Chloroanilines are intermediates in the industrial production of many herbicides, azo dyes, azo pigments, plastics, and pharmaceuticals [145, 196]. As a result of their use in industry, spills of chloroanilines can result in contamination of soil and aquatic systems. Additionally, chloroanilines are formed during biodegradation of several pesticides, such as the phenylcarbamate herbicides [124]. For example, 3-chloroaniline results from the hydrolysis of chlorpropham (isopropyl-N-(3-chlorophenyl)carbamate),

a herbicide and plant growth inhibitor that is mainly applied on soybean and for sprouting inhibition in stored potatoes (Fig. 1.14).

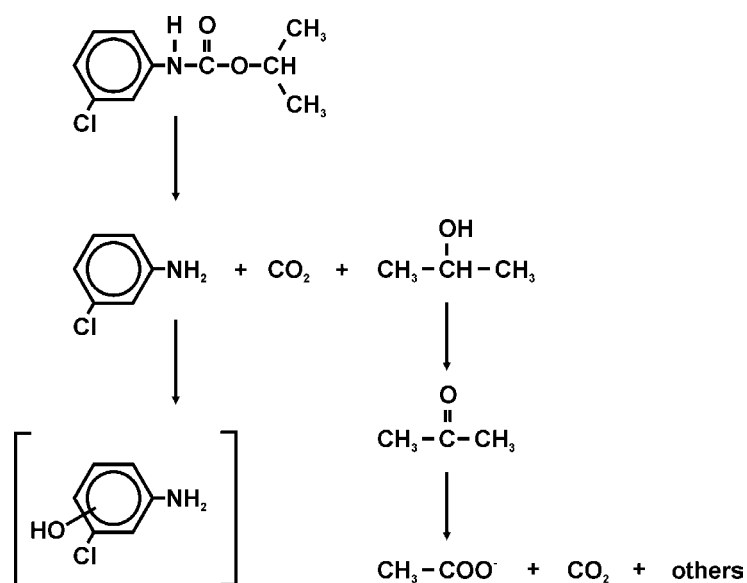


FIG 1.14: Microbial hydrolysis of chlorpropham (reprinted from Kaufman and Kearny, 1965 [144])

Toxic effects of chloroanilines include an inhibitory and uncoupling action on the respiratory chain [60] and accumulation in biological membranes, where they alter the lipid bilayer structure and thereby perturb its functioning [6]. Some (chloro)anilines are known carcinogens [175].

In soil and aquatic environments, chloroanilines frequently become unextractable through the formation of condensation products and incorporation into humus polymers [226, 251] or are transformed into more persistent chemicals such as substituted biphenyls, azobenzenes and triazines [17]. Nevertheless, biodegradation does occur, and various strains utilizing mono- and/or disubstituted chloroanilines as sole sources of carbon have been isolated. Such chloroaniline degrading organisms were found within the α -Proteobacteria (*Brevundimonas diminuta*, *Paracoccus denitrificans*, and *Aquaspirillum* sp. [269, 270]), β -Proteobacteria (*Delftia acidovorans*, *Comamonas testosteroni*, *Acidovorax* sp., and *Achromobacter* sp. [26, 33, 73, 172]), and γ -Proteobacteria (*Pseudomonas* sp. and *Moraxella* sp. [73, 155, 331]). The white rot fungus, *Phanerochaete chrysosporium*, has also been observed to degrade chloroanilines, even when these chemicals were tightly complexed to lignin and other plant cell wall components [7, 185, 242]. Bacterial degradation of chloroanilines occurs in a way that is similar to that of other chlorinated aromatic compounds (Fig. 1.15). Peripheral enzymes catalyse an oxidative deamination of chloroanilines, resulting in chlorinated catechols that are subsequently metabolised through the modified *ortho*-cleavage pathway [126, 332]. However, the existence of a *meta*-cleavage pathway for the mineralization of 3-chlorocatechol has also been shown [143, 182]. Recent studies indicate that the genes involved in the biodegradation of chloroanilines are at least partly located on transferable plasmids [27, 73], but no genes or gene clusters dedicated to chloroaniline metabolism have been described yet.

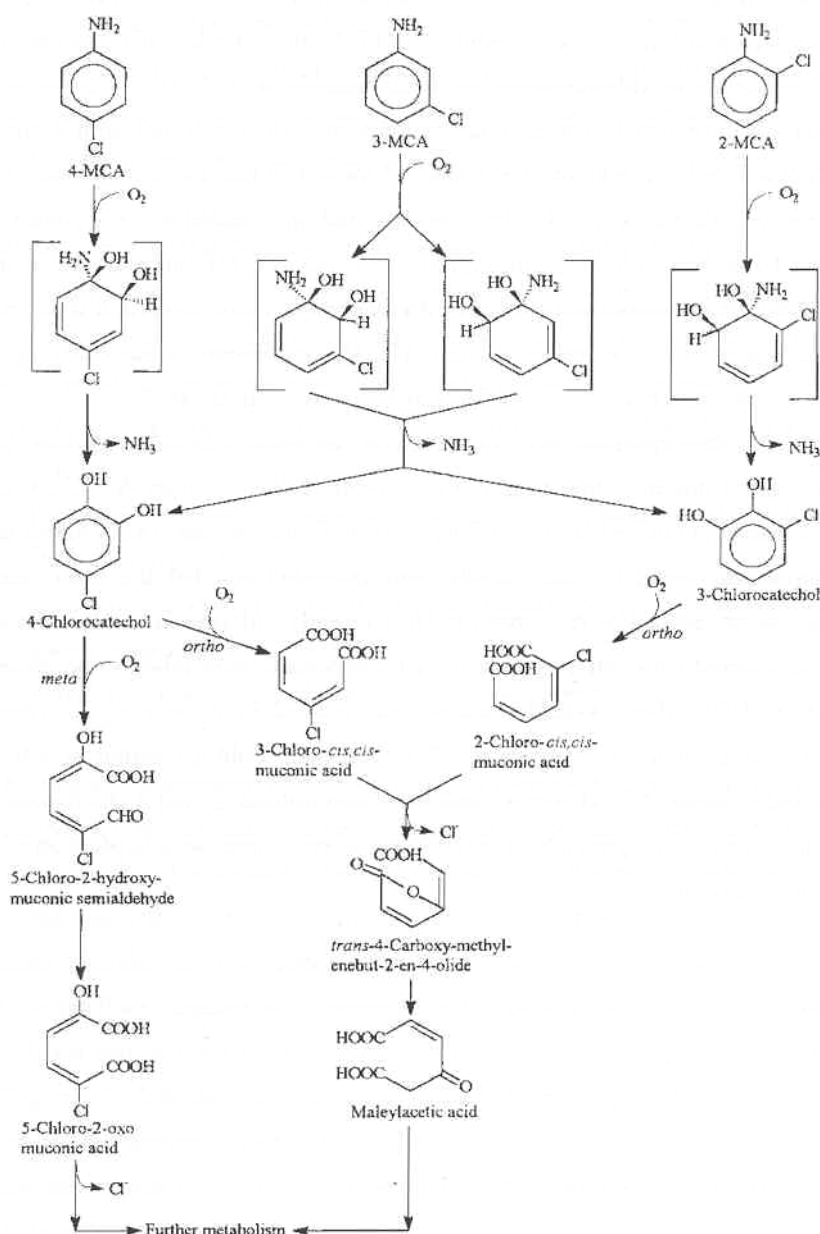


FIG. 1.15: The proposed pathways for the bacterial degradation of monochloroanilines under aerobic conditions (reprinted from van Agteren et al., 1998 [294]).

1.4.3 Bioremediation strategies

1.4.3.1 Natural attenuation, biostimulation and bioaugmentation

In some cases, an anthropogenic pollution can be cleaned up within a reasonable period of time by the natural contaminant degradation processes of indigenous bacteria. This phenomenon is referred to as natural attenuation or intrinsic bioremediation. Manipulations can, in such cases, be limited to a detailed sampling and characterization of the site. On the basis of the predicted contaminant migration and degradation pattern, exposure and health risk to potential receptors may then be assessed. To confirm

continued attenuation, a long-term monitoring strategy should be implemented [131]. Intrinsic bioremediation has been successfully applied to fuel hydrocarbon plumes in groundwater [15, 66], where indicators of microbial activity (e.g. dissolved oxygen, concentration of other electron acceptors, pH, methane concentration, etc.) can be linked to contaminant removal processes. For contaminants such as chlorinated aromatics however, such direct relationships cannot be inferred. When the kinetics of the biodegradation process at a given site are slower than desired from public health or environmental considerations, measurements need to be taken. Such measurements can include biostimulation, i.e. the stimulation of *in situ* degradation capacities by supplementing nutrients or additional electron acceptors.

For recalcitrant pollutants, it is conceivable that either the catabolic potential is not present in the contaminated ecosystem or that the indigenous micro-organisms adapt too slowly to the pollutant. In such conditions, bioaugmentation can present a feasible strategy. Bioaugmentation is described as the application of indigenous or foreign wild-type or genetically modified organisms to polluted hazardous waste sites or bioreactors to accelerate the removal of undesired compounds [299]. The inoculum is often a bacterial strain or an unspecified bacterial community that has been enriched (possibly from samples taken at the contaminated site) by supplying the pollutant as sole source of carbon and energy. Alternatively, bacteria can be genetically engineered for increased bioremediation performance [40, 225, 278]. Different strategies exist, such as the combination of catabolic segments from different organisms within one recipient strain (i.e. 'patchwork assembly') [230], site-directed mutagenesis to alter substrate specificities of enzymes [129], or the replacement of a native, regulated promoter by a strong, constitutive equivalent [217]. Due to public concern with the release of genetically modified organisms, field-scale applications are in general poorly documented [243]. Several cases of successful bioaugmentation for laboratory- or pilot-scale treatment of polluted soil (e.g. [86, 146, 236]) and activated sludge (e.g., [26, 215, 249]) have been described in the literature. However, results are not always predictable and success depends also on associated parameters such as pollutant characteristics (e.g. bioavailability, toxicity), physico-chemical conditions (e.g. organic matter content, pH), availability of co-substrates, presence of predating protozoa, aggregation behaviour of the inoculum (in activated sludge), inoculum concentration, etc. [299, 312]. The major bottleneck in many bioaugmentation strategies is the survival or retention of the inoculum in the system. In nearly all cases, a fairly rapid decline of the original high inoculum densities is observed, often concomitant with a decrease in biodegradation activity. Low inoculant survival has been attributed to the scarcity of available nutrients and to the hostility of the environment in which it was introduced [25, 302]. To circumvent the need for prolonged inoculum survival, ecosystems can be inoculated with strains encoding the relevant catabolic genes on mobile genetic elements, which can be horizontally transferred to well-adapted indigenous bacteria. Using this strategy, the survival of the introduced donor strain is no longer required once the catabolic genes are transferred and expressed in members of the indigenous bacterial community [285].

1.4.3.2 Bioaugmentation through horizontal transfer of catabolic genes

A separation of bioaugmentation processes into procedures that rely on inoculation with degrading strains and procedures employing horizontal gene transfer might be somewhat artificial since both principles can overlap e.g. when the donor of catabolic genes itself is able to degrade the pollutant or when the biodegrading inoculum carries catabolic information partly on mobile genetic elements. Despite this possible overlap, bioaugmentation through horizontal transfer of catabolic genes will be discussed in more detail under a separate header because a significant part of the present study is related to the latter subject. A recent review on the application of catabolic genetic elements for bioaugmentation is given by Top and co-authors [285].

Horizontal gene transfer and catabolic plasmids

Horizontal gene transfer between bacteria can be defined as the non-parent-to-offspring exchange of genetic material between donor and recipient cells and occurs through one of three basic mechanisms: transformation, transduction, and conjugation. In these processes, mobile genetic elements such as bacteriophages, plasmids, and transposons provide key vehicles for gene transfer between bacteria [322]. It is now generally accepted that horizontal gene transfer is not just a laboratory artefact, but in fact constitutes a major driving force in bacterial adaptation, evolution, and speciation [69, 164, 180, 216].

One of such bacterial adaptation mechanisms is the relatively recent development of degradation routes for anthropogenic substances previously considered to be recalcitrant. The genetic information encoding these degradation reactions is often found on plasmids or other mobile elements [284, 288]. The present overview will focus only on catabolic plasmid transfer. Catabolic plasmids that encode degradation of xenobiotics, such as chloroaromatics, are rather large plasmids (>50 kb) and mostly belong to the incompatibility group IncP-1 [284]. These broad host range plasmids are the most promiscuous self-transmissible plasmids known to date and, obviously, this promiscuity is an important factor in the evolution of new metabolic pathways by recruiting catabolic genes or gene clusters from different organisms into a suitable host [18].

Methods to study catabolic plasmid transfer

The dissemination of catabolic plasmids can be studied in several ways. An often-applied method consists of adding a plasmid donor strain to an ecosystem such as soil or activated sludge, followed by isolation of indigenous bacteria that took up the plasmid (transconjugants) by plating on agar surfaces. Especially at the onset of such an experiment, the donor strain is numerically dominant over the transconjugants and, therefore, a system to counter-select the donor is needed. This can be achieved through the application of a donor-specific bacteriophage [254] or by employing a mineral medium that does not support the growth of an auxotrophic donor [74]. Attention has to be paid to possible overestimation of transconjugant numbers due to plate matings [253, 315].

Alternatively, plasmid transfer in ecosystems can be studied through exogenous plasmid isolation, in which a plasmid-free recipient strain is mixed with a natural

bacterial community and, after mating, recipients that gained a new marker are selected for [12]. This method allows the isolation of conjugative plasmids without culturing the original host. In triparental exogenous plasmid isolation, a donor of a non-conjugative ($\text{Tra}^- \text{Mob}^+$) vector, a recipient strain and a natural bacterial community are mixed. The isolation of plasmids in this procedure is solely based on their ability to mobilize the Mob^+ vector and is therefore not restricted to the plasmid markers used in the experiment [125, 281]. In such triparental matings, chromosomally or plasmid-encoded genes can be transferred from the original recipient back into the Mob^+ plasmid donor, a phenomenon that has been designated shuttle transfer or retrotransfer [193, 272].

Catabolic plasmids can be detected by the degradative phenotype they confer to their hosts. However, not all bacteria acquire the corresponding degrading capacities upon receiving a catabolic plasmid. The use of other ‘naturally’ encoded selection markers on the plasmid can provide a valuable alternative, e.g. in case of the mercury resistance genes encoded on pJP4 [79]. If no such markers are available, catabolic plasmids can be labelled to facilitate their detection and consequently also the study of their transfer. Several labelling systems are available, most of them based on the miniTn5 vectors developed by de Lorenzo and co-workers [70]. In the *LacZ* system, transconjugants produce β -galactosidase, encoded by the reporter gene, that forms a blue complex in the presence of a substrate [134]. The use of the gene encoding green fluorescent protein (*gfp*) as a reporter [38], circumvents the need for a substrate and allows *in situ* visualization of bacteria with an epifluorescence microscope as well as detection by flow cytometry [280]. Furthermore, GFP detection can be combined with the use of rRNA-directed fluorescent probes [84]. A system for the visualisation of conjugation events has been developed in which the plasmid-encoded *gfp* gene is controlled by a *lac* promoter and repressed in the donor strain by a chromosomally located *lacI^f* gene [43]. This versatile marking system has proven to be useful in the study of bacterial conjugation in marine environments [63, 64], in activated sludge [97], and on plant leaf surfaces [213]. A recent review on the *in situ* detection of horizontal transfer, providing a more comprehensive overview of available marker genes, is presented by Haagensen and co-authors [113].

Examples of bioaugmentation through transfer of catabolic plasmids

The first papers in which the hypothesis was raised that inoculation of mobile genetic elements into polluted soils and aquatic systems and their subsequent transfer to indigenous populations may be a feasible bioaugmentation strategy, were published about twelve years ago [32, 95]. This hypothesis was examined in subsequent studies in which the degradation capacities of lab-scale ecosystems inoculated with donors carrying catabolic plasmids and non-inoculated systems were compared.

A clear positive effect on biphenyl degradation in soil was observed to result from transfer of plasmid RP4::Tn4371 [71]. Transfer of the 2,4-D degrading plasmid pJP4 in soil was the subject of several studies [61, 62, 74, 78, 204-207], with effects on the 2,4-D degradation varying from undetectable to very significant. For plasmid pEMT1, which is different from pJP4 but also contains 2,4-D degradative genes [282], an enhanced degradation was observed upon inoculation of two different donors harbouring this plasmid [74, 283, 286]. The bioaugmentation by introduction of bacteria

with catabolic plasmids into activated sludge is less studied and in most cases the exact role of transconjugants in accelerated removal was not investigated in detail. However, a positive effect of the transfer of the mobile *clc* element, which codes for chlorocatechol degradation, was suggested [99, 260].

Although differences in experimental design (xenobiotic concentration, bioreactor size, soil origin, donor strain, etc.) hamper a direct comparison of the obtained results, a few general factors influencing the success of a given approach can be identified: (i) xenobiotic degradation by the donor can obscure the contribution of transconjugants, therefore a donor that dies off quickly in soil or one that is unable to degrade the component under study should be used preferentially (ii) selective pressure i.e. the presence of a xenobiotic, affects the extend of gene spread, most probably due to proliferation of transconjugants (iii) in order to have an effect on degradation, transconjugants must reach sufficiently high population sizes, hence, they must have a selective advantage over the other indigenous bacteria (iv) the use of relatively easily degradable model compounds possibly impairs the selective advantage conferred by the plasmid, as the catabolic potential is already present within the indigenous population.

In conclusion, plasmid-mediated bioaugmentation could be a valuable strategy in these cases where the catabolic potential is not present indigenously. More research, in soil and especially in activated sludge systems, is necessary to increase our basic knowledge about factors influencing the dissemination and fate of introduced catabolic genes and about the interactions between plasmid genes and host genomic background.

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CHAPTER 2
GOALS OF THE THESIS

“First things first, but not necessarily in that order”

Doctor Who

CHAPTER 2 GOALS OF THE THESIS

The goals of this study comprise i) the evaluation and validation of molecular techniques currently in use in bacterial systematics, ii) the application of these techniques to unravel the systematics of bacteria with bioremediation potentialities, and iii) tracing horizontal gene transfer of catabolic genes involved in the degradation of chloroaromatic xenobiotics.

As DNA-DNA hybridisations of whole bacterial genomes form a cornerstone of the bacterial species concept, they are often a prerequisite for species delineation in taxonomic studies. However, due to the laborious and specialised nature of “traditional” hybridisation protocols they often form a bottleneck in studies on bacterial systematics. A number of methods have been described more recently, offering DNA hybridisations at a smaller scale and associated higher throughput. Very often, however, these newer methods were not calibrated against more established methods for a statistically significant number of hybridisations. This is essential for the consistency of the bacterial species concept whenever results obtained with previous methods are compared with those obtained with newly developed methods. Therefore, a thorough evaluation of such a microscale DNA-DNA hybridisation protocol would not only contribute to the present study, but also to other taxonomic studies performed in the Laboratory of Microbiology Gent or elsewhere.

The base composition of a genome is an important descriptive parameter in bacterial systematics. Concomitant with the introduction of a new microscale DNA-DNA hybridisation technique, the method for determination of nucleotide composition should also be updated, according to the newly described enzymatic methods.

Bioremediation could be a valuable tool for the clean-up of environmental pollutants such as toxic metals and chloroaromatics. Many strains with potential applications in bioremediation are extremely well-studied with regard to their biotechnological potential but are taxonomically poorly characterised. The genera *Ralstonia* and *Burkholderia* contain several of such examples. Both genera consist of functionally extremely versatile organisms and comprise besides environmental strains also plant pathogens, human pathogens, and human opportunistic pathogens e.g. for cystic fibrosis patients. From the public health perspective, it is obvious that biotechnologically important strains, predestined for a large-scale environmental release, should be distinct from detrimental organisms. Therefore, a sound classification and correct identification of these organisms is important. This study contributes to improve the systematics of biotechnologically interesting organisms within the genera *Ralstonia* and *Burkholderia*. Firstly, the taxonomic situation of several biotechnologically important strains with a long history of research is still unclear. This results in confusion about nomenclature and often the same strain is referred to differently in different publications. Scientific communication benefits from a definite taxonomic allocation of such strains. Secondly, newly isolated strains with interesting bioremediation potential are classified in a reliable way according to the improved taxonomic situation in this group of bacteria.

Finally, the application of taxonomic tools, and especially typing techniques, may shed new light on transfer of catabolic plasmids. Horizontal gene transfer is an

important evolutionary mechanism. It is believed to be one of the key factors in the evolution of pathways for the degradation of xenobiotic compounds. Furthermore, the dissemination of catabolic genes located on mobile genetic elements could possibly be exploited as a bioaugmentation tool. Detailed typing and taxonomic analysis of transconjugants obtained from matings of a donor strain with indigenous bacterial populations in soil and activated sludge could reveal new information about the host range of catabolic plasmids and their transfer behaviour in these ecosystems. As such, it may contribute in general to improve our knowledge on horizontal gene transfer and for triggering successful bioaugmentation studies and applications.

CHAPTER 3
MICROPLATE DNA-DNA
HYBRIDISATIONS

“Experience is what you get when you don't get what you want”

Dan Stanford

CHAPTER 3 MICROPLATE DNA-DNA HYBRIDISATIONS

3.1 INTRODUCTION

As discussed in Chapter 1, bacterial species are currently defined based upon DNA-DNA hybridisation data [38, 49]. It is therefore self-evident that the accurate determination of DNA reassociation and ΔT_m (for a definition of these two parameters, see below) is extremely important in bacterial systematics. In this paragraph, some general principles concerning hybridisation will be outlined and an overview of the existing methods for whole-genome DNA-DNA hybridisation will be given.

When native DNA in aqueous solution is heated, the double stranded molecules will dissociate into single strands. This denatured state can be maintained by rapid cooling of the mixture, although considerable non-specific base pairing occurs. Upon slow cooling, however, complementary strands will reassociate to their original conformations. This process is called renaturation. The denaturation temperature (or melting temperature; T_m) of a DNA duplex is defined as the midpoint of the denaturation curve i.e. the point at which half of the DNA duplexes are denatured. When two denatured DNA preparations of different biological origin are mixed together prior to renaturation, heteroduplexes or hybrid DNA molecules can be formed. The extent to which these heteroduplexes are formed in a hybridisation reaction reflects the similarity between the two DNA's. This general principle can be applied for both DNA and RNA and has found countless applications in molecular biology. In bacterial systematics, DNA reassociation values between different strains (heterologous reaction) are expressed as a percentage of the homologous reaction and ΔT_m is defined as the difference in T_m (in °C) between the hybrids formed in the heterologous and the homologous reaction.

Methods for whole-genome DNA-DNA reassociation were described for the first time in the 1960's. The membrane hybridisation procedure of Denhardt [10], has become the basis for numerous hybridisation protocols in molecular biology, still in use today. In this procedure, denatured unlabelled DNA is immobilized on a nitrocellulose filter. The filter is then blocked to prevent the binding of more DNA (prehybridisation) and incubated with radioactively labelled single stranded DNA. After a washing step, the amount of DNA reassociation is estimated by measuring the radioactivity of the filter. DNA relatedness can often be more precisely determined in competition experiments. In this method, immobilized and probe DNA are from the same source and hybridisation is performed in the absence or presence of an excess of unlabelled competitor DNA [16]. An alternative membrane hybridisation procedure, which determines the thermal stability of DNA hybrids in bacterial lysates directly immobilized on a nitrocellulose filter, was described by Tjernberg et al. [44].

A number of methods were described that all rely on the same principle: the formation of duplexes between a radioactively labelled and an unlabelled denatured DNA sample in solution, followed by the separation of the duplexes from residual single-stranded DNA and measurement of their radioactivity. These different methods diverge principally in the way of separating double stranded DNA from single strands. In the method of Brenner et al. [2] and Lachance [26], hydroxyapatite is used to

selectively absorb the double stranded DNA. Alternatively, the enzyme S1 nuclease can be employed to specifically digest the residual single stranded DNA. The double stranded DNA is subsequently precipitated with trichloroacetic acid [6] or adsorbed to a DEAE-cellulose filter [34]. One of the major advantages of these free-solution methods is the ability to accurately determine the ΔT_m [16].

No labelled DNA (radioactive or otherwise) is required in the initial renaturation rate method of De Ley et al. [9]. As the rate of renaturation in the initial stages depends on the molecular complexity of the denatured mixture, reassociation values can be calculated from renaturation curves, determined in a specially designed spectrophotometer. The method has also been used to estimate genome sizes of phages [13] and bacteria [14].

Whole-genome DNA-DNA hybridisation procedures are generally rather time-consuming and often represent a bottleneck in the completion of taxonomic studies. In addition, most of the “classical” hybridisation protocols require large quantities of DNA, which are sometimes hard to obtain e.g. in case of fastidious, slow growing organisms or organisms that are difficult to lyse. Therefore, much effort has been invested in the development of high-throughput hybridisation methods that are performed either as slot-blots obtained with microfiltration apparatuses or in the 96-well microplate format. Nearly all the high-throughput methods described below make use of a non-radioactive labelling with biotin or – more often – photobiotin. The latter reagent is a photo-activatable analogue of biotin, that can bind covalently to amine groups present in DNA upon irradiation with visible light [12]. The structure of photobiotin consists of a biotin moiety, connected through a linker with a photo-activatable aryl azide group (Fig. 3.1). Photobiotin-labelled DNA can be detected through the addition of avidin- or streptavidin-enzyme complexes and suitable substrates.

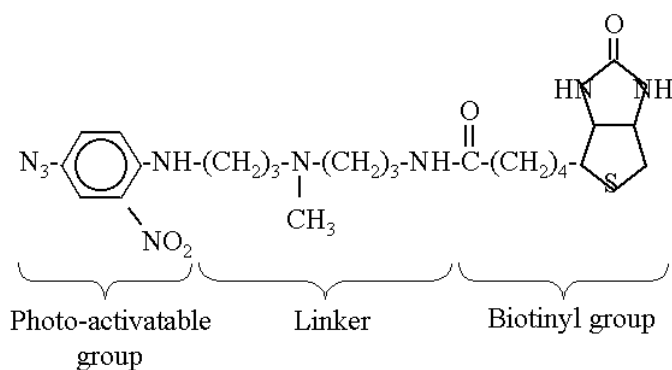


FIG. 3.1: Structure of photobiotin, a reagent for stable, non-radioactive labelling of DNA (after Forster et al., 1985 [12]).

In the dot-blot hybridisation method of Imaeda et al. [22], biotin-labelled probe DNA is revealed through a colorimetric reaction. Since the colour intensity of the dots is only visually evaluated, only semi-quantitative information is obtained and no reassociation values can be determined.

In 1989, Ezaki and co-workers described a method in which DNA is attached to microplates through non-covalent interactions and hybridised with photobiotin-labelled probe DNA. Quantification is done in a microplate reader with the aid of a streptavidin-

β -galactosidase complex and a fluorogenic substrate [11]. Several variants of this protocol were described, with either fluorescent or colorimetric detection of hybrid formation (e.g. [18, 25, 39]).

In other procedures, DNA is immobilized by covalent attachment of its 5' end to reactive groups on the surface of CovaLink (Nalge Nunc International) microplates [35]. Hybrid formation is quantified through an enzymatic reaction, generating either a luminescent [1] or coloured product [24]. Although the latter procedures were originally described as an improvement of the hybridisation method of Ezaki et al. [11], they did not gain as much popularity. Our own results indicate that with DNA of an undefined fragment length (as it is the case for genomic DNA), the reproducibility of the fixation is in fact lower than with non-covalent fixation (E. De Clerck, J. Goris, P. De Vos, unpublished results). Perhaps a recently proposed method, which uses covalent DNA attachment to the more efficient NucleoLink (Nalge Nunc International) plates, can improve the reproducibility [3].

A “hybrid” protocol has been described by Jahnke [23], in which slot-blot hybridisation on a nitrocellulose filter is followed by a colorimetric probe DNA quantification in a microplate, but it has not gained much popularity.

Currently, no consensus exists among bacterial taxonomists about which DNA-DNA hybridisation method is to be preferred. Although microplate methods (especially the method of Ezaki et al.) are increasingly used today, older methods such as the hydroxyapatite, membrane filter, S1 nuclease, and renaturation rate method (and numerous variants of the original protocols) are still frequently employed. The choice for a certain method often merely depends on the experience of the laboratory with this method. Different DNA-DNA hybridisation methods, however, do not necessarily yield comparable reassociation values and caution should be exercised when basing taxonomic conclusions on results obtained with different methods.

In the present study, the microplate method of Ezaki et al. [11] was compared with the more established initial renaturation method of De Ley et al. [9] (paragraph 3.3), in order to provide a bench-mark that would facilitate future comparisons of DNA-DNA hybridisation results obtained with both methods. Since the microplate has considerable advantages over the initial renaturation method in terms of lower amounts of DNA required and higher throughput, the microplate method was subsequently introduced in the Laboratory of Microbiology Gent. A detailed experimental protocol is provided in paragraph 3.2 and some additional remarks on the use of the microplate hybridisation method are presented in paragraph 3.4.

3.2 EXPERIMENTAL PROCEDURE

The procedure for DNA-DNA hybridisations that was used in the present work was slightly modified from the protocol of Ezaki et al. [11]. A detailed description is given below.

3.2.1 Immobilization of DNA in microplate wells

Pure DNA of the strains to be hybridised is dissolved in 0.1 x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0 ± 0.2) and diluted to a concentration corresponding to an optical density at 260 nm (OD₂₆₀) of 2. The DNA is then denatured in a heat block at 100°C for 10 min, followed by rapid cooling on ice. The samples are then ten times diluted in phosphate buffered saline with MgCl₂ (PBS-Mg; 8 mM NaH₂PO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.1 M MgCl₂, pH 7.2). Hundred µl portions of this solution are transferred to the wells of a polystyrene microplate (MaxiSorp, FluoroNunc™, Nalge Nunc International). The microplate is subsequently sealed with self-adhesive vinyl tape (Nalge Nunc International) and incubated for 4 h at 30°C in a ShellLab Model 1004 hybridisation oven (Sheldon Manufacturing Inc., Oregon). During this time, non-covalent DNA adsorption to the polystyrene wells takes place. Afterwards, the microplates are washed once with 300 µl PBS per well, using a Wellwash Ascent microplate washer (Labsystems, Finland) and dried at 45°C for 15 min. DNA that has been immobilized this way can be stored under dry conditions for several weeks or even months. The procedure for non-covalent DNA immobilization is schematically presented in Fig. 3.2.

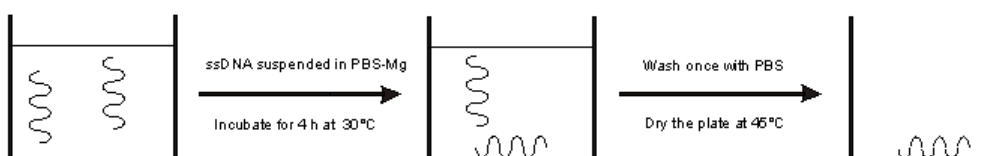


FIG. 3.2: Procedure for non-covalent adsorption of denatured DNA in MaxiSorp FluoroNunc™ (Nalge Nunc International) microplate wells.

3.2.2 Preparation of probe DNA

Ten µl DNA solution (with an OD₂₆₀ of 10) is mixed with 10 µl photobiotin solution (0.5 µg photobiotin (Sigma) per µl water) in an 1.5 ml Eppendorf reaction vial. The open vials are kept upright in a cooling block on ice while being illuminated for 30 min under a 400 W mercury vapor lamp. The labelled probe DNA is diluted by adding 185 µl of 0.1 M Tris-HCl (pH 9.0) and the remaining free photobiotin is removed by extracting twice with 200 µl 1-butanol, saturated with 0.1 M Tris-HCl (pH 9.0). The probe DNA is then fragmented by ultrasonic treatment for 30 sec at 10 W (Misonix Microson™ ultrasonic cell disruptor), denatured at 100°C for 10 min and immediately cooled on ice. The labelling procedure is schematically presented in Fig. 3.3.

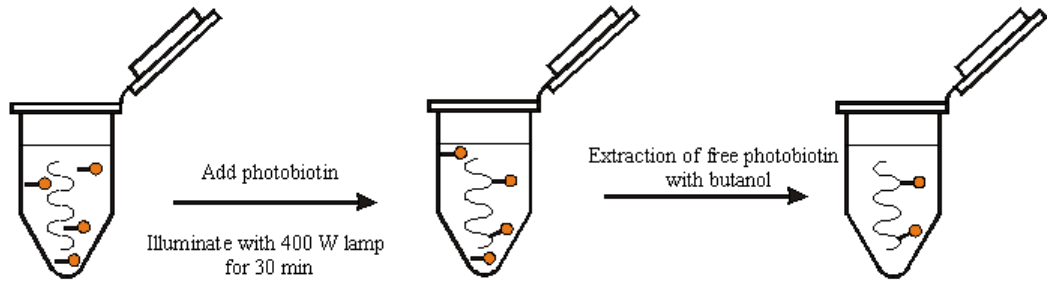


FIG. 3.3: Labelling of probe DNA with photobiotin.

3.2.3 Prehybridisation

Two hundred μl prehybridisation solution (2 x SSC, 5 x Denhardt solution [10], 50% formamide, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA) is added per well of a microplate containing the immobilized DNA's. The microplate is sealed with vinyl tape and incubated for at least 30 min at the appropriate hybridisation temperature. The latter temperature is always taken equal to or slightly higher than the optimal renaturation temperature (T_{OR}), which is calculated from the average %G+C by the formula $T_{OR} = [0.51 \times (\%GC) + 47] - 36^\circ\text{C}$, with 36°C being the correction for the presence of 50% formamide [15, 31].

3.2.4 Actual hybridisation

The labelled probe DNA is mixed with hybridisation solution (prehybridisation solution plus 2.5% dextran sulphate) in a ratio of 1:20. After removal of the prehybridisation solution from the microplate, 100 μl of the probe – hybridisation solution mixture is added per well. The microplate is again sealed with vinyl tape and incubated for 3 h at the appropriate hybridisation temperature. The hybridisation procedure is schematically presented in Fig. 3.4.

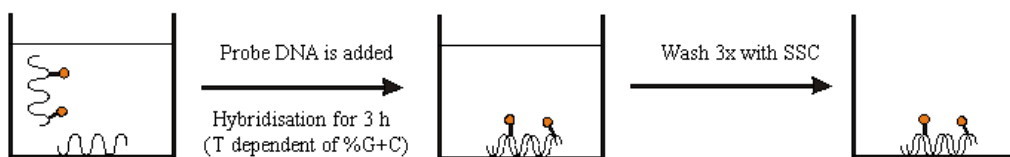


FIG. 3.4: Hybridisation of photobiotin labelled probe DNA with immobilized DNA in microplate wells.

3.2.5 Enzymatic development of the hybridisation reaction

After hybridisation, the microplate is rinsed three times with 300 μl of 1 x SSC per well, using the microplate washer. To each of the emptied wells, 100 μl of streptavidin- β -D-galactosidase (GibcoBRL) solution (0.5 U/ml in PBS plus 0.5% bovine serum albumin) is added. The microplate is then covered with a preheated empty microplate and incubated for 10 min at 37°C . Subsequently, the plate is again rinsed three times with 300 μl 1x SSC per well. Finally, 100 μl of 4-methylumbelliferyl- β -D-

galactopyranoside (Sigma) solution (0.1 mg/ml in PBS plus 1 mM MgCl₂) is added per well and the plate is incubated at 37°C. The latter substrate is converted by β-D-galactosidase into the fluorophore 4-methylumbelliferone (excitation max. 360 nm, emission max. 465 nm). This fluorophore is quantified using an HTS 7000 Bio Assay Reader (Perkin Elmer) at 15, 30 and 45 min after addition of the substrate. The principle of the enzymatic development is given in Fig. 3.5.

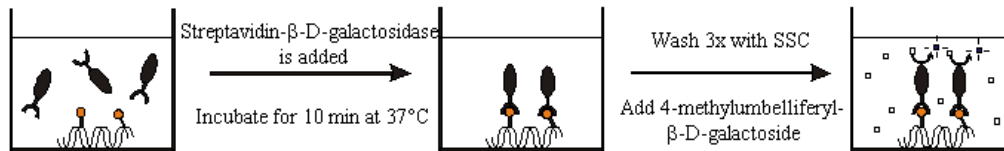


FIG. 3.5: Procedure for enzymatic detection of photobiotin labelled DNA, using streptavidin-β-D-galactosidase.

3.2.6 Calculation of reassociation values

For all hybridisations four duplicate wells are used and reassociation values are calculated as a mean of these four experiments, with aberrant values being eliminated from further calculations. Hybridisation reactions of bacterial probe DNA with salmon sperm DNA are used as estimations of background fluorescence and unspecific hybridisations. All fluorescence values are corrected by subtracting the corresponding blank values. The homologous reaction is regarded as representing 100% reassociation and reciprocal hybridisations (different hybridisations using the same DNA's A and B, but once with A as probe DNA and once with B as probe DNA) are always performed.

3.3 EVALUATION OF A MICROPLATE DNA-DNA HYBRIDISATION METHOD COMPARED TO THE INITIAL RENATURATION METHOD[§]

3.3.1 Summary

Previously reported hybridisation data, obtained with the initial renaturation method [9], were compared with data obtained with a microplate DNA-DNA hybridisation method [11]. The comparison was done for more than 82 hybridisations, within 4 sets of *Bacillus / Virgibacillus*, *Myroides*, *Vibrio* and *Xanthomonas / Stenotrophomonas* strains, comprising DNA base compositions ranging from 34 to 65 %. Under the experimental conditions used, both methods were in very good correlation. With the microplate method, the variation between reciprocal hybridisations and repeated experiments was around 7 %, while for the initial renaturation method a 5 % variation was calculated. It is concluded that the microplate method can be used as a reliable taxonomic tool.

3.3.2 Introduction

In bacterial taxonomy, a 70 % overall chromosomal relatedness is recommended for species delineation [49]. Different methods to measure this DNA relatedness between bacteria are currently used. Examples are the hydroxyapatite method [2], the S1-nuclease method [6, 17], the membrane filter hybridisation [10] and the initial renaturation method [9, 21].

A comparison between two variants of the S1-nuclease method and the hydroxyapatite method was done by Grimont et al. [17]. These authors concluded that different methods resulted in different relative binding ratios, but similar percent divergence (ΔT_m) values. The initial renaturation method was compared with the membrane filter hybridisation method by Huss et al. [21]. Both methods were shown to agree very well above a degree of 20-30 % binding.

More recently, protocols for DNA-DNA hybridisation in microplates have been developed. Reference DNA is immobilized in a microplate and hybridised with a labelled probe-DNA, whereupon the amount of hybridised probe-DNA is quantified. Different variants of this method exist. Reference DNA can either be immobilized non-covalently by non-specific interactions with the microplate polystyrene [11] or covalently to a specially treated microplate [1, 24]. The probe-DNA can either be radioactively [35] or non-radioactively labelled. In the latter case photobiotin [12] is most often used. The reaction is developed with a streptavidin-enzyme complex converting a substrate to a coloured, fluorogenic or chemiluminescent product. Quantification is obtained via colorimetric [18, 24], fluorimetric [11, 39] or luminometric [1] technology, respectively.

[§] Redrafted from **Goris, J., K. Suzuki, P. De Vos, T. Nakase, and K. Kersters**. 1998. Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**:1148-1153.

The microplate method has the advantage of being fast and less DNA consuming than the more "classical" methods. Therefore, the application of the microplate hybridisation method is becoming increasingly popular in taxonomic studies (e.g. [37, 40]). Nevertheless, no extensive study on the comparison of the microplate method with the initial renaturation hybridisation method is reported in the literature so far.

In this paper, we compared the results obtained with the microplate hybridisation method of Ezaki et al. [11] with those obtained with the initial renaturation method of De Ley et al. [9] for different species, covering a large % G+C range. We performed DNA-DNA hybridisations within and between species of which the values obtained with the initial renaturation method are already published [45, 48] or are in preparation ([19]; Vandenberghe, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Bolland, A., Balladares, M., Gomez-Gil, B., Sorgeloos, P., Calderon, J., and Swings, J., unpublished data).

3.3.3 Materials and methods

3.3.3.1 Strains

DNA of the following bacterial strains was used: *Bacillus* sp. LMG 17892; *Bacillus sporothermodurans* LMG 17894^T; *Bacillus* sp. LMG 17883; *Virgibacillus pantothenicus* LMG 7129^T; *Virgibacillus* sp. LMG 17368; *Myroides odoratus* LMG 1233^T, LMG 4028, LMG 12841 and LMG 13344; *Myroides odoratimimus* LMG 4029^T, LMG 12837, LMG 14973, LMG 12839 and LMG 14961; *Vibrio harveyi* LMG 4044^T, LMG 18298 and LMG 18299; *Vibrio alginolyticus* LMG 18300, LMG 18301 and LMG 18302; *Xanthomonas fragariae* LMG 706 and LMG 708^T; *Xanthomonas populi* LMG 5743^T; *Xanthomonas axonopodis* LMG 9181, LMG 682 and LMG 538 t1^T; *Stenotrophomonas maltophilia* LMG 958^T.

3.3.3.2 DNA extraction

DNA was isolated according to the protocol of Marmur [30], except for the *Bacillus* strains, of which DNA was isolated by a combination of protocols of Marmur [30] and Pitcher et al. [33]. Biomass was harvested from 10-15 Roux-flasks, washed with resuspension buffer (0.15 M NaCl, 0.01 M EDTA; pH 8.0) and treated with lysozyme (25 mg/ml) in 10 ml TE buffer (1.21 g Tris.HCl, 2 ml 0.5 M EDTA; pH 8.0 in 1 l MilliQ-water) for 30 min at 37 °C. TE buffer is added to a total volume of 50 ml. A 20 ml volume of GES reagent (600 g guanidiniumthiocyanate, 200 ml 0.5 M EDTA; pH 8, 10 g sarcosyl in 1 l MilliQ-water) is added and the suspension is mixed until cell lysis occurs and left on ice for 10 min. A 17.5 ml volume of 5 M NaCl is added and, after vigorous shaking, 36 ml 7.5 M ammonium acetate is added. The suspension is then mixed and again left on ice for 10 min. Chloroform extraction and DNA precipitation is performed according to the unmodified protocol of Marmur [30].

3.3.3.3 Determination of the G+C content

The direct HPLC method of Tamaoka and Komagata [41] was used.

3.3.3.4 DNA-DNA hybridisation

Microplate hybridisations were performed according to Ezaki et al. [11], using a Model FL-2575 microplate reader (Towa Scientific Co., Ltd., Tokyo, Japan) and black MaxiSorp FluoroNunc microplates (Nunc A/S, Roskilde, Denmark). After biotinylation, the probe DNA was sheared by an ultrasonic treatment (30 s; duty cycle 50; output control 1) with a Branson Sonifier Model 450 (Carouge-Genève, Switzerland) equipped with a microcup horn. Hybridisation temperatures were 40 °C, 37 °C, 45 °C and 55 °C for experiments within *Bacillus* / *Virgibacillus*, *Myroides*, *Vibrio* and *Xanthomonas* / *Stenotrophomonas*, respectively. Salmon sperm DNA was used as the negative control in all experiments.

The initial renaturation method was performed as described by De Ley et al. [9], using a Uvikon 940 spectrophotometer. Hybridisation temperatures were calculated from the G+C content with the formula of De Ley [8] and were 66.8 °C, 64.3 °C, 70.6 °C and 80.8 °C for experiments within *Bacillus* / *Virgibacillus*, *Myroides*, *Vibrio* and *Xanthomonas* / *Stenotrophomonas*, respectively.

3.3.3.5 Numerical interpretation

The DNA relatedness percentages determined with the microplate method are presented here as average values, based on 2 to 5 independent hybridisation experiments. Reciprocal reactions (e.g. A x B and B x A) were also considered as independent hybridisation experiments. The standard deviation (SD) was used as best estimation of the absolute error on the DNA relatedness percentage.

Hybridisations with the initial renaturation method were performed in duplicate or in triplicate. A SD based on two values only is statistically not significant. Nevertheless we used these SD values to calculate an average SD based on 941 DNA hybridisation data available in the lab. The obtained 5 % value was regarded as the best estimation of the absolute error for all relatedness data obtained with the initial renaturation method.

3.3.4 Results

3.3.4.1 *Bacillus* / *Virgibacillus* hybridisations

Hybridisation results with *Bacillus* / *Virgibacillus* DNA are presented in Table 3.1. Although only few hybridisation values with the initial renaturation method have been obtained, one could expect only the hybridisation between DNA's of *B. sporothermodurans* LMG 17894^T and *Bacillus* sp. LMG 17883 to show high relatedness values with the microplate method. The obtained experimental data are indeed in accordance with these expectations, except perhaps for the hybridisation between *Bacillus* sp. LMG 17892 and *B. sporothermodurans* LMG 17894^T, which revealed significantly lower values if the microplate method was applied.

3.3.4.2 *Myroides* hybridisations

The hybridisations with *Myroides* DNA are presented in Table 3.2. Both DNA hybridisation methods clearly allow to separate *M. odoratus* from *M. odoratimimus*, as

was already shown for the initial renaturation method by Vancanneyt et al. [45]. Moreover, the intermediate hybridisation values of *M. odoratus* LMG 13344 versus all other *M. odoratus* representatives, were confirmed with the microplate method.

Remarkably, microplate hybridisations performed with the DNA from *M. odoratimimus* LMG 12839 show very high standard deviations, because of the large difference between hybridisation values of reciprocal reactions (e.g. 205 % for LMG 14973 x LMG 12839 and 38 % for LMG 12839 x LMG 14973). Experiments with immobilized DNA of *M. odoratimimus* LMG 12839 showed abnormal low fluorescence values. Control of DNA integrity on an agarose gel (results not shown) indicated that *M. odoratimimus* LMG 12839 DNA was fragmented. The large differences between the reciprocal reactions can be explained by assuming that fragmented DNA is not adsorbed on the microplate in the same amount as native DNA. Since the same DNA batch was used as in the experiments of Vancanneyt et al. [45], the fragmentation could also explain the aberrant % G+C value (30 % compared with 34 % for the other strains of this species), determined via the thermal denaturation method. Indeed, it is reported in the literature [16] that extensively fragmented DNA has a lower T_m than native DNA has.

TABLE 3.1: Hybridisation results with the microplate and the initial renaturation method^a (in bold face) from the *Bacillus* and *Virgibacillus* strains.

| | % G+C | LMG 17892 | LMG 17894 ^T | LMG 17883 | LMG 7129 ^T | LMG 17368 |
|--|-------|-------------|------------------------|-----------------|-----------------------|-----------------|
| <i>Bacillus</i> sp. LMG 17892 | 38.1 | 100 | 33±5 (2) | | | |
| <i>B. sporothermodurans</i> LMG 17894 ^T | 36.4 | 8.5±0.4 (3) | 100 | 83±5 (2) | | |
| <i>Bacillus</i> sp. LMG 17883 | 36.6 | 10±2 (2) | 78±9 (3) | 100 | | |
| <i>V. pantothenicus</i> LMG 7129 ^T | 37.5 | 4.8±0.5 (3) | 5.3±0.3 (4) | 6±2 (3) | 100 | 24±5 (2) |
| <i>Virgibacillus</i> sp. LMG 17368 | 36.8 | 5.1±0.6 (3) | 6.1±0.5 (4) | 7±1 (3) | 16±2 (4) | 100 |

Note: Values are means ± standard deviations. The number of hybridisation experiments is indicated between brackets.

^aValues taken from Heyndrickx et al. [19]

TABLE 3.2: Hybridisation results with the microplate and the initial renaturation method^a (in bold face) from the *Myroides* strains.

| | % G+C ^b | LMG 1233 ^T | LMG 4028 | LMG 12841 | LMG 13344 | LMG 4029 ^T | LMG 12837 | LMG 14973 | LMG 12839 | LMG 14961 |
|--|--------------------|-----------------------|-----------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------|-----------------|
| <i>M. odoratus</i> LMG 1233 ^T | 35.6 [38] | 100 | 75±5 (2) | 71±5 (2) | 55±5 (2) | 14±5 (2) | | | | 14±5 (2) |
| <i>M. odoratus</i> LMG 4028 | 35.8 [38] | 81±8 (4) | 100 | 85±5 (2) | 57±5 (2) | | 9±5 (2) | | | |
| <i>M. odoratus</i> LMG 12841 | 35.6 [37] | 87±12 (4) | 90±6 (4) | 100 | 55±5 (2) | | | | | |
| <i>M. odoratus</i> LMG 13344 | 35.6 [37] | 65±12 (4) | 61±7 (4) | 63±2 (4) | 100 | | | | | |
| <i>M. odoratimimus</i> LMG 4029 ^T | 33.9 [34] | 11±3 (4) | 7±4 (4) | 14±1 (4) | 16±2 (4) | 100 | 98±5 (2) | | | 80±5 (2) |
| <i>M. odoratimimus</i> LMG 12837 | 34.2 [34] | 13±5 (4) | 8±2 (4) | 12±2 (4) | 13±1 (4) | 97±14 (4) | 100 | 91±5 (2) | 89±5 (2) | 81±5 (2) |
| <i>M. odoratimimus</i> LMG 14973 | 34.3 [34] | 15±5 (4) | 12±7 (4) | 14±3 (4) | 15±4 (4) | 101±10 (4) | 92±12 (4) | 100 | 89±5 (2) | 83±5 (2) |
| <i>M. odoratimimus</i> LMG 12839 | 34.0 [30] | 13±18 (3) | 9±21 (3) | 13±16 (3) | 15±17 (3) | 117±124 (3) | 100±108 (3) | 94±97 (3) | 100 | 83±5 (2) |
| <i>M. odoratimimus</i> LMG 14961 | 34.1 [35] | 14±5 (3) | 9±8 (3) | 13±4 (3) | 13±3 (3) | 99±4 (3) | 90±10 (3) | 89±7 (3) | 120±122 (2) | 100 |

Note: Values are means ± standard deviations. The number of hybridisation experiments is indicated between brackets.

^aValues taken from Vancanneyt et al. [45]

^bValues between [] are from Vancanneyt et al[45]

3.3.4.3 *Vibrio* hybridisations

The results obtained for the *Vibrio* strains with the two hybridisation methods are presented in Table 3.3. Both methods clearly separate *V. harveyi* from *V. alginolyticus*. Without exception, microplate values are slightly higher than values obtained with the initial renaturation method. Hybridisations with the DNA from *V. alginolyticus* LMG 18301 showed higher standard deviations. Integrity of this DNA could not be confirmed by agarose gel electrophoresis.

3.3.4.4 *Xanthomonas* / *Stenotrophomonas* hybridisations

The hybridisation results with *Xanthomonas* / *Stenotrophomonas* DNA are presented in Table 3.4. Strains in this group were selected from the work of Vauterin et al. [48] because they showed intermediate DNA relatedness values with the initial renaturation method. Again there is a good correlation between both methods, although standard deviations are somewhat higher than in the other groups.

3.3.4.5 Global comparison

A linearity plot is presented in Fig. 3.6. The hybridisations with the - fragmented - DNA of *M. odoratimimus* LMG 12839 and the hybridisation of *Bacillus* sp. LMG 17892 DNA with *Bacillus sporothermodurans* LMG 17894^T DNA were omitted. With the mathematical relation between the microplate method (y) and the initial renaturation method (x) given by $y = a.x + b$, a and b turned out to be 1.05 and 2.77, respectively. For a perfect agreement, of course, $a = 1$ and $b = 0$. The correlation between the theoretical model and the experimental results was 97.1 %. The DNA relatedness data from the microplate hybridisation method were thus slightly higher than those from the initial renaturation method under the conditions used. The high background values in the 20-30 % DNA relatedness range as shown for the initial renaturation method [21], was not undoubtedly confirmed in the plot of our experimental data (Fig. 3.6).

TABLE 3.3: Hybridisation results with the microplate and the initial renaturation method^a (in bold face) from the *Vibrio* strains.

| | % G+C | LMG 4044 ^T | LMG 18298 | LMG 18299 | LMG 18300 | LMG 18301 | LMG 18302 |
|---|-------|-----------------------|-----------------|-----------------|-----------------|-----------|-----------------|
| <i>V. harveyi</i> LMG 4044 ^T | 45.0 | 100 | 79±5 (2) | 87±5 (2) | 22±5 (2) | | |
| <i>V. harveyi</i> LMG 18298 | 44.8 | 87±7 (6) | 100 | 85±5 (2) | 25±5 (2) | | 11±5 (2) |
| <i>V. harveyi</i> LMG 18299 | 44.9 | 90±5 (5) | 90±3 (5) | 100 | 17±5 (2) | | 25±5 (2) |
| <i>V. alginolyticus</i> LMG 18300 | 44.4 | 34±3 (5) | 33±2 (5) | 35±3 (4) | 100 | | 55±5 (2) |
| <i>V. alginolyticus</i> LMG 18301 | 46.4 | 31±13 (5) | 34±11 (5) | 32±10 (4) | 75±36 (4) | 100 | 72±5 (2) |
| <i>V. alginolyticus</i> LMG 18302 | 44.8 | 25±6 (4) | 23±6 (4) | 24±4 (3) | 57±14 (3) | 85±25 (2) | 100 |

Note: Values are means ± standard deviations. The number of hybridisations is indicated between brackets.

^aValues taken from Vandenberghe et al. (in preparation)

TABLE 3.4: Hybridisation results with the microplate and the initial renaturation method^a (in bold face) from the *Xanthomonas* and *Stenotrophomonas* strains.

| | % G+C ^b | LMG 706 | LMG 708 ^T | LMG 5743 ^T | LMG 9181 | LMG 682 | LMG 538 t1 ^T | LMG 958 ^T |
|--|--------------------|-----------|----------------------|-----------------------|-----------------|-----------------|-------------------------|----------------------|
| <i>X. fragariae</i> LMG 706 | 62.2 | 100 | 91±5 (2) | | | 24±5 (2) | 32±4 (2) | |
| <i>X. fragariae</i> LMG 708 ^T | 61.9 [63.3] | 101±8 (5) | 100 | 30±5 (2) | 20±5 (2) | 19±5 (2) | | 5±5 (2) |
| <i>X. populi</i> LMG 5743 ^T | 63.0 | 39±14 (5) | 39±13 (4) | 100 | 37±5 (2) | 40±5 (2) | 21±5 (2) | 10±5 (2) |
| <i>X. axonopodis</i> LMG 9181 | 64.6 | 27±5 (5) | 28±9 (4) | 29±12 (4) | 100 | 57±5 (2) | 55±5 (2) | 15±5 (2) |
| <i>X. axonopodis</i> LMG 682 | 64.5 | 26±8 (5) | 28±9 (4) | 28±11 (4) | 77±17 (4) | 100 | 47±5 (3) | 5±5 (2) |
| <i>X. axonopodis</i> LMG 538 t1 ^T | 64.1 [65.0] | 31±14 (5) | 29±12 (4) | 30±12 (4) | 62±21 (4) | 70±19 (4) | 100 | 13±5 (2) |
| <i>S. maltophilia</i> LMG 958 ^T | 65.9 [65.5] | 6±3 (5) | 7±4 (4) | 7±4 (4) | 8±4 (4) | 11±5 (4) | 12±6 (4) | 100 |

Note: Values are means ± standard deviations. The number of hybridisation experiments is indicated between brackets.

^aValues taken from Vauterin et al. [48]

^bValues between [] are from Vauterin et al. [48]

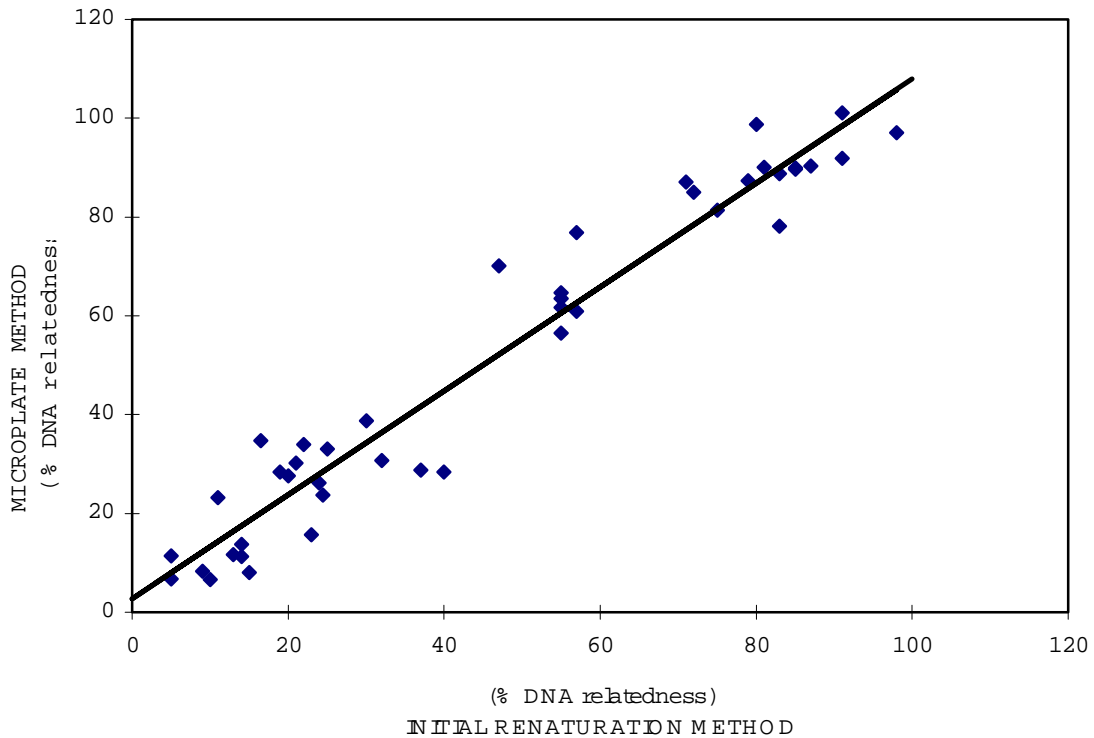


FIG. 3.6: Correlation between microplate and initial renaturation DNA relatedness data.

In Fig. 3.7, results are presented as a bar diagram with error flags. Only for *Bacillus* sp. LMG 17892 x *Bacillus sporothermodurans* LMG 17894^T, *Vibrio harveyi* LMG 4044^T x *Vibrio alginolyticus* LMG 18300, *V. harveyi* LMG 18299 x *V. alginolyticus* LMG 18300 and *Myroides odoratimimus* LMG 4029^T x *M. odoratimimus* LMG 14961, significantly different DNA relatedness values were obtained for both methods.

In our hands, the microplate DNA-DNA hybridisation method showed an average standard deviation of $\pm 7\%$, calculated from 74 hybridisations as the results of 291 individual experiments. This is slightly higher than the 5% standard deviation as calculated for the initial renaturation method.

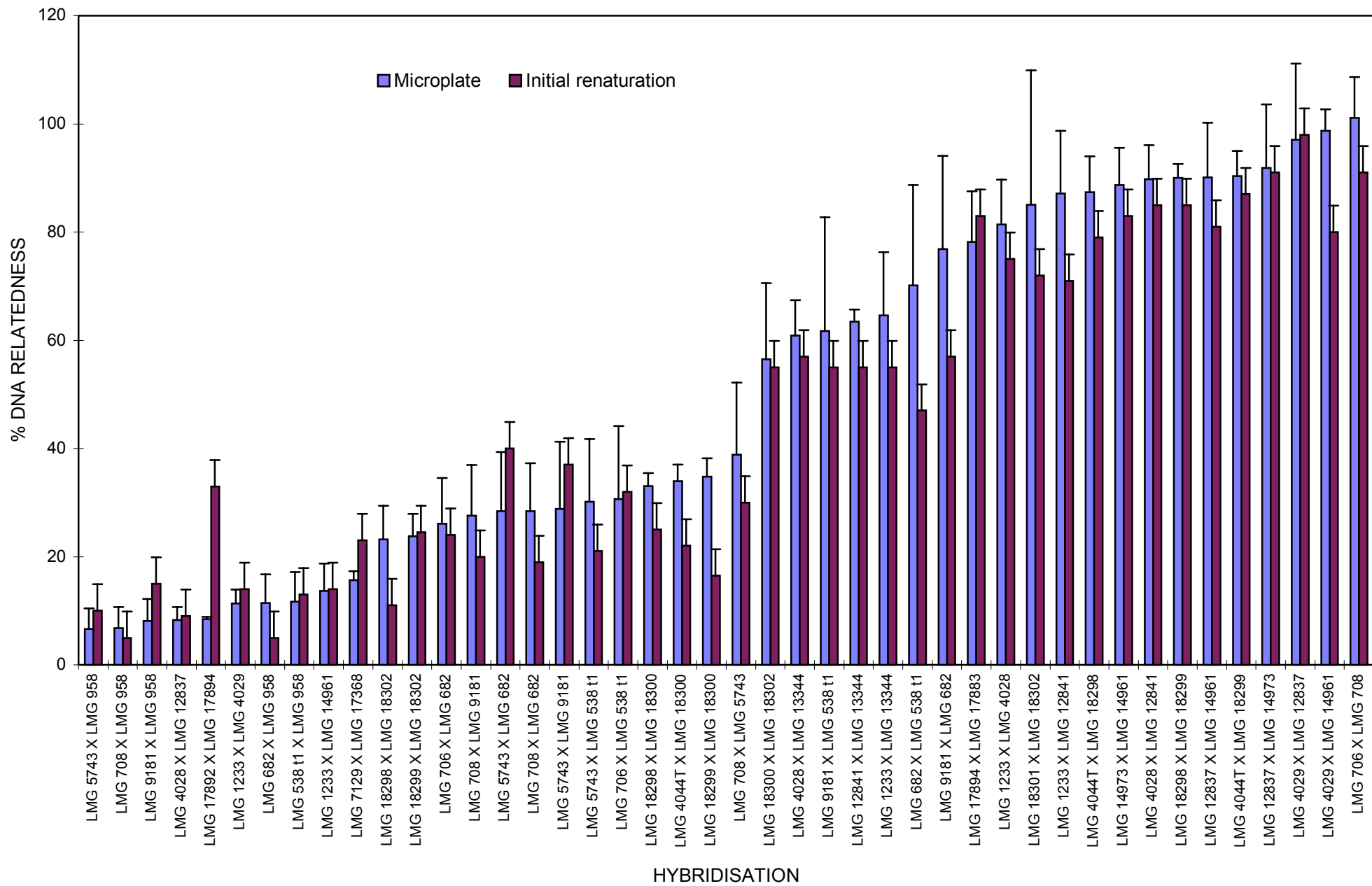


FIG. 3.7: Percentage DNA relatedness as determined with the microplate DNA-DNA hybridisation method and the initial renaturation method. With the microplate method, the values are averages of 2 to 5 independent experiments. The standard deviation is presented as an error flag. Hybridisations with the initial renaturation method were performed in duplicate and 5 % was assumed to be the error on the determination.

3.3.5 Discussion

Under the experimental conditions used, the microplate method is reproducible and shows a very good correlation with the initial renaturation method. The microplate method was already shown to correlate very well with a radioisotope membrane filter method for a limited number of hybridisations [18] and with the S1 nuclease method [52]. Extensively fragmented DNA cannot be used with the microplate method. Unreliable data caused by DNA fragmentation can easily be identified by careful examination of the raw fluorescence values or by discrepancies between reciprocal reactions. Reliable DNA relatedness measurements can be obtained between organisms with low or high % G+C DNA provided that the hybridisation temperature is adjusted properly.

The microplate method can thus be applied as a reliable taxonomic tool. Because of its high speed in generating data, in combination with the limited amount of DNA needed, the number of hybridisation reactions can easily be extended, in order to provide a more complete overview of the genetic relatedness of a bacterial group.

3.4 FURTHER EXPERIENCE WITH MICROPLATE DNA-DNA HYBRIDISATIONS

3.4.1 Possible sources of error

3.4.1.1 Effect of DNA quality

Although the microplate DNA-DNA hybridisation method generally yields reproducible and reliable data, problems can arise when DNA preparations of low quality are utilized. The quality of a DNA preparation is reflected in (i) the average fragment size of the DNA molecules and (ii) its purity, i.e. the amount of contaminating proteins, polysaccharides, and RNA. The use of sheared or impure DNA negatively affects the hybridisation results, principally by reducing the efficiency of non-covalent DNA adsorption to the microwells. Problems of this kind are revealed through significant divergences of the reciprocal hybridisation values. Conversely, small differences between reciprocal values are an indication for reliable data and can therefore be considered as a “quality control parameter” in DNA-DNA hybridisation experiments.

3.4.1.2 Enzymatic development of the hybridisation reaction

The amount of photobiotin-labelled probe DNA that hybridised to the immobilized DNA in the microwell is quantified using a streptavidin- β -D-galactosidase complex and a fluorogenic substrate, 4-methylumbelliferyl- β -D-galactopyranoside (4-MUF). The streptavidin- β -D-galactosidase complex binds readily and quantitatively to the biotin moieties on the probe DNA. Provided that unspecific adsorption of the enzyme complex is prevented by adequate blocking of free adsorption sites on the polystyrene, no problems are to be encountered in this step. As explained below, the fluorogenic enzymatic reaction requires more attention.

In Fig. 3.8, a typical enzymatic development of a hybridisation reaction is presented. The solid lines represent the fluorescent signal directly after substrate addition (0 min) and at 5 min intervals thereafter of the homologous reaction (■) and the heterologous reaction (●). Bars represent the reassociation values (signal of the heterologous reaction / signal of the homologous reaction), calculated at a given time interval after substrate addition. Dashed lines are extrapolations from the initial fluorescence measurements at 5, 10, and 15 min. At time 0 some fluorescence is detected already, because in practice, some time (several seconds) elapses between substrate addition and the actual measurement. Because of the relatively error associated with these determinations, reassociation values measured at 0 min are further not taken into account. Fluorescence values increase linear with time, until at a given point (in this example, after approx. 15 min for the homologous reaction and 30 min for the heterologous reaction) the slope of the line starts decreasing. As the slope homologous reaction decreases faster than the slope of the heterologous reaction, the calculated reassociation values increase with time (Fig. 3.8). This phenomenon can be explained as follows. In the initial phases of the enzymatic reaction, substrate is in

excess and the rate of transformation is determined solely by the enzyme concentration. With time, more and more substrate is converted and the enzymatic reaction slows down due to decreased substrate availability. Obviously, since we want to measure the amount of enzyme in the microwells (as this is an indirect parameter for the amount of hybridised probe DNA), fluorescence values from the linear part of the graphs (e.g. after 15 min) need to be used.

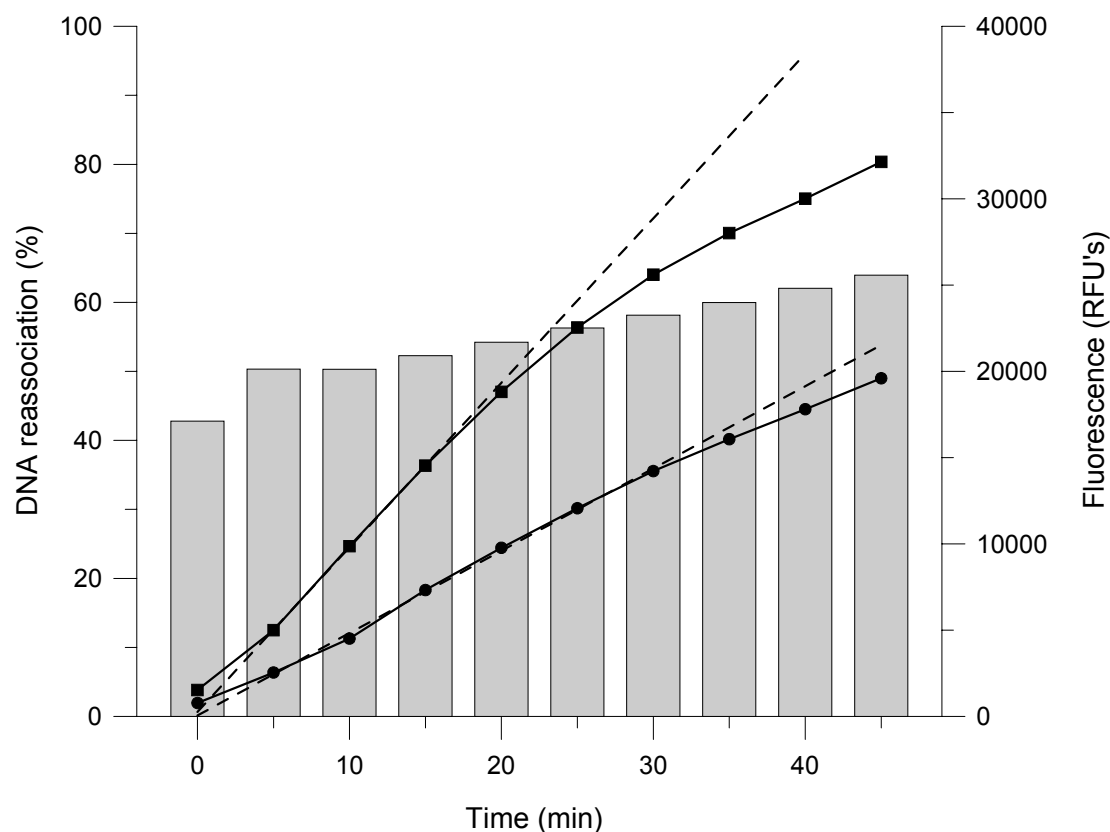


FIG. 3.8: DNA reassociation values (bars) and fluorescent signal (lines) in function of the time elapsed after addition of substrate. Fluorescence values of the homologous reaction are presented by squares (■) and values of the heterologous reaction by dots (●). Dashed lines are extrapolations from the initial fluorescence values (5, 10, and 15 min after addition of substrate).

3.4.2 Influence of hybridisation temperature

DNA-DNA hybridisations with the microplate method were always performed under stringent conditions, i.e. the hybridisation temperature was always equal or slightly higher than the calculated optimal renaturation temperature (T_{OR}). For practical reasons, experiments within a given group of organisms (representing a genus or a group of related genera), were always done at the same temperature. As a consequence, hybridisation temperatures up to 10°C above the calculated T_{OR} were sometimes applied.

The influence of the hybridisation temperature, in an interval that represents stringent conditions, was assessed with four *Myroides* strains. The calculated T_{OR} for these strains was 29°C and hybridisations were performed at 28, 33, 37, 40, and 44°C (Fig. 3.9).

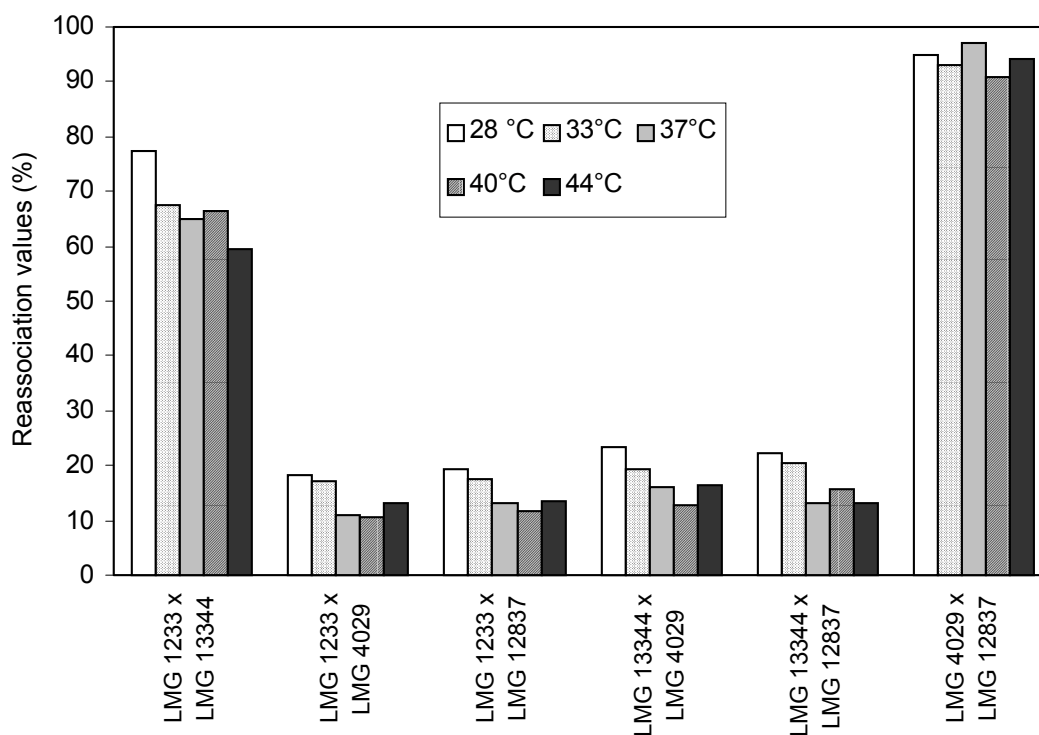


FIG. 3.9: Effect of hybridisation temperature on DNA reassociation values, as determined with *Myroides odoratus* LMG 1233^T, *M. odoratus* LMG 13344, *M. odoratimimus* LMG 4029^T, and *M. odoratimimus* LMG 12837. The optimal renaturation temperature for these strains is 29°C.

As can be derived from Fig. 3.9, all except the highest (LMG 4029^T x LMG 12837) hybridisation values, decrease slightly with increasing hybridisation temperature. However, considering the standard deviation of about 7% on the reassociation values, this decrease is probably not significant. Obviously, all measured fluorescence values markedly decreased with increasing hybridisation temperatures (on the average, about 25% lower fluorescence values were measured at 44°C compared to values at 28°C; data not shown).

3.4.3 Applications of the microplate DNA-DNA hybridisation technique

The microplate DNA-DNA hybridisation method as discussed above was applied in the present study for the classification of metal resistant *Ralstonia* (Chapter 5) and *Burkholderia* species able to degrade PCB's (Chapter 6) or 2,4-D (Chapter 7).

The microplate DNA-DNA hybridisation method was also used for the proposal of several *Ralstonia*, *Burkholderia*, and related species not directly connected to the present study [4, 5, 7, 46, 47] and for taxonomic studies of aerobic endospore formers [20, 27-29, 36], fish-associated bacteria [42, 43], symbiotic nitrogen fixers [50, 51], cellulose degrading bacteria [32], and various other taxonomical studies performed in the Laboratory of Microbiology Gent.

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CHAPTER 4
DETERMINATION OF NUCLEOTIDE
COMPOSITION VIA HPLC

"Not everything that can be counted counts, and not everything that counts can be counted"

Albert Einstein

CHAPTER 4 DETERMINATION OF NUCLEOTIDE COMPOSITION VIA HPLC

4.1 INTRODUCTION

The nucleotide composition of a bacterial genome, expressed as mole %G+C, is an important descriptive parameter. It varies between 24% and 76% in the bacterial world, but the range observed within a well-defined species is generally no more than 3% and no more than 10% within a well-defined genus [14]. Determination of the mole %G+C is considered mandatory by the “ad hoc committee for the re-evaluation of the species definition in bacteriology” for the type strain of the type species of a new genus [13]. Furthermore, essentially all proposals for “minimal standards” state that %G+C values should be part of the description of a new species (e.g. [1, 3, 4]).

The first methods for determination of the DNA base composition were developed in the 1950's and were based on chemical degradation (using formic acid) of DNA followed by paper chromatography. The findings that the ratios dA/T and dG/dC are essentially one [2] and that G+C values have taxonomic significance had a great impact on biology. However, it was difficult to achieve quantitative hydrolysis with formic acid without decomposition. The procedure was rather laborious and due to the many steps involved, the results were not always reliable.

After the discovery of the simple relationships between DNA base composition and denaturation temperature [8] and buoyant density in a CsCl gradient [12], physical methods for mole% G+C determination became popular. These indirect methods were faster and gave more reproducible results than the chemical methods, but were still relying on standards of which the base composition was chemically determined. As a consequence, no higher level of accuracy could be obtained. Furthermore, DNA fragment size and presence of modified bases influenced the results. One analysis typically required 10–50 microgram of DNA [8].

In 1984, three Japanese research groups independently presented a method based on nuclease P1 mediated DNA degradation and separation by HPLC. Enzyme-mediated degradation avoids the side reactions involved with formic acid decomposition. Nuclease P1 is an endo- and exonuclease isolated from *Penicillium citrinum* with a high specific activity towards RNA and single-stranded DNA. The enzyme is unusually heat-stable and has a pH optimum of about 5.3. In contrast to nuclease S1, nuclease P1 does not require the addition of Zn^{2+} for its activity, although this improves enzyme stability. The nucleotide mixture, obtained after treatment of DNA with nuclease P1, can be analysed using ion-exchange liquid chromatography [7]. Alternatively, the nucleotides can be dephosphorylated by alkaline phosphatase and then subjected to reversed-phase HPLC [15]. Detection of nucleosides is done via UV absorption at 270 nm, which corresponds to the absorption maximum of thymidine, the nucleoside with the lowest extinction coefficient of the normal nucleosides [15]. The level of accuracy required for the mole% G+C determination is high in comparison to other biochemical and microbiological experiments. Since relative amounts of nucleosides or nucleotides are measured, incorporation of a standard is essential. Accurate preparation of standard nucleotide solutions is complicated by the water content of the reagents, especially

dTMP is highly hygroscopic. Nucleosides are also hygroscopic and, in addition, largely differ in their water solubility. A standard equimolar mixture of four dNMPs is commercially available (Yamasa Shoyu Co. Ltd.). Perhaps an even more elegant solution is the use of a DNA preparation of an organism of which the complete genome sequence is known e.g. bacteriophage lambda DNA [9]. This reference DNA can be treated in exactly the same way as the unknown DNA samples.

Recently, Xu and co-workers presented a modern update of the denaturation temperature method using a Light Cycler real-time PCR apparatus combined with SYBR Green fluorescent detection of double-stranded DNA [16]. Although the procedure is slightly less reproducible than the HPLC method, it is very rapid and only requires extremely small amounts of DNA. The error previously associated with the use of an external standard is now overcome, since renaturation curves of bacterial genomes from which the complete sequence is known can be determined and used for calibration.

It was reported previously that enzymatic DNA digestion, followed by HPLC analysis of the resulting nucleoside mixture, provides a more reliable and much faster alternative for common physical methods [5]. In the present study, the determination of %G+C through melting temperature determination in the Laboratory of Microbiology Gent was therefore replaced by a HPLC-based method. A detailed description of the novel experimental protocol is presented in paragraph 4.2 and this protocol is evaluated based on the results of more than 2300 analyses in paragraph 4.3.

4.2 EXPERIMENTAL PROCEDURE

The here-employed method for determination of nucleoside composition was based on protocols of Mesbah et al. [9] and Tamaoka and Komagata [15].

Bacterial DNA was dissolved in 25 μ l 1 x TE (Tris-EDTA buffer), 0.1 x SSC (Standard Saline Citrate buffer) or double distilled water at a concentration of 350 μ g/ml ($OD_{260} = 7$). For each analysis series, a tube containing equal amounts of non-methylated bacteriophage lambda DNA (Sigma) and *E. coli* LMG 2093 DNA was included as external standard and control sample, respectively. The DNA solution was denatured by heating 10 min at 100°C in a water bath or heat block and immediately cooled on ice. After addition of 50 μ l 30 mM sodium acetate buffer (pH 5.3), 5 μ l 20 mM ZnSO₄; 3 μ l nuclease P1 solution (1 mg nuclease P1 (Sigma), dissolved in 1 ml 30 mM sodium acetate buffer + ZnSO₄; this equals 3 units nuclease P1 per reaction), the solution was vortexed, briefly centrifuged, and incubated for 1 h at 37°C in a water bath. Subsequently, 10 μ l phosphatase solution (7 μ l bacterial alkaline phosphatase solution (Sigma) added to 193 μ l 0.1 M glycine buffer (pH 10.4); this equals 10 units phosphatase per reaction) was added and after vortexing and short centrifugation, the mixture was incubated for 6 h at 37°C in a water bath. The principle of the enzymatic degradation is presented in Fig. 4.1.

After centrifugation for 2 min at 13000 rpm, 80 μ l of the nucleoside mixture was transferred to polypropylene conical HPLC vials (Alltech) for injection and subsequent separation by high-performance liquid chromatography. The HPLC configuration consisted of a solvent delivery system (Waters WISP model 501), an automatic injector (Waters WISP model 710 B), an UV-VIS absorbance detector (Waters model 484), and an integrator (Intersmat model II CR) and was equipped with a Waters SymmetryShield RP8 column assembly (analytical column and pre-column) thermostated at 37°C. As mobile phase 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile was used at a flow rate of 1 ml/min. The nucleoside composition, expressed as mole % G+C, was calculated using bacteriophage lambda DNA as the calibration reference. The genome of this phage was sequenced and the %G+C determined to be 49.858% [11].

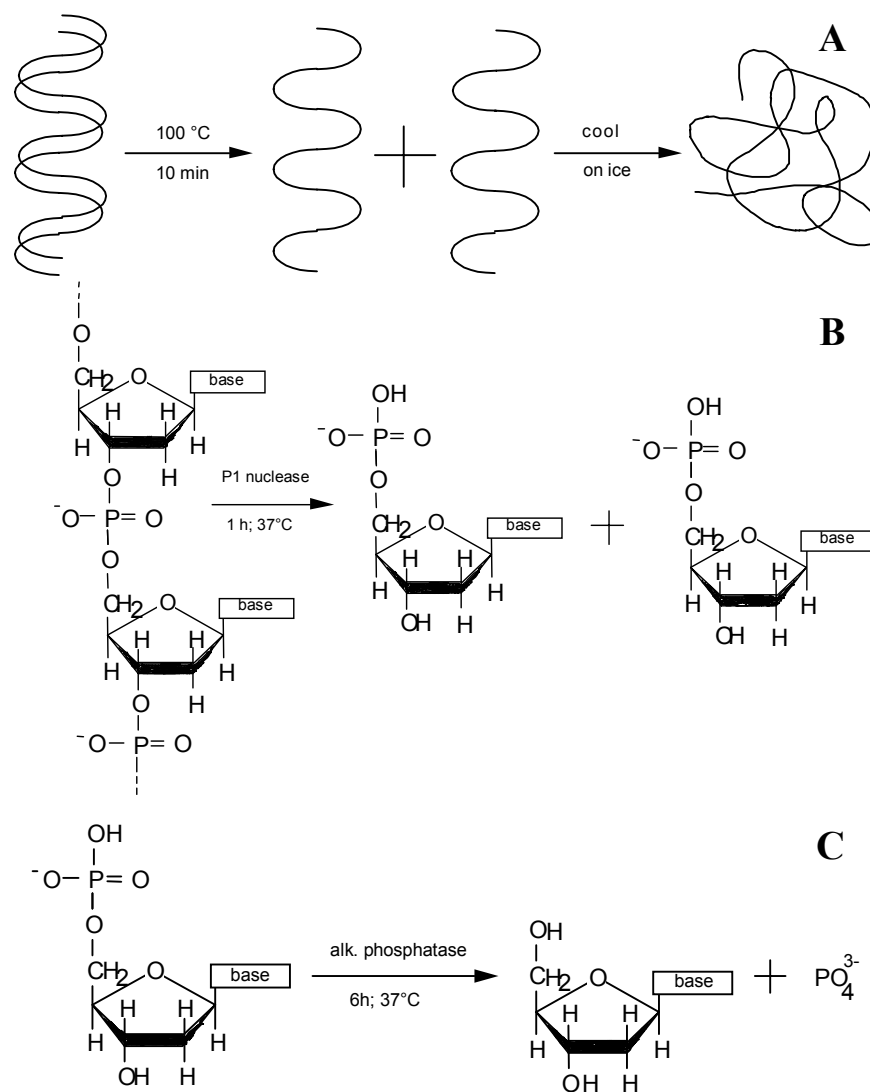


FIG. 4.1: Principle of the enzymatic DNA degradation. Complete conversion of DNA to a nucleoside mixture is performed in three steps: (A) heat-denaturation of double-stranded DNA into single-stranded DNA; (B) DNA degradation by nuclease P1 into nucleotides; (C) dephosphorylation of nucleotides to nucleosides.

4.3 EVALUATION OF THE METHOD

Comments on the determination of the nucleoside composition of DNA through HPLC analysis can be divided in aspects related to (i) HPLC separation of the nucleosides, (ii) enzymatic degradation of DNA, and (iii) quantification of the nucleosides. The various modifications made to the original protocol will be discussed under these headings. In the time period from March 1998 to December 2002 more than 2300 %G+C analyses of over 1500 different strains were performed in 103 analysis series. Conclusions that can be drawn from these data and from separate experiments will also be discussed.

4.3.1 HPLC separation of nucleosides.

Since a Waters CosmoSil 5C18 HPLC column as used in RIKEN [15] was not commercially available in Europe, a new protocol for HPLC separation of nucleosides was designed, based on a Waters SymmetryShield RP8 column. In the latter reversed-phase column, silanol groups on the silica surface are end-capped, which improves the separation of polar analytes. The retention time of nucleosides is strongly pH-dependent [5] and of course also depends on the hydrophobicity of the mobile phase. For an accurate %G+C determination, the four major deoxynucleosides (deoxycytidine dC, deoxyguanosine dG, thymidine T, and deoxyadenosine dA) need to be clearly separated from each other and from modified nucleosides that are possibly present in bacterial DNA. Furthermore, deoxynucleosides need to be separated from RNA nucleosides and from nucleotides (nucleoside monophosphates) to allow %G+C determination in the presence of contaminating RNA and to detect possible incomplete enzymatic degradation, respectively. The designed protocol meets all these requirements. In Fig. 4.2, chromatograms of pure DNA and an artificial mixture of all relevant nucleosides and nucleotides are presented.

Since the mole% G+C can be calculated from the ratio dG/T (see below), special emphasis was placed on the optimisation of the separation of these two peaks and to assure that no other components eluted in the same time interval. Since RNA nucleosides elute at different times than DNA nucleosides (Fig. 4.2), the %G+C of a DNA sample can be determined in the presence of contaminating RNA. In order to keep the retention times as constant as possible, the column assembly was thermostated at 37°C using a water jacket.

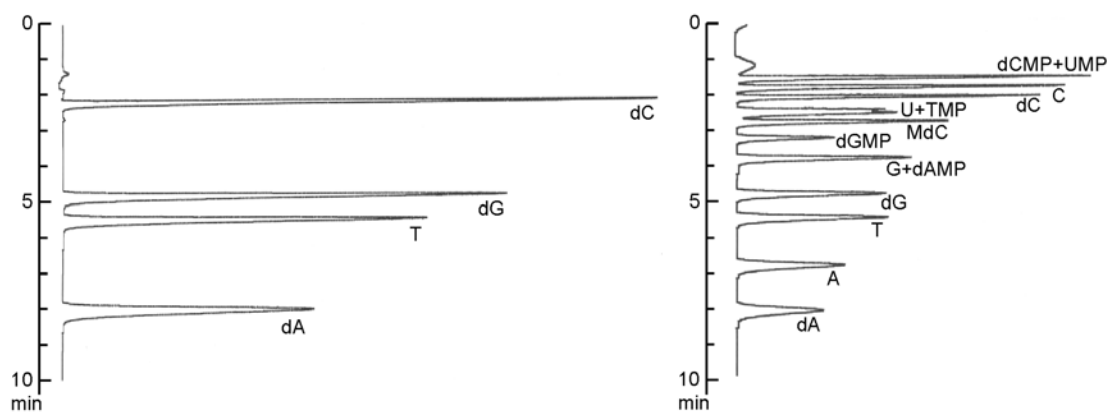


FIG. 4.2: HPLC separation of the four major deoxynucleosides from pure DNA (left) and of an artificial nucleoside and nucleotide mixture (right). dCMP: deoxycytidine monophosphate; UMP: uridine monophosphate; C: cytidine; dC: deoxycytidine; U: uridine; TMP: thymidine monophosphate; MdC: 5'-methyldeoxycytidine; dGMP: deoxyguanosine monophosphate; G: guanosine; dAMP: deoxyadenosine monophosphate; dG: deoxyguanosine; T: thymidine; A: adenosine; dA: deoxyadenosine

4.3.2 Enzymatic degradation of DNA.

Because the nucleotides are released from the DNA at different rates, incomplete degradation may produce significant errors. As pointed out by Mesbah and co-workers [9], the activity of nuclease P1 is affected by pH, salt concentration, and buffer composition. However, with purified DNA dissolved in standard low-salt buffers such as 1 x TE, 0.1 x TE or 0.1 x SSC or even dissolved in distilled water, no problems were encountered. The activity of bacterial alkaline phosphatase also shows different reaction rates towards the different nucleotide monophosphates, and its activity is sensitive to salt concentration and pH. An incubation of 6 h at 37°C is necessary to ensure complete hydrolysis [9].

The influence of the DNA amount on the ratios of nucleosides released from the DNA after a standard enzymatic treatment is shown in Fig. 4.3. The DNA amount of *Burkholderia* sp. LB400 (62.5 mole% G+C) had a clear impact on the ratios of released nucleosides (Fig. 4.3). Below 2.5 µg, the determination was not accurate due to the small size of the peaks in the HPLC chromatogram. Above 10 µg, relatively less dC was released from the DNA. At even higher concentrations (15 µg), also the ratios of dG to T and dA decreased. Therefore, with strains of high %G+C, a too high DNA concentration will very often result in an underestimation of the %G+C value. The DNA of *Bacillus sporothermodurans* LMG 17894^T (36.5 mole% G+C), showed a much less pronounced effect on the nucleoside ratios in the DNA concentration range tested (Fig. 4.3). The nucleoside ratios were stable up to 20 µg DNA. At higher amounts, the release of both dC and T from the DNA was decreased. Small amounts of nucleotides (monophosphates) were detected in the chromatograms of the samples with higher DNA concentrations (data not shown). From the combined results of both degradation experiments, it appears that dC released from DNA at the slowest rate.

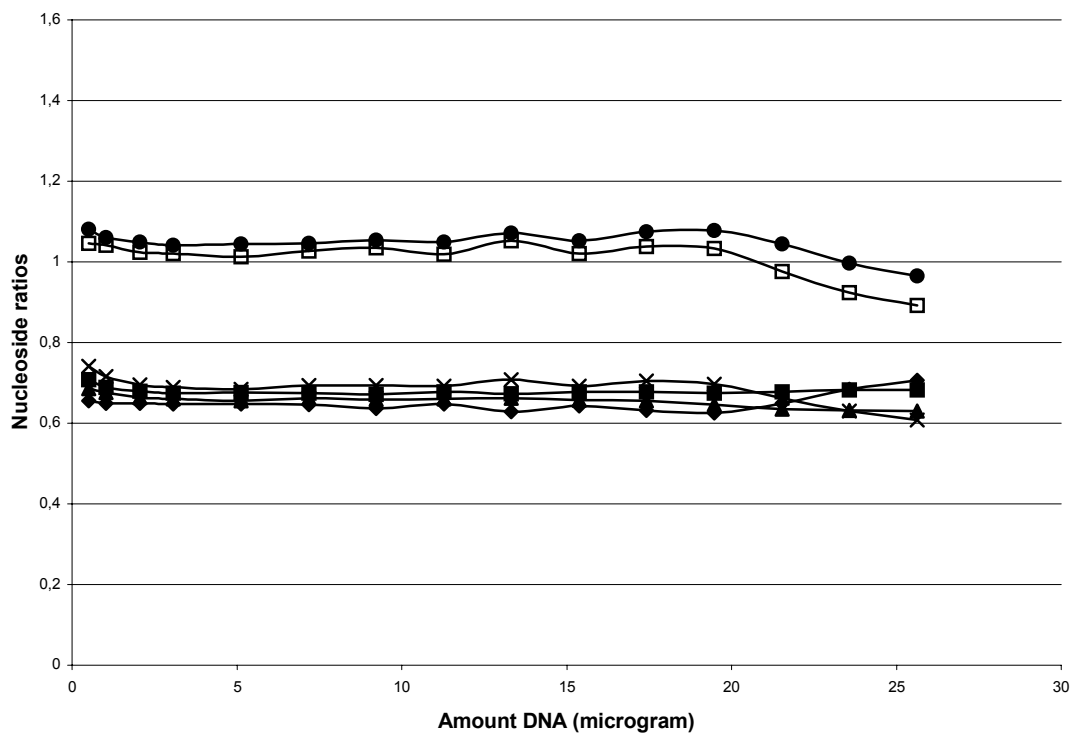
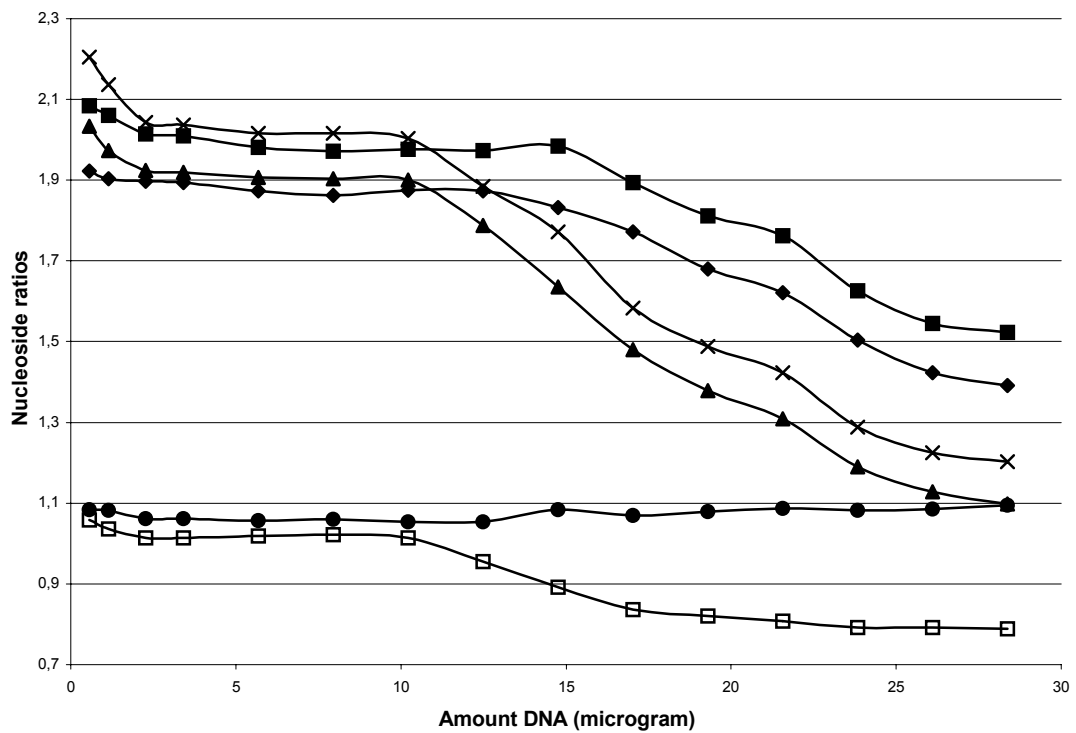


FIG. 4.3: Nucleoside ratios in function of the DNA amount in samples from *Burkholderia sp. LB400* (above) and *Bacillus sporothermodurans* LMG 17984^T (below). Symbols: [dG]/[T] (◆), [dG]/[dA] (■), [dC]/[T] (▲), [dC]/[dA] (×), [T]/[dA] (●), and [dC]/[dG] (□) with [dG], [dC], [T], and [dA] representing the peak areas of deoxyguanosine, deoxycytidine, thymidine, and deoxyadenosine, respectively.

Preferential substrate utilisation was previously found by Mesbah and co-workers [9], although in their experiments with nuclease P1 combined with bovine intestinal mucosa alkaline phosphatase, deoxyguanosine monophosphate was hydrolysed at the slowest rate. This indicates possibly a different substrate preference of bovine intestinal mucosa alkaline phosphatase compared to the bacterial alkaline phosphatase used in our experiments, since the other experimental conditions were identical.

In general, with amounts of purified DNA between 2.5 and 10 µg dissolved in 1 x TE, 0.1 x TE, 0.1 x SSC or distilled water, no problems are to be expected due to incomplete enzymatic degradation of DNA.

4.3.3 Quantification of nucleosides and calculation of %G+C.

Each sample is analysed in triplicate, while the standard is injected five times per series. Calculations are based on average values of the replicate injections. The mole% G+C can be calculated simply from the sum of the relative concentrations (in %) of dG and dC. However, since dC and dA are frequently modified in bacteria, a better approach is to calculate the %G+C from the ratio dG to T, thereby assuming that dA = T and dG = dC [10]. The formula used is given below.

$$\text{mole}\%G + C = \frac{1}{Y \cdot \frac{[T]}{[dG]}} \quad \text{with} \quad Y = \frac{[dG]_{STD}}{[T]_{STD}} \cdot \frac{\text{mole}\%T}{\text{mole}\%dG}$$

[T] and [dG] are the peak areas for T and dG, respectively. The mole% T and mole% dG are calculated from the sequence of the standard DNA [10].

Originally, an equimolar nucleotide standard mixture (Yamasa Shoyu Co. Ltd.) was used for calibration. Because this seemed to contribute to the variability associated with the determination (data not shown) and also for practical reasons of availability and cost, the nucleotide mixture was replaced by bacteriophage lambda DNA. This DNA is sequenced and commercially available in a non-methylated form from several suppliers. As an extra control, an *E. coli* LMG 2093 DNA sample was included in each analysis series. The variability of the [T]/[dG] ratio of bacteriophage lambda DNA compared to DNA from *E. coli* LMG 2093 is illustrated in Fig. 4.4.

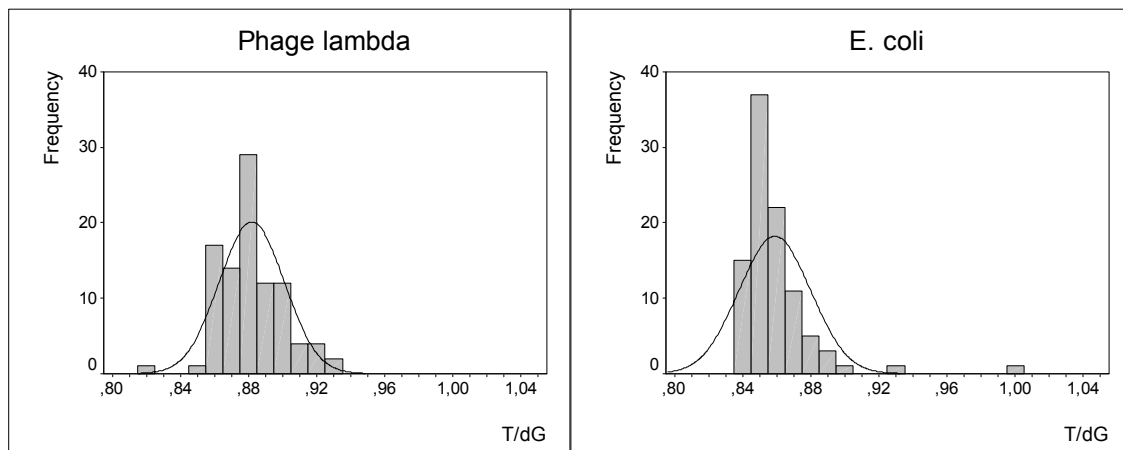


FIG. 4.4: Variability of the ratio $[T]/[dG]$ of bacteriophage lambda (left) and *E. coli* LMG 2093 DNA (right). Both DNA's were analysed on 96 occasions and the presented values were always based on three injections per sample. The curve superimposed on the histograms is a normal distribution calculated from the measurements.

Both histograms show a normal (or Gaussian) distribution and therefore, standard deviations can be used as a measure of the variability. The standard deviations of the $[T]/[dG]$ ratio from phage lambda and *E. coli* DNA are 0.0192 and 0.0212, respectively. Therefore, it can be concluded that the relatively small size of the bacteriophage lambda genome (48,502 bp) compared to a bacterial genome ($\pm 5.10^6$ bp) does not adversely affect the variability of the determination. A 0.02 deviation of the $[T]/[dG]$ ratio of bacteriophage lambda DNA corresponds to approximately 0.44 – 0.55% difference in mole% G+C calculated based on this standard.

A measure for the overall reproducibility of the %G+C determination with this method can be calculated in two different ways, either based on the *E. coli* LMG 2093 DNA control sample or based on replicate analyses of other strains at separate occasions. The average difference of the mole% G+C determined for *E. coli* (95 analyses, average calculated from 4465 differences) was 0.40 mole%, compared with 0.42 mole% for the other strains that were analysed more than once (350 strains, analysed 2 to 6 times, average calculated from 1061 differences). These values are nearly identical to the values reported by Kaneko and co-workers [5]).

With increasingly more complete bacterial genome sequences coming available (for an overview, see <http://wit.integratedgenomics.com/GOLD/>), %G+C values based on sequencing data can be used to evaluate the accurateness of the HPLC method. However, no systematic comparative study was undertaken and from the more than 1500 strains analysed by HPLC, a complete genome sequence was available only for *Bradyrhizobium japonicum* USDA110 [6] and for *Burkholderia cenocepacia* LMG 16656 (http://www.sanger.ac.uk/Projects/B_cepacia/). Calculated differences in nucleotide composition between the values obtained with the HPLC method as compared with sequencing results were 0.0 and 0.2 mole% G+C, respectively. Therefore, no evidence for the occurrence of systematic errors in the %G+C determination by HPLC could be found.

4.4 CONCLUSIONS

The HPLC-based protocol presented here is a modification of existing procedures that offers a relatively fast, robust, and accurate determination of the mole% G+C of bacterial genomes. In comparison with the “classical” %G+C determination by thermal denaturation, it is faster, more accurate and requires less sample. The method is furthermore not affected by the presence of RNA in the sample, small variations in salt concentration, DNA fragmentation, nor by the presence of modified nucleosides. A similar reproducibility was calculated as reported for other HPLC-based protocols. The usefulness of the procedure has been shown by the determination of the %G+C of over 1500 different bacterial strains.

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CHAPTER 5
METAL-RESISTANT *RALSTONIA*

“The trouble with normal is it always gets worse”

Bruce Cockburn

CHAPTER 5 METAL-RESISTANT *RALSTONIA*

5.1 CLASSIFICATION OF METAL-RESISTANT BACTERIA FROM INDUSTRIAL BIOTOPES AS *RALSTONIA CAMPINENSIS* SP. NOV., *RALSTONIA METALLIDURANS* SP. NOV., AND *RALSTONIA BASILENSIS* STEINLE ET AL. 1998 EMEND.[§]

5.1.1 Summary

Thirty-one heavy metal-resistant bacteria isolated from industrial biotopes were subjected to a polyphasic characterization, including 16S rDNA sequence analysis, DNA-DNA hybridisations, biochemical tests, whole-cell protein and fatty acid analyses. All strains were shown to belong to the *Ralstonia* branch of the β -subclass of the *Proteobacteria*. Whole-cell protein profiles and DNA-DNA hybridisations revealed two clearly distinct groups, showing low similarity with known *Ralstonia* species. These two groups of respectively 8 and 17 isolates were assigned to two new species for which the names *Ralstonia campinensis* and *Ralstonia metallidurans* are proposed. Type strains are WS2 (= LMG 19282 = CCUG 44526) and CH34 (= LMG 1195 = DSM 2839), respectively. Six isolates were allocated to *Ralstonia basilensis*, presently containing only the type strain, and therefore an emendation of the latter species description is proposed.

5.1.2 Introduction

Metal-resistance has been reported for a number of gram-negative bacteria belonging to the *Ralstonia* lineage of the β -*Proteobacteria* [9, 18, 25, 26]. The resistance to Cd^{2+} , Zn^{2+} , Co^{2+} has been shown to be located on a *czc* operon of the plasmid pMOL30 (240 kb), while Co^{2+} combined with Ni^{2+} resistance is located on the *cnr* operon of the plasmid pMOL28 (163 kb) [14, 18, 20, 27]. Other plasmid-borne resistances to metals include resistance to copper (*cop*), lead (*pbr*), and mercury (*mer*) on pMOL30 and resistance to chromate (*chr*) and mercury on pMOL28 (for a review see [17]). Hybridisation with a *czc* gene probe showed the spread of the former resistance types over a variety of closely related taxa [1].

The application of metal-resistant bacteria in bioremediation processes offers attractive perspectives for biomonitoring (via biosensors), treatment of wastewater and the recycling of polluted soils [3, 8]. In the case of the *Ralstonia* strains mentioned above, the knowledge of the exact taxonomic position is of utmost importance. Indeed, apart from organisms that can be used for bioremediation of polluted soils, the genus *Ralstonia* contains also an important plant pathogen (*Ralstonia solanacearum* [12]) and several opportunistic human pathogens such as *Ralstonia pickettii* [24], *Ralstonia mannitolilytica* [5], *Ralstonia gilardii* [2], and *R. paucula* [30]. *Ralstonia* strains with

[§] Redrafted from Goris, J., P. De Vos, T. Coenye, B. Hoste, D. Janssens, H. Brim, L. Diels, M. Mergeay, K. Kersters, and P. Vandamme. 2001. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov., and *Ralstonia basilensis* Steinle et al. 1998, emend. Int J Syst Evol Microbiol **51**:1773-1782.

potential large-scale applications for bioremediation should obviously be distinct from (opportunistic) pathogens for humans and plants. Recent taxonomic studies describing new *Ralstonia* species not only highlight the unsuspected biodiversity of the genus but also its weak and incomplete internal classification. The present report deals with the polyphasic classification of 31 metal-resistant strains, isolated from diverse industrial locations. Six strains could be allocated to *Ralstonia basilensis*, while two groups of respectively 8 and 17 strains are representatives of new *Ralstonia* species, for which we propose the names *Ralstonia campinensis* and *Ralstonia metallidurans*.

5.1.3 Materials and methods

5.1.3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 5.1. After being checked for purity, subcultures of all strains were maintained on slants of Tryptic Soy Agar (TSA; Trypticase Soy Broth from Becton & Dickinson supplemented with 15 g per liter Bacto Agar from Difco) in the refrigerator, and lyophilized for long-term storage.

TABLE 5.1: List of strains studied

| Strain number as received | Other strain designation | Source |
|------------------------------|--|--|
| <i>Ralstonia campinensis</i> | | |
| DS185 | LMG 19284 | Zinc desert, Lommel, Belgium |
| LH103 | LMG 19285 | Non-ferrous industry, Tienen, Belgium |
| LH104 | | Non-ferrous industry, Tienen, Belgium |
| LH105 | LMG 19283 | Non-ferrous industry, Tienen, Belgium |
| LH106 | | |
| WS2 ^T | LMG 19282 ^T , CCUG 44526 ^T | Zinc desert, Lommel, Belgium |
| WS3 | | |
| WS5 | | Zinc desert, Lommel, Belgium |
| <i>Ralstonia eutropha</i> | | |
| ATCC 17697 ^T | LMG 1199 ^T , TF93 ^T | Soil (USA, 1957) |
| ATCC 17698 | LMG 1200 | Garden pond, sludge, Germany |
| ATCC 17699 | LMG 1201, H16 | Sludge, Weende-Quelle, Göttingen, Germany |
| ATCC 17700 | LMG 1202, H20 | Soil, USA |
| ATCC 17702 | LMG 1203 | Soil |
| ATCC 17704 | LMG 1204 | Soil |
| ATCC 17705 | LMG 1205 | Soil |
| ATCC 17706 | LMG 1206 | |
| ATCC 17709 | LMG 1209 | Soil, USA |
| ATCC 17710 | LMG 1210 | USA |
| ATCC 25207 | LMG 1211 | |
| ATCC 33178 | LMG 1190 | Paddy field soil, Japan |
| N9A | LMG 1196 | |
| <i>Ralstonia basilensis</i> | | |
| DSM 11853 ^T | LMG 18990 ^T , LMG 19474 ^T , RK1 ^T | Freshwater pond sediment, Amponville, France |
| ER8 | LMG 19287 | Non-ferrous industry, Overpelt, Belgium |
| ER107 | | Non-ferrous industry, Overpelt, Belgium |
| ER121 | | Non-ferrous industry, Overpelt, Belgium |
| ER122 | LMG 19286 | Non-ferrous industry, Overpelt, Belgium |

| Strain number as received | Other strain designation | Source |
|----------------------------------|--|---|
| KS1 SV884 | LMG 19289 LMG 19288 | Zinc desert, Lommel, Belgium |
| <i>Ralstonia metallidurans</i> | | |
| 31A | LMG 19293 | Metal factory, Holzminden, Germany |
| AB2 | | Mine area, Kipushi, Congo |
| AS2 | LMG 19295 | Mine area, Likasi-Sud, Congo |
| AS167 | LMG 19291 | Mine area, Likasi-Sud, Congo |
| AS168 | | Mine area, Likasi-Sud, Congo |
| CH34 ^T | LMG 1195 ^T , CCUG 38400 ^T , DSM 2839 ^T , ATCC 43123 ^T | Wastewater zinc factory "Métallurgie de Prayon", Liège, Belgium |
| CH42 | | Polluted sediments, "Métallurgie de Prayon", zinc factory, Liège, Belgium |
| CH45 | | Polluted sediments, "Métallurgie de Prayon", zinc factory, Liège, Belgium |
| CH79 | LMG 19296 | Polluted sediments, "Métallurgie de Prayon", zinc factory, Liège, Belgium |
| Sh2-1 | LMG 19294 | Mine area, Shituru, Congo |
| SV661 | LMG 19290 | Non-ferrous industry, Beerse, Belgium |
| VA1 | | Mine area, Likasi-Sud, Congo |
| VA2 | | Mine area, Likasi-Sud, Congo |
| VA3 | | Mine area, Likasi-Sud, Congo |
| VA7 | LMG 19297 | Mine area, Likasi-Sud, Congo |
| VA10 | LMG 19292 | Mine area, Likasi-Sud, Congo |
| VA11 | | Mine area, Likasi-Sud, Congo |
| <i>Ralstonia pickettii</i> | | |
| CCUG 3318 ^T | LMG 5942 ^T , ATCC 27511 ^T | Patient after tracheotomy, Los Angeles, USA |
| <i>Ralstonia solanacearum</i> | | |
| NCPPB 325 ^T | LMG 2299 ^T , ATCC 11696 ^T | <i>Lycopersicon esculentum</i> , USA |
| <i>Ralstonia gilardii</i> | | |
| API 141-2-84 ^T | LMG 5886 ^T | Whirlpool |
| <i>Ralstonia paucula</i> | | |
| CDC E6793 ^T | LMG 3244 ^T | Human, respiratory tract, USA |
| CIP 62.31 | LMG 3413, CCUG 13723 | |
| <i>Ralstonia oxalatica</i> | | |
| Ox1 ^T | LMG 2235 ^T , DSM 1105 ^T | Indian earthworm |
| <i>Ralstonia mannitolilytica</i> | | |
| NCIB 10805 ^T | LMG 6866 ^T | Hospital outbreak, St. Thomas, London, UK |
| <i>Ralstonia</i> sp. | | |
| ATCC 17707 | LMG 1207 | Soil |
| JMP134 | LMG 1197 | Agricultural soil, Australia |

API, Appareils et Procédés d'Identification, Montalieu-Vercieu, France; ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Sweden; CDC, Center for Disease Control, Atlanta, GA, USA; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, United Kingdom.

5.1.3.2 PAGE of whole-cell proteins

Cells were grown for 48 hours on TSA at 37 °C. The SDS protein extracts were prepared and separated by electrophoresis as described by Pot *et al.* [23]. The collected data were interpreted as described by Vauterin & Vauterin [32], using the GelCompar 4.2 software (Applied Maths). The similarity between all pairs of electrophoresis patterns was calculated by the Pearson product-moment correlation coefficient, expressed as a percentage.

5.1.3.3 DNA-DNA hybridisations

DNA was prepared by the method of Marmur [16], as modified by Vandamme *et al.* [31], or by a modification of the protocol of Pitcher *et al.* [22]. Briefly, 500 - 750 mg cell mass was harvested from agar surfaces, washed with 15 ml RS buffer (0.15 M NaCl, 0.01 M EDTA; pH 8.0), centrifuged and resuspended in 3 ml TE buffer (1.21 g Tris.HCl, 2 ml 0.5 M EDTA; pH 8.0 in 1 l MilliQ-water). Fifteen ml GES reagent (600 g guanidinium thiocyanate, 200 ml 0.5 M EDTA; pH 8.0, 10 g sarcosyl in 1 l MilliQ-water) was added and the suspension was mixed gently until cell lysis occurred, and left on ice for 15 min. Ammonium acetate (7.5 ml, 7.5 M) was added, mixed again and left on ice for 15 min. Ten ml chloroform-isoamylalcohol (24/1) was added and mixed vigorously. After 20 min of centrifugation at 17,000 g, the upper phase was transferred to a plastic recipient and 0.54 volumes isopropanol were added. The DNA was spooled onto a glass rod, washed in a series of ethanol solutions (70, 80 and 90% (v/v)), dried to the air and dissolved overnight in 5 ml TE buffer. When the DNA was completely dissolved, 25 µl of RNase solution (2 mg/ml) was added and incubated for 1 h at 37 °C. After RNase treatment, 625 µl acetate-EDTA (3 M sodium acetate, 1 mM EDTA; pH 7.0) was added and the chloroform extraction repeated. The supernatant was again transferred to a plastic recipient and DNA was precipitated with 2 volumes ethanol, spooled onto a glass rod, washed in an ethanol series and dried to the air. Finally, the DNA was dissolved in 1 ml 0.1 SSC (1 SSC contains 0.15 M NaCl and 0.015 M sodium citrate).

DNA-DNA hybridisations were either performed by the initial renaturation rate method of De Ley *et al.* [6] or with the microplate method as described by Ezaki *et al.* [10]. In the latter case, a HTS7000 Bio Assay Reader (Perkin Elmer) was used for the fluorescence measurements in white MaxiSorp (Nunc) microplates. The hybridisation conditions were 81 °C in 2 SSC and 50 °C in 2 SSC with 50% (v/v) formamide for the initial renaturation method and the microplate method, respectively. Results obtained by both methods were shown previously to correlate very well [11].

5.1.3.4 DNA base compositions

DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* [19]. The analysis parameters were as reported in Logan *et al.* [15].

5.1.3.5 16S rDNA sequencing

16S rDNA sequencing was performed using an Applied Biosystems 377 DNA sequencer as described by Coenye *et al.* [2]. Partial sequences were assembled using the

program Auto AssemblerTM (Applied Biosystems) and phylogenetic analysis was performed using the BioNumerics 1.5 software package (Applied Maths).

The nucleotide accession numbers for the 16S rDNA sequence of strains WS2^T, ER121 and DSM 11853^T are AF312020, AF312021 and AF312022, respectively.

5.1.3.6 Gas chromatographic analysis of methylated fatty acids

Cells were grown for 24 hours on TSA at 28 °C and fatty acid methyl esters (FAMES) were extracted, prepared, separated and identified using the Microbial Identification System (Microbial ID) as described before [33].

5.1.3.7 Phenotypic tests

Classical phenotypic tests were performed as described previously [7]. API 20 NE and API ZYM (bioMérieux) were utilized according to the protocol supplied by the manufacturer.

5.1.4 Results

5.1.4.1 PAGE of whole-cell proteins

The result of a numerical analysis based on whole-cell protein profiles is shown in Fig. 5.1. A zone corresponding to molecular masses between 32.1 and 38 kDa was omitted from the analysis. This zone comprised a distorted band in the profile of some cluster I (*R. campinensis*) strains, e.g. WS2^T (Fig. 5.2). Six (I –VI) major clusters and twelve strains holding a distinct position in the dendrogram (Fig. 5.1) were differentiated above a correlation level of 85%. The *R. eutropha* reference strains formed a single homogeneous cluster (cluster III), grouping above 86.1% similarity. The type strains of *R. pickettii*, *R. gilardii*, *R. paucula*, *R. solanacearum*, *R. mannitolilytica*, and *R. oxalatica* [34] occupied distinct positions. Six isolates grouped together with the type strain of *R. basiliensis* in cluster II above 88.1% similarity. The other metal-resistant isolates were found scattered over clusters IV, V and VI, whereas the isolates AB2, AS2, VA1 and VA11 occupied separate positions in the dendrogram. Yet, the protein patterns of the latter strains were visually very similar, in apparent contradiction to their location in the dendrogram. Reproducible protein band distortions were indeed observed above the 116 kDa molecular weight region for the cluster IV strains, and around the 60 kDa region for strains VA10 and VA7 (forming cluster VI), as well as for strains AB2, AS2, VA1, and VA11 (Fig. 5.2). Little or no band distortions were observed in the protein patterns of the isolates grouping above 85% correlation in cluster V (e.g. the pattern of *R. metallidurans* CH34^T in Fig. 5.2). The presence of reproducible strain specific distortions has been described within other species of *Ralstonia* [30]. DNA-DNA hybridisation experiments confirmed that these distortions represent intra-species variability and not inter-species variability (see below; [30]).

Ralstonia sp. JMP134 and ATCC 17707 occupied separate positions in the dendrogram. Both strains were originally received as *R. eutropha*, but since the whole cell protein profiles differed clearly from those of the other *R. eutropha* strains (cluster

III), we consider them here as “*Ralstonia eutropha*-like” organisms pending further taxonomic analyses.

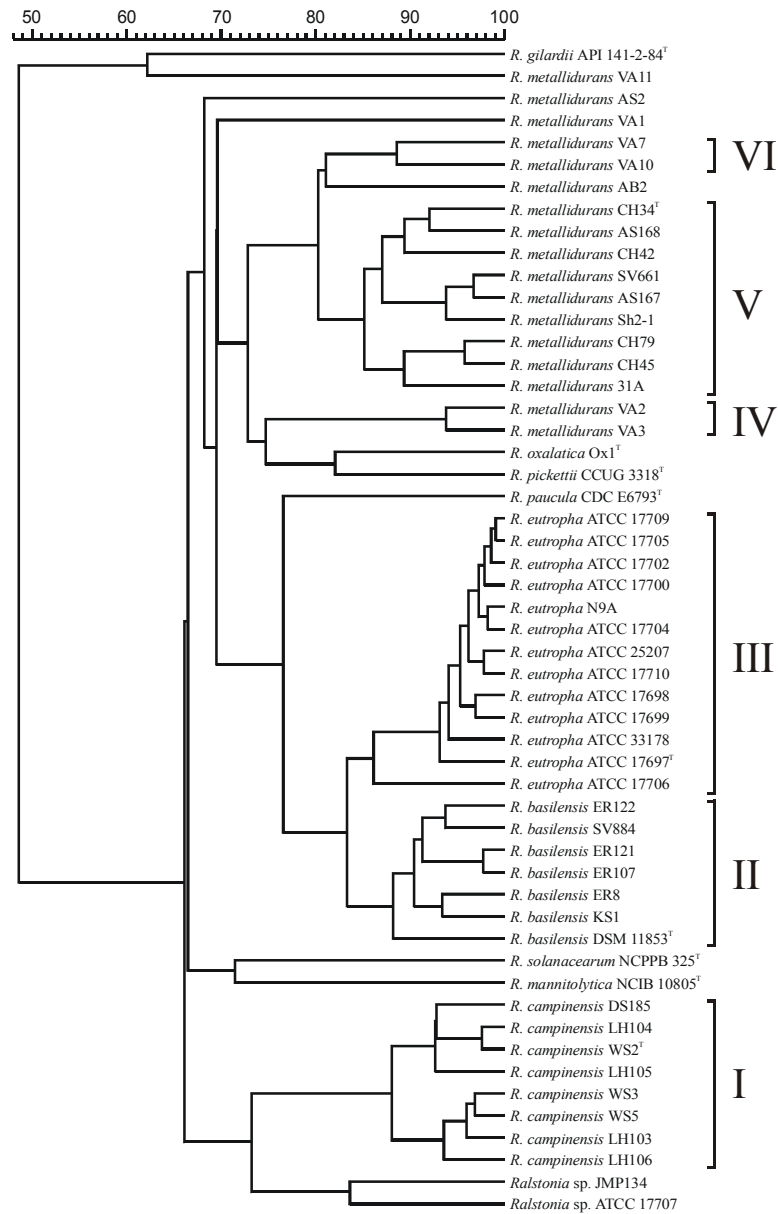


FIG. 5.1: Dendrogram based on UPGMA clustering of SDS-PAGE analyses of whole cell proteins of *Ralstonia* isolates. The zones used for numerical analysis were between 18.6 – 32.1 kDa and 38 – 107 kDa. Similarities are expressed as a percentage.

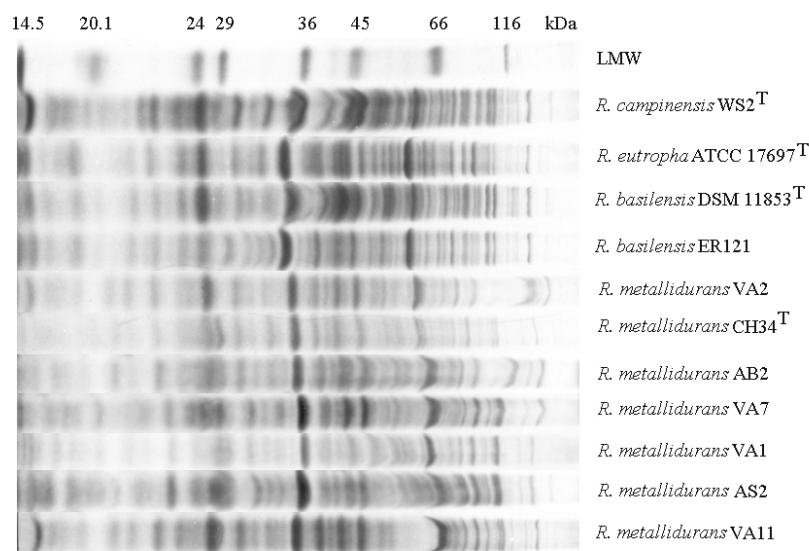


FIG. 5.2: Photograph of whole cell proteins patterns of *Ralstonia* strains illustrating the presence of reproducible strain specific distortions. The molecular mass markers used were (from left to right) lysozyme (14.5 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and β -galactosidase (116 kDa).

5.1.4.2 DNA-DNA hybridisations and DNA base compositions

Strains representing the protein electrophoretic types were hybridised with type strains of the valid *Ralstonia* species. DNA-DNA reassociation values and nucleotide compositions are shown in Table 5.2. The type strain of *R. basilensis* showed high DNA reassociation values (61 – 78%) with strains ER121 and ER8. These three strains are representatives of protein electrophoretic cluster II and showed only low to intermediate DNA reassociation values (2 – 37%) with representatives of other *Ralstonia* species. Hybridisation values between *R. campinensis* WS2^T and LH103 (representatives of protein electrophoretic cluster I) were high (100%), while low to intermediate DNA relatedness (11 – 41%) was found between the above mentioned *R. campinensis* strains and representatives of the other *Ralstonia* species. The metal-resistant strains CH34^T, CH79 and VA7, representing protein electrophoretic clusters V and VI, form a homogenous DNA-reassociation group (83 – 88%; *R. metallidurans*), showing low to intermediate DNA relatedness (0 – 39%) with representatives of other *Ralstonia* species. The high DNA relatedness observed between *R. metallidurans* strains from clusters V and VI confirmed that the detected protein electrophoretic differences reveal intra-species and not inter-species diversity.

The nucleotide compositions presented in Table 5.2 are in good agreement with those reported for the genus and are not in contradiction with the grouping based on whole cell protein profiles and DNA-DNA hybridisations.

TABLE 5.2: DNA-DNA reassociation values and nucleotide compositions of *Ralstonia campinensis*, *Ralstonia metallidurans*, *Ralstonia basilensis* and *Ralstonia* reference strains.

| Strain | PAGE cluster | %G+C | %DNA-DNA reassociation with: | | | | | | | | | | | | | |
|--|--------------|-------------------|------------------------------|--------------------|---------------------------------|-----------------|------------------------|--------------------|-------------------|-----|--------------------|-------------------|--------------------------------|------------------------|----------------------------|------------------------|
| | | | WS2 ^T | LH103 | CDC E6793 ^T | CIP 62.31 | DSM 11853 ^T | ER8 | ER121 | VA7 | CH79 | CH34 ^T | ATCC 17697 ^T | NCPBB 325 ^T | API 141-02-84 ^T | CCUG 3318 ^T |
| <i>Ralstonia campinensis</i> WS2 ^T | I | 66.6 | 100 | | | | | | | | | | | | | |
| <i>Ralstonia campinensis</i> LH103 | I | 66.8 | 100* | 100 | | | | | | | | | | | | |
| <i>Ralstonia paucula</i> CDC E6793 ^T | - | 64.2 | 31 [‡] | | 100 | | | | | | | | | | | |
| <i>Ralstonia paucula</i> CIP 62.31 | - | 65 [§] | 25* | | 82 [§] | 100 | | | | | | | | | | |
| <i>Ralstonia basilensis</i> DSM 11853 ^T | II | 65.0 | 18 [‡] | | 17 [‡] | 100 | | | | | | | | | | |
| <i>Ralstonia basilensis</i> ER8 | II | 65.5 | 14*22 [‡] | | 20*19 [‡] | 78 [‡] | 100 | | | | | | | | | |
| <i>Ralstonia basilensis</i> ER121 | II | 65.4 | | 32 [‡] | 3* | | | 61* | 100 | | | | | | | |
| <i>Ralstonia metallidurans</i> VA7 | VI | | | | | 29* | | | | 100 | | | | | | |
| <i>Ralstonia metallidurans</i> CH79 | V | 63.9 | | 29* | 31* | 37 [‡] | | | | 83* | 100 | | | | | |
| <i>Ralstonia metallidurans</i> CH34 ^T | V | 63.7 | 26 [‡] | 37 [‡] | 35 [‡] | 39 [‡] | 16 [‡] | 17*16 [‡] | 2*25 [‡] | | 84*88 [‡] | 100 | | | | |
| <i>Ralstonia eutropha</i> ATCC 17697 ^T | - | 66.6 | 18*26 [‡] | 41 [‡] | 21 [‡] | 24 [§] | 20 [‡] | 22*25 [‡] | 8*37 [‡] | 0* | | 28 [‡] | 100 | | | |
| <i>Ralstonia solanacearum</i> NCPBB 325 ^T | - | 67.0 | 24*11 [‡] | | 16 [§] 10 [‡] | 19* | 8 [‡] | 11 [‡] | 17* | | 18* | 1*10 [‡] | 1*13 [‡] | 100 | | |
| <i>Ralstonia gilardii</i> API 141-02-84 ^T | - | 68.3 [†] | | 26 [‡] | 21 [§] | | | | 22 [‡] | | | 20 [‡] | 0 [†] 40 [‡] | | 100 | |
| <i>Ralstonia pickettii</i> CCUG 3318 ^T | - | 63.8 | 20* | 21*18 [‡] | 13 [§] | | | 20* | 5*17 [‡] | | | 3*20 [‡] | 35*25 [‡] | 32* | 21 [‡] | 100 |

* Initial renaturation rate method; values are averages from at least two independent reactions

[†] Data from Coenye *et al.* [2]

[‡] Microplate method; values are averages from at least two reciprocal reactions

[§] Data from Vandamme *et al.* [30]

5.1.4.3 16S rDNA sequencing

A phylogenetic tree including all *Ralstonia* species is given in Fig. 5.3. Compared with known representatives of validly described *Ralstonia* species, the 16S rRNA genes of *R. campinensis* WS2^T and *R. metallidurans* CH34^T showed the highest sequence similarity (98.2 and 98.8%, resp.) with the 16S rRNA gene of *Ralstonia paucula* LMG 3413 (= CIP 62.31; accession number AF085226), a close relative (82% DNA reassociation) of *R. paucula* CDC E6793^T (Table 5.2).

The 16S rDNA sequence determined in the present study for *R. basilensis* DSM 11853^T (= LMG 18990^T = LMG 19474^T = RK1^T; accession number AF312022) differed in 30 base positions (97.9% sequence similarity) from the sequence that was originally deposited for this strain (accession number AJ002302).

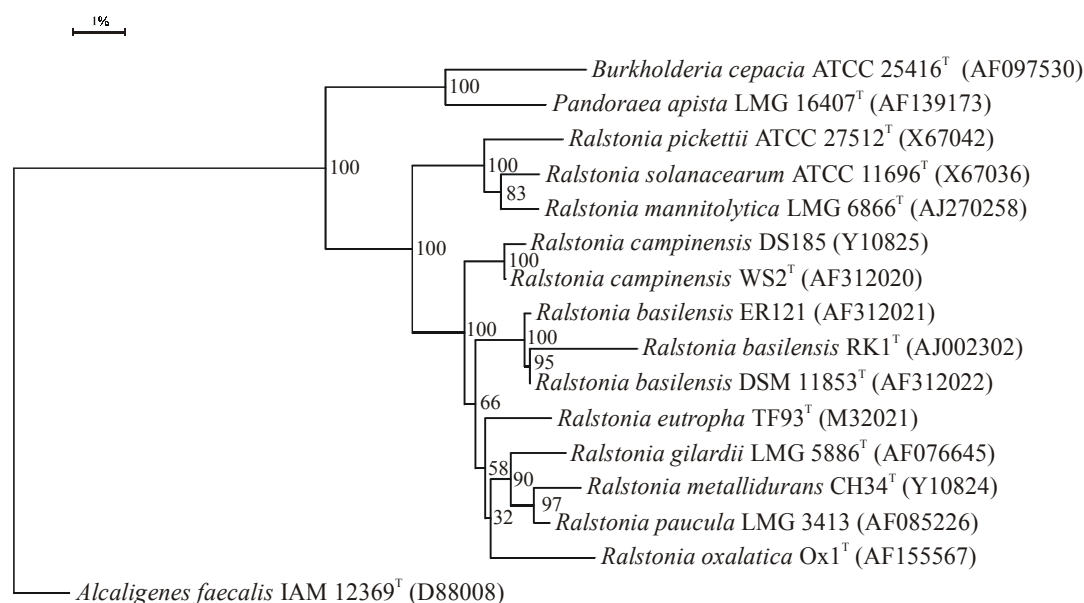


FIG. 5.3: Neighbour-joining phylogenetic tree of all described *Ralstonia* species based on 16S rDNA sequence comparisons. Bootstrap values obtained with 500 repetitions are indicated at all branches. The 16S rDNA sequences of *Alcaligenes faecalis*, *Burkholderia cepacia*, and *Pandoraea apista* were included as outgroups.

5.1.4.4 Cellular fatty acids

Table 5.3 shows the average fatty acid profiles for the *Ralstonia* species examined, which are characterized by large amounts of 16:0 (16.8 – 25.4%), 18:1 ω 7c (18.5 – 36.4%), summed feature 3 (27.8 – 36.6%), moderate amounts of 14:0 (2.6 – 5.0%) and summed feature 2 (7.7 – 15.4%).

Discrimination between the different *Ralstonia* species is mainly based on percentages of fatty acids present in trace to very low amounts or completely absent. Furthermore, 18:1 ω 7c seems to be present in *R. gilardii* LMG 5886^T in a significantly higher amount (36.4%) than in all other *Ralstonia* strains examined (13.9 – 27.9%; raw data not shown).

TABLE 5.3: Comparative fatty acid patterning of *Ralstonia* species

| | <i>R. campinensis</i> (8 strains) | <i>R. basilensis</i> (8 strains) | <i>R. metallidurans</i> (16 strains) | <i>R. eutropha</i> ATCC 17697 ^T | <i>R. solanacearum</i> NCPFB 325 ^T | <i>R. pickettii</i> CCUG 3318 ^T | <i>R. gilardii</i> API 141-2-84 ^T | <i>R. oxalatica</i> Ox1 ^T |
|------------------|--------------------------------------|-------------------------------------|---|---|--|---|---|---|
| 12:0 | | | | | | Tr | Tr | |
| 14:0 | 5.0±0.3 | 4.5±0.1 | 4.4±0.2 | 2.9 | 4.2 | 4.5 | 4.1 | 2.6 |
| 15:0 | | | | Tr | Tr | Tr | Tr | Tr |
| 15:0 iso | | | | | | Tr | | |
| 16:0 | 24.6±1.7 | 20.6±0.9 | 20.9±1.4 | 20.6 | 22.1 | 25.4 | 17.7 | 16.8 |
| 16:0 iso | | | Tr | | | | | |
| 17:0 | | | | Tr | 1.5 | Tr | Tr | Tr |
| 17:0 cyclo | 6.1±3.5 | 1.3±0.9 | 3.2±2.1 | 2.0 | 2.1 | Tr | 1.2 | 2.0 |
| 18:0 | 1.5±4.9 | Tr | Tr | Tr | Tr | Tr | Tr | Tr |
| 15:1 ω6c | | | | Tr | Tr | Tr | | Tr |
| 16:1 ω5c | | | | Tr | | Tr | | Tr |
| 18:1 ω7c | 19.5±1.8 | 24.7±1.3 | 19.8±2.9 | 18.5 | 19.2 | 20.0 | 36.4 | 20.7 |
| 19:0 cyclo ω8c | Tr | | Tr | | | | Tr | |
| 14:0 2OH | 2.1±0.2 | | Tr | 3.2 | | Tr | Tr | 3.8 |
| 16:1 2OH | Tr | 1.8±0.8 | Tr | Tr | 1.3 | 1.2 | | |
| 16:0 2OH | Tr | Tr | 3.3±0.4 | | Tr | Tr | Tr | |
| 16:0 3OH | | | | Tr | | | | |
| 18:1 2OH | 2.7±0.5 | 4.8±0.4 | 1.4±0.4 | Tr | 4.0 | 3.3 | 2.0 | |
| Summed feature 2 | 8.9±1.4 | 9.0±0.5 | 11.3±2.5 | 13.7 | 9.8 | 7.7 | 8.1 | 15.4 |
| Summed feature 3 | 28.4±4.0 | 32.9±1.4 | 34.2±2.9 | 36.5 | 32.7 | 34.2 | 27.8 | 36.6 |

Summed feature 2 comprises 14:0 3OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928, 12:0 ALDE or any combination of these fatty acids
Summed feature 3 comprises 16:1 ω7c, 15 iso 2OH, 15:0 iso 2OH, 16:1 ω7c or any combination of these fatty acids
Tr: fatty acid content below 1%

5.1.4.5 Phenotypic tests

All strains examined grew at 20, 30 and 37 °C and assimilated D-gluconate, adipate, and L-malate; glucose, L-arabinose, D-mannose, D-mannitol, and maltose were never assimilated. Indole production from tryptophan and acid formation from glucose was never observed. Enzyme activities detected in all strains examined: alkaline phosphatase, C4 esterase, esterase lipase, leucine arylamidase, and acid phosphatase. None of the examined strains showed protease, lipase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, or α -fucosidase activity. Urease activity and reduction of nitrate were variable. Biochemical features, enabling differentiation between the four *Ralstonia* species associated with industrially polluted soils are given in Table 5.4.

TABLE 5.4: Differentiating biochemical characters between *R. campinensis*, *R. basilensis*, *R. metallidurans*, and *R. eutropha*.

| | <i>R. campinensis</i> | <i>R. basilensis</i> | <i>R. metallidurans</i> | <i>R. eutropha</i> |
|---------------------------|-----------------------|----------------------|-------------------------|--------------------|
| Nitrate reduction | + | -* | +* | +† |
| Nitrite reduction | - | - | +* | -† |
| Denitrification | - | - | +* | -† |
| Urease (after 48h) | + | - | - | -† |
| Assimilation of citrate | - | + | + | + |
| Growth at 41°C | + | - | - | + |
| Acid phosphatase activity | +* | w | + | + |

w: weak reaction

* One or a few strains showed a different reaction

† The type strain showed a different reaction

5.1.5 Discussion

A variety of metal-resistant bacteria related to CH34, a *R. eutropha*-like bacterium [18], has been isolated from various industrial metallurgical biotopes (Table 5.1; [17]). Most of these isolates have been examined by means of amplified ribosomal DNA restriction analysis (ARDRA), and can be subdivided into two major clusters [1]. A first cluster comprised six isolates obtained from different locations in Belgium, whereas the second cluster comprised all other isolates, reference strains of *R. paucula*, and several isolates that were received as “*Pseudomonas oxalaticus*”.

5.1.5.1 *R. campinensis* sp. nov.

In the present study, the six isolates from ARDRA cluster I [1] and two additional ones, formed protein electrophoretic cluster I (Fig. 5.1). Protein electrophoretic cluster I strains were shown to be a homogenous DNA hybridisation group (Table 5.2) and could be further differentiated from strains belonging to other *Ralstonia* species by growth at 41 °C, urease activity, and citrate assimilation. The DNA base ratio (Table 5.2) and the phylogenetic analysis (Fig. 5.3) of isolates from cluster I confirmed the allocation of

this taxon to the genus *Ralstonia*. We therefore propose the name *R. campinensis* sp. nov. to accommodate these eight isolates.

The 16S rDNA sequence of *R. campinensis* WS2^T (AF312020) is nearly identical (99.9%) to the 16S rDNA sequence of *Ralstonia* sp. CT14, deposited in the EMBL database under accession number D88001. It is therefore likely that strain CT14 also belongs to the new species *R. campinensis*, although DNA hybridisation data are still necessary for a final assignment at the species level [28].

5.1.5.2 Differentiation between *R. basilensis*, *R. paucula*, and *R. metallidurans* sp. nov.

In the study of Brim *et al.* [1], the majority of metal-resistant bacteria formed a single ARDRA cluster, including the reference strains of *R. paucula*. In the present study, these isolates distribute into five protein electrophoretic clusters (II to VI), while the type strain of *R. paucula* occupies a clearly distinct position (Fig. 5.1). Cluster II comprises six metal-resistant strains in addition to the *R. basilensis* type strain, an organism that was described recently [29]. DNA-DNA hybridisations reveal high DNA-DNA relatedness values between cluster II strains, while low to intermediate values were measured towards reference strains of the other taxa studied (Table 5.2). Furthermore, strains of cluster II have very similar whole-cell fatty acid and biochemical profiles. Therefore, we consider all metal-resistant bacteria belonging to protein electrophoretic cluster II as *R. basilensis*. The DNA base ratio of the *R. basilensis* strains examined conformed to that of the genus. The 16S rDNA sequence of *R. basilensis* DSM 11853^T in the present study, and of that of a metal-resistant isolate (ER121) revealed 99.7% sequence identity. However, a reproducible difference of 30 bases was observed between our sequence of the *R. basilensis* type strain and the one deposited in EMBL by Steinle *et al.* [29]. We received twice a subculture of *R. basilensis* DSM 11853^T (once as LMG 18990^T and once as LMG 19474^T) from the “Deutsche Sammlung von Mikroorganismen und Zellkulturen”. Since both subcultures yielded identical whole cell protein profiles, partial 16S rDNA sequences, and repetitive element PCR-profiles obtained with the REP and BOX primers (data not shown), we suspect sequencing errors in the sequence deposited by Steinle. Alternatively, the sequence deposited by Steinle may not correspond to the strain that was deposited at DSMZ. The results from the biochemical analysis seem to favour the last hypothesis, because RK1^T was reported to be arginine dihydrolase and urease positive by Steinle *et al.* [29], while in our tests no such enzyme activities were found for DSM 11853^T.

The 16S rRNA gene of *R. basilensis* DSM 11853^T (accession number AF312022) showed very high sequence similarities (99.7%, 99.9% and 99.6%) with strains *Ralstonia* sp. BKME-6 (accession number AJ011503), KN1 (accession number AB031995) and MBIC 3902 (accession number AB017488), respectively. Polyphasic taxonomical analysis and/or DNA-DNA hybridisation data are necessary to elucidate the exact taxonomic relationship between these isolates and *R. basilensis*.

The remaining metal-resistant bacteria that clustered together with *R. basilensis* and *R. paucula* strains in the ARDRA analysis [1], formed protein electrophoretic clusters IV, V, and VI or occupied distinct positions in the dendrogram (strains AB2, AS2, VA1, and VA11). Visual comparison of their protein profiles (Fig. 5.2) and DNA-

DNA hybridisations (Table 5.2) clearly show that these strains represent a new species, for which we propose the name *R. metallidurans* sp. nov. *R. metallidurans* can be differentiated from *R. campinensis*, *R. basilensis*, and *R. eutropha* by the presence of the fatty acid 16:0 2OH and from *R. campinensis*, *R. basilensis*, *R. eutropha* and *R. paucula* by the ability to reduce nitrate to nitrogen. *R. metallidurans* CH34^T was previously included in a taxonomy study of *R. eutropha*-like bacteria by Jenni *et al.* [13]. These authors reported a low DNA-DNA hybridisation level between strain CH34^T and the *R. eutropha* type strain, which was confirmed in the present study.

5.1.5.3 Taxonomic status of other *R. eutropha*-like strains

Strains JMP134 [21] and ATCC 17707 [4] were originally classified as *Alcaligenes eutrophus*, now *Ralstonia eutropha*. As already reported by Jenni *et al.* [13], JMP134 shows only low DNA reassociation values (21%) with H20 (= ATCC 17700), a genuine *R. eutropha* strain (cluster III, Fig. 5.1). This is in good agreement with our results of whole cell protein profiles (Fig. 5.1), where the former strain occupied a distinct position in the dendrogram, clearly different from the protein profiles of the *Ralstonia eutropha* strains. The same observation holds for ATCC 17707. Therefore, we consider both isolates as *R. eutropha*-like organisms until additional taxonomic data are available.

5.1.5.4 Description of *Ralstonia campinensis*, sp. nov.

(cam.pin.ensis' L. adj., named after the geographical region [North-East Belgium] where the strains were originally isolated). *R. campinensis* cells are short, motile rods (0.8 x 1.2 - 1.8 μ m) single, in pairs or in short chains, forming round (sometimes with slightly scalloped margin), smooth, convex and transparent colonies of about 0.5 mm diameter after 24 h incubation on TSA at 30 °C. Oxidase and catalase positive. Growth at 20, 30, 37 and 41 °C, but not at 4 °C. Nitrate reduced, nitrite not. No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: urease, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, α -glucosidase (except for strain LH104), protease, β -galactosidase, lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Assimilation of D-gluconate, caprate, adipate, L-malate and phenylacetate. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose and citrate. The DNA base ratio is 66.6 – 66.8 mol%. Major fatty acid components are 14:0 (5.0%), 16:0 (24.6%), 17:0 cyclo (6.1%), 18:0 (1.5%), 18:1 ω 7c (19.5%), 14:0 2OH (2.1%), 18:1 2OH (2.7%), summed feature 2 (8.9%), and summed feature 3 (28.4%).

The type strain WS2 (= LMG 19282 = CCUG 44526) was isolated from a zinc-desertified area in Lommel, Belgium. Its DNA base ratio is 66.6 mol% and its phenotypic characteristics are as described above for the species. The accession number of its 16S rRNA gene sequence is AF312020.

5.1.5.5 Description of *Ralstonia metallidurans*, sp. nov.

(me.tal.li.du'rans L. n. *metallum* metal; L. pres. part. *durans* enduring; M. L. part. adj. *metallidurans* to indicate that these strains are able to survive high heavy metal concentrations). *R. metallidurans* cells are short, motile rods (0.8 x 1.2 - 2.2 μm) single, in pairs or in short chains, forming round (sometimes with slightly scalloped margin), smooth, flat, convex or umbonate and transparent colonies of about 0.5 mm diameter after 24 h incubation on TSA at 30 °C. Oxidase and catalase positive. Growth at 20, 30 and 37 °C, but no growth detected at 4 or 41 °C. An exception is AS2, which does grow at 41 °C. Nitrate and nitrite are reduced (CH34^T reduces nitrate, but not nitrite; VA1 and AS2 reduce neither nitrate nor nitrite). No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, urease (AS2 and AS168 show a weak reaction after 48h), α -glucosidase, protease, β -galactosidase, lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Assimilation of D-gluconate, caprate (except CH34^T), adipate, L-malate and citrate (except CH34^T). Most strains seem to be able to assimilate phenylacetate after 48h of incubation (exceptions are AB2 and VA2 [weak assimilation even after 48h] and CH34^T [no assimilation]). No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine or maltose. The DNA base ratio is 63.7 – 63.9 mol%. Major fatty acid components are 14:0 (4.4%), 16:0 (20.9%), 17:0 cyclo (3.2%), 18:1 ω 7c (19.8%), 16:0 2OH (3.3%), 18:1 2OH (1.4%), summed feature 2 (11.3%), and summed feature 3 (34.2%).

The type strain CH34 (= LMG 1195 = DSM 2839) was isolated from the wastewater from a zinc factory at Liège, Belgium. Its DNA base ratio is 63.7 mol% and its phenotypic characteristics are as described above for the species, unless stated otherwise. The accession number of its 16S rRNA gene sequence is Y10824.

5.1.5.6 Emended description of *Ralstonia basilensis* Steinle et al., 1998 VP

Cells are short, motile rods (0.8 x 1.2 - 2.2 μm) single, in pairs or in short chains, forming round (sometimes with slightly scalloped margin), smooth, convex and transparent colonies of about 0.5 mm diameter after 24 h incubation on TSA at 30 °C. Oxidase and catalase positive. Growth at 4, 20, 30 and 37 °C, but no growth detected at 41 °C. No nitrate or nitrite reduction (except strain SV884 which reduces nitrate, not nitrite). No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase (weak) and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, urease (ER8 and KS1 give a weak reaction after 48h), α -glucosidase, protease, β -galactosidase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Assimilation of D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine or maltose. The DNA base ratio is 65.0 – 65.5 mol%. Major fatty acid

components are 14:0 (4.5%), 16:0 (20.6%), 17:0 cyclo (1.3%), 18:1 ω 7c (24.7%), 16:1 2OH (1.8%), 18:1 2OH (4.8%), summed feature 2 (9.0%), and summed feature 3 (32.9%).

The type strain RK1 (= LMG 18990 = LMG 19474 = DSM 11853) was isolated from a freshwater pond sediment at Amponville, France, after enrichment in a fixed-bed reactor with 2,6-dichlorophenol as the sole carbon and energy source. Its DNA base ratio is 65.0 mol% and its phenotypic characteristics are as described above for the species. The accession number of the 16S rRNA gene sequence of DSM 11853^T is AF312022.

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CHAPTER 6
PCB-DEGRADING *BURKHOLDERIA*

“Like a No. 2 pencil, I always got a point”

Jimmy Pop

CHAPTER 6 PCB-DEGRADING *BURKHOLDERIA*

6.1 CLASSIFICATION OF THE PCB- AND BIPHENYL DEGRADING STRAIN LB400 AND RELATIVES AS *BURKHOLDERIA XENOVORANS* SP. NOV.[§]

6.1.1 Summary

Strain LB400 is the best-studied polychlorinated biphenyl (PCB) degrader. This organism has previously been allocated in the genus *Burkholderia* through 16S rDNA sequence analysis. However, since confusion still exists in scientific literature about its species affiliation, a polyphasic study was undertaken to clarify the exact taxonomical position of this biotechnologically important organism. Two strains, one recovered from a blood culture vial and one coffee plant rhizosphere isolate, were found to resemble LB400 in patterns obtained through whole-cell protein profiling and repetitive-element PCR. DNA-DNA hybridisations revealed a relationship at the species level for these three strains. Furthermore, low DNA reassociation values with type strains of the closest related *Burkholderia* species support a classification as a novel species, for which we propose the name *Burkholderia xenovorans*. Strains of this novel species can be differentiated from other *Burkholderia* species by several phenotypic tests. The type strain of *B. xenovorans* is LB400 (= LMG 21463 = CCUG 46959).

6.1.2 Introduction

The genus *Burkholderia* is a phylogenetically well-defined group of organisms, occupying very diverse ecological niches. The more than 30 currently described *Burkholderia* species comprise soil and rhizosphere bacteria as well as plant pathogens, human pathogens, and human opportunistic pathogens [5]. Several *Burkholderia* strains have gained interest for their ability to degrade xenobiotic compounds, such as halogenated aromatics. One of the best-studied examples is *Burkholderia* sp. strain LB400. This strain co-metabolises many polychlorinated biphenyl (PCB) congeners when grown on biphenyl [11]. The pathways for degradation of PCB's by LB400 have been extensively characterized both at the genetic and molecular level (e.g. [6, 14]) and have become a model system for the bacterial breakdown of these very persistent environmental contaminants (for a recent review on bacterial PCB degradation, see [10]).

Strain LB400 was originally identified as a *Pseudomonas* species [3], but was referred to in more recent scientific literature as *Burkholderia* sp. or *Burkholderia cepacia* (e.g. [2, 15, 19]). Its allocation to the genus *Burkholderia* was confirmed in a recent taxonomic characterization performed by Fain and Haddock [9]. Furthermore, evidence provided by these authors clearly excluded this strain to be a member of *B. cepacia*. The exact species affiliation of strain LB400, however, remains unclear.

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The finding that two additional strains exhibit striking similarities with LB400 at the phenotypic (whole-cell protein patterns) and genotypic level (repetitive element PCR), prompted a polyphasic study to clarify the taxonomic position of these organisms. Evidence based on DNA-DNA hybridisation data and phenotypic tests is presented here that support the assignment of strain LB400 and its relatives to a novel *Burkholderia* species, for which we propose the name *Burkholderia xenovorans*.

6.1.3 Materials and methods

6.1.3.1 Bacterial strains and growth conditions

Strain LB400 (= LMG 21463 = CCUG 46959) was isolated from PCB-contaminated soil in upstate New York, USA [3]. Strain CCUG 28445 (= LMG 16224) was retrieved in 1991 from a blood culture vial, containing blood of a 31-year old woman in Göteborg, Sweden. Strain CAC-124 (= LMG 21720 = CCUG 46958) is a coffee plant rhizosphere isolate from Coatepec, Veracruz State, Mexico [7]. All three strains were grown aerobically on tryptic soy agar plates at 28°C.

6.1.3.2 SDS-PAGE of whole-cell proteins

Whole-cell protein analysis was performed as described by Pot et al. [17], with cultivation conditions and analysis parameters as reported by Coenye et al. [4].

6.1.3.3 Phylogenetic analysis

A nearly complete (1466 bp) 16S rDNA sequence of strain LB400 was previously determined by Lau and Bergeron (unpublished data) and deposited in the EMBL sequence database under accession number U86373. This sequence was compared with those of other *Burkholderia* species with the BioNumerics software package version 2.0 (Applied Maths). A phylogenetic tree was constructed based on the neighbour-joining method.

6.1.3.4 DNA-DNA hybridisations and %G+C determinations

DNA was prepared as described previously [12]. The microplate DNA-DNA hybridisation protocol of Ezaki et al. [8] was used, with minor modifications as explained in Chapter 3, paragraph 3.2 and at a hybridisation temperature of 55°C. The DNA base composition was determined through the HPLC-based protocol presented in Chapter 4, paragraph 4.2.

6.1.3.5 Repetitive-element PCR fingerprinting

The protocol of Rademaker and de Bruijn for repetitive-element PCR fingerprinting with the BOXA1R primer was used [18], with some previously described modifications [13].

6.1.3.6 Phenotypic descriptive parameters

Cells were grown for 24 hours on TSA at 28 °C and fatty acid methyl esters (FAMES) were extracted, prepared, separated and identified using the Microbial Identification System (Microbial ID) as reported before [24]. Classical phenotypic tests were performed as described previously [22]. API 20 NE and API ZYM (bioMérieux) were utilized according to the protocol supplied by the manufacturer.

6.1.4 Results and discussion

It was known from the study of Fain and Haddock [9] that strain LB400 should be allocated in the genus *Burkholderia*. Fig. 6.1 presents an updated phylogenetic tree, based on 16S rDNA sequences, including all currently recognised *Burkholderia* species. As observed by Fain and Haddock [9], strain LB400 is located in a separate cluster together with *B. phenazinium*, *B. caribensis*, and *B. graminis*. Additionally, several recently described species also belong to this cluster: *B. phymatum*, *B. hospita*, *B. tuberum*, *B. kururiensis*, *B. sacchari*, *B. fungorum*, *B. caledonica*, and *B. terricola*. Strain LB400 showed the highest 16S rDNA sequence similarity (98.6%) to the type strains of *B. graminis* and *B. terricola* and to *Burkholderia* sp. N3P2. The latter strain was isolated from a soil contaminated with polycyclic aromatic hydrocarbons [16] and was not identified at the species level.

Strains LMG 16224 and LMG 21720, isolated from very diverse origins, showed a striking similarity with LB400 based on SDS-PAGE patterns of their whole-cell proteins (data not shown) and BOX-PCR patterns (Fig. 6.2). Although BOX-PCR patterns obtained for the latter three strains are relatively similar (between 60 and 66% as calculated by the Pearson correlation coefficient; numerical comparison not shown), they differ from each other in a few bands, e.g. at positions corresponding with 1200, 970, and 790 bp (Fig. 6.2). Because of these differences, no clonal relationship between the isolates should be assumed.

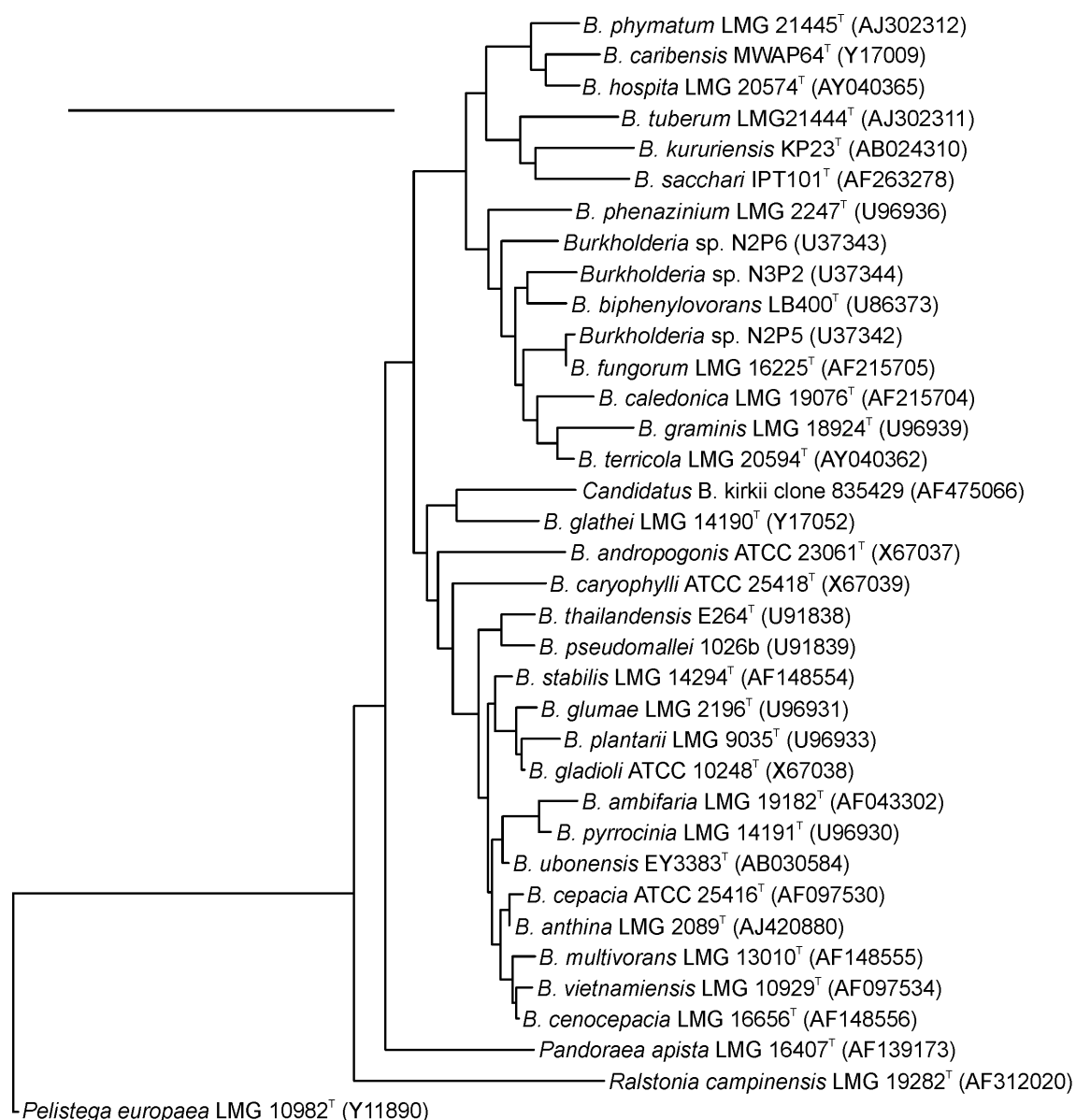


FIG. 6.1: The phylogenetic position of *Burkholderia xenovorans* as revealed by 16S rDNA sequence comparisons. The bar represents 5% sequence divergence.

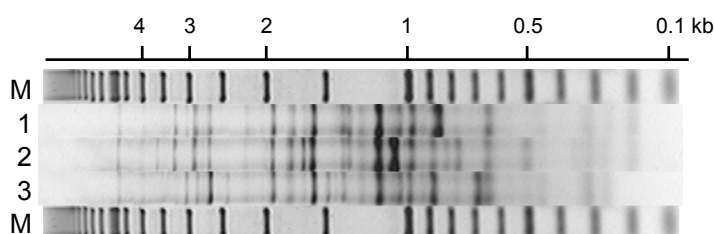


FIG.6.2: Normalized BOX-PCR patterns of *B. xenovorans* LMG 21720 (1); LMG 21463 (2); LMG 16224 (3). Marker lanes (M) contain a mixture of Molecular Ruler 500 (BioRad; 5000 to 500 bp DNA fragments in 500 bp intervals) and Molecular Ruler 100 (BioRad; 1000 to 100 bp DNA fragments in 100 bp intervals).

To clarify taxonomic relationships at the species level, DNA-DNA hybridisation experiments were performed with strains LB400, LMG 16224, LMG 21720 and type strains of the closest related *Burkholderia* strains, as evidenced by 16S rDNA sequence data (Table 6.1). DNA reassociation values between the former three strains are all above 70%, indicating a relationship at the species level [20, 26]. In contrast, low to intermediate reassociation values (average of reciprocal values $\leq 35\%$) were obtained in hybridisations of LB400 (= LMG 21463) with type strains of related *Burkholderia* species. Furthermore, as would be expected, %G+C values of *Burkholderia* type strains and internal DNA reassociation values as shown in Table 6.1 were very similar to those previously reported [1, 4, 13, 25]. These DNA-DNA hybridisation data (Table 6.1), together with the 16S rDNA sequence analysis (Fig. 6.1), support the classification of strains LB400, LMG 16224, and LMG 21720 into a separate, novel *Burkholderia* species, for which we propose the name *Burkholderia xenovorans* below.

TABLE 6.1: DNA-DNA reassociation values between *B. xenovorans* strains and closest relatives.

| Strain: | %G+C | % reassociation with probe-DNA from: | | | | | | | | | |
|---|------|--------------------------------------|-----------|-----------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-----------------------|
| | | LMG 21463 ^T | LMG 16224 | LMG 21720 | LMG 20598 ^T | LMG 20594 ^T | LMG 18924 ^T | LMG 18531 ^T | LMG 16225 ^T | LMG 19076 ^T | LMG 2247 ^T |
| <i>B. xenovorans</i> LMG 21463 ^T | 62.6 | 100 | 76 | 83 | 12 | 33 | 29 | 13 | 23 | 22 | 15 |
| <i>B. xenovorans</i> LMG 16224 | 62.5 | 69 | 100 | 86 | | | | | | | |
| <i>B. xenovorans</i> LMG 21720 | 62.9 | 83 | 94 | 100 | | | | | | | |
| <i>B. hospita</i> LMG 20598 ^T | 62.0 | 18 | | | 100 | 14 | 16 | 55 | 11 | 15 | 12 |
| <i>B. terricola</i> LMG 20594 ^T | 63.9 | 37 | | | 15 | 100 | 34 | 15 | 25 | 28 | 19 |
| <i>B. graminis</i> LMG 18924 ^T | 62.8 | 41 | | | 22 | 39 | 100 | 21 | 25 | 48 | 24 |
| <i>B. caribensis</i> LMG 18531 ^T | 62.4 | 24 | | | 65 | 20 | 21 | 100 | 16 | 20 | 17 |
| <i>B. fungorum</i> LMG 16225 ^T | 61.8 | 39 | | | 15 | 36 | 26 | 14 | 100 | 24 | 24 |
| <i>B. caledonica</i> LMG 19076 ^T | 62.0 | 35 | | | 17 | 36 | 42 | 15 | 21 | 100 | 20 |
| <i>B. phenazinium</i> LMG 2247 ^T | 62.5 | 18 | | | 11 | 21 | 16 | 11 | 16 | 15 | 100 |

Strains LMG 21463, LMG 16224, and LMG 21720 were phenotypically characterized (data reported in the species description below) and compared with previously published phenotypic data that were obtained under identical experimental conditions [4, 13, 23]. All three strains showed very similar phenotypic characteristics, which is in agreement with their classification into a single species. Their average cellular fatty acid pattern (included in the species description) is similar to the previously reported pattern of strain LB400 [9]. Phenotypic traits, useful for the differentiation of *B. xenovorans* from closely related *Burkholderia* species, are summarized in Table 6.2. Remarkably, *B. xenovorans* strains differ from nearly all *Burkholderia* strains in their inability to assimilate L-arabinose.

TABLE 6.2: Phenotypic tests useful for the differentiation of *B. xenovorans* from its closest relatives.

| | <i>B. xenovorans</i> | <i>B. hospita</i> * | <i>B. terricola</i> * | <i>B. graminis</i> † | <i>B. caribensis</i> † | <i>B. fungorum</i> † | <i>B. caledonica</i> † | <i>B. tuberum</i> ‡ | <i>B. phymatum</i> ‡ | <i>B. phenazinium</i> † |
|-----------------------------|----------------------|---------------------|-----------------------|----------------------|------------------------|----------------------|------------------------|---------------------|----------------------|-------------------------|
| L-arabinose assimilation | - | + | + | + | + | v | + | + | + | + |
| Citrate assimilation | + | v | v | + | + | + | - | - | - | - |
| Sucrose assimilation | - | - | + | + | - | - | v | ND | ND | + |
| Growth in O/F with D-xylose | + | - | - | v | - | v | + | ND | ND | v |
| β-galactosidase activity | - | + | + | - | + | - | - | + | + | - |

* data from Goris et al. [13]

† data from Coenye et al. [4]

‡ data from Vandamme et al. [23]

+, -: phenotypic trait present, resp., absent; v: variable reaction; ND: not determined

6.1.4.1 Description of *Burkholderia xenovorans* sp. nov.

Burkholderia xenovorans [xe.no'vor.ans. Gr. adj. Xenos foreign; L. part pres. vorans devouring, digesting; N.L. part adj. xenovorans digesting foreign (xenobiotic) compounds]. *B. xenovorans* cells are Gram-negative, motile, non-sporulating, straight rods. Growth is observed at 28°C, not at 42°C. Growth in O/F medium is observed with D-glucose, D-fructose or D-xylose as C-source, but not with maltose or adonitol. No growth was observed on cetrimide, on 10% lactose, in the presence of acetamide, or in the presence of 4.5 or 6.0% NaCl. Growth in the presence of 1.5 or 3.0% NaCl was strain dependent. The cells grew on blood agar at 30°C and on Drigalski agar, but growth on blood agar at 37°C was strain dependent. Nitrate and nitrite reduction is variable. No fermentation of D-glucose. No liquefaction of gelatin or hydrolysis of aesculin. Hydrolysis of Tween 80 is positive. Haemolysis of horse blood was not observed. No production of acid or H₂S in triple-sugar-iron agar, no indole or pigment produced. Assimilation of D-glucose, D,L-norleucine, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-gluconate, caprate, adipate, L-malate, citrate, phenyl acetate, D,L-lactate, and D,L-lactate with methionine, but not of trehalose, L-arabinose, maltose, and sucrose. Variable assimilation of L-arginine. Catalase, oxidase, alkaline and acid phosphatase, esterase C4, ester lipase C8, leucine arylamidase, and phosphoamidase activity is present. Amylase, DNase, lipase C14, tryptophanase, lysine decarboxylase, ornithine decarboxylase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α- and β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, and arginine dihydrolase activity was not detected. Reactions of urease, valine arylamidase and cystine arylamidase were strain dependent. The whole-cell fatty acid profile of *B. xenovorans* strains comprised 14:0 (4.7%), 14:0 3OH (8.5%), 16:1 ω7c (19.1%), 16:0 (18.2%), 17:0 cyclo (5.1%), 16:1 2OH (2.2%), 16:0 2OH (2.2%),

16:0 3OH (7.1%), 18:1 ω 7c (27.3%), 18:0 (0.5%), 19:0 cyclo ω 8c (3.6%), and 18:1 2OH (0.9%) as major components (summed feature 2 [comprising 14:0 3OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928, or 12:0 ALDE, or any combination of these fatty acids], and summed feature 3 [comprising 16:1 ω 7c or 15 iso 2OH or both] are mentioned above as 14:0 3OH and 16:1 ω 7c, respectively, as these fatty acid have been reported in *Burkholderia* species [21]). The G+C content varies between 62.4 - 62.9 mol%. The three available *B. xenovorans* strains LMG 21463, LMG 16224, and LMG 21720 were isolated from PCB-contaminated soil in New York (USA), from a blood culture vial of a 31-year old woman in Göteborg (Sweden), and from coffee plant rhizosphere in Coatepec (Mexico), respectively.

The type strain is LB400 (= LMG 21463 = CCUG 46959), an organism of which the PCB and biphenyl metabolism has been extensively investigated. Its G+C content is 62.6 mol% and its 16S rDNA sequence was previously deposited in the GenBank database under accession number U86373 (P.C.K. Lau and H. Bergeron, unpublished data).

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CHAPTER 7
TRANSFER OF PLASMIDS PJP4 AND PEMT1
IN SOIL

“Lateral gene transfer is not just a molecular phylogenetic nuisance supported in evidence by a few anecdotal cases. Instead, it is a major force, at least in prokaryotic evolution.”

W. Ford Doolittle and John M. Logsdon

CHAPTER 7 TRANSFER OF PLASMIDS pJP4 AND pEMT1 IN SOIL

7.1 HOST RANGE OF PLASMID pEMT1

7.1.1 Introduction

The 2,4-D catabolic plasmid pEMT1 was isolated from soil by Top et al. through exogenous plasmid isolation [88]. Its 2,4-D degradative genes share a high degree of sequence similarity with their counterparts on plasmid pJP4 from *Ralstonia* sp. JMP134, but both plasmids do not belong to the same incompatibility class [88].

Previously, the transfer of Tn5Km1-labelled derivatives of pEMT1 was investigated in soil microcosms [89, 91]. A number of indigenous soil bacteria that had taken up the plasmid were isolated on a 2,4-D containing selective medium and subsequently identified as *Ralstonia eutropha*, *Burkholderia* sp., and *Stenotrophomonas maltophilia* [89, 91]. The actual host range of a 2,4-D degradative plasmid, however, is not limited to organisms in which the 2,4-D catabolic genes are efficiently expressed, but can instead be wider [23]. No in-depth study to determine the host range of plasmid pEMT1, independently from the 2,4-D degradative phenotype, has yet been published.

In the here-presented study, the host range of pEMT1::*lacZ*, a *lacZ*-labelled derivative of pEMT1 [21], was assessed by performing conjugations on agar surfaces (plate matings) with taxonomically well-characterized pure cultures. The recipients were chosen to represent different phylogenetic lineages and eventual transconjugants were selected for expression of the plasmid marker genes.

7.1.2 Materials and methods

The collection strains that were used as recipient for plasmid pEMT1::*lacZ* in plate matings and the different phylogenetic lineages they represent, are given in Table 7.1. The plasmid donor strains were *Escherichia coli* DH5 α (pEMT1::*lacZ*) and *Pseudomonas putida* (pEMT1::*lacZ*) [21].

For enumeration of recipients and transconjugants in the presence of the plasmid donor, growth of the latter strain needs to be suppressed. Different selective growth conditions were applied, such as the presence of antibiotics, a restrictive temperature, anaerobiosis, a minimal medium (for an auxotrophic donor strain) or a nitrogen-free medium. Antibiotics were applied in following concentrations: tetracycline (5 mg/l), rifampicin (100 mg/l), streptomycin (150 mg/l), cefoperazone (250 mg/l), ampicilin (64 mg/l), and nalidixic acid (200 mg/l). Medium 80 (M80) contained per litre distilled water 1 g yeast extract, 1 g sodium succinate, 5 mg Fe(III)-citrate, 0.5 g KH₂PO₄, 0.4 g MgSO₄.7H₂O, 0.4 g NaCl, 0.4 g NH₄Cl, 50 mg CaCl₂.2H₂O, 1 ml trace element solution (per litre: 0.1 g ZnSO₄.7H₂O; 30 mg MnCl₂.4H₂O; 0.3 g H₃BO₃; 0.2 g CoCl₂.6H₂O; 10 mg CuCl₂.H₂O; 20 mg NiCl₂.6H₂O; 30 mg Na₂MoO₄.2H₂O), 0.5 ml ethanol, and 15 g agar (pH 5.7). M80 agar plates were incubated in an anaerobic cabinet and illuminated with a tungsten lamp to allow anaerobic photoheterotrophic growth of *Rhodobacter capsulatus* LMG 5161. MMO mineral medium was prepared as described

by Stanier [81]. The nitrogen-free medium 10 (M10) contained per litre distilled water 10 g glucose, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 0.9 g KH₂PO₄, 5 g CaCO₃, 10 mg FeSO₄·7H₂O, 5 mg Na₂MoO₄·2H₂O, and 15 g agar (pH 7.3). Double-selective medium (for selective enumeration of transconjugants) contained additionally 50 mg/l kanamycin and 20 mg/l X-gal. The latter substrate is converted to a blue product by LacZ, encoded on plasmid pEMT1::lacZ.

Transfer frequencies were calculated as the number of colonies that appeared on double-selective medium (transconjugants), divided by the number of colonies on single-selective medium (total recipients, i.e. with or without plasmid).

For each conjugation, a putative transconjugant (a blue colony on double-selective medium) was isolated and further characterized. Strain identity was confirmed by repetitive-element PCR fingerprinting with the REP- and GTG₅ primer set [76]. The presence of the pEMT1::lacZ plasmid was verified by plasmid isolation [74], either directly or after transfer of the plasmid to *E. coli* DH5 α . Finally, it was assessed whether the transconjugant had acquired the capacity for 2,4-D degradation through growth tests in liquid minimal medium (supplemented with 50 mg/l 2,4-D as sole C-source), followed by HPLC analysis of the spent medium [21].

7.1.3 Results and discussion

The results of the plate mating experiments are summarized in Table 7.1. Plasmid pEMT1::lacZ could be transferred to representatives of the α -Proteobacteria (*Rhizobium radiobacter* and *Blastomonas natatoria*, but not to *Rhodobacter capsulatus*), β -Proteobacteria (*Ralstonia eutropha*), and γ -Proteobacteria (*Escherichia coli* and *Azomonas macrocytogenes*). No transfer was detected in conjugations with representatives of the ϵ -Proteobacteria, Firmicutes low %G+C, Firmicutes spore formers, and Flavobacteriaceae. However, this observation does not exclude that the plasmid could be transferred to other representatives of the latter phyla. A 2,4-D degradative phenotype, resulting from acquisition of pEMT1::lacZ, was solely observed for *Ralstonia eutropha* and *Azomonas macrocytogenes* transconjugants (Table 7.1). The transfer frequencies calculated here are in the same order of magnitude as reported for plasmid pJP4 [23].

All putative transconjugants showed REP- and GTG₅-fingerprints identical to those of pure cultures of the corresponding recipient strains (data not shown). It can therefore be excluded that the isolates are medium contaminants or donor strain mutants growing on the double-selective medium. Plasmids with a size of ca. 97 kb (corresponding to the size of pEMT1::lacZ) were effectively isolated from all putative transconjugants that were picked from the double-selective medium, except for *Rhizobium radiobacter* (data not shown). Therefore, only the latter isolates could not be confirmed unambiguously as “true” pEMT1::lacZ transconjugants.

In conclusion, plasmid pEMT1::lacZ could be transferred in plate matings to representatives of the α -, β -, and γ -Proteobacteria, with efficient expression of the 2,4-D degradative genes limited to (some) representatives of the β - and γ -Proteobacteria. These findings are very similar to the reported host range of plasmid pJP4 [23].

TABLE 7.1: Transfer of plasmid pEMT1::lacZ in conjugations with recipient strains on agar surfaces.

| Donor | Recipient | Phylogenetic position recipient | Counter-selection† | Transfer frequency | 2,4-D‡ |
|---|--|---------------------------------|--------------------|----------------------|--------|
| <i>Escherichia coli</i> DH5α (pEMT1::lacZ) | <i>Rhizobium radiobacter</i> LMG 221 | α-Proteobacteria | tetracycline | 4,5.10 ⁻⁴ | - |
| | <i>Rhizobium radiobacter</i> R-14122 | α-Proteobacteria | rifampicin | 4,5.10 ⁻⁸ | - |
| | <i>Blastomonas natatoria</i> LMG 17322 ^T | α-Proteobacteria | streptomycin | 1,7.10 ⁻⁵ | - |
| | <i>Escherichia coli</i> R-14120 | γ-Proteobacteria | rifampicin | 3,3.10 ⁻¹ | - |
| | <i>Arcobacter butzleri</i> LMG 10828 | ε-Proteobacteria | cefoperazone | no transc. | |
| | <i>Empedobacter brevis</i> LMG 4013 | Flavobacteriaceae | ampicilin | no transc. | |
| | <i>Myroides odoratus</i> LMG 13344 | Flavobacteriaceae | ampicilin | no transc. | |
| | <i>Bacillus sporothermodurans</i> R-6710 | Firmicutes, spore formers | 52°C | no transc. | |
| <i>Pseudomonas putida</i> UWC3 (pEMT1::lacZ) | <i>Rhizobium radiobacter</i> LMG 221 | α-Proteobacteria | MMO | 8,1.10 ⁻⁵ | - |
| | <i>Rhodobacter capsulatus</i> LMG 5161 | α-Proteobacteria | M80 an, ph | no transc. | |
| | <i>Azomonas macrocytogenes</i> LMG 1266 ^T | γ-Proteobacteria | M10 | 1,3.10 ⁻² | + |
| | <i>Listeria innocua</i> LMG 19458 | Firmicutes, low %G+C | 42°C | no transc. | |
| | <i>Ralstonia eutropha</i> JMP228 | β-Proteobacteria | nalidixic acid | ND | + |
| | <i>Lactococcus lactis</i> LMG 19460 | Firmicutes, low %G+C | nalidixic acid | no transc. | |

† Agent or conditions used to prevent the growth of the plasmid donor on selective plates. An, ph: anaerobic photoheterotrophic conditions. For the composition of the media and applied antibiotics concentrations, see text.

‡ 2,4-dichlorophenoxyacetic acid degradation capacity of a selected transconjugant in liquid medium

ND, not determined

7.2 EFFECT OF THE DISSEMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) DEGRADATIVE PLASMIDS ON 2,4-D DEGRADATION AND ON THE BACTERIAL COMMUNITY STRUCTURE IN TWO DIFFERENT SOIL HORIZONS[§]

7.2.1 Summary

Transfer of the 2,4-D (2,4-dichlorophenoxyacetic acid) degradative plasmids pEMT1 and pJP4 from an introduced donor strain *Pseudomonas putida* UWC3 to the indigenous bacteria of two different horizons (A-horizon: 0-30 cm; B-horizon: 30-60 cm depth) of a 2,4-D contaminated soil was investigated as a means of bioaugmentation. When the soil was amended with nutrients, plasmid transfer and enhanced degradation of 2,4-D were observed. This was most striking in the B-horizon, where the indigenous bacteria were unable to degrade any of the 2,4-D (100 mg/kg soil) during at least 22 days, while inoculation with either of the two plasmid donors resulted in complete 2,4-D degradation within 14 days. In contrast, in soils not amended with nutrients, inoculation of donors in the A-horizon and subsequent formation of transconjugants (10^5 CFU/g soil) could not increase the 2,4-D degradation rate compared to the non-inoculated soil. However, donor inoculation in the non-amended B-horizon soil resulted in complete degradation of 2,4-D within 19 days, while no degradation at all was observed in non-inoculated soil during 89 days. In the case of plasmid pEMT1, this enhanced degradation seemed to be only due to transconjugants (10^5 CFU/g soil), since the donor was already undetectable when degradation started. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes showed that inoculation of the donors was followed by a shift in the microbial community structure of the non-amended B-horizon soils. The new 16S rRNA gene fragments in the DGGE-profile corresponded with the 16S rRNA genes of 2,4-D degrading transconjugant colonies isolated on agar plates. This indicates that the observed change in the community was due to proliferation of transconjugants formed in soil. Overall, this work clearly demonstrates that bioaugmentation can constitute an effective strategy for clean-up of soils which are poor in nutrients and microbial activity, such as those of the B-horizon.

7.2.2 Introduction

The highly diverse microbial communities present in fresh and marine waters, sewage, and soils are able to transform a wide range of organic chemicals. However, many synthetic organic compounds, although biodegradable, may persist in nature for a long time because the required catabolic capacity is not present or because the populations of micro-organisms bringing about their destruction are not large or active

[§] Redrafted from **Dejonghe, W., J. Goris, S. El Fantroussi, M. Höfte, P. De Vos, W. Verstraete, and E. M. Top.** 2000. Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. *Appl Environ Microbiol* **66**:3297-3304.

enough. One way to enhance breakdown of these chemicals is bioaugmentation by inoculation of a habitat with micro-organisms that are either known to readily metabolise the chemicals or that have been equipped with the necessary degradative genes in the laboratory [39, 78]. This bioaugmentation of polluted soils has already been studied by different authors [6, 11, 16, 17, 30, 53]. However, in many cases the introduced bacteria fail to degrade the pollutants due to their poor survival or low activity in the environment caused by abiotic and biotic stresses which are not encountered in the laboratory environment [1, 2, 39, 78, 93, 95]. An alternative approach involves the introduction of appropriate plasmid-encoded catabolic genes into the well established and competitive indigenous bacterial populations. Here, the survival of the introduced donor strain is no longer needed once the catabolic genes are transferred to the indigenous bacteria. In addition, the plasmid transfer may result in vertical movement of the catabolic genes through the soil, resulting in the dissemination of the desired catabolic activity into the deeper soil layers. This is especially important since the indigenous metabolic activity in these subsurface soils is lower, which could result in contamination of groundwater [35]. Also, the high costs of mixing the soil layers to bring the bacteria in close proximity of the pollutant could be avoided. To date, a large number of studies have reported the occurrence of conjugative gene transfer between bacteria in soil [26, 48], however, there is only little information about transfer of catabolic plasmids as a means of bioaugmentation [9, 20, 22, 66, 89, 91]. Research in our laboratory has shown before that this approach accelerated the degradation of biphenyl or 2,4-dichlorophenoxyacetic acid (2,4-D) in soil, although the introduced donor strains survived only between 3 and 14 days [20, 89, 91]. Other groups have shown less pronounced effect of 2,4-D degradation, using the same strategy [22, 66].

A model compound for the study of plasmid-encoded catabolic genes is the herbicide 2,4-D, which is a commercially used chlorinated aromatic compound. Genes encoding 2,4-D degradation are often located on conjugative plasmids [7, 23, 57, 88, 90] but have recently been found to be also chromosomally located [58, 85]. The most extensively studied plasmid is pJP4 from *Ralstonia* sp. JMP134 [23], which has become a model for the study of 2,4-D degradation. Plasmid pEMT1 [88] carries virtually the same degradative genes in a similar organisation as plasmid pJP4 but does probably not belong to the same incompatibility group as pJP4 (IncP β) [43, 88]. Nothing is known about the difference in their transfer frequency and host range in soil.

In this study we have investigated the transfer of the 2,4-D degradative plasmids pEMT1 and pJP4, from an introduced donor strain *Pseudomonas putida* UWC3 to the indigenous bacteria of the A- and B-horizon of a 2,4-D contaminated sandy-loam soil. Compared to the soil from the A-horizon (0-30 cm), the soil of the B-horizon (30-60 cm) has a different texture, organic matter content and probably also a different microbial community, with a different metabolic activity. The impact of these differences on the 2,4-D degradation capacity of the soils, the plasmid transfer rates and the subsequent accelerated 2,4-D degradation was investigated. In addition we examined if the formation of indigenous transconjugants in soil had an impact on the overall microbial community structure, as revealed by the culture-independent technique DGGE (Denaturing Gradient Gel Electrophoresis) of 16S rRNA genes.

7.2.3 Materials and methods

7.2.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 7.2. The 2,4-D degradative plasmids pEMT1 and pJP4 were tagged with a mini-transposon, mini-Tn5 *KmlacZ* [19] as previously described [90] and were designated pEMT1::*lacZ* and pJP4::*lacZ*. In brief, a triparental conjugation was performed with *E. coli* CC118 λ pir (pUT-mini-Tn5 *KmlacZ*) as donor strain [47], *E. coli* HB101 (pRK2013) as helper strain [34] and *Ralstonia* sp. JMP134, containing the plasmid pJP4 [23], or *R. eutropha* JMP228 (pEMT1) as recipients. The mating mixture was resuspended in MMO mineral medium [81] with 1000 mg/l 2,4-D, 50 mg/l Km, and 20 mg/l X-gal, the latter as a chromogenic substrate for detection of β -galactosidase activity. Cultures were shaken at 150 rpm at 28°C. Tagged plasmids were subsequently transferred to *E. coli* DH5 α [44] and further to *P. putida* UWC3, which was used as a donor in the transfer experiments. Marking of the plasmids had no measurable effect on their transfer frequencies, nor on the 2,4-D degradation capacity of the host strain *R. eutropha* JMP228 (data not shown). We have chosen *P. putida* UWC3 as a donor strain because *P. putida* UWC3 (pEMT1) has been shown to degrade 2,4-D poorly or not at all in mineral medium with or without additional C-source [89]. Additional degradation tests with autoclaved soil showed that *P. putida* UWC3 (pEMT1::*lacZ*) degraded 100 mg/kg 2,4-D in 15 days in nutrient amended soil but no degradation of 2,4-D, even after 80 days, was observed in non-amended soil. This poor degradation capacity of *P. putida* UWC3 (pEMT1::*lacZ*) seemed to be due to instability of the plasmid. *P. putida* UWC3 (pJP4::*lacZ*) on the contrary degraded 100 mg/kg 2,4-D both in the presence (5 days) and absence of nutrients (21 days) (data not shown).

TABLE 7.2: Most important bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristic(s) ^a | Reference or source |
|-----------------------------------|---|---------------------|
| Strains | | |
| <i>Pseudomonas putida</i> UWC3 | Rif ^r , ilva ⁻ | [49] |
| <i>Ralstonia eutropha</i> JMP228 | Rif ^r | [23] |
| <i>Ralstonia eutropha</i> JMP228n | Rif ^r , Nx ^r | [88] |
| Plasmids | | |
| pUT-mini-Tn5 <i>KmlacZ</i> | Km ^r , LacZ ⁺ | [19]13 |
| pEMT1 | Tfd ⁺ | [88] |
| pJP4 | Tfd ⁺ , 3-CBA ⁺ , Hg ^r | [23] |
| pEMT1:: <i>lacZ</i> | Tfd ⁺ , Km ^r , LacZ ⁺ | This study |
| pJP4:: <i>lacZ</i> | Tfd ⁺ , 3-CBA ⁺ , Hg ^r , Km ^r , LacZ ⁺ | This study |

^a Rif^r, Nx^r, Km^r, and Hg^r, resistance to rifampin, nalidixic acid, kanamycin, and mercury, respectively; ilva⁻, auxotrophic for isoleucine, leucine and valine; Tfd⁺ and 3-CBA⁺, ability to degrade 2,4-D and 3-chlorobenzoate, respectively; LacZ⁺, β -galactosidase activity.

7.2.3.2 Media and culture conditions

P. putida UWC3 was grown on MKB agar [79] with 100 mg/l rifampin. The MKB-medium for the donors *P. putida* UWC3 (pEMT1::lacZ) and *P. putida* UWC3 (pJP4::lacZ) contained besides 100 mg/l rifampin, also 50 mg/l Km as selection pressure for the plasmids and 20 mg/l X-gal. *R. eutropha* JMP228 (pEMT1) and *R. eutropha* JMP228 (pJP4) were maintained on MMO mineral medium supplemented with 500 mg/l 2,4-D [88] and 40 mg/l bromothymol blue, a pH-indicator [56]. All other strains were grown on LB [79] medium with the appropriate antibiotics (100 mg/l rifampin, 200 mg/l nalidixic acid, or 50 mg/l kanamycin (Km)) and 20 mg/l X-gal. All plates were incubated at 28°C.

7.2.3.3 Soils

The soils used in this study were collected from a farm (Pittem, Belgium), and have previously not been exposed to 2,4-D. The A-horizon was sampled at a depth of 0-30 cm and was characterized as a sandy loam soil (7.3% clay, 16.8% loam, 75.9% sand). This soil has an organic matter content of 1.38 %, a pH of 5.6 (KCl) and a moisture content at field capacity of 15.9 % ± 0.9 % on dry soil [94]. The microbial activity in this A-horizon was 1.97 mg CO₂-C/kg soil.day as determined by CO₂ respiration measurements [70]. The B-horizon (30-60 cm) belongs to the sandy texture class (7.6% clay, 9.2% loam, 83.2% sand) with an organic matter content of 0.38 %, a pH of 5.3 (KCl), a moisture content at field capacity of 11.8 % ± 0.8 % on dry soil [94] and a microbial activity of 0.52 mg CO₂-C/kg.day. The heterotrophic plate counts (R2A agar, Difco, Detroit, MI) of both the A- and B-horizon varied between ca. 10⁷ and 10⁸ CFU/g fresh weight of soil. Soils were stored at room temperature.

7.2.3.4 Microcosm design and treatments

Transfer experiments were performed in 500 ml glass microcosms which contained 100 g of the A- or B-horizon soil. An overnight culture of the donors *P. putida* UWC3 (pEMT1::lacZ) and *P. putida* UWC3 (pJP4::lacZ) in LB with 50 mg/l Km was washed twice in 0.85 % sterile saline. When transfer experiments were performed in the presence of nutrients the pellet was resuspended in 5 ml of five times concentrated LB (5 × LB), otherwise in 5 ml of saline. One ml of this bacterial suspension was mixed thoroughly with 100 mg/kg of 2,4-D in 100 g of the A- or the B-horizon. To non-inoculated control soils only 2,4-D and either 5 × LB or saline, but no donor cultures were added. After all amendments were made, the moisture content was adjusted with water to 75 % of the water-holding capacity. Soil microcosms were incubated at room temperature in a plastic container covered with aluminium foil in which the relative humidity was kept high by means of an open beaker filled with tap water. All treatments were performed in duplicate using independent microcosms.

The survival of the donor strain in the soil was followed in function of time by plating 0.1 ml samples of serial ten-fold dilutions of 1 g of soil in saline on MKB agar that contained 100 mg/l rifampin and 200 mg/l cycloheximide (MKBRC), a medium on which the donor is fluorescent under U.V.-light. The survival of the donor with the plasmid was monitored by plating on the previous medium with in addition 50 mg/l Km (MKBRCK) to select for the tagged plasmids. Transconjugants were detected as blue

colonies on MMO mineral medium with 500 mg/l 2,4-D as the sole C-source, 20 mg/l X-gal, 50 mg/l Km, and 200 mg/l cycloheximide (MXK2,4-D). Since the *P. putida* UWC3 donors require isoleucine, leucine and valine they cannot grow on this medium. The detection limit for donors and transconjugants was 10^2 CFU/g soil. All plates were incubated at 28°C, and plate counts (CFU/g soil) are expressed per g fresh weight of soil.

2,4-D was extracted from 1 g of soil with 2 ml of sterile demineralised water. Its concentration was determined by High Performance Liquid Chromatography analysis (Kontron) using an Alltima C18 reversed phase column (250 mm x 8 mm ID; 5 µm particle size; Alltech, Deerfield, Ill.), a methanol/0.1 % aqueous phosphoric acid (85:15) mixture as an eluent at a flow rate of 0.8 ml/min and a Kontron Diode Array Detector set at 230 nm for detection and quantification of 2,4-D [91].

7.2.3.5 Confirmation of the potential transconjugants

Blue colonies which developed from the highest dilution on MMO plates with 2,4-D, X-gal and Km (MXK2,4-D) were transferred to LB, MKBRCK and MXK2,4-D plates by replica plating to compare the colony morphology of the transconjugants, and to eliminate false positives or potential donor cells, forming colonies on MXK2,4-D. Putative transconjugants with different morphologies were mated with *R. eutropha* JMP228n [61] with selection on LB plates containing 50 mg/l Km, 200 mg/l nalidixic acid and 20 mg/l X-gal. The presence of the plasmids was confirmed by a modified Kado & Liu plasmid extraction [50, 87, 88]. Plasmids were visualised on a 0.8 % agarose gel [79] and compared with the plasmids extracted from the donors. This procedure also suggested that transconjugants formed in soil were able to act as donors of the plasmids. We also confirmed that the pJP4 and pEMT1 plasmid-borne *tfdA* gene was present in unique isolates by performing a PCR with the primers TVU and TVL as previously described [92]. The 2,4-D degradation capacities of some of the positive transconjugants were compared with those of the donors, *R. eutropha* JMP228 (pEMT1::lacZ) and *R. eutropha* JMP228 (pJP4::lacZ) (positive controls) and *P. putida* UWC3 and *R. eutropha* JMP228n (negative controls) by transferring one colony of each strain in 5 ml of liquid MMO mineral medium with 100 mg/l 2,4-D and when appropriate 50 mg/l Km. After 3 days of shaking at 150 rpm at 28°C the remaining amount of 2,4-D was quantified by HPLC-analysis [91].

7.2.3.6 DNA extraction and denaturing gradient gel electrophoresis

For pure cultures, the PCR template was obtained by boiling a colony for 10 min in 200 µl of MilliQ-water. For DGGE analysis of colony mixtures resuspended from plates (referred to as plate DNA), colonies were scraped off with a loop after which the plates were washed with 2 ml of water. Two hundred µl of this suspension was boiled for 10 min and used as template in the PCR reaction. For soil analyses, samples (2 g) were taken at regular intervals from one single microcosm per treatment. Total DNA (referred to as soil DNA) was extracted based on the protocol described previously [31, 32], followed by purification with the Wizard[®] DNA Clean-Up System (Promega, Madison, WI). In all cases 1 µl of the template was used for PCR with the bacterium-specific 16S rRNA forward primer P63f and the reverse primer P518r, based on a

universally conserved region, as previously described [32]. The PCR product contains a GC-clamp of 40 bases, added to the forward primer and has a total length of 531 bp (based on the reference strain *Escherichia coli* K12). PCR products were subjected to DGGE based on the protocol of Muyzer et al. [63] and El Fantroussi et al. [32]. In brief, PCR samples were run for 17 h at 50 V on a 6% (wt/vol) polyacrylamide gel with a denaturing gradient ranging from 50-65% (where 100% denaturant contains 7 M urea and 40% formamide). After electrophoresis the gels were stained with SYBR GreenI nucleic acid gel stain and photographed. The pictures presented in this paper are negative images.

Some bands were excised from the gel and incubated overnight at 4°C in 20 µl of MilliQ-water. Subsequently, 1 µl of this solution was reamplified in 25 µl PCR mix, and the amplified products were cloned into the PCR-TOPO 2.1 cloning vector (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Recombinant (white) colonies were screened by using a two-stage procedure to ensure recovery of the DGGE band of interest. First, plasmid inserts (8 for each band) were reamplified by PCR with vector-specific primers (M13 reverse and T7; Invitrogen Corp.). The PCR products were immediately reamplified with the 16S-specific PCR primers described above [32] and subjected to DGGE analysis. Sequences that comigrated with the original band of interest were selected and sequenced by Eurogentec (Liège, Belgium). The partial sequences, of approximately 400 bp., were aligned to 16S rRNA sequences obtained from the National Centre for Biotechnology Information database by using the BLAST 2.0 search program [3].

7.2.3.7 Nucleotide sequence accession numbers

The nucleotide sequences for bands 1 till 5 have been deposited in the GenBank database under accession no. AF247780 till AF247784.

7.2.4 Results

Horizontal transfer of the mini-Tn5 *KmlacZ* tagged plasmids pEMT1 and pJP4 from an introduced donor strain *P. putida* UWC3, to indigenous bacteria of the A- and B-horizon of a sandy loam soil was studied. The effect of this transfer on the degradation of 2,4-D and on the microbial community structure was investigated.

7.2.4.1 Transfer of plasmids and degradation of 2,4-D in nutrient-amended soil

Approximately 10^5 CFU/g soil of *P. putida* UWC3 (pEMT1::*lacZ*) and 10^6 CFU/g soil of *P. putida* UWC3 (pJP4::*lacZ*) were inoculated separately in duplicate independent microcosms containing either the nutrient-amended A- or B-horizon, spiked with 2,4-D (100 mg/kg). The microcosms were sampled on day 0, and 2, 6, 14, 22, and 50 days after inoculation. In the A-horizon soil, 2,4-D degrading transconjugants with plasmid pEMT1::*lacZ* and pJP4::*lacZ* were first detected after 6 days ($4,2 \times 10^4$ CFU/g soil and $3,0 \times 10^3$ CFU/g soil, respectively), and their numbers remained quite stable throughout the experiment. On day 0 and day 2 counts of putative transconjugant colonies on plates did not exceed those of the background growth of non-inoculated control soil (ca. 10^3 CFU/g soil) (data not shown). Randomly picked

transconjugants from day 6 and later contained a plasmid of the same size as pEMT1::lacZ or pJP4::lacZ, gave a positive PCR signal with the *tfdA* primers, and degraded 100 mg/l of 2,4-D in liquid mineral medium in 3 days while no degradation at all was observed during this period for the donors.

In non-inoculated nutrient amended soil from the A-horizon indigenous bacteria degraded 100 mg/kg of 2,4-D in 14 days, a period that was shortened when *P. putida* UWC3 (pEMT1::lacZ) and *P. putida* UWC3 (pJP4::lacZ) were added (Fig. 7.1). This shows that inoculation of the donors enhanced the degradation of 2,4-D.

In the nutrient-amended inoculated soil from the B-horizon, transconjugants were also first detected on day 6, but in slightly higher numbers than in the A-horizon ($8,3 \times 10^5$ CFU/g soil for pEMT1::lacZ and 2.9×10^5 CFU/g soil for pJP4::lacZ), and they also remained constant for at least 50 days. Background on transconjugant selective plates was the same as in the A-horizon (data not shown). Randomly isolated and purified transconjugants were confirmed to be true transconjugants as described above.

Interestingly, indigenous bacteria of the B-horizon only started to degrade the 2,4-D after 22 days, while in the inoculated soils 80-90 % of the 2,4-D was degraded in 6 days and 100 % between day 6 and 14 (Fig. 7.1). Hence, the inoculation of donors in this B-horizon enhanced the degradation of 2,4-D in an even more pronounced way than in the A-horizon.

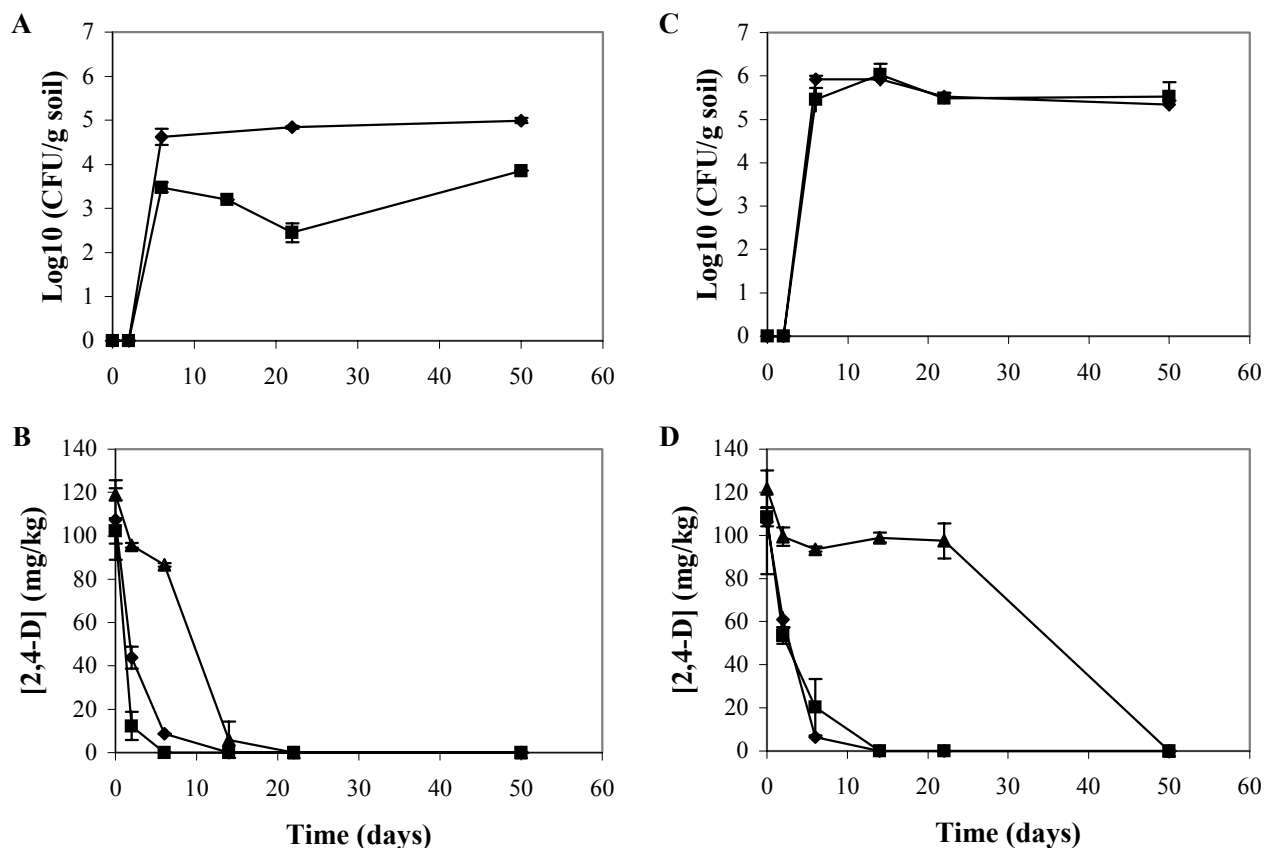


FIG. 7.1: Effect of donor inoculation and subsequent plasmid transfer on the degradation of 2,4-D in the nutrient-amended A- horizon (panel a and b) and B-horizon (panel c and d). The data points and error bars show the means and standard deviations based on data from duplicate microcosms. (▲) non-inoculated soil + 2,4-D; (◆) soil + 2,4-D + pEMT1::lacZ; (■) soil + 2,4-D + pJP4::lacZ.

7.2.4.2 Transfer of plasmids and degradation of 2,4-D in soil without nutrients

In order to mimic more closely the natural soil conditions, the same experiment was performed as described above, but without addition of nutrients to the soils (non-amended soil).

In the A-horizon soil, spiked with 2,4-D, transconjugants with either pEMT1::lacZ or pJP4::lacZ were first observed on day 7, and slightly increased in numbers until day 34 (Fig. 7.2a). On day 0 and day 4 counts of transconjugants on plates did not exceed these of the background growth of non-inoculated control soil (ca. 10^2 CFU/g soil). For each plasmid donor fifty putative transconjugants were picked from the transconjugant selective plates of days 7, 12, 22, and 34. Of all colony types, a representative was selected (22 for pEMT1::lacZ, 14 for pJP4::lacZ) and confirmed to be true transconjugants, as described above.

In the A-horizon the number of plasmid-containing donors declined rapidly, especially for *P. putida* UWC3 (pEMT1::lacZ) (Fig. 7.2a). This seemed due to the instability of the plasmid, since counts of *P. putida* UWC3 on plates without selection for the plasmid (kanamycin) remained quite constant (10^4 CFU/g soil) (data not shown).

In this non-amended A-horizon, the addition of donors and formation of transconjugants did not result in an enhanced 2,4-D degradation. The 100 mg/kg of 2,4-D was degraded as fast in the non-inoculated control soil as in the inoculated soils (Fig. 7.2b). When 100 mg/kg of 2,4-D was added to the soil for a second time at day 21, it was completely degraded in less than 4 days in both the A-horizon soil with and without the donors.

In the B-horizon soil, spiked with 2,4-D and inoculated with approximately the same numbers of donors as the A-horizon, transconjugants with either plasmid were first detected on day 7 (Fig. 7.2c) and further increased by 2 log units. Background on transconjugant selective plates was the same as in the A-horizon. For each donor fifty transconjugants were picked from the selective plates on days 7, 12, 22, and 34. A selection of these transconjugants was confirmed to contain the corresponding plasmid and to degrade 2,4-D (36 transconjugants for pEMT1::lacZ, 14 for pJP4::lacZ).

As in the A-horizon, a poor survival of the plasmid containing donors was observed, especially for *P. putida* UWC3 (pEMT1::lacZ). On day 12 the number of this donor had already decreased below the detection limit (Fig. 7.2c).

Interestingly, unlike the indigenous bacteria in the non-amended A-horizon, those in the non-amended B-horizon control soil were not able to degrade any of the 100 mg/kg of 2,4-D over the first 21 days. However, in the B-horizon soils inoculated with *P. putida* UWC3 (pEMT1::lacZ) and *P. putida* UWC3 (pJP4::lacZ) the 2,4-D was completely degraded by 19 and 15 days, respectively (Fig. 7.2d). A second addition of 100 mg/kg 2,4-D on day 21 was completely degraded in less than 4 days in the soils inoculated with donors, but still no degradation occurred in the non-inoculated B-horizon soil (Fig. 7.2d). Even 68 days after the second amendment of 2,4-D there was still 174 of the initial 200 mg/kg of 2,4-D present in this control soil (data not shown). These results demonstrate a clear success of bioaugmentation in the B-horizon.

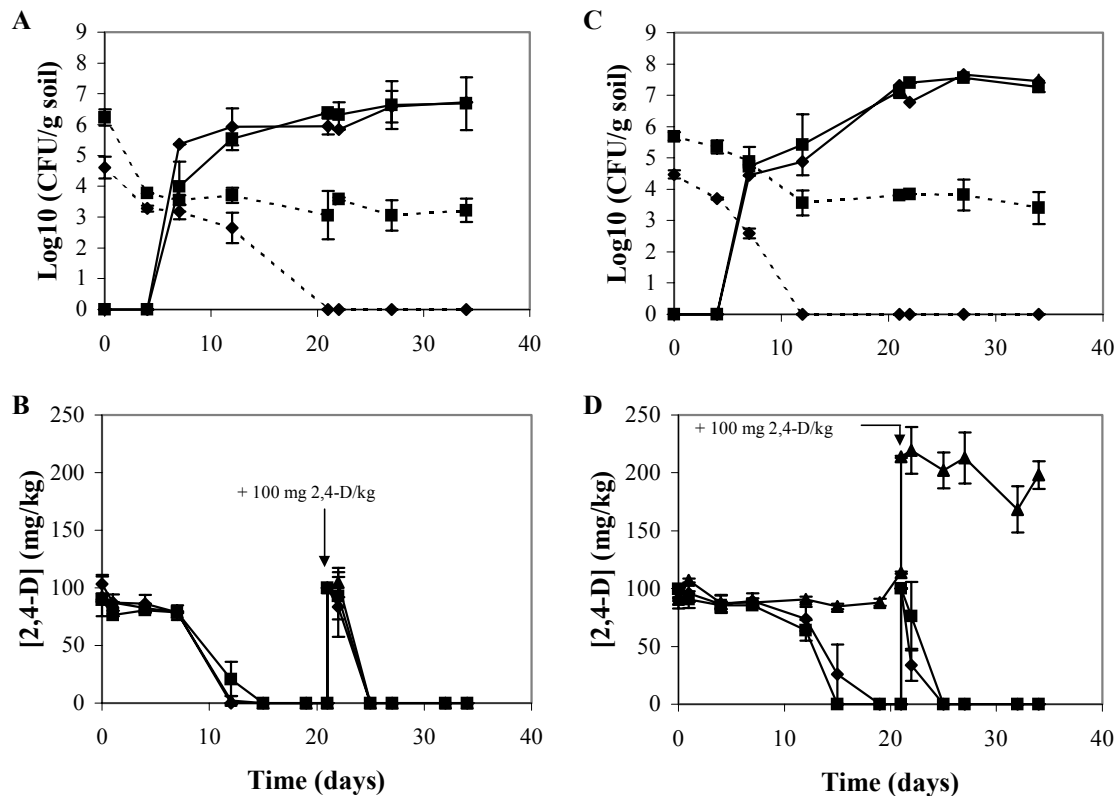


FIG. 7.2: Transfer of the plasmids pEMT1::lacZ and pJP4::lacZ in the non-amended A- and B-horizon. (a and c) Survival of donors (dotted lines) and formation of transconjugants (solid lines). (b and d) Effect of donor inoculation and subsequent plasmid transfer on the degradation of 2,4-D. The data points and error bars show the means and standard deviations based on data from duplicate microcosms. ▲, non-inoculated soil plus 2,4-D; ◆, soil plus 2,4-D plus pEMT1::lacZ; ■, soil plus 2,4-D plus pJP4::lacZ. The broken straight line represents the detection limit of plate counts. The arrow indicates the second amendment of 100 mg 2,4-D/kg soil on day 21.

7.2.4.3 Revelation of transconjugants by Denaturing Gradient Gel Electrophoresis

To study the effect of the inoculation of the *P. putida* donors on the structure of the microbial community, DGGE of 16S rRNA genes was used, based on total soil DNA. More specifically, the aim was to investigate if the transconjugants, detected in high numbers by selective plating (Fig. 7.2a and 7.2c) would also be revealed as members of the numerically dominant populations in soil using a non-cultivation based approach. Since the effect of donor inoculation and subsequent plasmid transfer on the 2,4-D degradation was most profound in the non-amended B-horizon soil, this experiment was selected to be studied in more detail.

In the non-inoculated non-amended B-horizon soil the addition of 100 mg/kg 2,4-D did not cause clear changes in the DGGE profiles (Fig. 7.3). When the *P. putida* donors were added to this soil, four bands corresponding to the donor, as shown in DGGE patterns of the strain UWC3, always were very dominant (data not shown). The intensity of these four bands diminished gradually from day 21, and this change concurred with the appearance of new dominant bands as shown for soil with *P. putida*

UWC3 (pEMT1::lacZ) in Fig. 7.3. These bands remained clearly dominant throughout the experiment and most were not visible in the patterns of the non-inoculated soil.

To investigate if the new bands observed in the DGGE pattern of the non-amended B-horizon soil, inoculated with *P. putida* UWC3 (pEMT1::lacZ), corresponded to transconjugants, the DGGE patterns from total soil DNA extracted on days 0, 4, 7, 12, 21, 27 and 34 (= "soil DNA") were first compared to the DGGE patterns of the mixture of transconjugant colonies, resuspended from the transconjugant selective agar plates (= "plate DNA") (see Fig. 7.2c). Fig. 7.3 shows that the different DGGE-profiles of soil and colony suspensions matched very well. Interestingly, the bands that corresponded with putative transconjugants were detected earlier in the DGGE-profiles of the colony suspension than in the DGGE-profile from total soil DNA (day 7 compared to day 21). The bands in the DGGE pattern of plate DNA of day 0 and day 4 correspond to false negatives since these two bands are also visible in the DGGE pattern inoculated with 2,4-D but without the donor strain (Fig. 7.3), and since on day 0 and day 4 counts of transconjugants on plates did not exceed these of the background of non-inoculated control soil (see Fig. 7.2c).

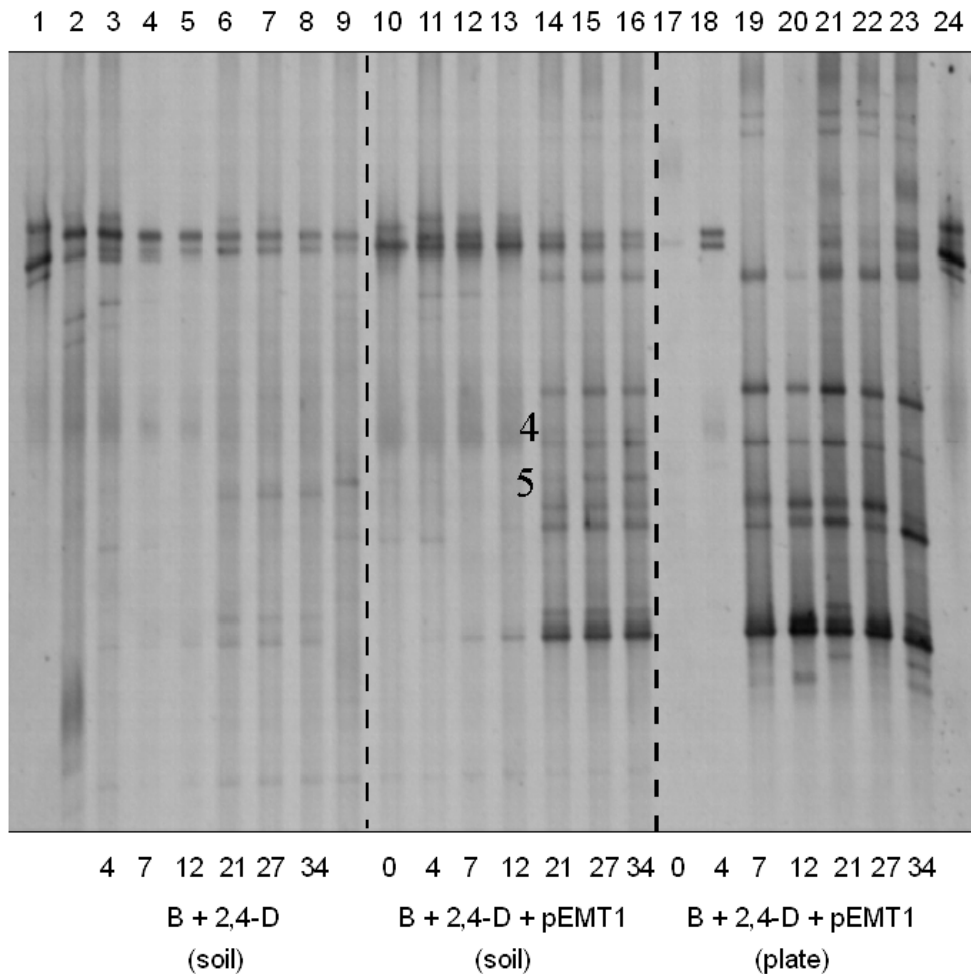


FIG. 7.3: Comparison of the DGGE profiles on days 0, 4, 7, 12, 21, 26 and 34 of 16S rRNA gene fragments amplified from DNA of the non-amended B-horizon (soil DNA) treated with 2,4-D and with or without the donor *P. putida* UWC3

(pEMT1::lacZ), and of DNA extracted from a mixture of colonies resuspended from transconjugant selective plates (plate DNA). Bands 4 and 5 are present in the DGGE profile of soil DNA, but not in that of plate DNA. Lane 1 and 24, *P. putida* UWC3 (pEMT1::lacZ), lane 2 and 9, native B-horizon without 2,4-D and donor, day 0 and 89, respectively; lane 3 through 8, soil DNA from non-inoculated B-horizon + 2,4-D; lane 10 through 16, soil DNA from B-horizon + 2,4-D + pEMT1::lacZ; lane 17 through 23, plate DNA from transconjugant selective plates.

To confirm that the new bands in the DGGE patterns of soil and plate DNA were indeed derived from real transconjugants, the partial 16S rRNA genes of a few purified and confirmed transconjugant colonies with different colony morphology isolated on day 7 and 34 were loaded on the same DGGE gel (Fig. 7.4). Most of the DGGE bands in the patterns of soil and plate DNA corresponded with bands of purified transconjugants. The DNA sequences of two bands from a different origin (soil DNA, lane 10; transconjugant colony, lane 14) but with the same position in the DGGE pattern (see band 1 and 2 on Fig. 7.4) were almost identical (99.8 % similarity) and had a high nucleotide sequence similarity (96 %) with *Burkholderia graminis*. This clearly indicates that the lowest band in the DGGE profile from soil corresponds with a real transconjugant.

As can be seen in Fig. 7.4, there were at least 4 major groups of transconjugants on day 34 based on the position of the brightest band in the DGGE gel (lane 12, lane 13, lane 14 through 17, lane 18 through 20). In a separate study, these transconjugants were identified as *Ralstonia eutropha*-like organisms (lanes 12 and 13), *Burkholderia graminis*-like (lanes 14 through 17) and *Burkholderia caribensis*-like (lanes 18 through 20) [40]. This was confirmed by sequence analysis of the DGGE bands of two of these transconjugants (Fig. 7.4, band 2: *B. graminis* (96 %), band 3: *B. caribensis* (96.6 %)). These results indicate that the plasmid transferred to different species within at least two different genera.

Two additional bands were detected in the DGGE pattern of the soil DNA that were absent in the pattern of plate DNA and also invisible in the pattern of non-inoculated soil (band 4 and 5 Fig. 7.3). The sequence of band 4 matched most closely (98 %) with *Bradyrhizobium* sp., while band 5 showed high sequence similarity to a Grassland soil clone (98 %) [60] and to a *Sphingomonas* sp. (97 %). This could suggest that some transconjugants which may not be detected by plating can be observed by DGGE analysis.

The DGGE patterns of soil and plate DNA from the non-amended B-horizon with *P. putida* UWC3 (pEMT1::lacZ) were compared with those of the same soil inoculated with the other donor, *P. putida* UWC3 (pJP4::lacZ). It was clear that the DGGE patterns on day 34 were almost identical for both plasmids, and that also in the case of pJP4::lacZ, two additional bands were present in the DGGE profile of soil DNA, which were not visible in the pattern of the plate DNA (data not shown).

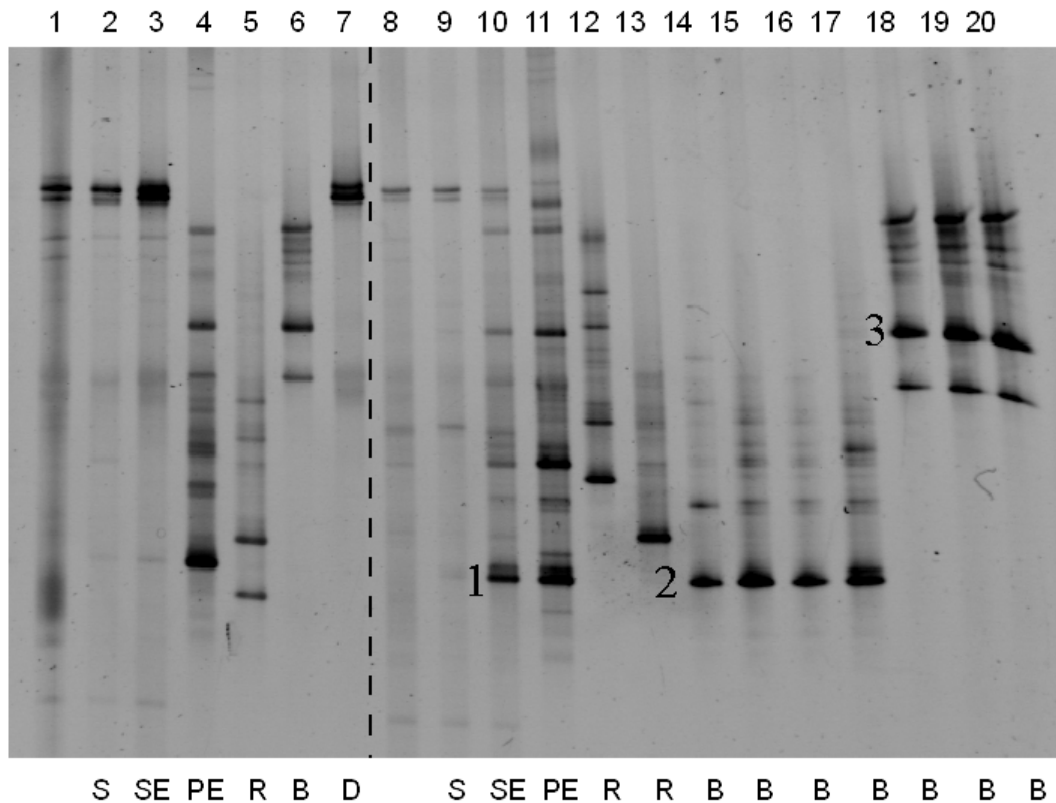


FIG. 7.4: Comparison of the DGGE patterns of soil DNA of the B-horizon soil with 2,4-D and with or without the donor *P. putida* UWC3 (pEMT1::lacZ) (soil DNA), with DNA of a mixture of colonies resuspended from transconjugant selective plates (plate DNA), and of single colonies of purified and confirmed transconjugants on day 7 and 34. Band 1 and 2 are very similar and most related to *B. graminis* (96 %). Band 3 matched most closely *B. caribensis* (96.6 %). Lane 1 and 8, native B-horizon without 2,4-D and donor, day 0 and 89, respectively; lane 2 and 9, soil DNA from non-inoculated B-horizon + 2,4-D, day 7 and 34, respectively (= S); lane 3 and 10, soil DNA from B-horizon + 2,4-D + pEMT1::lacZ, day 7 and 34, respectively (= SE); lane 4 and 11, plate DNA from transconjugant selective plates, day 7 and 34, respectively (= PE); lane 5, 6, and lanes 12 through 20, single colonies of purified transconjugants, day 7 and 34, respectively (B = *Burkholderia* species; R = *Ralstonia eutropha*-like organism); lane 7, *P. putida* UWC3 (pEMT1::lacZ) (= D).

7.2.5 Discussion

Plasmid transfer and a clearly increased rate of 2,4-D degradation were observed when *P. putida* UWC3 was added as donor of the 2,4-D degradative plasmids pEMT1::lacZ and pJP4::lacZ to nutrient-amended A- and B-horizon (Fig. 7.1). However, the enhanced 2,4-D degradation was probably mainly due to the activity of the donor strains, and not to the transconjugants since by day 2, ca. 50 % or more of the added 2,4-D was already degraded, while no transconjugants could be detected yet at that point in time, and the donors were still present at ca. 10^6 CFU/g soil (data not shown). This corresponds with the degradation results in sterile nutrient-amended soil, where the two donor strains could degrade 50 % of the added 2,4-D in 2 days (data not shown). However, we cannot totally exclude the involvement of the transconjugants in

the 2,4-D degradation, since their numbers rose drastically between day 2 and day 6, during which the remaining 50 mg/kg of 2,4-D was almost completely degraded.

In contrast, in the case of bioaugmentation of the non-amended B-horizon soil inoculated with *P. putida* UWC3 (pEMT1::lacZ) the observed 2,4-D degradation must be due to transconjugants, since they were already detected when the 2,4-D degradation started at day 7, and since most of the *P. putida* UWC3 donor cells had already lost their plasmid by then (Fig. 7.2 c and d). This is supported by the results obtained in sterile soil experiments, where *P. putida* UWC3 (pEMT1::lacZ) was not able to degrade 2,4-D due to the instability of its plasmid (data not shown). In the case of plasmid pJP4::lacZ, the observed disappearance of 2,4-D in the non-amended B-horizon was probably due to both transconjugants and donors, since *P. putida* UWC3 (pJP4::lacZ) was still present at 10^4 CFU/g soil when 2,4-D degradation started, and was able to degrade 2,4-D in sterile soil (100 mg/kg in 21 days). Since the number of transconjugants of both plasmids increased dramatically while the 2,4-D was degraded, they were probably involved in the degradation in both cases.

The more striking effect of bioaugmentation in the B-horizon compared to the A-horizon was due to the poor 2,4-D degradation capacity of the indigenous bacteria in this deeper soil layer (Fig. 7.1, Fig. 7.2d). A similar decrease in 2,4-D degradation activity with depth was observed by Veeh et al. [98] in a silt loam and silt clay soil. This was explained by the fact that microbial plate counts, which are positively correlated with the soil organic carbon content, declined in function of depth. In our study, the total plate count of the B-horizon was not different from that of the A-horizon, but its microbial activity, as shown by the respiration rate (1.97 mg CO₂-C/kg soil.day compared to 0.52 mg CO₂-C/kg.day) was clearly lower. The microbial capacity to degrade 2,4-D seemed to be present in the B-horizon of our soil since 2,4-D was degraded in the nutrient amended soil after a lag period of more than 22 days. Audus [5] and Veeh et al. [98] also observed this lag phase which increased with depth. They characterised it as a period of adaptation during which the enzymes needed for decomposition of the substrate and its metabolites are synthesised.

The transfer of the 2,4-D degradative plasmid pEMT1 in nutrient-amended soil has been shown before by our group, first with *E. coli* X11Blue (pEMT1k) as a donor strain [91]. Transconjugants were detected at similar numbers as in this study, and the transfer also resulted in enhanced degradation of 2,4-D. A more recent study showed transfer of plasmid pJP4 from an *E. coli* strain to different indigenous bacteria in some but not all tested soils, and in the presence of 500 or 1000 mg/kg 2,4-D. The effect of transfer on accelerated 2,4-D degradation was very small and only shown in one of the four soils studied [66]. Since *E. coli* is not a suitable strain for bioremediation purposes, Top et al. [89] extended their study [91] in the same soil without nutrients, with *P. putida* UWC3 as donor of plasmid pEMT1k. A similar transfer rate was found as in our current study, causing an enhanced degradation of 2,4-D. However, since the donor strain was still present when degradation started (10^4 CFU/g soil), its involvement in the degradation of 2,4-D could not be totally excluded. As far as we know, our current study is the first one to demonstrate so clearly that bioaugmentation can be effective in soils with low microbial activity, such as those of the B-horizon.

It was clear that the transfer rate of the plasmids was independent of the kind of plasmid (pEMT1 or pJP4) and soil (A- or B-horizon) but that the addition of nutrients to the soil did not increase the number of transconjugants. This is in contrast with many studies such as those of Top et al. [87] and Götz and Smalla [42], who showed that addition of nutrients to soil enhanced the number of transconjugants per gram soil. A possible explanation why the transconjugant numbers in the nutrient-amended soil were not higher than in the non-amended soil is that 2,4-D was degraded much faster in the nutrient-amended soil. Due to this, the stimulating effect of 2,4-D on the transconjugants was diminished. Top et al. [91] showed that few or no transconjugants with plasmids pEMT1k and pEMT3k were detected in soil without 2,4-D, compared to the high numbers in 2,4-D contaminated soil. Also Neilson et al. [64] and Newby et al. [66] could only detect the transfer of the plasmid pJP4 from an introduced donor to the indigenous bacteria of non-sterile soil when 2,4-D was added.

From the comparison of DGGE patterns and the sequence information it can be concluded that the new bands in the DGGE pattern of the B-horizon soil, first visible on day 21 most probably correspond to transconjugants. The fact that the formation of transconjugants could be revealed by DGGE analysis means that these transconjugants formed very dominant populations in this soil. This was also clear by plating, since the transconjugant numbers at day 21 were ca. 10^7 CFU/g soil, as high as the total plate count. Interestingly, transconjugants were not yet detected in the DGGE profile of soil DNA on day 7, when ca. 10^5 CFU/g soil were counted by plating. Since the DGGE pattern of soil DNA represents a profile of the numerically dominant populations in the soil, transconjugants can only be visualised if they are present in sufficiently high numbers. These high numbers were rather due to growth of transconjugants than to additional plasmid transfer into new species, since no change occurred in the DGGE pattern of soil and plate DNA in function of time.

The heterogeneity of the 16S rRNA genes, observed in the *P. putida* UWC3 strain and in some transconjugants, has been reported previously for other species [10, 68, 77]. Also the clear visibility of the DGGE bands of the inoculum *P. putida* UWC3, corresponds with other studies that were able to track inocula in soil, aquifers and sludge [28, 29, 83] by their prominent DGGE band(s). The strong DGGE signal of the donor could be explained by the fact that the initial donor concentration represented ca. 10 % of the total heterotrophic plate count of the B-horizon soil. Over time the number of donors decreased while those of transconjugants increased, resulting in dominant transconjugant bands and faint to invisible donor bands in the DGGE pattern. Also Stephen et al. [83] found that DGGE analysis of amplified 16S rDNA fragments from soil was a useful method of tracking the survival of introduced bacteria while they provided only 2% of the viable biomass.

The possibility that some transconjugants which may not be detected by plating can be observed by DGGE analysis of 16S rRNA is in agreement with other authors who found that certain bacteria which do not grow on synthetic media can be visualised by 16S rRNA analysis [4]. The strain most related to *Bradyrhizobium* sp. may indeed have acquired the 2,4-D degradative plasmids pEMT1 and pJP4 since Kinkle et al. [53] have demonstrated transfer of the plasmid pJP4 between *B. japonicum* USDA438 and several *Bradyrhizobium* sp. strains in non-sterile soil.

The very similar DGGE profiles of the B-horizon inoculated with either pEMT1::lacZ or pJP4::lacZ indicate that the numerically dominant transconjugants of each plasmid belonged to the same species. This is in agreement with the identification of several transconjugants isolated on agar plates, which revealed that most transconjugants were *Burkholderia* species and *Ralstonia eutropha*-like organisms, both in the case of pEMT1::lacZ and pJP4::lacZ [40]. This suggests that the host range for transfer and expression of the degradative genes in soil is very similar for both plasmids. Transconjugants of plasmids pEMT1 and pJP4 obtained in other transfer studies in surface soils (A-horizon) were also identified as *Ralstonia* or *Burkholderia* species [22, 66, 89, 91]. Since the transfer range of pJP4 is a lot broader than these two genera [23], the limited diversity of transconjugants found in soil, when selected for growth on 2,4-D as sole C-source, must be due to the limited ability of several transconjugants to mineralise 2,4-D.

In conclusion, bioaugmentation of a 2,4-D contaminated soil was very successful in soil from the B-horizon, where the indigenous community could not, or only very slowly, degrade the herbicide. In the case of plasmid pEMT1::lacZ, this success must be due to the transfer of the plasmid to the indigenous bacteria. To our knowledge, such a clear effect of bioaugmentation through the spread of natural catabolic genes in a soil community has not yet been reported in literature. In addition, DGGE analysis of the soil 16S rRNA gene pool was used for the first time to show that the *in situ* formation and subsequent proliferation of high numbers of 2,4-D degrading transconjugants caused clear changes in the soil microbial community structure.

7.3 DIVERSITY OF TRANSCONJUGANTS THAT ACQUIRED PLASMID pJP4 OR pEMT1 AFTER INOCULATION OF A DONOR STRAIN IN THE A- AND B-HORIZON OF AN AGRICULTURAL SOIL AND DESCRIPTION OF *BURKHOLDERIA HOSPITA* SP. NOV. AND *BURKHOLDERIA TERRICOLA* SP. NOV.[§]

7.3.1 Summary

We examined the diversity of transconjugants that acquired the catabolic plasmids pJP4 or pEMT1, which encode degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), in microcosms with agricultural soil inoculated with a donor strain [21]. Using repetitive element PCR fingerprinting, eight different rep-clusters and six separate isolates could be discriminated among 95 transconjugants tested. Representative isolates were identified using 16S rDNA sequencing, cellular fatty acid analysis, whole-cell protein analysis and/or DNA-DNA hybridisations. Plasmids pJP4 and pEMT1 appeared to have a similar transfer and expression range, and were preferably acquired and expressed in soil by indigenous representatives of *Ralstonia* and *Burkholderia*. Two rep-clusters were shown to represent novel *Burkholderia* species, for which the names *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. are proposed. When easily degradable carbon sources were added together with the plasmid-bearing donor strain, also a significant proportion of *Stenotrophomonas maltophilia* isolates were found. The transconjugant collections isolated from A- (0 - 30 cm depth) and B-horizon (30 - 60 cm depth) soil were similar, except for *B. terricola* transconjugants, which were only isolated from the B-horizon.

7.3.2 Introduction

The presence of man-made chemicals such as chlorinated aromatic compounds in soil and water ecosystems has become a major environmental problem. One way to deal with this kind of pollution is mineralization through bioremediation. The "naturally" available remediation potential of an ecosystem can be enhanced by inoculation with a strain with known biodegradation capacities (bioaugmentation). Although acceleration of the degradation was shown in several cases [8, 14, 15, 28, 62], the effect is usually limited in time since catabolic activity decreases along with a decrease in survival of the inoculated strain and eventually even comes to a halt [8, 15, 28, 67].

As an alternative to the introduction of a catabolic strain, a number of studies have focussed on the dissemination of a self-transferable catabolic plasmid as a tool in bioremediation [18, 20-22, 27, 65, 66, 89, 91]. The plasmid can be transferred from an introduced donor strain to the indigenous bacteria, thereby expanding the catabolic range of the latter. Indigenous bacteria are well adapted to their environment and

[§] Redrafted from Goris, J., W. Dejonghe, P. Vandamme, E. De Clerck, B. Geeraerts, A. Willems, E. M. Top, and P. De Vos. 2002. Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Syst Appl Microbiol* **25**:340-352.

therefore remain in the system for extended periods compared with an often quick decline in the numbers of inoculated strains. As a consequence, survival of the donor is only required for a short period, during which conjugation can take place.

The herbicide 2,4-dichlorophenoxyacetate (2,4-D) has been widely used over the past 50 years for weed control in cereals and other crops. Unlike many other synthesised chemicals released in the environment, it has a rather short half-life in soil [69]. The most extensively studied 2,4-D-degradative plasmid is pJP4 from *Ralstonia* sp. JMP134 [23]. It encodes for the degradation of 2,4-D and for the resistance against mercuric ions and phenyl mercury acetate. The plasmid is partially sequenced, the catabolic pathway is elucidated and the degradation genes and regulatory genes are localized and characterized [24, 25, 36-38, 45, 51, 52, 59, 72, 84]. Recently, a second *tfd* gene cluster was identified that also resides on this plasmid [54].

Plasmid pEMT1 was isolated by exogenous plasmid isolation from a 2,4-D treated agricultural soil [88]. This 2,4-D degradative plasmid shows a high sequence similarity with the *tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE*, *tfdF* and *tfdR* genes of pJP4, but differs from the latter in size (ca. 97 kb compared to 80 kb for pJP4), restriction pattern, localisation of *tfdA* relative to *tfdCDEFtfdB*, mercury resistance and incompatibility class (pJP4 belongs to IncP-1 incompatibility class, pEMT1 does not) [88].

In a previous study [21], both pJP4 and pEMT1 were marked with the mini-transposon mini-Tn5 *KmlacZ*, and transferred to *Pseudomonas putida* UWC3. *P. putida* UWC3 strains harbouring either plasmid pJP4 or pEMT1 were subsequently inoculated in microcosms with either A- or B-horizon soil treated with 2,4-D (100 mg/kg), and either amended with nutrients (Luria Broth) or not amended. At different time points (6 to max. 34 days after inoculation) transconjugants were detected in both horizons and for both plasmids, while a positive effect of plasmid transfer on 2,4-D degradation was most pronounced in the B-horizon soil. PCR-DGGE analysis of 16S rDNA amplified from total soil community DNA revealed a clear shift in the bacterial community of the B-horizon after inoculation with the donor of plasmid pEMT1. The 16S rRNA gene sequences of newly appearing bands corresponded to those of 2,4-D-degrading transconjugant colonies that were isolated on agar plates [21]. The current report focuses on the identification and the diversity of the isolated transconjugants resulting from these experiments.

7.3.3 Materials and methods

7.3.3.1 Plasmid labelling and isolation of transconjugants

The 2,4-D degradative plasmids pJP4 and pEMT1 were tagged previously with the mini-transposon mini-Tn5 *KmlacZ*. Neither the transfer frequencies of the plasmids, nor their 2,4-D degradation potential was affected by this labelling [21]. Therefore, and for convenience, plasmids pJP4::*lacZ* and pEMT1::*lacZ* are designated throughout the manuscript as pJP4 and pEMT1, respectively. The isolation procedure for the transconjugants was described in detail by Dejonghe et al. [21]. Briefly, A-horizon (0 - 30 cm depth) and B-horizon (30 - 60 cm depth) soil was collected from an agricultural plot, which had no previous exposure to 2,4-D. In a first experiment (referred to as nutrient amended soil), a donor strain (*Pseudomonas putida* UWC3) harbouring either

plasmid pJP4 or pEMT1, was suspended in 1 ml 5x LB broth and added together with 10 mg 2,4-D to 500 ml glass microcosms containing 100 g A- or B-horizon soil. The experiment was repeated but the 5x LB broth was replaced by saline (referred to as non-amended soil). Samples were taken on day 0, 2, 6, 14, 22, and 50 and on day 0, 4, 7, 12, 22, and 34 for the nutrient-amended and the non-amended experiment, respectively. Hundred microliters of serial 10-fold dilutions of 1 g soil were plated on MMO mineral medium [81] with 500 mg of 2,4-D per litre as the sole carbon source, 200 mg of cycloheximide per litre, 50 mg of kanamycin per litre, and 20 mg of X-Gal per litre. Transconjugants were detected as blue colonies on this medium, while growth of the donor strain was prevented, since *P. putida* UWC3 is auxotrophic for the amino acids isoleucine, leucine and valine. The colonies that developed from the highest dilutions were transferred via replica plating to LB and MMO medium and representatives for the different colony morphologies were isolated from the MMO medium. The presence of the plasmids in the putative transconjugants was confirmed with a modified Kado & Liu [50] plasmid extraction after transfer to *Ralstonia eutropha* JMP228n and by a specific PCR detection of the *tfdA* gene with the primers TVU and TVL [92]. An overview of the transconjugants obtained in this study is given in Table 7.3.

TABLE 7.3: List of transconjugants.

| Isolate no. | Isolate code* | Identification | Rep-type† |
|--|-----------------|-------------------------------------|-----------|
| Nutrient-amended experiment, pJP4-harboursing, B-horizon: | | | |
| R-4957 | N B pJP4 d6.1 | <i>Burkholderia hospita</i> | I |
| R-4959 | N B pJP4 d6.2 | <i>Ralstonia campinensis</i> | VI |
| R-4983 | N B pJP4 d6.3 | <i>Stenotrophomonas maltophilia</i> | IV |
| Nutrient-amended experiment, pEMT1-harboursing, A-horizon: | | | |
| R-4932 | N A pEMT1 d6.1 | <i>Stenotrophomonas maltophilia</i> | IV |
| R-4933 | N A pEMT1 d6.2 | <i>Stenotrophomonas maltophilia</i> | IV |
| R-4934 | N A pEMT1 d6.3 | <i>Stenotrophomonas maltophilia</i> | VIII |
| LMG 20563 | N A pEMT1 d6.4 | <i>Stenotrophomonas maltophilia</i> | VIII |
| R-4936 | N A pEMT1 d6.5 | <i>Stenotrophomonas maltophilia</i> | VIII |
| LMG 21262 | N A pEMT1 d6.6 | <i>Burkholderia</i> sp. | - |
| R-4943 | N A pEMT1 d6.7 | <i>Ralstonia campinensis</i> | VI |
| LMG 20565 | N A pEMT1 d6.8 | <i>Stenotrophomonas maltophilia</i> | - |
| LMG 20567 | N A pEMT1 d6.9 | <i>Ralstonia campinensis</i> | VI |
| LMG 20568 | N A pEMT1 d6.10 | <i>Ralstonia campinensis</i> | VI |
| LMG 20569 | N A pEMT1 d6.11 | <i>Stenotrophomonas maltophilia</i> | - |
| LMG 20574 | N A pEMT1 d6.12 | <i>Burkholderia hospita</i> | I |
| R-4965 | N A pEMT1 d6.13 | <i>Burkholderia hospita</i> | I |
| LMG 20576 | N A pEMT1 d6.14 | <i>Ralstonia campinensis</i> | VI |
| LMG 20575 | N A pEMT1 d22.1 | <i>Stenotrophomonas maltophilia</i> | IV |
| R-4971 | N A pEMT1 d22.2 | <i>Ralstonia campinensis</i> | VI |
| Nutrient-amended experiment, pEMT1-harboursing, B-horizon: | | | |
| R-4950 | N B pEMT1 d6.1 | <i>Burkholderia hospita</i> | I |
| R-4964 | N B pEMT1 d6.2 | <i>Burkholderia hospita</i> | I |
| R-4969 | N B pEMT1 d22.1 | <i>Ralstonia campinensis</i> | VI |
| Non-amended experiment, pJP4-harboursing, A-horizon: | | | |
| R-6386 | S A pJP4 d12.1 | <i>Ralstonia campinensis</i> | VI |

| Isolate no. | Isolate code* | Identification | Rep-type† |
|-------------|----------------|------------------------------|-----------|
| LMG 20585 | S A pJP4 d21.1 | <i>Ralstonia campinensis</i> | V |
| R-6405 | S A pJP4 d21.2 | <i>Ralstonia campinensis</i> | VI |
| R-6376 | S A pJP4 d22.1 | <i>Ralstonia campinensis</i> | V |
| LMG 20580 | S A pJP4 d22.2 | <i>Burkholderia</i> sp. | - |
| R-6383 | S A pJP4 d22.3 | <i>Ralstonia campinensis</i> | V |
| R-6390 | S A pJP4 d22.4 | <i>Ralstonia campinensis</i> | V |
| R-6406 | S A pJP4 d22.5 | <i>Ralstonia campinensis</i> | V |
| R-6408 | S A pJP4 d22.6 | <i>Ralstonia campinensis</i> | V |
| LMG 20587 | S A pJP4 d27.1 | <i>Burkholderia glathei</i> | III |
| R-6411 | S A pJP4 d27.2 | <i>Ralstonia campinensis</i> | V |
| LMG 20597 | S A pJP4 d34.1 | <i>Burkholderia hospita</i> | I |
| LMG 20599 | S A pJP4 d34.2 | <i>Burkholderia hospita</i> | I |

Non-amended experiment, pJP4-harboring, B-horizon:

| | | | |
|-----------|----------------|-------------------------------|-----|
| R-8120 | S B pJP4 d7.1 | <i>Burkholderia terricola</i> | II |
| R-8375 | S B pJP4 d7.2 | <i>Burkholderia hospita</i> | I |
| R-8376 | S B pJP4 d7.3 | <i>Burkholderia hospita</i> | I |
| R-8377 | S B pJP4 d7.4 | <i>Burkholderia hospita</i> | I |
| R-8378 | S B pJP4 d7.5 | <i>Burkholderia hospita</i> | I |
| LMG 20583 | S B pJP4 d21.1 | <i>Burkholderia terricola</i> | II |
| LMG 21261 | S B pJP4 d21.2 | <i>Burkholderia glathei</i> | III |
| R-8379 | S B pJP4 d22.1 | <i>Burkholderia hospita</i> | I |
| R-6372 | S B pJP4 d27.1 | <i>Burkholderia terricola</i> | II |
| LMG 20593 | S B pJP4 d34.1 | <i>Burkholderia terricola</i> | II |
| LMG 20594 | S B pJP4 d34.2 | <i>Burkholderia terricola</i> | II |

Non-amended experiment, pEMT1-harboring, A-horizon:

| | | | |
|-----------|-----------------|------------------------------|-----|
| R-8309 | S A pEMT1 d7.1 | <i>Burkholderia hospita</i> | I |
| R-8373 | S A pEMT1 d7.2 | <i>Burkholderia hospita</i> | I |
| R-15266 | S A pEMT1 d21.1 | <i>Ralstonia campinensis</i> | V |
| R-6371 | S A pEMT1 d21.2 | <i>Ralstonia campinensis</i> | VII |
| LMG 20579 | S A pEMT1 d21.3 | <i>Ralstonia campinensis</i> | V |
| LMG 20586 | S A pEMT1 d21.4 | <i>Burkholderia hospita</i> | I |
| R-6403 | S A pEMT1 d21.5 | <i>Burkholderia hospita</i> | I |
| LMG 20588 | S A pEMT1 d21.6 | <i>Ralstonia campinensis</i> | V |
| R-6385 | S A pEMT1 d22.1 | <i>Ralstonia campinensis</i> | VII |
| R-6393 | S A pEMT1 d22.2 | <i>Ralstonia campinensis</i> | VII |
| R-6394 | S A pEMT1 d22.3 | <i>Burkholderia hospita</i> | I |
| R-6402 | S A pEMT1 d22.4 | <i>Ralstonia campinensis</i> | VII |
| R-6412 | S A pEMT1 d22.5 | <i>Ralstonia campinensis</i> | VII |
| R-6377 | S A pEMT1 d27.1 | <i>Burkholderia hospita</i> | I |
| LMG 20584 | S A pEMT1 d27.2 | <i>Ralstonia campinensis</i> | VII |
| R-8650 | S A pEMT1 d34.1 | <i>Ralstonia campinensis</i> | VII |
| R-8651 | S A pEMT1 d34.2 | <i>Burkholderia hospita</i> | I |
| LMG 20600 | S A pEMT1 d34.3 | <i>Burkholderia hospita</i> | I |
| R-8653 | S A pEMT1 d34.4 | <i>Ralstonia campinensis</i> | VII |

Non-amended experiment, pEMT1-harboring, B-horizon:

| | | | |
|-----------|-----------------|-------------------------------------|----|
| LMG 20595 | S B pEMT1 d7.1 | <i>Ralstonia basiliensis</i> | - |
| R-8301 | S B pEMT1 d7.2 | <i>Ralstonia campinensis</i> | V |
| LMG 20596 | S B pEMT1 d7.3 | <i>Stenotrophomonas maltophilia</i> | - |
| R-8368 | S B pEMT1 d7.4 | <i>Burkholderia hospita</i> | I |
| R-8093 | S B pEMT1 d12.1 | <i>Burkholderia terricola</i> | II |
| LMG 20592 | S B pEMT1 d12.2 | <i>Burkholderia terricola</i> | II |
| R-8372 | S B pEMT1 d12.3 | <i>Burkholderia hospita</i> | I |
| R-6379 | S B pEMT1 d21.1 | <i>Burkholderia hospita</i> | I |
| R-6381 | S B pEMT1 d21.2 | <i>Ralstonia campinensis</i> | VI |
| LMG 20581 | S B pEMT1 d21.3 | <i>Burkholderia terricola</i> | II |

| Isolate no. | Isolate code* | Identification | Rep-type† |
|-------------|------------------|-------------------------------|-----------|
| LMG 20589 | S B pEMT1 d21.4 | <i>Burkholderia terricola</i> | II |
| R-6382 | S B pEMT1 d22.1 | <i>Ralstonia campinensis</i> | VI |
| R-6407 | S B pEMT1 d22.2 | <i>Burkholderia terricola</i> | II |
| LMG 20590 | S B pEMT1 d22.3 | <i>Ralstonia campinensis</i> | VI |
| R-8090 | S B pEMT1 d22.4 | <i>Burkholderia terricola</i> | II |
| R-8091 | S B pEMT1 d22.5 | <i>Burkholderia terricola</i> | II |
| R-8098 | S B pEMT1 d22.6 | <i>Ralstonia campinensis</i> | VI |
| R-8369 | S B pEMT1 d22.7 | <i>Burkholderia hospita</i> | I |
| R-8370 | S B pEMT1 d22.8 | <i>Burkholderia hospita</i> | I |
| R-8371 | S B pEMT1 d22.9 | <i>Burkholderia hospita</i> | I |
| R-8086 | S B pEMT1 d34.1 | <i>Burkholderia terricola</i> | II |
| R-8087 | S B pEMT1 d34.2 | <i>Burkholderia terricola</i> | II |
| R-8088 | S B pEMT1 d34.3 | <i>Burkholderia terricola</i> | II |
| R-8089 | S B pEMT1 d34.4 | <i>Burkholderia terricola</i> | II |
| R-8095 | S B pEMT1 d34.5 | <i>Ralstonia campinensis</i> | V |
| R-8096 | S B pEMT1 d34.6 | <i>Ralstonia campinensis</i> | VII |
| R-8097 | S B pEMT1 d34.7 | <i>Burkholderia terricola</i> | II |
| R-8365 | S B pEMT1 d34.8 | <i>Burkholderia hospita</i> | I |
| LMG 20598 | S B pEMT1 d34.9 | <i>Burkholderia hospita</i> | I |
| R-8367 | S B pEMT1 d34.10 | <i>Burkholderia hospita</i> | I |

*: The isolate code consists of an indication of the experiment type (N: donor added together with nutrients; S: donor added in saline), A- or B-horizon, type of plasmid (pJP4 or pEMT1), day of isolation and serial number.

†: A hyphen (-) denotes a unique rep-type.

7.3.3.2 Repetitive element genomic fingerprinting

Template DNA was prepared according to the protocol of Pitcher et al. [73]. The REP2I-, REP1R-, and BOXA1R-primer sequences and PCR conditions for repetitive element genomic fingerprinting were as described by Rademaker and De Bruijn [76]. GoldStar Taq DNA polymerase (EuroGentec) was used in a Perkin Elmer 9600 thermocycler. The resulting amplicons were separated on a 15 x 20 cm agarose gel containing 1.5% RESult TE agarose gel (BioZym) in 1 x Tris-acetate-EDTA (TAE) buffer. The gel was mounted in a Sub-cell GT (Bio-Rad) electrophoresis tank connected to a PowerPac 300 (Bio-Rad) power supply, generating an electrical field of 1.9 V/cm for 15 h at 4°C. A mixture of Molecular Rulers 500 and 100 (Bio-Rad) was used as a molecular weight marker and to allow for intra- and inter gel comparison. Numerical analysis was done using the BioNumerics 2.0 software package (Applied Maths). The digitised REP- and BOX-PCR patterns were linearly combined, assigning the same weight to each analysis. Comparisons were made using the Pearson product moment correlation coefficient and for dendrogram construction the UPGMA method was employed.

7.3.3.3 16S rDNA sequencing

16S rDNA sequencing was performed using an Applied Biosystems 377 DNA sequencer as described by Coenye et al. [12]. Nearly complete sequences (corresponding to positions 8 to 1541 of the *E. coli* numbering system) were assembled from partial sequences using the program Auto Assembler™ (Applied Biosystems). For

a number of isolates, only partial (394 - 476 bases) sequences obtained with primer 16R519 (= PD) were determined. The FASTA program [71] was applied to find the most similar sequences from the EMBL database. The nucleotide accession numbers for the sequences determined in this study can be found in Table 7.4.

7.3.3.4 *Whole-cell protein analysis*

Preparation of the cell-free extracts for whole-cell protein analysis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Pot et al. [75]. Whole-cell protein profiles from transconjugants were numerically compared to profiles from different databases available in the lab by using the GelCompar 4.2 software package (Applied Maths). The result of the numerical comparison was always verified by visual comparison. Culture conditions were 48 h at 28°C on buffered nutrient agar (per litre distilled water: 1 g Lab Lemco powder, 2 g yeast extract, 5 g bacteriological peptone, 5 g NaCl, 0.45 g KH₂PO₄ and 2.39 g Na₂HPO₄·12H₂O (pH 6.8), and 20 g agar), 48 h at 37°C on Tryptic Soy Agar (BBL) for comparison with the *Burkholderia* and *Ralstonia* laboratory databases [13], respectively.

7.3.3.5 *Gas chromatographic analysis of methylated fatty acids*

Cells were grown for 24 hours on TSA at 28 °C and fatty acid methyl esters (FAMES) were extracted, prepared, and separated as described before [97]. Identification was done using the Sherlock Microbial Identification System version 3.10 (MIDI).

7.3.3.6 *DNA-DNA hybridisations and determination of DNA base composition*

Protocols for large-scale DNA preparation and mole % G+C determination were described previously [55]. DNA-DNA hybridisations were performed at 55°C with photobiotin-labelled probes in microplate wells as described by Ezaki et al. [33], using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. A detailed description of the DNA-DNA hybridisation procedure can be found in Willems et al. [100].

7.3.3.7 *Biochemical and phenotypic analyses*

APIZYM and API20NE tests were performed according to the recommendations of the manufacturer (bioMérieux). Classical phenotypic analyses were done as described by Vandamme et al. [96].

7.3.4 **Results**

7.3.4.1 *Repetitive element-based genomic fingerprinting*

A numerical analysis based on combined REP and BOX fingerprints is shown in Fig. 7.5. Discriminatory profiles were obtained for all isolates with both primer sets, although with REP-PCR a less complex pattern with a lower background amplification

level was observed compared with BOX-PCR. Eight rep-clusters (designated I to VIII) and 6 isolates with unique positions in the dendrogram (a total of 14 rep-types) could be differentiated at a similarity level of 55%. At this similarity level, rep-profiles that differed in a few bands were still regarded as belonging to the same cluster. Nevertheless, the rep-clusters were expected to contain highly related organisms, an expectation that was supported by the results of the other taxonomic methods applied (see below). The reproducibility of the method was good, since repeated analyses always clustered above 85% (data not shown).

Representatives of the different rep-types were tentatively identified by partial or nearly complete 16S rDNA sequencing, and depending on the result, appropriate taxonomic techniques (whole-cell protein analysis, cellular fatty acid analysis, and DNA-DNA hybridisations) were selected for further identification.

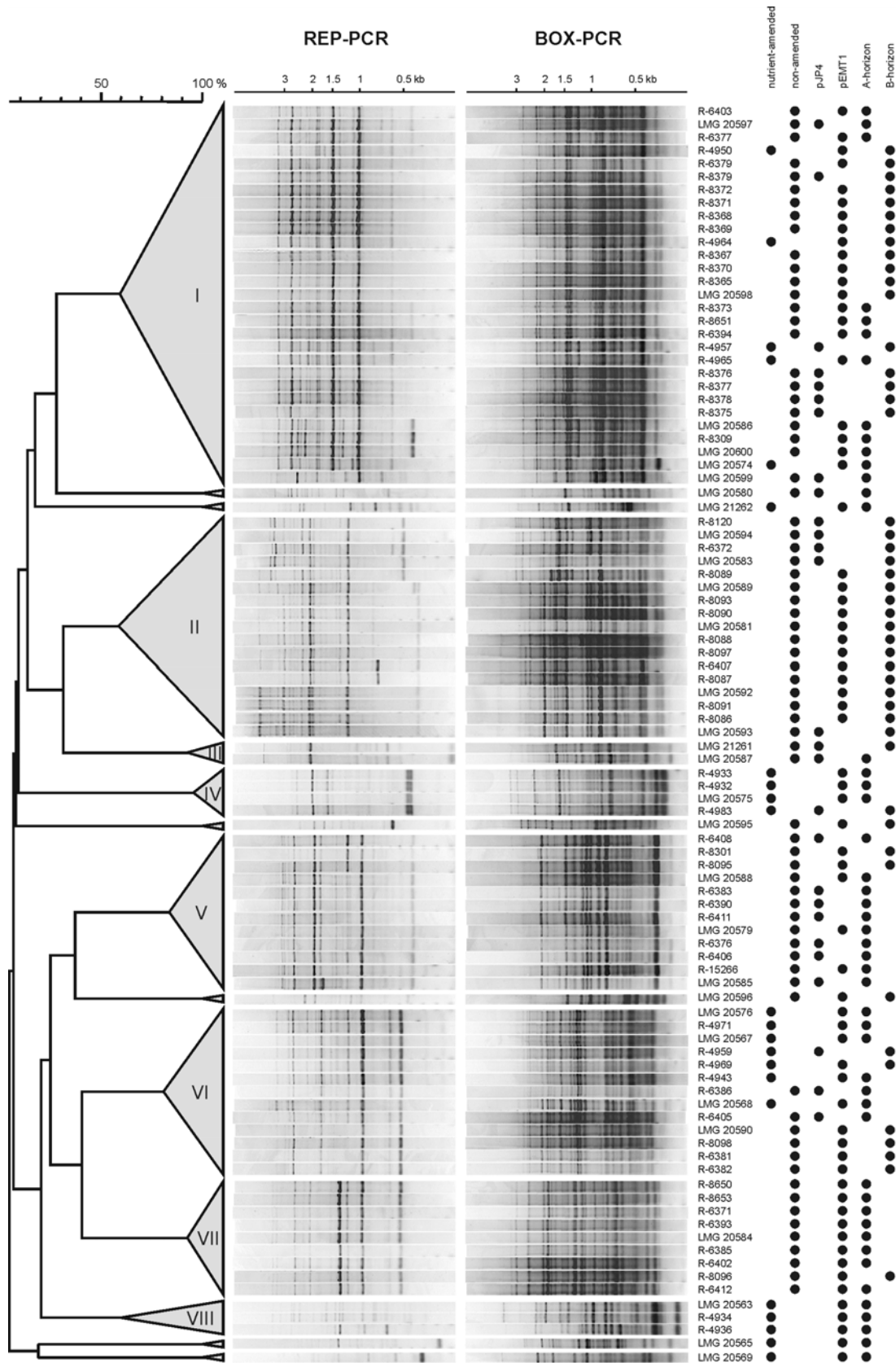


FIG. 7.5: Clustering based on linearly combined REP- and BOX-PCR profiles of all transconjugants in this study. The origin of the transconjugants is also given.

TABLE 7.4: 16S rDNA sequences determined in this study.

| Strain no. | Rep-type* | Seq. length | Accession no. | Best match (accession no.)† | Similarity (%) |
|------------|-----------|-------------|---------------|--|----------------|
| LMG 20574 | I | 1483 | AY040353 | <i>Burkholderia caribensis</i> MWAP64 ^T (Y17009) | 98.3 |
| LMG 20598 | I | 1484 | AY040365 | <i>Burkholderia caribensis</i> MWAP64 ^T (Y17009) | 98.6 |
| LMG 20599 | I | 423 | AY040366 | <i>Burkholderia caribensis</i> MWAP64 ^T (Y17009) | 96.7 |
| LMG 20594 | II | 1497 | AY040362 | <i>Burkholderia graminis</i> C4D1M ^T (U96939) | 98.3 |
| LMG 21261 | III | 463 | AF452131 | <i>Burkholderia glathei</i> LMG 14190 ^T (U96935) | 100 |
| LMG 20575 | IV | 467 | AY040354 | <i>Stenotrophomonas maltophilia</i> LMG 10991 (AJ131115) | 99.5 |
| LMG 20579 | V | 1484 | AY040358 | <i>Ralstonia campinensis</i> LMG 19282 ^T (AF312020) | 99.8 |
| LMG 20568 | VI | 1484 | AY040349 | <i>Ralstonia campinensis</i> LMG 19282 ^T (AF312020) | 100 |
| LMG 20576 | VI | 1505 | AY040355 | <i>Ralstonia campinensis</i> LMG 19282 ^T (AF312020) | 100 |
| LMG 20584 | VII | 448 | AF452133 | <i>Ralstonia campinensis</i> LMG 19282 ^T (AF312020) | 99.4 |
| LMG 20563 | VIII | 394 | AY040346 | <i>Stenotrophomonas maltophilia</i> VUN10075 (AF137357) | 99.9 |
| LMG 20565 | - | 469 | AY040347 | <i>Stenotrophomonas maltophilia</i> e-p10 (AJ293463) | 100 |
| LMG 20569 | - | 476 | AY040350 | <i>Stenotrophomonas maltophilia</i> e-p10 (AJ293463) | 100 |
| LMG 20580 | - | 969 | AY040359 | <i>Burkholderia fungorum</i> LMG 16225 ^T (AF215705) | 98.8 |
| LMG 20595 | - | 468 | AY040363 | <i>Ralstonia basilensis</i> LMG 19474 ^T (AF312021) | 99.3 |
| LMG 20596 | - | 406 | AY040364 | <i>Stenotrophomonas maltophilia</i> e-p20 (AJ293466) | 99.9 |
| LMG 21262 | - | 1483 | AF452132 | <i>Burkholderia fungorum</i> LMG 16225 ^T (AF215705) | 98.1 |

*: A hyphen (-) denotes a unique rep-type.

†: EMBL database entry with a species designation that showed the highest similarity to the 16S rDNA sequence of the transconjugant.

7.3.4.2 Identification of the transconjugants

Rep-cluster I contained twenty-nine transconjugants, of which twelve originated from the A-horizon (two harbour pJP4 and ten harbour pEMT1) and seventeen from the B-horizon (six harbour pJP4 and eleven harbour pEMT1). Isolates LMG 20574, LMG 20598 and LMG 20599 were randomly chosen as representatives. From these three isolates, the partial 16S rDNA sequences were determined and compared with the EMBL database (Table 7.4). Their 16S rDNA sequences were almost identical (above 99.5% similarity) and have *Burkholderia caribensis* MWP64^T as the nearest named neighbour (respectively 98.3, 98.6, and 96.7% sequence similarity) in the EMBL database. A phylogenetic tree that shows the allocation of LMG 20574 and LMG 20598 to the genus *Burkholderia* is given in Fig. 7.6. The whole-cell protein profiles of cluster I representatives were different from those of *B. caribensis* LMG 18531^T (Fig. 7.7) and from all other entries in the *Burkholderia* database, which includes all currently named *Burkholderia* species, except *B. mallei*. DNA reassociation values (averages of the two reciprocal reactions) of cluster I representatives with *Burkholderia* reference strains are all lower than 60% (Table 7.5). These data clearly show that cluster I isolates represent a new *Burkholderia* species, for which we propose the name *Burkholderia hospita* below.

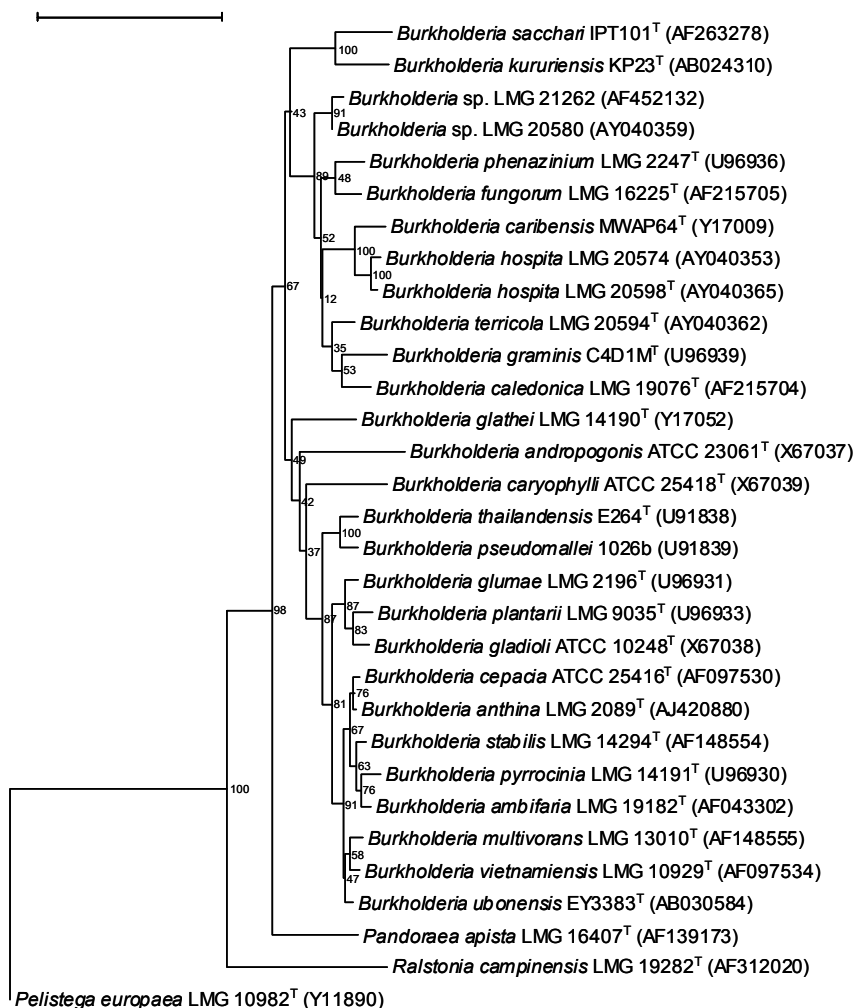


FIG. 7.6: Neighbour-joining tree showing the phylogenetic position of *Burkholderia hospita* and *Burkholderia terricola*, based on 16S rDNA sequence comparisons. Bootstrap values obtained with 500 repetitions are indicated as percentages. Bar represents 5% sequence divergence.

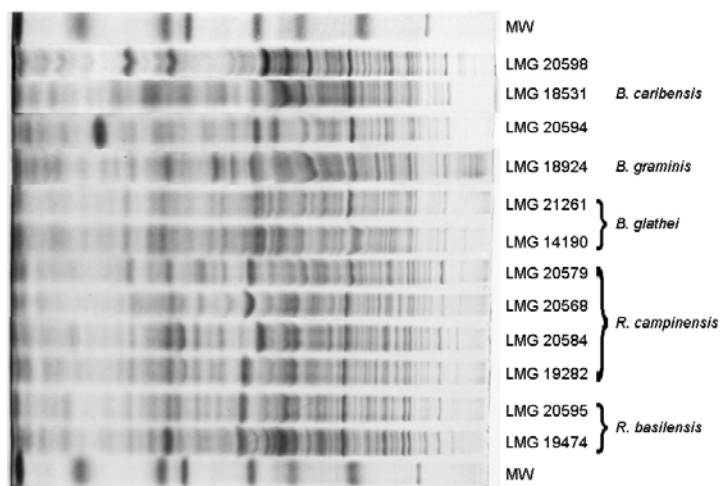


FIG. 7.7: Whole-cell protein profiles of selected transconjugants and reference strains. The culture conditions, as applied for the different databases, were as described in Material and Methods. The exception was *Ralstonia basilensis* LMG 20595, which was unable to grow at 37°C and was therefore cultivated under conditions as applied for the *Burkholderia* laboratory database. MW: molecular weight markers (from left to right) lysozyme (14.5 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and β-galactosidase (116 kDa).

Rep-cluster II comprised five pJP4- and twelve pEMT1 containing transconjugants, all originating from the non-amended B-horizon soil. Cluster II isolate LMG 20594 showed 98.3% 16S rDNA sequence similarity with *Burkholderia graminis* C4D1M^T (Table 7.4). The phylogenetic position of LMG 20594 is also shown in Fig. 7.6. The whole-cell protein profiles of cluster II representatives showed no match with *B. graminis* profiles (shown for LMG 20594 in Fig. 7.7) or any other *Burkholderia* database entries. DNA-DNA hybridisation experiments revealed low DNA reassociation values (average of the two reciprocal reactions max. 39%) of cluster II representatives with *Burkholderia* reference strains (Table 7.5). All these data indicate that cluster II isolates represent a new *Burkholderia* species, for which we propose the name *Burkholderia terricola* below.

Rep-cluster III only contained two pJP4-harboured transconjugants, LMG 20587 (from non-amended A-horizon) and LMG 21261 (from non-amended B-horizon). The partial 16S rDNA sequence of the latter was identical to the sequence of *Burkholderia glathei*_LMG 14190^T (Table 7.4). Since this identification was in very good agreement with the results of the whole-cell protein patterns (Fig. 7.7), the cluster III isolates were identified as *B. glathei*.

TABLE 7.5: DNA-DNA hybridisation values and mole% guanosine + cytosine of transconjugants and *Burkholderia* reference strains.

| | %G+C | Reassociation values with probe-DNA from: | | | | | | | | | | | | | | | | |
|--|------|---|------------------------|-----------|------------------------|-----------|-----------|-----------|-----------|-----------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-----------------------|
| | | LMG 20574 | LMG 20598 ^T | LMG 20583 | LMG 20594 ^T | LMG 20592 | LMG 20593 | LMG 20589 | LMG 20580 | LMG 21262 | LMG 14190 ^T | LMG 18531 ^T | LMG 18924 ^T | LMG 19447 ^T | LMG 19450 ^T | LMG 16225 ^T | LMG 19076 ^T | LMG 2247 ^T |
| <i>B. hospita</i> LMG 20574 | 62.2 | 100 | 92 | 18 | 24 | 14 | 25 | | | | | 69 | 23 | | | | | |
| <i>B. hospita</i> LMG 20598 ^T | 62.0 | 92 | 100 | 17 | 15 | 17 | 21 | 15 | 17 | 11 | 65 | 22 | 11 | 11 | 15 | 17 | 11 | |
| <i>B. terricola</i> LMG 20583 | 63.4 | 16 | 5 | 100 | 104 | 86 | 96 | | | | 9 | 26 | 50 | | | | | |
| <i>B. terricola</i> LMG 20594 ^T | 63.9 | 16 | 14 | 90 | 100 | 92 | 85 | 40 | 40 | 9 | 20 | 39 | 16 | 12 | 36 | 36 | 21 | |
| <i>B. terricola</i> LMG 20592 | 63.6 | 18 | 15 | 95 | 94 | 100 | 98 | | | | 10 | 24 | 49 | | | | | |
| <i>B. terricola</i> LMG 20593 | 63.8 | 15 | 11 | 70 | 82 | 101 | 100 | | | | 9 | 15 | 25 | | | | | |
| <i>B. terricola</i> LMG 20589 | 62.7 | | | | | | | 100 | 64 | | | | | | | | | |
| <i>Burkholderia</i> sp. LMG 20580 | 62.5 | | 16 | | 34 | | | 68 | 100 | 53 | | 21 | 37 | | 35 | 30 | 19 | |
| <i>Burkholderia</i> sp. LMG 21262 | 62.7 | | 16 | | 29 | | | | 45 | 100 | | 21 | 33 | | 35 | 29 | 17 | |
| <i>B. glathei</i> LMG 14190 ^T | 64.1 | | 8 | 21 | 13 | 15 | 14 | | | | 100 | 14 | 12 | | | | | |
| <i>B. caribensis</i> LMG 18531 ^T | 62.4 | 49 | 55 | 17 | 15 | 17 | 19 | 14 | 15 | 13 | 100 | 21 | | | 14 | 15 | 11 | |
| <i>B. graminis</i> LMG 18924 ^T | 62.8 | 13 | 16 | 28 | 34 | 29 | 35 | 35 | 30 | 14 | 21 | 100 | | | 26 | 42 | 16 | |
| <i>B. kururiensis</i> LMG 19447 ^T | 64.3 | | 16 | | 20 | | | | | | | | | 100 | 19 | | | |
| <i>B. sacchari</i> LMG 19450 ^T | 63.9 | | 11 | | 12 | | | | | | | | | 14 | 100 | | | |
| <i>B. fungorum</i> LMG 16225 ^T | 61.8 | | 11 | | 25 | | | 28 | 27 | | 16 | 25 | | | | 100 | 21 | 16 |
| <i>B. caledonica</i> LMG 19076 ^T | 62.0 | | 15 | | 28 | | | 31 | 32 | | 20 | 48 | | | 24 | 100 | 15 | |
| <i>B. phenazinium</i> LMG 2247 ^T | 62.5 | | 12 | | 19 | | | 20 | 19 | | 17 | 24 | | | 24 | 20 | 100 | |

Rep-cluster IV contained three pEMT1-harboured transconjugants from A-horizon soil and one pJP4-harboured transconjugant from B-horizon soil, both amended with nutrients. The partial 16S rDNA sequence of cluster IV isolate LMG 20575 revealed the highest similarity (99.5%) with *Stenotrophomonas maltophilia* LMG 10991 (Table 7.4), a representative of sequence group I as defined by Hauben et al. [46]. Since representatives of the genus *Stenotrophomonas* and the related genus *Xanthomonas* have a very characteristic cellular fatty acid profile [82], FAME-analysis was performed to confirm this identification. All cluster IV isolates were identified by the MIS software (TSBA40 database) as *S. maltophilia*, with similarity values ranging from 0.713 to 0.870. The average fatty acid profiles of rep-cluster IV isolates and of all other isolates identified as *S. maltophilia* in this study (see below) were very similar and contained large amounts (>10%) of 15:0 iso, 15:0 anteiso, and summed feature 3 (comprises 16:1 ω 7c and 15 iso 2OH in an unknown ratio), low to intermediate amounts (between 2 and 10%) of 11:0 iso, an unknown fatty acid with an equivalent chain length of 11.799, 11:0 iso 3OH, 12:0 3OH, 14:0 iso, 14:0, 13:0 iso 3OH, 16:1 ω 9c, and 16:0, iso 17:1 ω 9c and small to trace amounts (<2%) of an unknown fatty acid with an equivalent chain length of 9.531, 10:0, 11:0 anteiso, 10:0 2OH, 10:0 3OH, 11:0 3OH, 13:0 iso, 13:0 anteiso, 12:0 iso 3OH, 12:1 3OH, 14:1 ω 5c, 13:0 2OH, 15:1 iso F, 15:1 ω 8c, 15:1 ω 6c, 15:0, 16:0 iso, 17:0 iso, 17:0 anteiso, 17:1 ω 8c, 17:0 cyclo, 18:1 ω 9c, and 18:1 ω 7c. This was in good agreement with the average fatty acid profile of *S. maltophilia* as reported by Stead [82] and Yang et al. [101]. Consequently, cluster IV isolates were identified as *S. maltophilia*.

Rep-cluster V contained only transconjugants from soil without added nutrients. Seven pJP4-harboured transconjugants were isolated from A-horizon microcosms and five pEMT1-harboured transconjugants were found in A-horizon (3) and B-horizon (2). The sequence of the 16S rRNA gene of LMG 20579 showed the highest similarity (99.8%) with *Ralstonia campinensis* LMG 19282^T (Table 7.4). The whole-cell protein electrophoresis patterns of these cluster V isolates were similar to those of *R. campinensis* strains (Fig. 7.7). Finally, high DNA reassociation values with *R. campinensis* reference strains LMG 19282^T and LMG 19285 (Table 7.6) allowed an unambiguous identification of cluster V isolates as *R. campinensis*.

Rep-cluster VI contained three pJP4- and eleven pEMT1-harboured transconjugants, which were isolated from A-horizon (8) as well as from B-horizon soil (6) from both the nutrient amended and non-amended microcosms. The 16S rDNA sequence of cluster VI isolates LMG 20568 and LMG 20576 was identical to the sequence of *R. campinensis* LMG 19282^T (Table 7.4). The whole-cell protein electrophoresis patterns were again similar to that of *R. campinensis* LMG 19282^T (Fig. 7.7). In addition, cluster VI isolates LMG 20567 and LMG 20590 showed high DNA reassociation values with *R. campinensis* reference strains and with representatives of rep-cluster V (Table 7.6), confirming the identification of cluster VI isolates as *R. campinensis*.

Rep-cluster VII contained solely transconjugants that harbour plasmid pEMT1, mainly (seven out of eight) originating from non-amended A-horizon soil. The partial 16S rDNA sequence of LMG 20584 showed 99.4% similarity with the sequence of *R. campinensis* LMG 19282^T (Table 7.4). Their whole-cell protein electrophoresis patterns were also similar (Fig. 7.7) and DNA reassociation values between LMG 20584 and

Ralstonia reference strains (Table 7.6), confirmed the identity of cluster VII isolates as *R. campinensis*.

TABLE 7.6: DNA-DNA hybridisation values and mole% guanosine + cytosine of transconjugants and *Ralstonia* reference strains.

| | %G+C | % DNA-reassociation with probe-DNA from: | | | | | |
|---|------|--|----------------|-----------------|-----------------|------------------|------------------------|
| | | LMG 20585 V | LMG 20588 V | LMG 20567 VI | LMG 20590 VI | LMG 20584 VII | LMG 19282 ^T |
| <i>R. campinensis</i> LMG 20585 | 66.5 | 100 | 100 | 83 | 85 | 87 | 85 |
| <i>R. campinensis</i> LMG 20588 | 66.6 | 102 | 100 | | 89 | | |
| <i>R. campinensis</i> LMG 20567 | 66.3 | 91 | | 100 | | 98 | 94 |
| <i>R. campinensis</i> LMG 20590 | 66.2 | 89 | 94 | | 100 | | |
| <i>R. campinensis</i> LMG 20584 | 65.7 | 81 | | 88 | | 100 | 84 |
| <i>R. campinensis</i> LMG 19282 ^T | 66.3 | 86 | | 95 | | 100 | 100 |
| <i>R. campinensis</i> LMG 19285 | 66.8 | 74 | 85 | 91 | 85 | 78 | |
| <i>R. basilensis</i> ER121 | 65.4 | 18 | 28 | 27 | 21 | 27 | |
| <i>R. metallidurans</i> LMG 1195 ^T | 63.8 | 29 | 37 | 37 | 36 | 37 | |
| <i>R. eutropha</i> LMG 1199 ^T | 66.7 | 29 | 40 | 41 | 35 | 39 | |
| <i>R. solanacearum</i> LMG 2299 ^T | 67.0 | 18 | 23 | 24 | 18 | 25 | |
| <i>R. paucula</i> LMG 3244 ^T | 63.9 | 33 | 45 | 45 | 35 | 44 | |
| <i>R. gilardii</i> LMG 5886 ^T | 67.2 | 29 | 36 | 32 | 27 | 36 | |
| <i>R. pickettii</i> LMG 5942 ^T | 64.2 | 20 | 26 | 23 | 18 | 23 | |

Rep-cluster VIII comprised three pEMT1-containing transconjugants from A-horizon soil microcosms amended with nutrients. The partial 16S rDNA sequence of cluster VIII isolate LMG 20563 was almost identical (99.9% similarity; Table 7.4) to that of *S. maltophilia* VUN10075 and clusters within the *S. maltophilia* sequence group III of Hauben et al. ([46]; data not shown). Cellular fatty acid analyses were performed and all isolates of cluster VIII were identified with the MIS software as *S. maltophilia* with identification scores between 0.33 and 0.48. Consequently, we considered cluster VIII transconjugants as *S. maltophilia*.

Six transconjugants occupied distinct positions in the dendrogram (Fig. 7.5).

Although isolates LMG 21262 and LMG 20580 had a clearly different REP- and BOX-PCR pattern (Fig. 7.5), their partial 16S rDNA sequences were 99.7% similar. Their nearest neighbour with a species designation in the EMBL database was *Burkholderia fungorum* LMG 16225^T (Table 7.4; Fig. 7.6). Since neither LMG 21262 nor LMG 20580 could be identified to the species level by whole-cell protein profiles (data not shown) or DNA-DNA hybridisations (Table 7.5), they were provisionally identified as *Burkholderia* sp.

Isolates LMG 20565 and LMG 20569 had clearly different REP- and BOX-PCR profiles (Fig. 7.5). Nevertheless, these isolates showed identical partial 16S rDNA sequences, which were in turn identical to the sequence of *S. maltophilia* e-p10 (Table 7.4). Furthermore, a high sequence similarity (99.4%) was found with LMG 10857 (accession no. AJ131117), a representative of sequence group V from Hauben et al. [46]. The two transconjugants also showed a typical *Stenotrophomonas* cellular fatty acid profile (data not shown) and, although only a low identification score as *S. maltophilia* (0.15 - 0.21) was found in the MIDI database, they were considered as *S. maltophilia*.

The partial 16S rDNA sequence of LMG 20596 was 99.8% similar to the sequence of *S. maltophilia* LMG 20563 (a representative of rep-cluster VIII) and therefore also belonged to sequence cluster III of Hauben et al. [46]. In the EMBL database, the closest match was found with *S. maltophilia* e-p20 (Table 7.4). This identification based on 16S rDNA sequence was in agreement with the cellular fatty acid data; the MIS software identified LMG 20596 as *S. maltophilia* with an identification score of 0.35.

Isolate LMG 20595 showed a high similarity (99.3%) in partial 16S rDNA sequence with *Ralstonia basilensis* LMG 19474^T (Table 7.4). However, it was unable to grow at 37°C and therefore, cell-free protein extracts could not be prepared under the conditions used for *Ralstonia* strains. Despite this difference in culture conditions, the whole-cell protein profile of LMG 20595 was very similar to that of *R. basilensis* LMG 19474^T (Fig. 7.7). This observation, combined with the sequence data, supports the classification of LMG 20595 as *R. basilensis*.

The results of the biochemical and phenotypic tests were as reported in the description of *Burkholderia hospita* and *Burkholderia terricola* (see below).

7.3.5 Discussion

7.3.5.1 Transconjugant collection

The transconjugant collection characterised in this study (Table 7.3) originated from transfer experiments in which *Pseudomonas putida* UWC3 strains, harbouring either plasmid pJP4 or pEMT1, were used as donor in A- and B-horizon soil microcosms, treated with 100 mg/kg 2,4-D. Transconjugants that could degrade 2,4-D as a result of acquisition of pJP4 or pEMT1 were selected for by plating on mineral medium with 2,4-D as sole carbon source. To avoid that transconjugants formed during the plating procedure were taken into account, the collection was limited to transconjugants isolated from day 6 (for the nutrient-amended experiments) or 7 (for the non-amended experiments) on. Starting from these sampling days, transconjugant counts on 2,4-D-containing selective plates clearly exceeded the background level of non-inoculated soil [21] and the calculated number of donor cells on isolation plates was always below 10⁴, required for plate matings to occur [80].

Previously, using PCR-DGGE, a shift in the bacterial community of the B-horizon augmented with plasmid pEMT1 was noted, with the 16S rRNA gene fragments of newly appearing bands corresponding to those of 2,4-D-degrading transconjugant

colonies on agar plates. This was shown for *R. campinensis*, *B. terricola*, and *B. hospita* (at that time provisionally designated as *R. eutropha*-like, *B. graminis*-like, and *B. caribensis*-like, respectively) [21]. Furthermore, the 16S rDNA sequence of cluster II isolate LMG 20594 as determined in this study was 99.4% similar to the sequence of soil clone WD1 (accession no. AF247780). The latter sequence was determined on a 16S rDNA band excised from a DGGE profile of the soil microbial community of the non-amended B-horizon microcosm inoculated with the donor of pEMT1 [21].

These data suggest that the isolates in Table 7.3 correspond to 'true' *in situ* transconjugants.

7.3.5.2 Diversity and identification of the transconjugants

As a first step, repetitive element-based genomic fingerprinting (rep-PCR) was used to assess the diversity of the transconjugant collection. Since rep-PCR is a highly discriminatory technique that typically characterizes bacterial isolates at the infraspecific level [99], we assumed that each delineated rep-cluster represented a group of highly related organisms. The specificity of rep-PCR was clearly illustrated by DNA-DNA hybridisation experiments with isolates representing rep-clusters V, VI and VII. Although obviously different in terms of rep-PCR profile, representatives were shown to be highly related members of the same species, *R. campinensis* (above 84% average DNA-DNA reassociation). Rep-PCR also detected multiple genomic groups within *S. maltophilia* (clusters IV and VIII and unique rep-types LMG 20565, LMG 20569, and LMG 20596), and even discriminated within the sequence groups of *S. maltophilia* as defined by Hauben et al. [46]. Indeed, LMG 20565 and LMG 20569 represented unique rep-types, although both were assigned by partial 16S rDNA sequencing to sequence group V. From a practical point of view, rep-PCR allowed us to limit the detailed taxonomic analyses to selected representatives of the fourteen rep-types compared to the 95 isolates of the entire transconjugant collection.

The identification strategy for these representative isolates consisted of an initial partial or near complete 16S rDNA sequence determination and subsequent comparison with the EMBL database, followed by a second taxonomic technique that allowed identification at the species level. The choice for the latter technique was thereby depending on the initial identification result and on the availability of databases in our research group. In most cases, whole-cell protein profiling - possibly supported with DNA hybridisation data - was used, while for the *S. maltophilia* isolates fatty acid methyl ester analysis was applied.

Using the strategy described above, six clusters (III, IV, V, VI, VII, and VIII) and four unique rep-types (LMG 20565, LMG 20569, LMG 20595, and LMG 20596) could unambiguously be assigned at the species level. These transconjugants were identified as members of following taxa (Table 7.3): *Ralstonia campinensis* (34 isolates), *Stenotrophomonas maltophilia* (10 isolates, distributed over sequence groups I, III and V), *Burkholderia glathei* (2 isolates), and *Ralstonia basilensis* (1 isolate). Rep clusters I and II (comprising 29 and 17 isolates, respectively) were assigned to the genus *Burkholderia* via 16S rDNA sequencing, but subsequent whole-cell protein analysis and DNA-DNA hybridisations clearly showed that each of these two rep-clusters represent a novel species. Below we propose the names *Burkholderia hospita* and *Burkholderia*

terricola, respectively, for these two clusters. In the course of the study, rhizosphere isolates from Scotland and Italy [41] were identified as *B. terricola* as well.

Strains LMG 20580 and LMG 21262, representing two unique rep-types, were also shown to belong to the genus *Burkholderia*. Although whole-cell protein data and DNA reassociation values indicated that these strains probably also represent new species, no formal description is given here, awaiting the availability of additional strains.

7.3.5.3 *Comparison of transconjugants from A- and B-horizon soil, and effect of nutrients on transconjugant diversity*

The A- and B-horizon soil differs in terms of texture and organic matter content, and therefore probably also in microbial community and metabolic activity [21]. This raises the question whether similar or different organisms act as recipients for both catabolic plasmids in A- and B-horizon soil.

Six rep-clusters (I, III, IV, V, VI, and VII) out of eight consisted of transconjugants from both soil horizons. This observation suggests that in A- and B-horizon the same or very similar organisms were able to acquire pJP4 or pEMT1. Only rep-cluster VIII (3 isolates belonging to *S. maltophilia* sequence group III of Hauben et al. [46]) and rep-cluster II (17 *B. terricola* isolates) seemed to be specific for the A- and B-horizon, respectively. In the B-horizon, an isolate with a unique rep-type (LMG 20596) was found that also belonged to *S. maltophilia* sequence group III. These data suggest that *B. terricola* populations able to acquire and express the 2,4-D degradative genes were only prevalent in the B-horizon, and not in the A-horizon.

Remarkably, when nutrients were added to the soil (1 ml 5x LB in 100 g soil), a large proportion (nine out of twenty-two isolates; Table 7.3) of the isolated transconjugants were identified as *S. maltophilia*. In contrast, when the soil was not amended with nutrients, only one *S. maltophilia* (LMG 20596) was found among a total of seventy-three isolated transconjugants. Possibly the addition of easily degradable carbon sources present in LB medium selectively stimulated the outgrowth of *S. maltophilia* in the soil microcosms.

7.3.5.4 *Expression range of pJP4 and pEMT1*

Since microcosm soil samples were plated on minimal medium with 2,4-D as sole carbon source, those transconjugants able to completely degrade 2,4-D were selected for. However, not all strains that have received pJP4 or pEMT1 can degrade 2,4-D. This can be due to several reasons, including lack of transcription or expression, lack of transport of the 2,4-D molecules in the cell, etc. Therefore the transconjugants in this collection (Table 7.3) were limited to those isolates that could degrade 2,4-D as a result of acquisition of the plasmid.

Within the total of fourteen rep-types that could be discriminated among the ninety-five isolated transconjugants, twenty-seven pJP4 containing transconjugants were found to belong to seven rep-types, while sixty-eight pEMT1 containing transconjugants were distributed over twelve rep-types. Five (I, II, IV, V, and VI) rep-clusters comprised both pJP4- and pEMT1-containing transconjugants. This observation

suggests that both plasmids can be transferred to and expressed within highly related or even similar organisms. All isolated transconjugants belonged to the genera *Burkholderia* (50 isolates), *Ralstonia* (35 isolates) or *Stenotrophomonas* (10 isolates) and representatives of these three genera were found for pJP4 as well as for pEMT1. Furthermore, Dejonghe et al. [21] noted that the same changes in DGGE-profiles of the soil microbial community occurred when *P. putida* UWC3 (pJP4) or *P. putida* UWC3 (pEMT1) was added. All these results suggest that, although both plasmids belong to different incompatibility classes [88], they have a very similar transfer and expression range.

The finding that both plasmids under study are preferably transferred to and expressed within β -subclass (*Burkholderia* and *Ralstonia*) and γ -subclass (*Stenotrophomonas*) *Proteobacteria*, is in good agreement with our knowledge about the host range of pJP4 and pEMT1. Don and Pemberton [23] showed via plate matings that plasmid pJP4 could be transferred to α -, β - and γ -subclass *Proteobacteria*, but the ability to degrade 2,4-D was expressed only in representatives of the β - and γ -subclass. Furthermore, several authors reported on the identification of transconjugants that resulted from the inoculation of soil with various donors containing the plasmid pJP4. In these reports, pJP4-transconjugants were selected based on the plasmid-encoded mercury resistance and were identified as *Acidovorax* sp., *Acinetobacter* sp., *Agrobacterium* sp., *Pasteurella* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Burkholderia glathei*, *Burkholderia caryophylli*, *Burkholderia cepacia*, and *Burkholderia* sp. [18, 22, 66]. However, in all of the above studies, identifications were performed with BIOLOG, a technique to be considered only as indicative when it comes to identification of environmental isolates [86]. In the study of Newby and co-workers [65], transconjugants that resulted from inoculation of a sandy loam soil with *E. coli* D11 (pJP4) or *Ralstonia* sp. JMP134, were identified via 16S rDNA sequencing as *Burkholderia caribensis*, *Burkholderia glathei*, *Burkholderia graminis*, *Burkholderia kururiensis*, *Burkholderia phenazinium*, *Ralstonia eutropha* and *Ralstonia gilardii*, with sequence similarities ranging from 97 to 99%. *B. graminis* was the most prevalent transconjugant. Unlike for plasmid pJP4, no published data are available on the host range of pEMT1, although in plate matings a similar transfer and expression behaviour as for pJP4 was observed (C. Coucke, J. Goris, and P. De Vos, unpublished results). In the only previous study in which transconjugants that acquired pEMT1 in soil were isolated [89], the transconjugants were identified as *R. eutropha* and *Burkholderia* sp.; representatives of the two genera that were most frequently isolated in this study.

7.3.5.5 Description of *Burkholderia hospita* sp. nov.

Burkholderia hospita (hos'pi.ta L. fem. n. *hospita* hostess, to indicate that this organism can act as a host for plasmid pJP4 or pEMT1). *B. hospita* cells are Gram-negative, motile, non-sporulating, straight rods. Growth is observed at 28°C, not at 42°C. No growth in O/F medium with D-glucose, maltose, adonitol, D-fructose or D-xylose as C-source. No growth was observed on cetrимide, on 10% lactose, in the presence of acetamide, nor in the presence of 1.5, 3.0, 4.5 or 6.0% NaCl. Growth in the presence of 0.5% NaCl was strain dependent. The cells grew on Drigalski agar and blood agar at 30°C, but not on blood agar at 37°C. Nitrate is reduced, nitrite not. Tween

80 is hydrolysed. Haemolysis of horse blood was not observed. No production of acid or H₂S in triple-sugar-iron agar, no indole or fluorescent pigment produced. No fermentation of D-glucose. No liquefaction of gelatin or hydrolysis of aesculin. Assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-gluconate, phenyl acetate, L-arginine, D,L-lactate, but not of trehalose and sucrose. Variable assimilation of citrate, caprate, adipate, L-malate, D,L-norleucine, and maltose. Oxidase, catalase, alkaline and acid phosphatase, esterase C4, leucine arylamidase, phosphoamidase, and β -galactosidase activity is present; DNase, lipase C14, tryptophanase, arginine dihydrolase, valine and cystine arylamidase, lysine decarboxylase, ornithine decarboxylase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, α - and β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, and amylase activity was not detected. Reactions of ester lipase C8 and urease were strain dependent. The whole-cell fatty acid profile (averages and standard deviations were calculated based on profiles of 29 strains) of *B. hospita* strains comprised 12:0 (1.6% \pm 0.2), 14:0 (3.6% \pm 0.2), 14:0 3OH (8.1% \pm 1.3), 16:1 ω 7c (6.9% \pm 1.4), 16:0 (17.2% \pm 1.6), 17:0 cyclo (15.4% \pm 1.9), 16:1 2OH (3.3% \pm 0.7), 16:0 2OH (4.9% \pm 0.8), 16:0 3OH (5.9% \pm 0.7), 18:1 ω 7c (26.0% \pm 3.1), 18:0 (0.5% \pm 0.5), 19:0 cyclo ω 8c (5.3% \pm 1.4), and 18:1 2OH (1.3% \pm 0.2) as major components (summed feature 2 [comprising 14:0 3OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928, or 12:0 ALDE, or any combination of these fatty acids], and summed feature 3 [comprising 16:1 ω 7c or 15 iso 2OH or both] are listed as 14:0 3OH and 16:1 ω 7c, respectively, as these fatty acid have been reported in *Burkholderia* species [82]).

The G+C content is 61.7 - 62.2 mol%. *B. hospita* strains were isolated as pJP4 or pEMT1 containing transconjugants from the A-horizon (depth of 0 to 30 cm) and B-horizon (depth of 30 to 60 cm) of agricultural soil in Pittem, Belgium. The type strain is LMG 20598 (= CCUG 43658), isolated from B-horizon soil, and carries the 2,4-dichlorophenoxyacetic acid degrading plasmid pEMT1. Its G+C content is 62.0 mol% and its 16S rDNA sequence has been deposited in the GenBank database under accession number AY040365.

7.3.5.6 Description of *Burkholderia terricola* sp. nov.

Burkholderia terricola (ter.ri.co'la L. n. terra earth, soil; L. suff. -cola inhabitant, dweller L. n. terricola soil-dweller, referring to the isolation of these strains from soil). *B. terricola* cells are Gram-negative, motile, non-sporulating, straight rods. Growth is observed at 28°C, not at 42°C. Growth in O/F medium is observed with D-glucose as C-source, but not with D-fructose or D-xylose. Growth in O/F medium with maltose and adonitol were strain dependent. No growth was observed on cetrimide, in the presence of acetamide, nor in the presence of 1.5, 3.0, 4.5 or 6.0% NaCl. Variable growth on 10% lactose or in the presence of 0.5% NaCl. The cells grew on blood agar at 30°C, but growth on blood agar at 37°C and on Drigalski agar was strain dependent. Nitrate is reduced, nitrite not. Variable fermentation of D-glucose. No liquefaction of gelatin or hydrolysis of aesculin. Hydrolysis of Tween 80 is variable. Haemolysis of horse blood was not observed. No production of acid or H₂S in triple-sugar-iron agar, no indole or fluorescent pigment produced. Assimilation of D-glucose, L-arabinose, D-mannose, D-

mannitol, N-acetyl-D-glucosamine, D-gluconate, phenyl acetate, D,L-lactate, sucrose, but not of adipate, D,L-norleucine, and maltose. Variable assimilation of citrate, caprate, L-arginine, L-malate, and trehalose.

Catalase, alkaline and acid phosphatase, esterase C4, ester lipase C8, leucine arylamidase, phosphoamidase, and β -galactosidase activity is present; DNase, lipase C14, tryptophanase, valine and cystine arylamidase, lysine decarboxylase, ornithine decarboxylase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, α - and β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, and urease activity was not detected. Reactions of oxidase, amylase, and arginine dihydrolase were strain dependent. The whole-cell fatty acid profile (averages and standard deviations were calculated based on profiles of 17 strains) of *B. terricola* strains comprised 14:0 (4.8% \pm 0.8), 14:0 3OH (8.2% \pm 2.8), 16:1 ω 7c (9.1% \pm 4.9), 16:0 (14.3% \pm 1.3), 17:0 cyclo (14.0% \pm 5.2), 16:1 2OH (3.1% \pm 1.2), 16:0 2OH (3.5% \pm 1.4), 16:0 3OH (6.6% \pm 1.5), 18:1 ω 7c (27.8% \pm 8.8), 18:0 (0.7% \pm 0.6), 19:0 cyclo ω 8c (5.5% \pm 3.0), and 18:1 2OH (1.5% \pm 0.7) as major components (summed feature 2 [comprising 14:0 3OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928, or 12:0 ALDE, or any combination of these fatty acids], and summed feature 3 [comprising 16:1 ω 7c or 15 iso 2OH or both] are listed as 14:0 3OH and 16:1 ω 7c, respectively, as these fatty acid have been reported in *Burkholderia* species [82]).

The G+C content is 62.7 - 63.9 mol%. *B. terricola* strains were isolated as pJP4 or pEMT1 containing transconjugants from the B-horizon (depth of 30 to 60 cm) of agricultural soil in Pittem, Belgium. The type strain is LMG 20594 (= CCUG 44527) and carries the 2,4-dichlorophenoxyacetic acid degrading plasmid pJP4. Its G+C content is 63.9 mol% and its 16S rDNA sequence has been deposited in the GenBank database under accession number AY040362.

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CHAPTER 8
TRANSFER OF PLASMID PC1GFP IN
ACTIVATED SLUDGE

“The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly
fact”

Thomas H. Huxley

CHAPTER 8 TRANSFER OF PLASMID pC1GFP IN ACTIVATED SLUDGE

8.1 DIVERSITY OF ACTIVATED SLUDGE BACTERIA RECEIVING THE 3-CHLOROANILINE-DEGRADATIVE PLASMID pC1GFP[§]

8.1.1 Summary

Plasmid pC1 of *Delftia acidovorans* CA28, encoding the oxidative deamination of 3-chloroaniline (3-CA), was tagged with a mini-Tn5 transposon containing the *gfp* gene. The labelled plasmid, designated pC1gfp, was subsequently transferred to *Pseudomonas putida* UWC3 and the plasmid transfer from this donor to the bacterial community in activated sludge was studied. Conjugation experiments were performed both on LB agar plates or directly in liquid activated sludge. Green fluorescent colonies appearing on mineral medium containing 3-CA as sole nitrogen source were picked up and verified to be true pC1gfp-harbouring transconjugants. REP- and BOX-PCR genomic fingerprinting revealed a large diversity in the transconjugant collection, indicating the occurrence of multiple plasmid transfer events. Remarkably, LB agar plate conjugations yielded a different set of transconjugants compared to conjugations directly in liquid activated sludge. From the plate matings, mainly *Aeromonas* sp. were isolated, whereas *D. acidovorans* strains were predominant in the transconjugant collection from liquid activated sludge. The majority of the isolates showed 3-CA deamination, probably to 4-chlorocatechol, but several pC1gfp-harbouring transconjugants performed a rapid and complete 3-CA degradation.

8.1.2 Introduction

Most of the genes involved in the degradation of xenobiotics are part of operons encoded on broad-host range, conjugative, or mobilisable plasmids [35]. The degradation pathways they encode are believed to be evolved from existing enzymatic systems effective towards naturally occurring compounds, probably upon the introduction of xenobiotics in the biosphere. Plasmid pC1 is a 100 kb self-transmissible IncP-1 β broad-host range plasmid, isolated from *Delftia acidovorans* CA28 [3]. This strain was originally isolated from soil [21] and its aniline and monochloroaniline metabolism has been the subject of several studies [18-20]. Recently, it was shown that the oxidative deamination of 3-chloroaniline is encoded on the plasmid pC1 [3].

Since horizontal transfer of catabolic genes has an impact on the degradation of xenobiotic compounds, the deliberate dissemination of a catabolic plasmid in an ecosystem could possibly be exploited as a bioremediation tool (for a review, see [33]). This bioaugmentation approach has been evaluated mainly in soil microcosms, supplemented with a broad-host range catabolic plasmid such as RP4::Tn4371 [8, 38], pEMT1 [9, 32, 34], or pJP4 [9, 10, 23-25]. In nearly all of these studies, plasmid

[§] Redrafted from Goris, J., N. Boon, L. Lebbe, W. Verstraete, and P. De Vos. Diversity of activated sludge bacteria receiving the 3-chloroaniline-degradative plasmid pC1gfp. Submitted for FEMS Microbiology Ecology.

transfer from the introduced donor strain to indigenous bacteria was observed. Increased biodegradation as a result of plasmid dissemination was clearly shown in some cases, e.g. for plasmid pEMT1 in B-horizon agricultural soil (30-60 cm depth) [9]. When *in situ* formed transconjugants were identified, they most often belonged to the genera *Burkholderia*, *Ralstonia*, or *Pseudomonas* [10, 13, 23, 24]. In contrast to soil ecosystems, only few data are available on bioaugmentation through dissemination of catabolic plasmids in activated sludge. This approach might nevertheless provide a valuable strategy, since activated sludge flocs may present an ideal site for conjugation, given the high cell densities and presence of biodegradable organic matter [33].

The spread of a plasmid in an ecosystem can be traced by tagging it with a suitable marker gene, such as the gene encoding the green fluorescent protein (*gfp*). Labelling with *gfp* was previously applied to trace the TOL plasmid on semi-solid agar surfaces [5], in a defined benzyl alcohol utilising biofilm [4, 15], and on the phylloplane of the bush bean [26]. Using the same system, the transfer behaviour of three conjugative plasmids isolated from marine bacterial communities was studied [7]. Geisenberger and co-workers combined *gfp*-marking with rRNA targeted oligonucleotide probes to monitor the spread of plasmid RP4 from an introduced donor strain to indigenous activated sludge bacteria [12]. Using epifluorescence microscopy and confocal laser scanning microscopy, the majority of the RP4 harbouring transconjugants were *in situ* identified as members of the genus *Aeromonas* [12].

The objectives of this study were (i) to label the 3-chloroaniline degradative plasmid pC1 with *gfp* to facilitate the study of its transfer and for application in future bioaugmentation studies, (ii) to assess pC1*gfp* transfer to the microbial community present in activated sludge by determining the diversity of the indigenous pC1*gfp*-harbouring transconjugants, and (iii) to evaluate the expression range of pC1*gfp* in activated sludge through a detailed taxonomic characterisation of the isolated transconjugants.

8.1.3 Materials and methods

8.1.3.1 Bacterial strains, plasmids and culture conditions

A list of the bacterial strains and plasmids, with their relevant characteristics, is compiled in Table 8.1. Strains were routinely kept on Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar per litre distilled water) containing the appropriate selective markers. Unless otherwise stated, the applied concentrations of 3-chloroaniline (3-CA), rifampicin, nalidixic acid, kanamycin, and tetracycline were 150, 100, 200, 50, and 5 mg/l, respectively. The composition of mineral medium without C- and N-source (MMN) was described previously [3]. When added, the concentration of sodium pyruvate, sodium succinate or sodium acetate was always 1 g/l.

TABLE 8.1: Bacterial strains and plasmids used, with their relevant characteristics

| Strain or plasmid | Characteristics ^a | Source or reference ^b |
|--|--|----------------------------------|
| Strain: | | |
| <i>Delftia acidovorans</i> CA28 (=R-15893) | Rif ^R ; 3-CA ^{CN} | [21] |
| <i>Ralstonia eutropha</i> JMP228n (=R-15898) | Nal ^R ; 3-CA ⁻ | [31] |
| <i>Escherichia coli</i> S17-1 λ pir (pUTgfp)Km (=R-15900) | Amp ^R ; Km ^R ; GFP | [2] |
| <i>Ralstonia eutropha</i> JMP228n (pC1gfp) (=R-15962) | Nal ^R ; 3-CA ^N ; Km ^R ; GFP | This study |
| <i>Pseudomonas putida</i> UWC3 (=R-4973) | ILV ⁻ ; 3-CA ⁻ ; Tet ^R | [17] |
| <i>Pseudomonas putida</i> UWC3 (pC1gfp) (=R-16349) | ILV ⁻ ; 3-CA ⁻ ; Km ^R ; GFP; Tet ^R | This study |
| <i>Rhizobium radiobacter</i> | | LMG 221 |
| <i>Blastomonas natarioria</i> | | LMG 17322 ^T |
| <i>Escherichia coli</i> DH5 α (=R-4987) | | [16] |
| Plasmid: | | |
| pC1 | 3-CA ^N | [3] |
| pUTgfp | Km ^R ; GFP | [30] |
| pC1gfp | 3-CA ^N ; Km ^R ; GFP | This study |

^a ILV⁻: isoleucine, leucine, and valine auxotrophy; Rif^R, Nal^R, Amp^R, Km^R, Tet^R: resistance to rifampicin, nalidixic acid, ampicillin, kanamycin, and tetracycline, respectively; 3-CA⁻, 3-CA^N, 3-CA^{CN}: 3-chloroaniline not used, used as sole nitrogen source or used as sole carbon and nitrogen source, respectively; GFP: expressing green fluorescent protein

^b LMG: BCCMTM/LMG Bacteria Collection, Laboratory of Microbiology Gent, Belgium

8.1.3.2 Labelling of the pC1 plasmid

The pC1 plasmid was marked with the mini-Tn5 transposon from plasmid pUTgfp, constructed by Tombolini and co-workers [30]. The transposon contained the *gfp* gene (encoding green fluorescent protein) and the *npt* gene (encoding kanamycin resistance), both transcribed from their respective constitutive promoters. The construction principle of a GFP-tagged pC1 derivative was previously used for tagging the TOL plasmid [4, 5], as well as conjugative plasmids isolated from marine environments [6, 7]. The pC1-containing strain *Delftia acidovorans* CA28 was conjugated overnight with *Escherichia coli* S17-1 λ pir (pUTgfp) [3] on an LB agar plate at 28°C. The conjugation mixture was harvested and suspended in 1 ml saline (0.85% (w/v) in distilled water). Transconjugants were enriched by transfer of 50 μ l of this suspension to 5 ml liquid MMN supplemented with 3-CA, rifampicin and kanamycin, followed by incubation on a rotary shaker (140 rpm) at 28°C. When growth had occurred, as revealed by an increased turbidity, the culture was transferred (1% inoculum) to fresh medium with the same composition. *E. coli* S17-1 λ pir cannot grow on 3-CA as sole C- and N-source and in the presence of rifampicin, while non-marked *D. acidovorans* CA28 will be excluded by its kanamycin sensitivity. Therefore, *D.*

acidovorans CA28 cells that received the mini-Tn5 cassette either in the chromosome or in the plasmid were specifically enriched. To distinguish between chromosomally and plasmid-marked CA28, a second conjugation with the nalidixic acid-resistant *Ralstonia eutropha* JMP228n [31] was performed. The conjugation mixture was again harvested and suspended in 1 ml saline. Subsequently, 50 µl of this suspension was transferred to 5 ml of MMN supplemented with sodium pyruvate, 3-CA, nalidixic acid and kanamycin. In this selective medium, *D. acidovorans* CA28 was eliminated by nalidixic acid and *R. eutropha* JMP228n without an *npt* containing plasmid was eliminated by kanamycin and by its inability to grow on 3-CA as sole N-source. Thus, only *R. eutropha* JMP228n that contained the labelled pC1 plasmid was selected. After an turbidity increase of the medium, the culture was plated on agar plates of the same composition and the colonies showing green fluorescence (excitation at 396/476 nm, emission at 508 nm) under UV light (TLD 18W/08, Philips, The Netherlands) were picked up and further purified. Single colonies were tested for 3-CA degradation as described below.

8.1.3.3 Plasmid donor strain construction

Pseudomonas putida UWC3 is a suitable donor strain in plasmid transfer experiments, since it allows counter-selection using its isoleucine, leucine and valine auxotrophy (ILV⁻) [9]. In a plate mating on LB, the pC1gfp plasmid was transferred from *R. eutropha* JMP228n (pC1gfp) to *P. putida* UWC3. The selection for the latter was done on LB with tetracycline and kanamycin. Plasmid pC1gfp did not confer 3-CA degradation properties to *P. putida* UWC3 and therefore, the plasmid was transferred again to *R. eutropha* JMP228n to check the integrity of the expression of the metabolic genes on the plasmid.

8.1.3.4 Plate matings with collection strains

Transfer capacities of the pC1gfp plasmid were verified by plate matings on LB of the donor strain *P. putida* UWC3 (pC1gfp) with the recipients *Escherichia coli* DH5α, *Rhizobium radiobacter* LMG 221, and *Blastomonas natatoria* LMG 17322^T. Transconjugants were selected on MMN plates, supplemented with sodium pyruvate, (NH₄)₂SO₄ (300 mg/l), and kanamycin.

8.1.3.5 Transfer of pC1gfp in activated sludge

P. putida UWC3 (pC1gfp) was grown overnight in 5 ml LB medium supplemented with kanamycin and tetracycline at 28°C. Cells were harvested by centrifugation (7000 x g, 10 min) and suspended in 5 ml of sterile saline. A grab sample of mixed liquor was freshly collected from the recirculation stage of a local municipal wastewater treatment plant (Bourgoyen-Ossemeersen, Gent, Belgium). Upon arrival in the laboratory, the suspended solids were determined [14] and adjusted to 4 g/l. The sludge was then incubated for one hour at room temperature at a volumetric loading of 1 g/l of COD, supplemented as skim milk powder [2]. Conjugations between *P. putida* UWC3 (pC1gfp) and the bacterial community in activated sludge were either performed on LB agar surfaces or directly in liquid activated sludge. For the plate matings, 30 µl of the *P. putida* UWC3 (pC1gfp) suspension and 30 µl activated sludge were brought

together on the agar surface and incubated overnight at 28°C. For the conjugations in suspension, 100 µl of *P. putida* UWC3 (pC1gfp) was directly added to 5 ml activated sludge and incubated overnight at 28°C under constant agitation (90 rpm). Both plate matings and conjugations in liquid activated sludge were performed in quadruple. The plasmid donor was quantified on LB agar plates, supplemented with kanamycin and tetracycline. Transconjugants were selected on MMN plates supplemented with 3-CA, sodium pyruvate, and kanamycin. The donor strain was unable to grow on the latter medium, due to its ILV auxotrophy. Transfer frequencies were calculated in this experiment as transconjugant CFU/ml divided by donor strain CFU/ml.

8.1.3.6 Characterisation of pC1gfp-containing transconjugants

Putative transconjugants appeared on MMN plates with sodium pyruvate, 3-CA, and kanamycin as fluorescent colonies under UV-light. From both experiments (LB plate matings or conjugations in liquid activated sludge), as many different colony types as possible were picked up and purified. To verify if the obtained isolates indeed corresponded to transconjugants, they were tested for the presence of the *gfp* gene by PCR as described previously [2] and for 3-CA degradation in liquid medium (see below). Isolates that scored positive for both tests were considered to be ‘true’ transconjugants and were genomically typed using repetitive element PCR with the REP and BOX-primers as described previously [13]. Using the BioNumerics 2.0 software package (Applied Maths, Belgium), the obtained REP- and BOX-PCR profiles for each transconjugant were linearly combined and numerically analysed. Cluster analysis allowed subdivision of the transconjugant collection into distinct rep-types, defined here as subgroups of most probably highly related organisms with high internal similarity in combined REP- and BOX-PCR profiles (more than 65% Pearson correlation). From each rep-type, one representative strain was selected and all further analyses were limited to these representatives. As an additional confirmation for transconjugants, each of the representatives was subjected to plasmid isolation [28]. A combination of genotypic and phenotypic information was employed to identify the transconjugants. A continuous stretch of about 400 bp from the 16S rRNA gene of each representative strain was determined as previously described [13] (for accession numbers, see Table 8.2). Obtained sequences were compared with EMBL database entries using the FASTA search algorithm [27]. Initial identification results derived from partial 16S rDNA sequencing were confirmed and refined phenotypically. For isolates belonging to the *Enterobacteriaceae*, BIOLOG GN2 carbon source utilisation patterning was performed according to the instructions of the manufacturer (Biolog, Inc., USA). From *Aeromonas* isolates, fatty acid methyl esters were analysed [36] and compared with the Sherlock Microbial Identification System version 3.10 (MIDI, Inc., USA). Whole-cell protein analysis [29] was performed for β -*Proteobacteria*.

8.1.3.7 3-CA degradation experiments

Single colonies grown on MMN agar plates were transferred to 5 ml liquid MMN supplemented with sodium pyruvate and 3-CA. The test tubes were incubated for 7 days at 28°C and 140 rpm, followed by HPLC determination of the 3-CA concentration as described previously [3]. With sodium pyruvate as C-source, a variable amount of 3-CA

was reversibly converted to an unknown complex. Since this phenomenon rendered an accurate quantification of the remaining 3-CA more complicated, sodium pyruvate was replaced with sodium succinate for the determination of degradation kinetics. The latter experiments were performed in 250 ml Erlenmeyer flasks equipped with Klett-tubes and containing 100 ml liquid MMN supplemented with sodium succinate and 3-CA. Pre-cultures were grown on 5 ml liquid MMN with sodium succinate, 3-CA and $(\text{NH}_4)_2\text{SO}_4$ (75 mg/l) and were washed once in MMN (without NH_4^+) to avoid carry-over of NH_4^+ . On regular intervals, optical density was measured at 550 nm and samples for HPLC analysis were taken.

8.1.4 Results

8.1.4.1 Marking of pC1 with *gfp*

About 50 fluorescent colonies appeared on the selective medium after the labelling procedure. One of these colonies was picked up and the presence of the *gfp* gene revealed by *gfp*-PCR. The REP-PCR profile of this isolate was identical with the database profile of *R. eutropha* JMP228n (data not shown). To examine if the *gfp* insertion had an effect on the degradation capacities of pC1*gfp*, a 3-CA degradation assay was performed. The Erlenmeyer flasks, containing MMN supplemented with 3-CA and sodium succinate, were inoculated with 2×10^6 - 7×10^6 CFU/ml *R. eutropha* JMP228n (pC1*gfp*) or JMP228n cells. *R. eutropha* JMP228n (pC1*gfp*) completely degraded 150 mg/l 3-CA in ten days, while after the same time interval, 80% of the 3-CA was still present in cultures inoculated with plasmid-free *R. eutropha* JMP228n. In non-inoculated control reactors no significant decline in 3-CA concentration was observed even after 25 days of incubation (data not shown). When *R. eutropha* JMP228n (pC1*gfp*) or *P. putida* UWC3 (pC1*gfp*) were propagated on non-selective LB agar plates, no spontaneous plasmid loss was observed even after more than 10 successive inoculations.

8.1.4.2 Transfer of pC1*gfp* to collection strains

The pC1*gfp* plasmid could be transferred from the donor strain *P. putida* UWC3 (pC1*gfp*) to *Rhizobium radiobacter* LMG 221 and *Blastomonas natatoria* LMG 17322^T (both strains belong to the α -*Proteobacteria*) with transfer frequencies of respectively 5.7×10^{-2} and 7.9×10^{-3} transconjugants per recipient. The plasmid could also be transferred to *Escherichia coli* DH5 α (γ -*Proteobacteria*), but no transfer frequencies were determined for this experiment. For each plate mating, a green fluorescent transconjugant colony was picked up and was shown to react positive with the *gfp*-PCR.

8.1.4.3 Transfer of pC1*gfp* in activated sludge samples

Indigenous activated sludge bacteria that had taken up pC1*gfp* appeared as green fluorescent colonies on MMN agar plates, supplemented with 3-CA, kanamycin, and sodium pyruvate. Transfer frequencies were 4.7×10^{-5} for plate matings with activated sludge on LB and 2.2×10^{-5} for conjugations directly in liquid activated sludge. From the 91 putative transconjugants picked up from the isolation medium, two isolates were

unable to degrade 3-CA in liquid medium and one of these isolates also failed to produce an amplicon in the *gfp*-PCR test (data not shown). Two additional strains reacted negative in the *gfp*-PCR test and were also omitted. Therefore, from the original 91 isolates, 87 (67 from plate matings and 20 from conjugations in liquid activated sludge) were retained and considered 'true' transconjugants. REP- and BOX-PCR fingerprints were generated from all 'true' transconjugants (Fig. 8.1). None of the transconjugant fingerprints corresponded to fingerprints from the donor strain *P. putida* UWC3 (pC1gfp) or from the natural pC1 plasmid host *D. acidovorans* CA28 (data not shown). Through cluster analysis of the combined REP- and BOX-PCR profiles, 38 different rep-types (comprising 17 clusters and 21 unique profiles) were distinguished at a similarity level of 65% (Fig. 8.1). A representative strain was selected for each of the 38 rep-types. Plasmid extraction revealed the presence of a plasmid of the same size as pC1gfp (i.e. 100 kb) in all representatives (data not shown). Furthermore, the phylogenetic position of the rep-type representatives was determined by comparing their partial 16S rDNA sequences with EMBL sequence database entries (Table 8.2). This preliminary identification aided in the selection of an appropriate phenotypic identification technique (in this case BIOLOG, fatty acid analysis, or whole cell protein analysis). Data from 16S rDNA sequence comparisons as well as phenotypic results were considered to obtain a more definite consensus identification (Table 8.2), which was then used throughout the manuscript. Sequence data were always in agreement with phenotypic results at the genus level. In case both methods yielded different species identifications, consensus identifications were only reported at the genus level (Table 8.2). In Table 8.3, the identification results are summarised for all transconjugants originating from conjugations on agar surfaces and in liquid activated sludge.

TABLE 8.2: Identification results for representatives of the different rep-types.

| Rep-type | Isolate | Acc. No. | Best match EMBL ^a | Phenotypic identification ^b | Consensus identification |
|----------|---------|----------|---|---|----------------------------|
| I | R-17630 | AY178548 | 100% <i>Aeromonas punctata</i> ATCC 15468 ^T (X74674) | <i>Aeromonas bestiarum</i> / <i>A. hydrophila</i> (FA) | <i>Aeromonas</i> sp. |
| II | R-17488 | AY178536 | 99.6% <i>Aeromonas punctata</i> ATCC 15468 ^T (X74674) | <i>Aeromonas hydrophila</i> / <i>A. caviae</i> (FA) | <i>Aeromonas</i> sp. |
| III | R-17627 | AY178545 | 100% <i>Aeromonas punctata</i> ATCC 15468 ^T (X74674) | <i>Aeromonas caviae</i> / <i>A. hydrophila</i> (FA) | <i>Aeromonas</i> sp. |
| IV | R-17681 | AY178568 | 100% <i>Aeromonas salmonicida</i> ATCC 27013 ^T (X74680) | <i>Aeromonas hydrophila</i> (FA) | <i>Aeromonas</i> sp. |
| V | R-17653 | AY178560 | 100% <i>Aeromonas salmonicida</i> ATCC 27013 ^T (X74680) | <i>Aeromonas schubertii</i> / <i>A. hydrophila</i> (FA) | <i>Aeromonas</i> sp. |
| VI | R-17635 | AY178551 | 98.6% <i>Klebsiella oxytoca</i> JCM 1665 ^T (AB004754) | <i>Klebsiella terrigena</i> (BG) | <i>Klebsiella</i> sp. |
| VII | R-17644 | AY178557 | 99.3% <i>Serratia fonticola</i> DSM 4576 ^T (AJ233429) | <i>Serratia fonticola</i> (BG) | <i>Serratia fonticola</i> |
| VIII | R-17642 | AY178555 | 100% <i>Aeromonas media</i> ATCC 33907 ^T (X74679) | <i>Aeromonas sobria</i> / <i>A. media</i> (FA) | <i>Aeromonas</i> sp. |
| IX | R-17626 | AY178544 | 99.6% <i>Aeromonas media</i> ATCC 33907 ^T (X74679) | <i>Aeromonas caviae</i> / <i>A. encheleia</i> (FA) | <i>Aeromonas</i> sp. |
| X | R-17629 | AY178547 | 100% <i>Aeromonas media</i> ATCC 33907 ^T (X74679) | <i>Aeromonas caviae</i> / <i>A. hydrophila</i> (FA) | <i>Aeromonas</i> sp. |
| XI | R-17531 | AY178539 | 97.5% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| XII | R-17690 | AY178572 | 98.3% <i>Achromobacter xylosoxidans</i> subsp. <i>denitrificans</i> DSM 30026 ^T (AJ278451) | <i>Achromobacter</i> sp. (PA) | <i>Achromobacter</i> sp. |
| XIII | R-17668 | AY178564 | 97.2% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| XIV | R-17536 | AY178542 | 98.2% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| XV | R-17530 | AY178538 | 98.5% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| XVI | R-17628 | AY178546 | 100% <i>Aeromonas salmonicida</i> ATCC 27013 ^T (X74680) | <i>Aeromonas sobria</i> (FA) | <i>Aeromonas</i> sp. |
| XVII | R-17643 | AY178556 | 98.7% <i>Serratia fonticola</i> DSM 4576 ^T (AJ233429) | <i>Serratia fonticola</i> (BG) | <i>Serratia fonticola</i> |
| | R-17491 | AY178537 | 98.1% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17533 | AY178540 | 97.2% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17535 | AY178541 | 98.5% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17537 | AY178543 | 98.1% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17631 | AY178549 | 98.5% <i>Aeromonas caviae</i> NCIMB 13016 ^T (X60408) | <i>Aeromonas caviae</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17634 | AY178550 | 100% <i>Aeromonas media</i> ATCC 33907 ^T (X74679) | <i>Aeromonas sobria</i> / <i>A. caviae</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17636 | AY178552 | 98.5% <i>Aeromonas caviae</i> NCIMB 13016 ^T (X60408) | <i>Aeromonas sobria</i> / <i>A. caviae</i> (FA) | <i>Aeromonas</i> sp. |

| Rep-type | Isolate | Acc. No. | Best match EMBL ^a | Phenotypic identification ^b | Consensus identification |
|----------|---------|----------|--|--|----------------------------------|
| | R-17638 | AY178553 | 97.9% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17639 | AY178554 | 97.0% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17646 | AY178558 | 98.5% <i>Aeromonas caviae</i> NCIMB 13016 ^T (X60408) | <i>Aeromonas hydrophila</i> / <i>A. caviae</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17651 | AY178559 | 98.5% <i>Aeromonas caviae</i> NCIMB 13016 ^T (X60408) | <i>Aeromonas veronii</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17656 | AY178561 | 100% <i>Aeromonas salmonicida</i> ATCC 27013 ^T (X74680) | <i>Aeromonas bestiarum</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17658 | AY178562 | 100% <i>Aeromonas media</i> ATCC 33907 ^T (X74679) | <i>Aeromonas sobria</i> / <i>A. caviae</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17665 | AY178563 | 99.6% <i>Serratia ficaria</i> DSM 4569 ^T (AJ233428) | <i>Serratia liquefaciens</i> / <i>S. grimesii</i> (BG) | <i>Serratia</i> sp. |
| | R-17669 | AY178565 | 98.3% <i>Delftia acidovorans</i> LMG 1226 ^T (AF078774) | <i>Delftia acidovorans</i> (PA) | <i>Delftia acidovorans</i> |
| | R-17670 | AY178566 | 99.6% <i>Achromobacter piechaudii</i> ATCC 43552 ^T (AB010841) | <i>Achromobacter</i> sp. (PA) | <i>Achromobacter</i> sp. |
| | R-17671 | AY178567 | 99.0% <i>Achromobacter ruhlandii</i> ATCC 15749 ^T (AB010840) | <i>Achromobacter</i> sp. (PA) | <i>Achromobacter</i> sp. |
| | R-17682 | AY178569 | 100% <i>Aeromonas allosaccharophila</i> CECT 4199 ^T (S39232) | <i>Aeromonas sobria</i> (FA) | <i>Aeromonas</i> sp. |
| | R-17686 | AY178570 | 100% <i>Ochrobactrum tritici</i> SCII24 ^T (AJ242584) | <i>Ochrobactrum anthropi</i> (FA) ^c | <i>Ochrobactrum tritici</i> |
| | R-17688 | AY178571 | 99.8% <i>Brachymonas denitrificans</i> AS-P1 ^T (D14320) | ND | <i>Brachymonas denitrificans</i> |
| | R-17693 | AY178573 | 99.6% <i>Ochrobactrum anthropi</i> IAM 14119 ^T (D12794) | <i>Ochrobactrum anthropi</i> (FA) | <i>Ochrobactrum anthropi</i> |

^a Highest sequence similarity observed with the 16S rDNA sequence of a type strain in the EMBL sequence database.

^b Abbreviations for the phenotypic identification techniques: FA fatty acid analysis, PA whole-cell protein analysis, BG carbon source utilisation patterning using BIOLOG GN2 plates. For the identifications based on fatty acid analysis, two possibilities were given when the identification score (as calculated with the MIDI software) from the best matching profile in the database differed less than 0.15 from the second best matching profile. ND not determined.

^c Low identification score; *Ochrobactrum tritici* was not included in the MIDI database for aerobic bacteria.

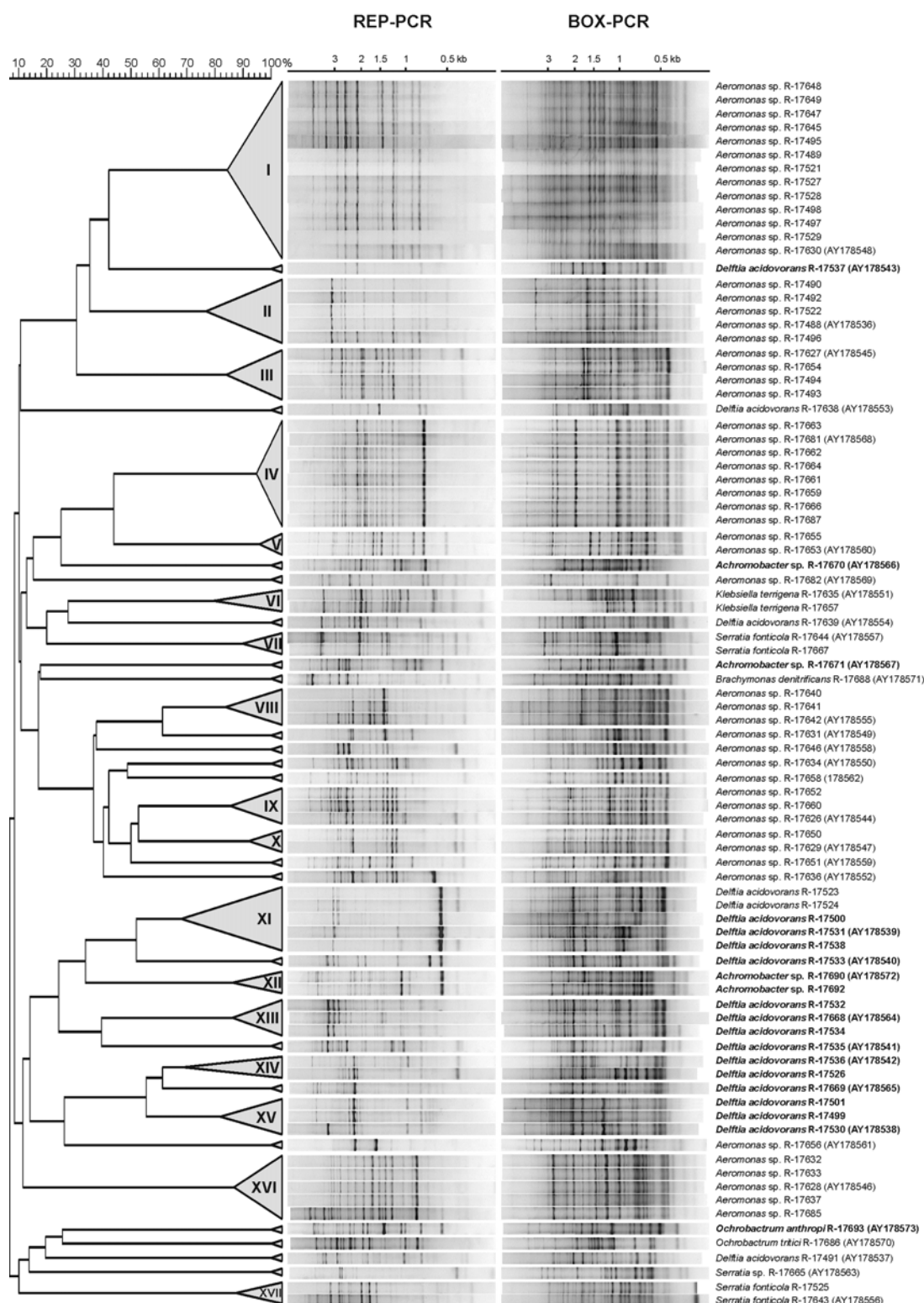


FIG. 8.1: Clustering based on linearly combined REP- and BOX-PCR profiles of pC1gfp-harbouring transconjugants from activated sludge. Transconjugants from conjugations in liquid activated sludge are printed in bold face, whereas those obtained from plate matings are presented in normal type. GenBank accession numbers of the 16S rDNA sequences are given between brackets.

TABLE 8.3: Summarised consensus identification results from indigenous activated sludge bacteria that acquired plasmid pC1gfp.

| Identification | Tot. # | Rep-types (# of isolates) |
|--|--------|--|
| Conjugations on LB agar plates: | | |
| <i>Aeromonas</i> sp. | 53 | I (13), II (5), III (4), IV (8), V (2), VIII (3), IX (3), X (2), XVI (5), R-17636, R-17646, R-17651, R-17656, R-17682, R-17631, R-17658, R-17634 |
| <i>Brachymonas denitrificans</i> | 1 | R-17688 |
| <i>Delftia acidovorans</i> | 5 | XI (2), R-17491, R-17638, R-17639 |
| <i>Klebsiella</i> sp. | 2 | VI (2) |
| <i>Ochrobactrum tritici</i> | 1 | R-17686 |
| <i>Serratia fonticola</i> | 4 | VII (2), XVII (2) |
| <i>Serratia</i> sp. | 1 | R-17665 |
| Conjugations in liquid activated sludge: | | |
| <i>Achromobacter</i> sp. | 4 | XII (2), R-17670, R-17671 |
| <i>Delftia acidovorans</i> | 15 | XI (3), XIII (3), XIV (2), XV (3), R-17537, R-17533, R-17535, R-17669 |
| <i>Ochrobactrum anthropi</i> | 1 | R-17693 |

8.1.4.4 3-CA degradation by pC1gfp transconjugants

Eighteen transconjugants identified as *Delftia acidovorans* and one *Achromobacter* sp. (R-17671) completely degraded all available 3-CA. No other aromatic products could be detected in the supernatant when grown in liquid MMN with 3-CA and sodium pyruvate. For the other 68 pC1gfp-containing transconjugants from activated sludge, only partial 3-CA degradation was observed. Although the 3-CA concentration significantly decreased during growth of these isolates, a brown precipitate was formed and peaks from other aromatic compounds appeared in the HPLC chromatogram.

The collection strains *R. radiobacter* LMG 221 and *B. natatoria* LMG 17322^T also acquired the capacity for partial 3-CA degradation upon transfer of pC1gfp. *E. coli* DH5 α (pC1gfp) however did not show any 3-CA degradation.

Fig. 8.2 shows 3-CA degradation in function of time for the transconjugants *D. acidovorans* R-17530 and R-17531 in comparison with the plasmid donor strain *P. putida* UWC3 (pC1gfp) and the natural host of plasmid pC1, *D. acidovorans* CA28. It can be seen that, although the donor strain itself does not show any degradation, the two indigenous activated sludge transconjugants degrade 3-CA at a rate only slightly lower than the original host of plasmid pC1.

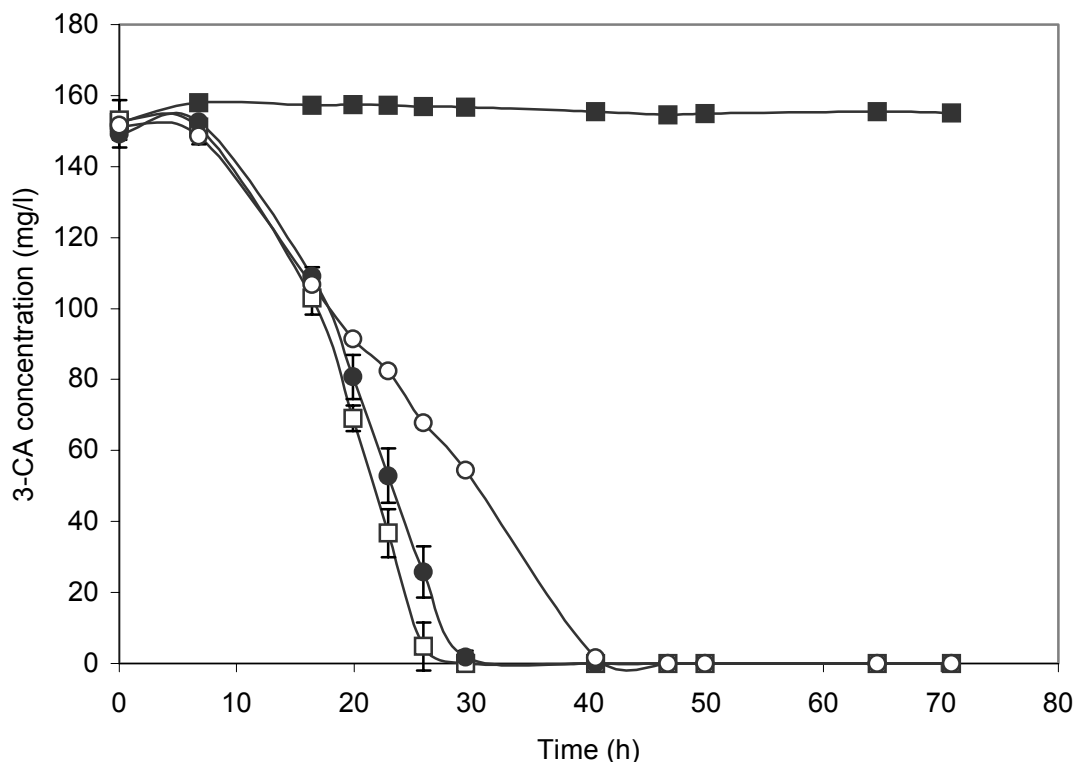


FIG. 8.2: 3-CA degradation capacities of pC1gfp-harboured transconjugants *Delftia acidovorans* R-17530 (○) and R-17531 (●), in comparison with *D. acidovorans* CA28 (□) and *Pseudomonas putida* UWC3 (pC1gfp) (■). Experiments were performed in duplicate in 250 ml Erlenmeyer flasks with 100 ml MMN supplemented with 1 g/l sodium succinate and 150 mg/l 3-CA. Error bars represent standard deviations.

8.1.5 Discussion

In order to follow the fate of the catabolic plasmid pC1, the plasmid was tagged with a mini-Tn5 transposon containing the gene encoding GFP. Insertion of the mini-Tn5 vector can cause inactivation of essential plasmid genes, especially since Tn5 preferably integrates in actively transcribing DNA regions [22]. The labelled pC1 plasmid (designated pC1gfp) was stably maintained in *R. eutropha* JMP228n and *P. putida* UWC3 and could be transferred from these hosts. Analogous to the observation made for the unlabelled pC1 plasmid [3], pC1gfp was shown in the present study to confer on *R. eutropha* JMP228n the ability to use 3-CA as sole nitrogen source. Consequently, no evidence was found for insertional inactivation of plasmid genes involved in replication, transfer or oxidative deamination of 3-CA.

Transfer of plasmid pC1gfp to members of the bacterial community present in activated sludge from a municipal wastewater treatment plant was investigated through isolation and subsequent characterisation of indigenous pC1gfp-harboured transconjugants. Isolates were considered to be true transconjugants if they (i) were able to grow on the isolation medium (MMN + sodium pyruvate + 3-CA + kanamycin), (ii) showed green fluorescence under UV-light and gave an amplicon of the correct size with the *gfp*-specific PCR, and (iii) showed partial or complete 3-CA degradation as

revealed by HPLC analysis. Additionally, transconjugants were also shown to contain a plasmid of approx. 100 kb, corresponding to the size of plasmid pC1gfp. Most probably this approach resulted in an underestimation of the diversity of transconjugants formed, since evidently transconjugants had to be cultivable in order to be detected. It is estimated that 1-15% of the bacteria in activated sludge are cultivable, a relatively large fraction compared to other ecosystems as soil or seawater [1]. Furthermore, transconjugants that did not efficiently express the genes for oxidative deamination of 3-CA would have been refractory to detection. Examples of such organisms are *E. coli* DH5 α (pC1gfp) and *P. putida* UWC3 (pC1gfp), transconjugants obtained in plate matings with pure bacterial cultures. These two strains obviously contained the pC1gfp plasmid, but did not show any 3-CA degradation. Despite the above-mentioned shortcomings, isolation of the formed transconjugants has the important advantage that the transconjugants are available for further analyses, e.g. for genotyping. Fingerprinting of a pure bacterial culture with a genomic typing technique has a much higher level of discrimination compared to in situ hybridisation with subclass- or even species-specific fluorescent probes. Using repetitive element PCR, we showed for example that the large number of isolates, identified as *Aeromonas* sp. (Table 8.2 and 8.3), was not due to repeated re-isolation of a single organism, but instead comprised at least 17 genotypically distinct organisms (Fig. 8.1). The obtained finer level of discrimination therefore allowed us to provide evidence for much more plasmid transfer events than would be feasible with an in situ technique. This is important, since plasmid transfer is believed to occur at a low frequency, in favourable conditions followed by the outgrowth of the transconjugants [4]. In total 38 distinct rep-types were distinguished within the transconjugant collection. A comparable high genotypic diversity was previously observed for pJP4 or pEMT1-containing transconjugants obtained from plasmid transfer experiments in soil [13, 23, 24]. Furthermore, from the 38 different rep-types discriminated, only rep-type XI contained transconjugants from plate matings as well as from conjugations in liquid activated sludge (Fig. 8.1 and Table 8.3). This observation suggests that conjugations on LB plates yield different taxonomic groups of transconjugants compared to conjugations in liquid activated sludge.

As shown in Table 8.3, the transconjugant collection obtained from plate matings contained mainly γ -Proteobacteria (*Aeromonas* sp., *Klebsiella* sp., *Serratia fonticola*, and *Serratia* sp.). Additionally, a few β -Proteobacteria (*Delftia acidovorans* and *Brachymonas denitrificans*), and one α -proteobacterium (*Ochrobactrum tritici*) were recovered. In contrast to the plate mating experiment, no γ -Proteobacteria were isolated from conjugations in liquid activated sludge. The latter transconjugant collection (Table 8.3) consisted solely of β -Proteobacteria (*Delftia acidovorans* and *Achromobacter* sp.) and one α -proteobacterium (*Ochrobactrum anthropi*). Previously, it has been demonstrated that a dramatic increase in γ -Proteobacteria occurred when activated sludge was plated on LB agar plates [12, 37]. The observed differences between our transconjugant collections, obtained respectively from LB plate matings and conjugations in liquid activated sludge, could therefore be explained by a selective enrichment on the LB agar conjugation plates of transconjugants belonging to the γ -Proteobacteria.

Geisenberger and co-workers previously studied the transfer of a GFP-tagged derivative of the IncP-1 α plasmid RP4 in liquid activated sludge from a municipal

wastewater treatment plant [12]. Using rRNA-targeted oligonucleotide probes, they found that more than 95% of the in situ transconjugants were γ -*Proteobacteria*, of which the majority belonged to the genus *Aeromonas* [12]. As mentioned above, no γ -*Proteobacteria* were isolated from our conjugations in liquid activated sludge and the majority of the pC1gfp-containing transconjugants from this experiment was identified as *Delftia acidovorans* (β -*Proteobacteria*). This apparent discrepancy could possibly be explained by differences in host range between the two plasmids. However, this hypothesis is not well supported by the data from plate matings with pure cultures. Indeed, plasmid pC1gfp (this study) as well as plasmid RP4 [12] could be transferred to collection strains belonging to the α -, β -, or γ -*Proteobacteria*. More probably perhaps, the observed differences could be attributed to the selection we applied for the isolation of transconjugants (growth on 3-CA as sole source of nitrogen). Since some γ -*Proteobacteria* (e.g. *E. coli* DH5 α and *P. putida* UWC3) apparently do not efficiently express plasmid pC1gfp, a considerable number of γ -*Proteobacteria* transconjugants may have escaped detection in our experiments. Previously, it was observed that the host range of the IncP-1 β plasmids pJP4 and pEMT3 and the non-IncP-1 plasmid pEMT1 is also much wider than the expression range of the 2,4-dichlorophenoxyacetic acid catabolic genes encoded on these plasmids [11, 31].

The 3-CA degrading capacity of the transconjugants isolated in this study was presumed to result from the acquisition of the pC1gfp plasmid. On theoretical bases, it can however not be excluded that these organisms were able to degrade 3-CA even before they received the pC1gfp plasmid. Nevertheless, the large number of 3-CA degrading isolates found in comparison with earlier experiments with the same activated sludge [2], strongly suggests an involvement of pC1gfp plasmid transfer. Boon and co-workers showed earlier that plasmid pC1 encodes the oxidative deamination of 3-CA, converting 3-CA to 4-chlorocatechol [3]. When grown on MMN medium with 3-CA as sole N-source, *R. eutropha* JMP228gfp (pC1) produced a brown precipitate, caused by polymerisation products of chlorocatechol. HPLC analysis subsequently revealed the formation of an aromatic intermediate, which was identified as 4-chloroaniline [3]. Since similar observations were made for the majority of the pC1gfp-containing transconjugants in this study, it seems very likely that these isolates also degraded 3-CA to 4-chlorocatechol. These organisms probably lacked the modified *ortho* cleavage pathway genes that convert 4-chlorocatechol to citric acid cycle intermediates [35], resulting in 4-chlorocatechol accumulation in the medium. From the 87 “true” transconjugants isolated, 18 *D. acidovorans* isolates and one *Achromobacter* sp. were able to degrade 3-CA completely. Two of these transconjugants (*D. acidovorans* R-17530 and R-17531) were tested in Erlenmeyer flasks and degraded 3-CA at a slightly lower rate than *D. acidovorans* CA28, the natural host of plasmid pC1 (Fig. 8.2). Furthermore, *D. acidovorans* R-17531 degraded 3-CA approximately at the same rate when supplemented with acetate, pyruvate or succinate as additional C-source and was even able to degrade 3-CA as sole source of carbon and nitrogen (data not shown). The above-mentioned results indicate a potential of plasmid pC1gfp to efficiently complement an existing modified *ortho* cleavage pathway in indigenous activated sludge bacteria, resulting in a rapid and complete 3-CA degradation. However, it would be premature to predict bioaugmentation success from the data presented above. In order to attain a sufficiently high degradation capacity, transconjugants should be able

to grow out and become numerically dominant members of the microbial community [33]. Such outgrowth will mainly depend on the selective advantage conferred by the catabolic plasmid. Since the competitive fitness of the transconjugants was not assessed here, more direct evidence for a successful bioaugmentation by transfer of plasmid pC1gfp should be demonstrated in experiments with lab-scale activated sludge reactors.

In conclusion, this study clearly demonstrated transfer of plasmid pC1gfp to indigenous activated sludge bacteria. High genotypic diversity within the transconjugant collection provided evidence for the occurrence of multiple plasmid transfer events. Insight was gained in the potential hosts for plasmid pC1gfp present in activated sludge and it was shown that the transconjugant population found in plasmid transfer studies can strongly depend upon the conjugation conditions. Further work will be required to assess the bioaugmentation potential of plasmid pC1gfp in lab-scale activated sludge reactors.

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CHAPTER 9
GENERAL CONCLUSIONS AND FUTURE
PERSPECTIVES

“Silence is golden when you can't think of a good answer”

Muhammed Ali

CHAPTER 9 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

9.1 INTRODUCTION

In this study, several taxonomical techniques were applied for the identification and classification of organisms that have a potential application in bioremediation. More specifically, the taxonomy of organisms resistant to toxic metals and degraders of chlorinated aromatic compounds was investigated and the diversity of transconjugants harbouring catabolic plasmids studied.

The major accomplishments of this work are:

1. Comparison and validation of the microplate DNA-DNA hybridisation technique and introduction of the latter technique and a method for determination of %G+C in the laboratory (Chapters 3 and 4).
2. Contributions to the taxonomy of *Ralstonia* and *Burkholderia* (Chapters 5, 6, and 7).
3. Improvement of scientific communication by formal classification of well-known biotechnologically important organisms (Chapters 5 and 6).
4. Contributions to our understanding of transfer and host range of catabolic plasmids (Chapters 7 and 8).

In next paragraphs, some general aspects of this work and perspectives for future studies will be discussed.

9.2 BACTERIAL SYSTEMATICS AND DNA-DNA HYBRIDISATIONS

The “biological” species concept, as it is used in classification systems of higher eukaryotes, cannot be applied in bacterial taxonomy. Instead, the bacterial species is pragmatically defined, based on DNA-DNA hybridisation data. An intrinsic disadvantage of DNA-DNA hybridisations is the comparative nature of the obtained data. Hence, when a new organism is added to a well-defined group, laborious cross-hybridisations have to be performed to allocate its position within this group. In contrast to e.g. sequence information, a central database cannot be constructed. Another disadvantage is that hybridisation protocols are often rather labour-intensive. Compared to older methods, such as the initial renaturation method, the microplate method of Ezaki et al. [6] is certainly an improvement regarding the higher throughput and the more modest quantities of DNA required, with the latter quality especially being important for the study of fastidious and slow-growing organisms. Its good correlation with the initial renaturation method allows comparison of older data with newly obtained values. Nevertheless, the microplate method still has its drawbacks. For example, it is more expensive compared to “traditional” protocols and automation of the method is not easily implemented. The major weakness of the method, however, is the fixation of DNA in the microwells. This problem is not unlike the “DNA-leaching” encountered in older protocols for filter hybridisations. In our experience, covalent DNA fixation did not appear to solve the problem, as previously suggested by Adnan et al. [1]. The efficiency of non-covalent DNA fixation is optimal with highly purified, high molecular weight DNA, but this is not easily obtained for all groups of bacteria. Indeed, for several genera wherein DNA-DNA hybridisations were performed with the microplate method (see literature cited in paragraph 3.4.3 for examples), DNA isolation procedures had to be adapted to yield DNA of a sufficient quality. The applied DNA preparation protocols differed in the procedure used for cell lysis (additional enzymatic treatments, mechanical means such as bead-beating), protection from endogenous DNase activity (EDTA concentrations), prevention of cell lysis in initial steps (buffering of the growth medium, salt concentration in the buffer), etc. Thus far, no “universal” DNA isolation procedure, allowing the preparation of sufficiently high quality DNA from all bacteria, has been described yet.

Some recent technological advances could prove useful in the field of whole-genome DNA-DNA hybridisations. For the further development of microplate methods, the design and evaluation of specially treated surfaces to optimise DNA immobilisation in the microwells could be valuable [3]. An alternative protocol that could be envisioned, would be a modern variant of the initial renaturation method, elaborated on the principles set out by Xu et al. [12]. In this method, hybridisations would be performed in capillary tubes in a Light Cycler real-time PCR apparatus. DNA renaturation could then be followed in function of time from the increase in SYBR Green fluorescence. A completely different approach of whole-genome DNA hybridisations could be provided by DNA micro-arrays [2]. In this method, randomly selected genome fragments of reference strains were spotted on a micro-array and hybridised with total genomic DNA of test strains. The obtained hybridisation profiles were useful for identification as well as for determination of genetic distances. Since a

micro-array can contain thousands of spots, it has the potential for a broad identification capacity. The technique could allow the construction of open database of hybridisation profiles and thereby circumvent the need for cross-hybridisations [2].

As many bacterial taxonomists are currently thinking about a new species concept, DNA-DNA hybridisation data as delineators for bacterial species will probably be replaced eventually with another set of descriptive parameters. However, such changes would take their time to be evaluated and generally accepted, so it can be foreseen that DNA-DNA hybridisation data will still remain important in bacterial taxonomy for the years to come.

9.3 THE GENERA *RALSTONIA* AND *BURKHOLDERIA*

The bacterial genera *Ralstonia* and *Burkholderia* have several features in common. These genera constitute two phylogenetically well-defined groups within the β -*Proteobacteria*, containing functionally and ecologically very diverse organisms. Both genera have clinical importance, as they comprise primary and/or opportunistic pathogens. At the same time, some of their representatives could have biotechnological values e.g. for bioremediation. Representatives are often found environmentally, in ecosystems such as soil and water. From an economical perspective, the plant-pathogenic species within these two genera are of primary importance. Perhaps the observed functional diversity within the genera *Ralstonia* and *Burkholderia* could partly be explained by genome plasticity. Indeed, genomes of representatives of both genera were shown to consist of multiple large replicons and contain several insertion sequences.

In transfer experiments in soil, representatives of the genera *Ralstonia* and *Burkholderia* were often found to act as recipients for broad-host range catabolic plasmids (e.g. [8, 9, 11], this study). Although this observation could be partially due to the limited expression range of the employed plasmids, it could also indicate an “eagerness” of these organisms to take up new genetic material that allows them to colonise novel ecological niches. The species *Ralstonia campinensis* is a notable example from the present study, which allows some speculation about the effect of plasmid acquisition on the ecological fitness of an organism. The name *R. campinensis* was proposed for a group of isolates from metal-polluted environments, showing plasmid-encoded resistance against toxic metals. Some of these strains (e.g. DS185) represented the dominant cultivable population in these habitats [5]. Later on, *R. campinensis* strains from agricultural soil were found to act as recipient for the catabolic plasmids pJP4 and pEMT1. It was shown using PCR-DGGE that this organism, upon acquisition of the catabolic plasmid, grew out to become a dominant member of the bacterial community in a 2,4-D contaminated soil microcosm [4]. Therefore, we could speculate that plasmid acquisition is a crucial factor that governs ecological success of this organism in two very different environments. Determination of the metal resistance and 2,4-D degrading capacities of available *R. campinensis* strains (and plasmid-cured derivatives) in a future study could give evidence for the latter hypothesis.

During recent years, considerable progress has been made in the elucidation of the taxonomy of *Ralstonia* and *Burkholderia*, with numerous new species described within these genera. It can be anticipated, however, that the whole diversity is not yet completely revealed. Especially the very recent discovery that representatives of the genera can live in symbiotic relationships with plants and animals, could trigger the search for additional *Ralstonia* and *Burkholderia* symbionts living in close association with other host plants and animals. This could lead to a more complete picture of the diversity of these intriguing organisms.

Bacterial genomes that have been completely sequenced and analysed so far, revealed the occurrence of horizontal gene transfer at a higher frequency than previously anticipated. As deduced from differences in average nucleotide composition,

gene transfer does not only occur between strains of the same species, but also between more distantly related organisms. Therefore, bacterial species should probably not be regarded as “biological” species i.e. as genetically isolated groups of organisms. Instead, the frequency of recombination seems to diminish continuously with genetic distance, without discontinuities that can be used in species identification [10]. Own results indicate that, with DNA-DNA hybridisations performed on a large number of phylogenetically related strains (as for *Ralstonia* and *Burkholderia*), more and more strains are found showing intermediate reassociation values (40-60%). The broad host range IncP-1 plasmids used in the present study (Chapters 7 and 8) obviously cross taxonomic boundaries at the species and even genus level, but can probably be lost as quickly as they were acquired. More important perhaps, is the frequency at which newly acquired genes become “stably” integrated in the host chromosome. The likelihood that newly acquired genes “fit” in the existing complex genetic and physiological background of the recipient cell and confer a selective advantage, is probably several orders of magnitude lower than the likelihood for the mechanism of gene transfer to occur.

Several whole-genome sequences of *Ralstonia* and *Burkholderia* species are currently available and several more are in progress or planned for the near future. Undoubtedly, analysis of these sequences will provide new insights in the genetic differences that determine the success of closely related organisms in a given ecological niche. For example, detailed analysis of the draft genome sequence of *R. metallidurans* CH34 indicated that this organism is highly specialised to metal-contaminated environments [7]. Moreover, the availability of a substantial set of genome sequences from closely related organisms could possibly allow taxonomists to determine what a bacterial species or genus means in terms of genome-wide sequence differences. Almost certainly, such analyses will shed a new light on the complex process of bacterial evolution.

9.4 TRANSFER OF CATABOLIC PLASMIDS

Horizontal gene transfer is a major driving force in bacterial evolution. The best-studied case of horizontal gene transfer is the spread of antibiotic resistance genes as a result of the widespread antibiotics use. However, the mechanism played an equally important role in the evolution of degradation pathways for xenobiotics. These pathways are believed to be evolved from enzyme systems attacking naturally occurring substances. Through “gene shuffling” by mobile genetic elements (such as plasmids, transposons, and insertion elements), hybrid pathways arose and degradation capacities were expanded.

Inoculation of an ecosystem with a donor strain, harbouring catabolic genes located on a mobile genetic element, could result in the spread of these catabolic genes to the indigenous microbial population and in accelerated pollutant degradation. The dissemination of catabolic mobile elements (especially plasmids) has therefore been studied by several groups for evaluation as a possible bioaugmentation tool. Effects on the degradation capacity vary from undetectable to very significant. In our own transfer experiments with plasmids pJP4 and pEMT1, a clear improvement of the degradation capacity was observed for B-horizon soil, suggesting successful bioaugmentation in soils with low metabolic activity. Furthermore, results reveal that the host range of the plasmids used in dissemination studies is often much wider than the expression range of the catabolic genes located on these plasmids. Using genomic typing, a large diversity was revealed within a collection of isolated transconjugants, indicating transfer of the catabolic plasmid to many distinct indigenous organisms. However, a pronounced effect on the degradation capacities can only be expected when transconjugants grow out and become a numerically dominant member of the community. This outgrowth is mainly governed by the selective advantage that the catabolic plasmid confers on the transconjugants. For example, when degradation pathways are incomplete and do not result in metabolites of the central metabolic routes (e.g. Krebs cycle), intermediates that are possibly even more toxic than the original compound, can accumulate in the cell. It can therefore be expected that such organisms will not become predominant. In practice, however, situations can arise when substrate (xenobiotic) concentrations are too low to maintain a selective advantage even for transconjugants that perform a complete degradation.

In conclusion, bioaugmentation by plasmid dissemination could provide a feasible strategy in ecosystems where the degradative capacity is absent or very low. More research is needed to better understand the conditions that stimulate gene transfer and to reveal which factors determine the competitive fitness of transconjugants.

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SUMMARY

In bacterial systematics, a whole array of phenotypic and genotypic methods is available, allowing the classification of bacteria in different hierarchical levels. Bacterial species, the basic units of taxonomy, are delineated based on whole-genome DNA-DNA hybridisation data. Despite their obvious importance, DNA reassociation experiments are not very popular among microbial taxonomists. This is mainly due to the laborious nature of the traditionally used protocols and their requirement for large amounts of purified DNA. More than a decade ago, the first protocols for DNA-DNA hybridisations in 96-well microplate format were described. Smaller sample volumes and higher throughput were the result of the adoption of this format. A few of the microplate hybridisation methods, and especially the method of Ezaki et al. [Ezaki, T., Hashimoto, Y., Yabuuchi, E. 1989 IJSB vol 39, p. 224-229], became relatively popular. However, the data generated with these newer methods were not yet compared with older data on a statistically sound base. This is important, especially since it was previously shown that different hybridisation methods may yield different DNA reassociation values. Therefore, we compared a large set of reassociation values obtained with the method of Ezaki et al. with previously reported values obtained with the more established initial renaturation rate method. Strains were selected to represent different phylogenetic lineages (Gram positives low %G+C, *Flavobacteria*, and *Proteobacteria*), with DNA base compositions ranging from 34 to 65 %. Both methods were shown to be in very good correlation. The reproducibility of the microplate and initial renaturation rate method were calculated to be 7 and 5%, respectively. DNA fragmentation and impurities in the DNA preparation negatively affect the efficiency of DNA adsorption to the microwells. Unreliable reassociation values caused by reduced DNA fixation can easily be identified by careful examination of the raw fluorescence values or by discrepancies between reciprocal reactions. It was concluded that the microplate DNA-DNA hybridisation method is a reliable taxonomic tool. The method has subsequently been applied in the here-presented study and in several other taxonomic studies conducted in the Laboratory of Microbiology Gent.

Concomitant with the introduction of the microplate DNA-DNA hybridisation method in the Laboratory of Microbiology, a HPLC-based protocol for the determination of the DNA nucleotide composition (expressed as mole% G+C) was introduced. The latter protocol has proven to be a reliable and accurate method for the determination of this important descriptive parameter. The %G+C of over 1500 bacterial strains has been analysed with this method on a routinely basis.

The above-mentioned taxonomic techniques, complemented with 16S rDNA sequencing, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, cellular fatty acid analysis, repetitive-element PCR fingerprinting, and conventional phenotypic tests, were applied in the taxonomic analyses of *Ralstonia* and *Burkholderia* strains derived from bioremediation studies. The latter two genera belong to the β -*Proteobacteria* and contain environmental organisms as well as plant pathogens, opportunistic human pathogens, and (for *Burkholderia*) primary human pathogens. Good classification and identification of these

organisms is important to assure that strains for potential bioremediation applications are distinct from detrimental strains. The present study focussed on metal-resistant *Ralstonia* strains and *Burkholderia* strains, capable of degrading polychlorinated biphenyls (PCB's) and 2,4-dichlorophenoxyacetic acid (2,4-D).

Thirty-one heavy metal-resistant *Ralstonia* isolated from industrial biotopes such as metal-polluted wastewater, sediments and soils, were subjected to a polyphasic characterization. Six metal-resistant isolates were identified as *Ralstonia basilensis*. Since the latter species previously contained only the type strain, an emendation of the species description was proposed. The remaining metal-resistant isolates were separated in two clearly distinct groups of 8 and 17 isolates by whole-cell protein profiles and DNA-DNA hybridisations. Reassociation values between representatives of these groups and type strains of known *Ralstonia* species were low. Therefore, these two groups were assigned to two new species for which the names *Ralstonia campinensis* and *Ralstonia metallidurans* are proposed. The type strains are WS2 and CH34, respectively. The latter strain has been studied extensively for its metal resistance mechanisms and has several possible biotechnological applications e.g. as biosensor and in bioreactors targeting the removal of toxic metals.

Strain LB400 is the best-studied degrader of PCB's. This organism was originally identified as a species of *Pseudomonas* but has recently been allocated in the genus *Burkholderia* through 16S rDNA sequence analysis. Since the exact species affiliation of strain LB400 remained unclear, a polyphasic study was undertaken to clarify the taxonomical position of this biotechnologically important organism. Through a screening with whole-cell protein profiling and repetitive-element PCR, two strains were found that exhibited striking similarities with strain LB400. One strain was recovered from a human blood culture in Sweden and the other was isolated from the rhizosphere of a coffee plant in Mexico. All three strains reacted very similar in phenotypic tests and DNA-DNA hybridisations revealed a relationship at the species level. Furthermore, low DNA reassociation values with type strains of related *Burkholderia* species supported a classification as a novel species, for which we proposed the name *Burkholderia biphenylovorans*. The type strain is LB400. Strains of this novel species can be differentiated from other *Burkholderia* species by several phenotypic tests.

The species *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. were proposed for two groups of pJP4- or pEMT1-harbouring bacteria with distinct REP- and BOX-PCR patterns. These organisms were isolated from soil microcosms to which 2,4-D and donor strain containing a 2,4-D degradative plasmid was added (see below). As for the taxonomic studies mentioned above, 16S rDNA sequences, DNA reassociation values, whole-cell protein patterns and phenotypic characteristics were in agreement with the placement of these organisms in separate, novel *Burkholderia* species.

Horizontal gene transfer is thought to be one of the key factors in the evolution of degradative pathways for xenobiotic compounds. Since the transfer of catabolic genes located on mobile genetic elements can stimulate the degradation of a pollutant, the deliberate dissemination of a catabolic plasmid in an ecosystem could possibly be

exploited as a bioaugmentation tool. The latter hypothesis was tested with the 2,4-D degradative plasmids pEMT1 and pJP4 in soil. Furthermore, the identification and typing at the infraspecific level of transconjugants that received catabolic plasmids in such dissemination experiments, reveals information about the *in situ* transfer of these plasmids. This was studied with the above-mentioned 2,4-D catabolic plasmids and with plasmid pC1gfp, encoding the oxidative deamination of 3-chloroaniline (3-CA).

The donor strain *Pseudomonas putida* UWC3, containing the 2,4-D degradative plasmids pEMT1 or pJP4, was inoculated in separate microcosms with 2,4-D contaminated soil from two different horizons (A-horizon: 0-30 cm; B-horizon: 30-60 cm depth). 2,4-D concentration and cell counts of donors and transconjugants were followed in function of time and compared with control experiments. A total of 95 transconjugants were picked up and purified. When the soil microcosms were amended with nutrients, plasmid transfer and enhanced degradation of 2,4-D were observed. This was most striking in the B-horizon microcosms, where indigenous bacteria were unable to degrade any of the 2,4-D during at least 22 days, while inoculation with either of the two plasmid donors resulted in complete 2,4-D removal within 14 days. In A-horizon soil microcosms not amended with nutrients, 2,4-D degradation occurred at the same high rate in the non-inoculated reactors as in the inoculated reactors. In contrast, donor inoculation in non-amended B-horizon soil microcosms resulted in complete degradation of 2,4-D within 19 days, while no degradation at all was observed in non-inoculated soil during 89 days. In the case of plasmid pEMT1, this enhanced degradation seemed to be only due to transconjugants (10^5 CFU/g soil), since the donor was already undetectable when degradation started. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes showed that inoculation of the donors was followed by a shift in the microbial community structure of the non-amended B-horizon soils. The new 16S rRNA gene fragments in the DGGE-profile corresponded with the 16S rRNA genes of 2,4-D degrading transconjugant colonies isolated on agar plates, indicating that the observed change in the community was due to proliferation of *in situ* formed transconjugants. Repetitive element PCR fingerprinting revealed eight different rep-clusters and six separate isolates within the collection of 95 transconjugants isolated on agar plates. Using a polyphasic approach, these transconjugants were identified as *Ralstonia campinensis* (34 isolates), *Burkholderia hospita* (29), *Burkholderia terricola* (17), *Stenotrophomonas maltophilia* (10 isolates), *Burkholderia glathei* (2 isolates), *Burkholderia* sp. (2 isolates), and *Ralstonia basilensis* (1 isolate). Dominant bands corresponding to 16S rDNA of the three species mentioned first were observed in the DGGE profiles of B-horizon soil microcosms, suggesting predominance of these species in the soil microbial community. *S. maltophilia* transconjugants were almost exclusively isolated from nutrient-amended microcosms. The transconjugant collections isolated from A- and B-horizon soil were similar, except for *B. terricola* transconjugants, which were only recovered from the B-horizon. Similar to results from plate matings plasmids pJP4 and pEMT1 appeared to have a similar transfer and expression range in soil. Overall, this work clearly demonstrates that bioaugmentation by dissemination of a catabolic plasmid can constitute an effective strategy for clean-up of soils which are poor in nutrients and microbial activity, such as those of the B-horizon.

Plasmid pC1gfp is a labelled derivative of pC1 from *Delftia acidovorans* CA28 and encodes the oxidative deamination of 3-chloroaniline (3-CA). Its transfer was studied from the donor *P. putida* UWC3 to the bacterial community in activated sludge. Conjugation experiments were performed both on LB agar plates or directly in liquid activated sludge. Eighty-seven green fluorescent colonies appearing on mineral medium containing 3-CA as sole nitrogen source were picked up and verified to be true pC1gfp-harbouring transconjugants. REP- and BOX-PCR genomic fingerprinting revealed 38 distinct rep-types. This large diversity suggests the occurrence of multiple plasmid transfer events, as opposed to a single transfer event followed by outgrowth of the transconjugant. Remarkably, LB agar plate conjugations yielded a different set of transconjugants compared to conjugations directly in liquid activated sludge. Transconjugants were identified as *Aeromonas* sp. (53 isolates), *Delftia acidovorans* (20 isolates), *Achromobacter* sp. (4 isolates), *Serratia fonticola* (4 isolates), *Klebsiella* sp. (2 isolates), *Brachymonas denitrificans* (1 isolate), *Ochrobactrum tritici* (1 isolate), *Serratia* sp. (1 isolate), and *Ochrobactrum anthropi* (1 isolate). From the plate matings, mainly *Aeromonas* sp. were isolated, whereas *D. acidovorans* strains were predominant in the transconjugant collection from liquid activated sludge. The majority of the isolates showed 3-CA deamination, probably to 4-chlorocatechol, but several pC1gfp-harbouring transconjugants performed a rapid and complete 3-CA degradation. The latter observation, together with the high diversity of transconjugants found, suggests a potential for bioaugmentation through dissemination of this catabolic plasmid.

SAMENVATTING

In bacteriële systematiek is een breed scala aan fenotypische en genotypische technieken beschikbaar voor het classificeren van bacteriën in verschillende hiërarchische niveaus. Het bacteriële species, de basiseenheid in taxonomie, wordt gedefinieerd op basis van DNA-DNA hybridisaties van volledige bacteriële genomen. Ondanks hun overduidelijk belang, zijn DNA reassociatie experimenten niet erg populair bij microbiële taxonomen. Dit is voornamelijk te wijten aan de arbeidsintensieve aard van de traditioneel toegepaste protocols en de grote hoeveelheden zuiver DNA die hiervoor vereist zijn. Reeds een veertiental jaar geleden werden de eerste protocols voor DNA-DNA hybridisaties in een 96-well microtiterplaat beschreven. Deze schaalverkleining liet toe te werken met kleinere staalvolumes en resulteert eveneens in een snellere generatie van data. Enkele van de microtiterplaat hybridisatie methoden en in het bijzonder de methode van Ezaki en medewerkers [Ezaki, T., Hashimoto, Y., Yabuuchi, E. 1989 IJSB vol 39, p. 224-229], werden vrij populair. Niettegenstaande werden de data gegenereerd met deze nieuwere methodes nog niet op een statistisch verantwoorde wijze vergeleken met eerder bekomen data. Dit is belangrijk, vooral omdat vroeger reeds werd aangetoond dat verschillende hybridisatie methodes kunnen leiden tot verschillende DNA reassociatiewaarden. Daarom werd in deze studie een grote dataset reassociatiewaarden, bekomen met zowel de methode van Ezaki et al. als met de meer gevestigde initiële renaturatie methode, vergeleken. Een zorgvuldig gekozen selectie van stammen was erop gericht diverse fylogenetische groepen (Gram positieven laag % G+C, *Flavobacteria*, en *Proteobacteria*) met uiteenlopende DNA basensamenstellingen van 34 tot 65 % te omvatten. Een zeer goede correlatie tussen beide methoden werd aangetoond. Een reproduceerbaarheid van 7 en 5 % werd berekend voor respectievelijk de microtiterplaat - en de initiële renaturatie methode. DNA fragmentatie en onzuiverheden hebben een negatieve invloed op de bindingsefficiëntie van DNA aan de microtiter cups. Onbetrouwbare reassociatiewaarden, veroorzaakt door een minder efficiënte DNA fixatie, kunnen eenvoudig opgespoord worden door het nauwkeurig nakijken van de ruwe fluorescentiewaarden of door discrepanties in de reciproque reacties. Er werd geconcludeerd dat de microtiterplaat DNA-DNA hybridisatie methode een betrouwbaar taxonomisch instrument is. Deze methode werd vervolgens toegepast in de hier voorgestelde studie, alsook in diverse andere taxonomische studies uitgevoerd in het Laboratorium voor Microbiologie Gent.

Gelijktijdig met de introductie van de microtiterplaat DNA-DNA hybridisatie techniek in het Laboratorium voor Microbiologie, werd een HPLC-gebaseerd protocol voor de bepaling van de DNA nucleotidesamenstelling (uitgedrukt als mol% G+C) ingevoerd. Dit protocol heeft bewezen een betrouwbare en accurate bepaling toe te laten van deze belangrijke beschrijvende parameter. De DNA nucleotidesamenstelling van meer dan 1500 bacteriële stammen werd op routinematige basis met deze methode geanalyseerd.

De hierboven vermelde taxonomische technieken, aangevuld met 16S rDNA sequentiebepaling, natrium dodecyl sulfaat polyacrylamide gel elektroforese (SDS-

PAGE) van volledige cel proteïnen, analyse van cellulaire vetzuren, repetitieve-element PCR typering en conventionele fenotypische testen, werden toegepast in taxonomische studies van *Ralstonia* en *Burkholderia* stammen, afkomstig uit bioremediatie experimenten. De twee laatstgenoemde genera behoren tot de β -*Proteobacteria* en omvatten zowel omgevingsisolaten als plant pathogenen, opportunistische - en (voor *Burkholderia*) primaire humane pathogenen. Een goede classificatie en identificatie van deze organismen zijn dan ook onontbeerlijk om te verzekeren dat stammen met potentieel voor bioremediatie toepassingen duidelijk verschillen van schadelijke stammen. Deze studie is toegespitst op metaalresistente *Ralstonia* stammen en *Burkholderia* stammen, die in staat zijn om polygechloreerde bifenylen (PCB's) en 2,4-dichlorophenoxyazijnzuur (2,4-D) af te breken.

Eénendertig metaal-resistente *Ralstonia*, geïsoleerd uit industriële biotopen zoals met metalen vervuild afvalwater, sedimenten en grond, werden onderworpen aan een polyfasische karakterisering. Zes metaal-resistente isolaten konden als *Ralstonia basilensis* geïdentificeerd worden. Omdat het laatstgenoemde species enkel de typestam bevatte, werd een verbeterde beschrijving voorgesteld. De overige metaal-resistente isolaten werden aan de hand van hun eiwitprofielen en interne DNA-DNA reassociatiewaarden ingedeeld in twee goed gedefinieerde groepen van respectievelijk acht en 17 isolaten. Reassociatiewaarden tussen vertegenwoordigers van deze twee groepen en typestammen van erkende *Ralstonia* species waren laag. Daarom werden deze twee groepen toegewezen aan twee nieuwe species, waarvoor de namen *Ralstonia campinensis* en *Ralstonia metallidurans* werden voorgesteld. De typestammen zijn respectievelijk WS2 en CH34. De laatstgenoemde stam, die uitgebreid bestudeerd werd voor zijn metaal resistentiemechanismen, bezit diverse mogelijke biotechnologische toepassingen, bijvoorbeeld als biosensor en in bioreactoren met als doel het verwijderen van toxische metalen.

De stam LB400 is de best bestudeerde afbreker van PCB's. Dit organisme was oorspronkelijk geïdentificeerd als een *Pseudomonas* species, maar werd meer recent toegewezen aan het genus *Burkholderia* aan de hand van 16S rDNA sequentie analyse. Omdat de exacte species status van LB400 onduidelijk bleef, werd een polyfasische studie gestart om de taxonomische positie van dit biotechnologisch belangrijk organisme op te helderen. Een screening op basis van eiwitprofielen en repetitieve-element PCR toonde aan dat twee stammen markante gelijkenissen vertoonden met LB400. Eén stam was afkomstig van een humane bloedcultuur uit Zweden en een tweede stam werd geïsoleerd uit de rhizosfeer van een koffieplant in Mexico. Deze drie stammen reageerden zeer gelijkaardig in fenotypische testen en DNA-DNA hybridisaties bevestigden een onderlinge relatie op het speciesniveau. Bovendien ondersteunden lage DNA reassociatiewaarden met typestammen van verwante *Burkholderia* species de classificatie als een nieuw species, waarvoor de naam *Burkholderia biphenylovorans* werd voorgesteld. De typestam is LB400. Verschillende fenotypische testen laten toe dit nieuw species de differentiëren van andere *Burkholderia* species.

De species *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. werden voorgesteld voor twee groepen van pJP4- of pEMT1 bevattende bacteriën met duidelijk verschillende REP- en BOX-PCR profielen. Isolatie van deze organismen gebeurde uit bodem microcosmen waaraan zowel 2,4-D als een donor stam met een 2,4-

D afbrekend plasmide waren toegevoegd (zie hieronder). Zoals bij de eerder vermelde taxonomische studies, waren ook hier 16S rDNA sequenties, DNA reassociatiewaarden, eiwitprofielen en fenotypische karakteristieken in overeenkomst met de indeling van deze organismen in een afzonderlijk, nieuw *Burkholderia* species.

Horizontale gentransfer wordt aanzien als één van de sleutelfactoren in de evolutie van degradatie reactiewegen voor xenobiotica. Aangezien de transfer van katabolische genen, gelokaliseerd op mobiele genetische elementen, de degradatie van een pollutant kan stimuleren, kan een doelbewuste “inzaaiing” van een katabolisch plasmide in een ecosysteem mogelijk als een bioaugmentatie techniek worden gebruikt. Deze laatste hypothese werd getest met de 2,4-D afbrekende plasmiden pEMT1 en pJP4 in grond. Bovendien liet de identificatie en typering op het intraspecies niveau van transconjuganten, die in dergelijke inzaaiingsexperimenten katabolische plasmiden hadden opgenomen, toe informatie in te winnen over de *in situ* transfer van deze plasmiden. Deze transfer werd bestudeerd met de hierboven vermelde 2,4-D katabolische plasmiden en met plasmide pC1gfp, die voor de oxidatieve deaminatie van 3-chloroaniline (3-CA) codeert.

De donor stam *Pseudomonas putida* UWC3, die het 2,4-D katabolisch plasmide pEMT1 of pJP4 bevat, werd ingeënt in afzonderlijke microcosmen met 2,4-D gecontamineerde grond van twee verschillende horizons (A-horizon: 0-30 cm; B-horizon: 30-60 cm diepte). Zowel de 2,4-D concentratie als het kiemgetal van donors en transconjuganten werden gevolgd in functie van de tijd. In totaal werden 95 transconjuganten opgepikt en vergeleken met het controle experiment. Wanneer extra nutriënten aan de grond microcosmen werden toegevoegd, werd zowel plasmide transfer als een verhoogde 2,4-D degradatie vastgesteld. Dit was vooral opvallend in de B-horizon microcosmen, waar de endogene bacteriën niet in staat bleken tot enige 2,4-D degradatie gedurende minstens 22 dagen, terwijl het toevoegen van eender welk van de twee plasmide donoren resulteerde in een volledige 2,4-D afbraak binnen de 14 dagen. In de A-horizon grond microcosmen die niet werden aangerijkt met nutriënten, gebeurde de afbraak van 2,4-D met dezelfde snelheid zowel in de niet-geïnoculeerde reactoren als in de geïnoculeerde reactoren. In de B-horizon daarentegen, resulteerde inoculatie van de donor in niet aangerijkte grond microcosmen in de volledige afbraak van 2,4-D binnen de 19 dagen, terwijl totaal geen afbraak werd vastgesteld in niet-geïnoculeerde bodem gedurende 89 dagen.

In het geval van het pEMT1 plasmide, bleek deze verhoogde degradatie enkel te wijten aan de ontstane transconjuganten (10^5 CFU/g bodem), aangezien de donor reeds ondetecteerbaar was wanneer de degradatie startte. “Denaturing Gradient Gel Electrophoresis” (DGGE) van 16S rRNA genen toonde aan dat inoculatie van de donors resulteerde in een verschuiving in de microbiële gemeenschapsstructuur van de niet aangerijkte B-horizon grond. De nieuw verschenen 16S rRNA genfragmenten in het DGGE-profiel kwamen overeen met de 16S rRNA genen van 2,4-D afbrekende transconjugante kolonies, geïsoleerd op agarplaten. Dit geeft aan dat de geobserveerde verschillen in de gemeenschap te wijten waren aan de proliferatie van *in situ* gegenereerde transconjuganten. Screening aan de hand van rep-PCR fijntypering van de

collectie van 95 transconjuganten geïsoleerd op agarplaten liet toe acht verschillende rep-clusters en zes afzonderlijke isolaten te onderscheiden. Door gebruik te maken van een polyfasische aanpak konden deze transconjuganten geïdentificeerd worden als *Ralstonia campinensis* (34 isolaten), *Burkholderia hospita* (29 isolaten), *Burkholderia terricola* (17 isolaten), *Stenotrophomonas maltophilia* (10 isolaten), *Burkholderia glathei* (2 isolaten), *Burkholderia* sp. (2 isolaten), en *Ralstonia basilensis* (1 isolaat). De dominante banden in de DGGE-profielen van de B-horizon grond microcosmen bleken te corresponderen met de 16S rDNA sequenties van de drie eerst vermelde species. Deze bevindingen suggereren de predominantie van deze species in de microbiële gemeenschap van de betreffende bodem. De *S. maltophilia* transconjuganten werden bijna uitsluitend geïsoleerd uit met nutriënten aangerijkte microcosmen. De transconjugant collecties geïsoleerd uit A- en B-horizon bodem waren grotendeels gelijkaardig, met uitzondering van *B. terricola* transconjuganten, die enkel in de B-horizon werden aangetroffen. Gelijkaardige resultaten betreffende de transfer- en de expressiefrequentie werden bekomen voor de conjugaties op agarplaten met plasmiden pJP4 en pEMT1 en voor grond. Samenvattend kan gesteld worden dat dit werk duidelijk aantoont dat bioaugmentatie door het “inzaaien” van katabolische plasmiden een duidelijke bijdrage kan leveren aan strategieën voor de sanering van bodems die arm zijn aan nutriënten en microbiologische activiteit, zoals deze van de B-horizon.

Het pC1gfp plasmide is een gemerkte variant van pC1 uit *Delftia acidovorans* CA28 en codeert voor de oxidatieve deaminatie van 3-chloroaniline (3-CA). De transfer van dit plasmide vanuit de donor *P. putida* UWC3 naar actief slib werd nader onderzocht. Conjugatie experimenten werden zowel op LB agarplaten als direct in slib uitgevoerd. Zevenentachtig groen fluorescerende kolonies, die verschenen op mineraal medium met 3-CA als enige stikstofbron werden opgepikt en geverifieerd als zijnde echte pC1gfp bevattende transconjuganten. Aan de hand van REP- en BOX-PCR genomische typering konden 38 verschillende rep-types worden onderscheiden. Deze grote diversiteit suggereert het voorkomen van meerdere plasmid transfers, eerder dan een enkelvoudige transfer gevolgd door proliferatie van de transconjugant. Opvallend was dat de conjugaties op LB agarplaten een andere set transconjuganten opleverde dan conjugaties rechtstreeks in actief slib. De bekomen transconjuganten uit actief slib konden geïdentificeerd worden als *Aeromonas* sp. (53 isolaten), *Delftia acidovorans* (20 isolaten), *Achromobacter* sp. (4 isolaten), *Serratia fonticola* (4 isolaten), *Klebsiella* sp. (2 isolaten), *Brachymonas denitrificans* (1 isolaat), *Ochrobactrum tritici* (1 isolaat), *Serratia* sp. (1 isolaat), and *Ochrobactrum anthropi* (1 isolaat). Uit de conjugaties op LB agarplaten werden voornamelijk *Aeromonas* sp. geïsoleerd, terwijl *D. acidovorans* predominant waren in de transconjuganten collectie uit actief slib. Het merendeel van de isolaten vertoonde deaminatie van 3-CA, waarschijnlijk tot 4-chlorocatechol, en verschillende pC1gfp-bevattende transconjuganten vertoonden een snelle en volledige 3-CA degradatie. Deze laatste observatie, samen met de hoge diversiteit van de bekomen transconjuganten, suggereert potentieel voor bioaugmentatie via inzaaiing van dit katabolisch plasmide.

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Graduate thesis:
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Assistance with the practical courses Microbiology for 3rd year students Biology, Biotechnology & Biochemistry.

Supervision of graduate theses:

- 2001-2002: Thierry Janssens – Transfer- and catabolic properties of the 3-chloroaniline-degrading plasmid pC1::*gfp*.
- 2000-2001: Christine Coucke – A study of the host range of the 2,4-D catabolic plasmid pEMT1::*lacZ* through conjugations on agar surfaces.
- 1999-2000: Ben Geeraerts – Genotypic characterisation of 2,4-dichlorophenoxy acetic acid (2,4-D) degrading transconjugants.
- 1998-1999: Elke De Clerck – Evaluation of the microplate DNA:DNA hybridisation method for identification of *Comamonas* and *Ralstonia* species.
- 1995-1996: Christophe Callewaert – Whey permeate as a model substrate for bacterial hydrogen gas production through a two-step fermentation.

Supervision of a trainee technician:

- 1998-1999: Karen Dierickx – Tracing of genotypic relationships between aerobic endospore formers with microplate DNA-DNA hybridisations.

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