# Characterization of Actinobacteria Degrading and Tolerating Organic Pollutants

Irina Tsitko

Division of Microbiology
Department of Applied Chemistry and Microbiology
Faculty of Agriculture and Forestry
University of Helsinki

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium 1015 at Viikki Biocentre, Viikinkaari 5, on January 12th, 2007, at 12 o'clock noon.

Helsinki 2007

Supervisor: Professor Mirja Salkinoja-Salonen
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Reviewers Doctor Anja Veijanen
Department of Biological and Environmental Science
University of Jyväskylä
Jyväskylä, Finland

Docent Merja Kontro
University of Helsinki
Department of Ecological and Environmental Sciences
Lahti, Finland

Opponent: Professor Edward R.B. Moore,
Department of Clinical Bacteriology
Sahlgrenska University Hospital,
Göteborg University
Göteborg, Sweden.

ISSN 1795-7079
ISBN 978-952-10-3634-7 (paperback)
ISBN 978-952-10-3635-4 (PDF)

Yliopistopaino
Helsinki, Finland 2007

**Front cover:** SEM picture of *R. opacus* GM-29 grown in mineral medium supplemented with toluene as sole carbon and energy source.

# Content


List of original publications 5
The author's contributions 5
Abbreviations 6
Abstract 7
1. Introduction 9
1.1 Classification of corynebacteria 9
1.1.1 The genus *Rhodococcus* 9
1.1.2 The genus *Gordonia* 10
1.1.3 The genus *Mycobacterium* 10
1.2 The polyphasic approach in taxonomy 10
1.2.1 Chemotaxonomic markers of bacteria in the suborder *Corynebacterineae* 12
1.2.2 Molecular targets used in taxonomy of bacteria in the suborder *Corynebacterineae* 13
1.3 Cell envelope structure of the bacteria of the suborder *Corynebacterineae* 14
1.4 Degradation power of corynebacteria 16
1.4.1 Degradation power of the genus *Rhodococcus* 16
1.4.2 Degradation power of the genus *Gordonia* 17
1.4.3 Degradation power of the genus *Mycobacterium* 22
1.5 Pathogenic potentials of the corynebacteria 22
1.5.1 Pathogenicity of environmental mycobacteria 22
1.5.2 Pathogenicity in the genus *Gordonia* 23
1.5.3 Pathogenicity in the genus *Rhodococcus* 23
1.6 Organic solvent toxicity toward microorganisms 24
1.6.1 Effects on membrane functioning 24
1.6.2 Solvent adaptations in microorganisms: regulation of membrane fluidity 24
2. Aims of this study 26
3. Materials and methods 27
3.1 Methods used in this thesis 27
3.2 Methods other than those described in I-V 27
3.2.1 Measurement of toluene and benzene in the aqueous phase 27
3.2.2 Conditions for assaying the tolerance of *Rhodococcus* to organic solvents 28
3.2.3 Assay for growth in liquid culture by ATP content measurement 29
3.2.4 Hydrophobicities of cells and solvents 29
3.2.5 Scanning electron microscopy 29
4. Results and discussion 30
4.1 Isolation history of the actinobacteria studied in this thesis 30
4.2 Characterization of the actinobacteria selected for this study 30
4.3 Conclusions and comments on the isolation and identification of the selected actinobacteria 39
4.4 Degradation and surviving properties of selected strains of *Rhodococcus, Mycobacterium* and *Gordonia* 40
4.4.1 Degradation of aromatic compounds by strains of *R. opacus*, *G. polyisoprenivorans* and *M. murale* 40
4.4.2 Tolerance of *R. opacus* strains to water-miscible and water-immiscible organic solvents 42
4.5 Growth of *R. opacus* and *G. polyisoprenivorans* in biphasic liquids 44

4.6 Toxic compound-induced changes in whole cell fatty acids of *R. opacus* GM-14 and GM-29 47
5. Conclusions 50
6. Acknowledgements 52
7. References 53

## List of original publications

I. Zaitsev G.M., Uotila J.S, **Tsitko I.V.,** Lobanok A.G. and Salkinoja-Salonen M.S. 1995. Utilization of halogenated benzenes, phenols and benzoates by *Rhodococcus opacus* GM-14. *Applied and Environmental Microbiology*. 61: 4191-4201.

II. Andersson M., **Tsitko I.,** Vuorio R. and Salkinoja-Salonen M.S. 1999. Mycobacteria and related genera are major colonizers of a wall in a children's day care center. In: Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control. E. Johanning (ed.), Eastern New York Occupational and Environmental Health Center, Albany New York, USA. pp 396-402.

III. **Tsitko I**., Rahkila R., Priha O., Ali-Vehmas T., Terefework Z., Soini H. and Salkinoja-Salonen M.S. 2006. Isolation and automated ribotyping of *Mycobacterium lentiflavum* from drinking water distribution system and clinical specimens. *FEMS Microbiology Letters*. 256:236-243.

IV. **Tsitko I.,** Golovlev E.L., Rainey F.A., Busse H.-J., Kroppenstedt R.M., Auling G. and Salkinoja-Salonen M.S. 200x. Characterization of *Gordonia polyisoprenivorans* B293 degrading phthalic esters (manuscript).

V. **Tsitko I.V.,** Zaitsev G.M., Lobanok A.G., and Salkinoja-Salonen M.S. 1999. Effect of aromatic compounds on cellular fatty acid composition of *Rhodococcus opacus*. *Applied and Environmental Microbiology.* 65: 853-855.

## The author's contributions

I. Irina Tsitko analysed the DNA content and menaquinones, planned and investigated the ability of the strain GM-14 to grow on aromatic substrates, carried out experiments on benzene and chlorobenzene degradation, interpreted the results and wrote these parts of the article together with co-authors.

II. Irina Tsitko designed and investigated the toluene-metabolizing capacities of *Mycobacterium murale* isolates, analysed the results and wrote parts of the article.

III. Irina Tsitko designed the protocol for cell pretreatment, executed the ribotyping and analysed the data. She is the corresponding author, interpreted the results and wrote the article.

IV. Irina Tsitko planned the experimental design, investigated the xenobiotic-metabolizing capacities of *Gordonia polyisoprenivorans* B293, interpreted the results and wrote the paper.

V. Irina Tsitko is the corresponding author. She planned and executed the experimental work, except for the isolation of strain GM29 and sequencing of the 16S RNA gene, and wrote the article.

The publications are included with kind permission of the publishers.

# Abbreviations

| | |
|---|---|
| AMYE | acetat-malt-yeast extract medium |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| bp | base pair |
| CB | chlorobenzene |
| CCUG | Culture Collection of the University of Göteborg |
| cfu | colony forming units |
| CP | chlorophenol |
| DSM | Deutsche Sammlung von Mikroorganismen und Zellkulture GmbH (German Collection of Microorganisms and Cell Cultures |
| EMBL | European Molecular Biology Laboratory |
| FESEM | field emission scanning electron microscopy |
| GC | gas chromatography |
| HSP | heat-shock protein |
| LAM | lipoarabinomannan |
| Log $K_{ow}$ | logarithm of octanol/water partition coefficient |
| MS | mass spectrometry |
| NCTC | National Collection of Type Cultures |
| NTM | nontuberculous mycobacteria |
| OD | optical density |
| PAH | polycyclic aromatic hydrocarbon |
| TBSA | tuberculostearic acid |
| SEM | scanning electron microscopy |
| TLC | thin-layer chromatography |
| UPGMA | unweighted pair group method with arithmetic averages |

## Abstract

Species of the genera *Rhodococcus*, *Gordonia* and *Mycobacterium* are known as degraders of recalcitrant pollutants. These bacteria are good survivors in harsh environments. Due to such properties these organisms are able to occupy a wide range of environmental niches. The members of these taxa have been suggested as tools for biotechnical applications such as bioremediation and biosynthesis. At the same time several of the species are known as opportunistic human pathogens. Therefore, the detailed characterization of any isolate that has potential for biotechnological applications is very important.

This thesis deals with several corynebacterial strains originating from different polluted environments: soil, water-damaged indoor walls, and drinking water distribution systems. A polyphasic taxonomic approach was applied for characterization of the isolates. We found that the strains degrading monoaromatic compounds belonged to *Rhodococcus opacus*, a species that has not been associated with any health problem. The taxonomic position of strain B293, used for many years in degradation research under different names, was clarified. We assigned it to the species *Gordonia polyisoprenivorans*. This species is classified under European Biohazard grouping 1, meaning that it is not considered a health hazard for humans. However, there are reports of catheter-associated bacteraemia caused by *G. polyisoprenivorans*. Our results suggested that the ability of the organism to grow on phthalate esters, used as softeners in medical plastics, may be associated with the colonization of catheters and other devices. In this thesis *Mycobacterium lentiflavum,* a new emerging opportunistic human pathogen, was isolated from biofilms growing in public drinking water distribution systems. Our report on isolation of *M. lentiflavum* from water supplies is the second report on this species from drinking water systems, which may thus constitute a reservoir of *M. lentiflavum.* Automated riboprinting was evaluated for its applicability in rapidly identifying environmental mycobacteria. The technique was found useful in the characterization of several species of rapidly and slowly growing environmental mycobacteria.

The second aspect of this thesis refers to characterization of the degradation and tolerance power of several *R. opacus*, *M. murale* and *G. polyisoprenivorans* strains. *R. opacus* GM-14 utilizes a wide range of aromatic substrates, including benzene, 15 different halobenzenes, 18 phenols and 7 benzoates. This study revealed the high tolerance of *R. opacus* strains toward toxic hydrophobic compounds. *R. opacus* GM-14 grew in mineral medium to which benzene or monochlorobenzene was added in amounts of 13 or 3 g $l^{-1}$, respectively. *R. opacus* GM-29 utilized toluene and benzene for growth. Strain GM-29 grew in mineral medium with 7 g $l^{-1}$ of liquid toluene or benzene as the sole carbon source, corresponding to aqueous concentrations of 470 and 650 mg $l^{-1}$, respectively.

Most organic solvents, such as toluene and benzene, due to their high level of hydrophobicity, pass through the bacterial membrane, causing its disintegration. In this

thesis the mechanisms of adaptation of rhodococci to toxic hydrophobic compounds were investigated. The rhodococcal strains increased the level of saturation of their cellular fatty acids in response to challenge with phenol, chlorophenol, benzene, chlorobenzene or toluene. The results indicated that increase in the saturation level of cellular fatty acids, particularly that in tuberculostearic acid, is part of the adaptation mechanism of strains GM-14 and GM-29 to the presence of toxic hydrophobic compounds.

# 1. Introduction

## 1.1 Classification of corynebacteria

The current hierarchic classification for *Actinobacteria* was proposed by Stackebrandt *et al*. (1997) based on nucleotide sequences of the 16S rRNA genes. According to this classification phylogenetically related genera are clustered into families, suborders, orders and subclasses, and finally grouped together into the new phylum *Actinobacteria*. Currently the phylum *Actinobacteria* comprises 6 orders, 39 families and more than 130 genera (Garrity and Holt, 2001; http://www.bacterio.cict.fr).

The order *Actinomycetales* is made up of Gram-positive organisms with a high ( > 55%) mole% G+C content of their DNA. The taxon includes genera with a wide range of morphology: some organisms form highly differentiated branched arial mycelia (*Streptomyces*), others have hyphal forms that fragment (e.g. *Nocardia*, *Rhodococcus*), whereas several have coccoid or rod-coccoid shapes (e.g. *Gordonia, Mycobacterium*).

Actinomycetes characterized by the presence of mycolic acids are classified in the suborder *Corynebacterineae*, which comprises eight families: *Corynebacteriaceae*, *Dietziaceae*, *Gordoniaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Tsukamurellaceae, Williamsiaceae* and `*Segniliparaceae´* that include the genera *Corynebacterium*, *Dietzia, Gordonia, Segniliparus*, *Skermania*, *Mycobacterium*, *Nocardia*, *Rhodococcus, Tsukamurella* and *Williamsia* (Stackebrandt *et al*., 1997; Butler *et al*., 2005). Members of these taxa form a separate lineage in the 16S rRNA gene tree of the *Corynebacterineae*.

### 1.1.1 The genus *Rhodococcus*

The genus name *Rhodococcus* was first used by Zopf in 1891 to describe two species of red-pigmented bacteria and was redefined in 1977 to contain the `rhodococcus complex´ which consisted of various species that did not belong to the genera *Nocardia*, *Mycobacterium* or *Corynebacterium* (Bell *et al.*, 1998). Currently, the genus *Rhodococcus* comprises more than 30 recognized species (http://www.bacterio.cict.fr/qr/rhodococcus.html).

In spite of the significant progress shown in recent years in determining the genome characteristics of members of the genus *Rhodococcus* (Gurtler *et al.*, 2004), the current taxonomic status of *Rhodococcus* remains unclear. Within the genus *Rhodococcus* there are at least six distinct groups (Gurtler *et al.*, 2004). Some authors suggested that the species *R. equi* should be recognized as a separate genus (McMinn *et al*., 2000; Gurtler *et al.*, 2004).

### 1.1.2 The genus *Gordonia*

The genus was initially known as *Gordona*. The present name, *Gordonia*, was proposed by Stackebrandt *et al*. (1997). The genus `Gordona´ was proposed in 1971 by Tsukamura for coryneform bacteria isolated from the sputa of patients with pulmonary disease or from soil (Tsukamura, 1971). Goodfellow and Alderson (1977), while rearranging the genus *Rhodococcus*, reclassified three original species (*G. bronchialis*, *G. rubra* and *G. terrae*) as *Rhodococcus*. However, based on results of chemotaxonomic analysis and 16S rRNA gene similarities, the genus *Gordona* was revived including the three species of *Rhodococcus* originally assigned *Gordona* (*G. bronchialis*, *G. rubripertincta* (formerly *G. rubra*) and *G. terrae*) and *G. sputi* (Stackebrandt *et al*., 1988).

Within the last 5 years seven new species were described (Kim *et al*., 2000, Brandao *et al*., 2001; Linos *et al*., 2002; Kim *et al*., 2003; Maldonado *et al*., 2003; Xue *et al*., 2003; Iida *et al*., 2005; Kageyama *et al*. 2006). Currently, the genus *Gordonia* contains 22 valid species (http://www.bacterio.cict.fr/g/gordonia.html).

### 1.1.3 The genus *Mycobacterium*

The genus *Mycobacterium* comprises more than 100 valid species (http://www.bacterio.cict.fr/m/mycobacterium.html). At least 30 novel species have been described within the last 5 years.

Several classifications have been used for mycobacteria in the last 50 years. The mycobacteria may be divided into four groups based on pigmentation and growth rate according to the classification proposed by Runyon (1959). The distinction between rapid and slow growth is based on the ability of strains to develop clearly visible colonies in less or more than 7 days. Group 1 consists of the photochromogenic slow growers whose pigmentation is light-dependent. Group 2 are the scotochromogenic slow growers whose pigmentation is light-independent. Group 3 consists of the nonchromogenic (nonpigmented) slow growers. Group 4 consists of the rapidly growing mycobacteria.

The division of mycobacteria into groups of slowly growing and rapidly growing organisms is supported by sequence analysis of 16S and 23S rRNA genes (Böddinghaus *et al*., 1990; Stahl and Urbance, 1990). Some authors suggested that the differences in 16S rRNA genes and high *in vivo* resistance of rapidly growing mycobacteria to antimycobacterial drugs (Primm *et al*., 2004) may justify placing them in a different genus (Brown-Elliott and Wallace, 2002).

## 1.2 The polyphasic approach in taxonomy

The modern polyphasic approach in bacterial taxonomy was first introduced 35 years ago (Colwell, 1970). The aim of polyphasic taxonomy is the integration of phenotypic and

genotypic data on microorganisms to generate well-defined taxonomic groups. Fig. 1.1 presents the main methods used for polyphasic characterization of microorganisms.

Even though the polyphasic approach in bacterial taxonomy is well established (Vandamme *et al*., 1996), there is still no generally accepted model of polyphasic species definition. The Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology recommends that a 70% DNA-DNA reassociation value is a threshold for defining a bacterial species (Stackebrandt *et al*., 2002). The analysis of 16S rRNA gene sequences is considered as a standard in bacterial classification (Stackebrandt *et al*., 2002).

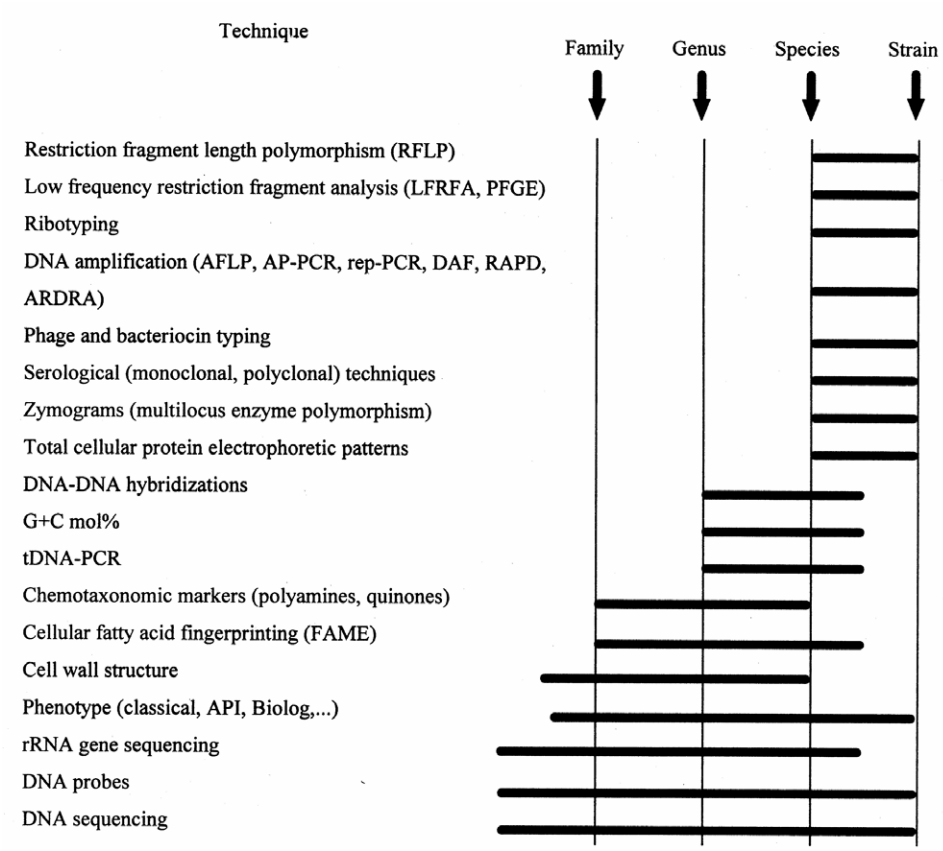



**Figure 1.1** Taxonomic resolution of some currently used techniques (reproduced from Vandamme et al. (1996) with kind permission of the American Society for Microbiology)
AFLP - amplified fragment length polymorphism; API -Apareils et Procedes d'Identification; AP-PCR - arbitrarily primed polymerase chain reaction; ARDRA - amplified ribosomal DNA restriction analysis; DAF-DNA amplification fingerprinting; FAME - fatty acid methyl ester; LFRFA - low-frequency restriction fragment analysis; PFGE - pulsed field gel electrophoresis; RAPD - random amplification of polymorphic DNA; Rep-PCR - repetitive extragenic palindromic polymerase chain reaction; RFLP - restriction fragment length polymorphism; tDNA-PCR - tDNA intergenic spacer PCR.

But the 16S rRNA gene sequence should not be used as the only method in species delineation (Stackebrandt and Goebel, 1994). The 16S rRNA gene sequence has been determined for a large number of strains. More than 300,000 16S rRNA gene sequences are available in the Nucleotide Sequence Database of European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/embl/) and in the Ribosomal Database Project (RDP) database (Kulikova *et al.*, 2004). Sequencing of total genomic DNA is an important source for discovering novel molecular characteristics that are useful for biochemical, taxonomic and phylogenetic purposes. However, it is not yet done routinely. During the last 10 years with the aid of the polyphasic approach, the classification of the *Corynebacterineae* has been changed markedly. The combination of chemotaxonomic and molecular techniques allowed scientists to establish several new genera and clarified the taxonomic positions of some previously described genera (Rainey *et al*., 1995; Kämpfer *et al*. 1999; Arenskötter, 2004; Gurtler *et al*., 2004; Butler *at al*., 2005).

### 1.2.1 Chemotaxonomic markers of bacteria in the suborder *Corynebacterineae*

Bacteria of the suborder *Corynebacterineae* have cell wall chemotype IV, meaning that the only diamino acid of the peptidoglycan is *meso*-diaminopimelic acid, and glucose and arabinose are the major cell wall sugars (Lechevalier and Lechevalier, 1970). The fatty acid composition of most corynebacteria is characterized by the presence of tuberculostearic acid (TBSA). The only exception is the genus *Corynebacterium,* in which this acid was reported for some species only. Table 1.1 summarizes the main chemotaxonomic characteristics of the genera of the suborder *Corynebacterineae.*

**Table 1.1** Differential chemotaxonomic characteristics of the mycolic acid-containing genera.

| Genus | Major menaquinone | Presence of TBSA | Mycolate size (number of carbons) | G+C content (mol%) |
|---|---|---|---|---|
| *Gordonia* | MK-9($H_2$) | + | 48-66 | 63-69 |
| *Skermania* | MK-8($H_4$ ω cyc) | + | 58-64 | 68 |
| *Corynebacterium* | MK-8($H_2$) | -[a] | 22-36 | 51-67 |
| *Segniliparus* | | + | | 68-72 |
| *Dietzia* | MK-8($H_2$) | + | 34-38 | 73 |
| *Mycobacterium* | MK-9($H_2$) | + | 60-90 | 70-72 |
| *Nocardia* | MK-8($H_4$ ω cyc) | + | 44-60 | 64-72 |
| *Rhodococcus* | MK-8($H_2$) | + | 34-52 | 63-73 |
| *Tsukamurella* | MK-9 | + | 64-78 | 67-68 |

[a] TBSA presents in *C. ammoniagenes, C. bovis, C. minutissimum, C. urealyticum* and *C. variabilis.*

All species of the suborder *Corynebacterineae* (with the exception of *Turicella otitidis* and *Corynebacterium amycolatum*) are characterized by the presence of mycolic acids in their cell envelopes (Funke *et al*., 1994; Sutcliffe, 1998). The mycolic acids are long-chain, *beta*-hydroxy fatty acids with an aliphatic side chain at the *alpha* position. They differ in the number of carbon atoms, ranging from 22 (in *Corynebacterium*) to 90 (in *Mycobacterium*) and in the presence of different functional groups. Structures of the mycobacterial mycolic acids have been studied in more detail than those of the other corynebacterial species. The mycolic acid pattern of the mycobacterial cell wall varies with the species; however, some mycolic acid patterns are shared by more than one mycobacterial species (Tortoli, 2003). Analysis of cleavage products of the mycolic acids together with whole cell fatty acids was shown to be useful in the identification of slowly growing environmental mycobacteria (Torkko *et al*., 2003).

Limited data are available on the structural diversity of mycolic acids in species other than those of *Mycobacterium* (Butler *et al*., 2005). In most cases only information on the number of carbon atoms in mycolic acids as determined by thin-layer chromatography or gas chromatography (GC) is available. Polyamines as chemotaxonomic markers of the corynebacteria were introduced in 1997 (Altenburger *et al*., 1997). However, the analysis of polyamines is not usually used in characterization and differentiation of taxa within the suborder *Corynebacterineae.*

### 1.2.2 Molecular targets used in taxonomy of bacteria in the suborder *Corynebacterineae*

The 16S rRNA gene is the most widely accepted gene used for bacterial taxonomic studies. Five to 15-base differences in the 16S rRNA gene was proposed for some microorganisms to indicate distinct taxa (Fox *et al*., 1992). Other authors proposed that 99% and higher similarity be used as a threshold in defining a species of Gram-positive rods and some coryneform bacteria (Bosshard *et al*., 2003). However, this cannot be applied for the genus *Mycobacterium*. The 16S rRNA gene sequences of some mycobacterial species differ in just a few nucleotides or are even identical (Tortoli, 2003; Vaerewijck *et al*., 2005). In contrast, some species present genetic heterogeneity (Tortoli, 2003).

Sequence analysis of the 65-kDa heat-shock protein (HSP) gene (*hsp*65) was shown to be useful in identifying mycobacteria (Ringuet *et al*., 1999; Kim *et al*., 2005). Restriction enzyme pattern analysis of a 441-bp sequence in *hsp*65 has been used more often than analysis of its sequence since the former was introduced in 1993 (Telenti *et al*., 1993). However, research by Adekambi and Drancourt (2004) done on a set of 19 species of fast-growing mycobacteria indicated that neither the 16S rRNA gene sequence nor the *hsp*65 gene and the *sod*A gene sequences discriminated between some species.

In 1995 Yamamoto and Harayama suggested that the *gyrB* gene (encoding the β-subunit of DNA gyrase) can be used as a phylogenetic marker in bacterial classification (Yamamoto and Harayama, 1995). Analysis of the *gyrB* sequence was applied for identification of 15 species of slowly growing mycobacteria (Kasai *et al*., 2000). Analysis of *gyrB* was recently reported to be useful for the taxonomy of *Gordonia*, providing a better discrimination of species within the genus *Gordonia* than the 16S rRNA gene (Shen *et al*., 2006).

Recently, the entire genomes of at least 12 different corynebacteria were sequenced: six strains of *Mycobacterium*, five of *Corynebacterium* and one strain of *Nocardia farcinica*. A *Rhodocccus equi* partial sequence was also released (Rahmana *et al*., 2003; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

## 1.3 Cell envelope structure of the bacteria of the suborder *Corynebacterineae*

The structure of the mycobacterial cell wall has been studied intensively during the last 20 years. Several models for the cell envelope organization have been proposed since the first model by Minnikin (1991). The general model for *Rhodococcus* cell envelope shown in Fig. 1.2 can also be applied to other members of the suborder *Corynebacterineae* (Sutcliffe, 1998).

The plasma membrane is the innermost layer surrounded by the cell wall envelope. The cell envelope consists of arabinogalactans and mycolic acids, which are covalently assembled into a peptidoglycan-arabinogalactan-mycolic acid matrix. The mycolic acids are attached to the arabinogalactan and form together with trehalose mycolates a lipid layer outside of the peptidoglycan-arabinogalactan cell wall matrix (Sutcliffe, 1998). The plasma membrane of rhodococci and mycobacteria is mainly composed of polar phospholipids (Goren, 1972; Sutcliffe, 1998). The main fatty acids in phospholipids are straight-chain saturated, monounsaturated and 10-methyl-branched (Goren, 1972; Sutcliffe, 1998).

Three different layers build the cell wall envelope. The first inside layer, common to all Gram-positive bacteria, is the thick peptidoglycan layer located next to the plasma membrane. The second layer is mostly represented by lipidated macroamphiphiles, the lipoglycans. Among them the most characterized are the lipoarabinomannans (LAMs) of mycobacteria (Nigou *et al*., 2003). LAM-like structures are present in the cell envelopes of *Dietzia maris*, *Gordonia rubropertincta*, *G. bronchialis, G. terrae*, *Rhodococcus rodnii*, *R. equi*, *R. ruber*, and *Tsukamurella paurometabolum* (Flaherty and Sutcliffe 1999; Sutcliffe, 2000; Garton *et al.*, 2002; Gibson *et al*., 2003, 2004; Garton and Sutcliffe, 2006). The LAMs have three domains in their structure: a mannosyl-phophatidyl-*myo*-inositol anchor, a polysaccharide backbone and cap structures. The structure of the LAM varies between genera and species, mostly due to differences in the capping motifs (Nigou *et al*., 2003). There are also variations in the number, positions and nature of fatty acids in the anchor.

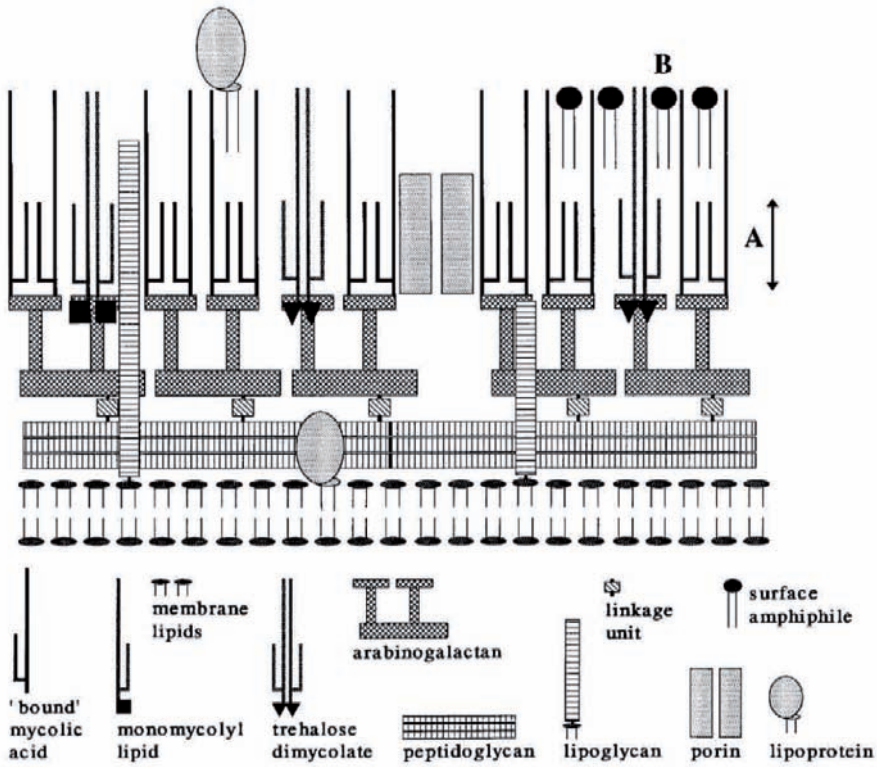



**Figure 1.2** A schematic representation of the proposed model for the cell wall organization of *Rhodococcus* sp. (reproduced from Sutcliffe (1998) with kind permission of Springer Science and Business Media).

The most abundant fatty acids in the LAM and LAM-like glycolipids are palmitic (hexadecanoic) and TBSA (10-methyl octadecanoic), although other acids such as palmitoleic (hexadecenoic), stearic (octadecanoic), oleic (octadecenoic), heneicosanoic, and margaric (heptadecanoic) are present in minor amounts (Nigou *et al*., 2003; Garton and Sutcliffe, 2006). The position of the LAM in the cell envelope is still a matter for discussion. There is evidence that the LAM is attached by a phosphodiester link to the peptidoglycan or plasma membrane and goes through the cell envelope towards the outer lipid layer (Sutcliffe, 1998).

Mycolic acids are characteristic components of the third outer layer of the cell envelope. In mycobacteria mycolic acids have very long chains (C60-90) and may be oxygenated or hydroxylated, whereas in nocardia, corynebacteria, rhodococci and gordonia the alkyl chain is shorter and consists of a mixture of saturated and unsaturated fatty acids. Mycolic acids are linked to arabinogalactan to form arabinogalactan mycolate or cell wall-bound mycolate. The esterification of one or two mycolic acids with a molecule of trehalose gives trehalose monomycolate and trehalose dimycolate, respectively, which are part of the extractable mycolate. Thus, the inner lipid layer of the mycolate layer is composed mainly of arabinogalactan mycolate and minor amounts of extractable mycolate, whereas the outer layer consists of trehalose monomycolate and

trehalose dimycolate (Daffé and Draper, 1998; Sutcliffe, 1998). In addition to trehalose mycolates, a number of mycolic acid-containing glycolipids, sulpholipids, phenolic glycolipids and others are located in the outer layer of the cell envelope of mycobacteria and related organisms (Daffé and Draper, 1998; Sutcliffe, 1998).

## 1.4 Degradation power of corynebacteria

The suborder *Corynebacterineae* harbours most of the xenobiotic degraders among the Gram-positive bacteria. Species of the genera *Gordonia*, *Rhodococcus* and *Mycobacterium* are well known for their metabolic versatility and capacity to degrade environmentally hazardous chemicals (Bell *et al*., 1998; Arenskötter *et al*., 2004; Larkin *et al*., 2005). Examples of the classes and types of organic compounds that can be degraded by the members of these three genera are compiled in Table 1.2.

### 1.4.1 Degradation power of the genus *Rhodococcus*

Members of the genus *Rhodococcus* are often isolated from contaminated soils. Due to their metabolic versatility, the metabolic pathways of several isolates have been studied in detail. The mono- and dioxygenases were characterized biochemically and genetically in several members of the genus (Gurtler *et al*., 2004).

The halogenated aliphatic and aromatic hydrocarbons are toxic chemicals, and resistant to biological degradation. Although many organisms are able to degrade monochlorinated benzene, only a few *Rhodococcus* can utilize this substrate (Zaitsev *et al*., 1993; Rehfuss and Urban, 2005). Degradation of phthalate esters by *Rhodococcus* has been documented (Aleshchenkova *et al*., 1996; Nalli *et al*., 2002; Li *et al*., 2006). Some of the organisms were isolated from soil heavily contaminated with phthalates and terephthalates (Aleshchenkova *et al*., 1996). Rhodococci are known as degraders of alkanes. Psychrotolerant rhodococci were isolated from Arctic and Antarctic soils contaminated with oil (Yakimov *et al*., 1999; Bej *et al*., 2000; Aislabie *et al*., 2004; Ruberto *et al*., 2005; Saul *et al*., 2005). A number of *Rhodococcus* isolates degrade a wide range of resistant compounds. For instance, *R. opacus* SAO101 grows on phenol, benzene, 4-nitrophenol, biphenyl, naphthalene, dibenzofuran and dibenzo-*p*-dioxin (Kimura and Urushigawa, 2001). *R. opacus* M213 degrades naphthalene, toluene, phenol and hydroxybenzoate (Uz *et al*., 2000).

The degradation versatility of rhodococci may be due to the presence of large linear plasmids that carry genes for the degradation of different compounds (van der Geize and Dijkhuizen, 2004; Konig *et al*., 2004). The presence of multiple pathways and genes also contributes to the catabolic versatility of the rhodococci (Larkin *et al*., 2005). An example of such organisms is *Rhodococcus* sp. RHA1, which uses multiple enzyme systems, including at least three ring-hydroxylating dioxygenases, to degrade biphenyl (Kitagawa *et*

*al*., 2001) and has a duplicate set of genes encoding the degradation of phthalate (Patrauchan *et al*., 2005).

### 1.4.2 Degradation power of the genus *Gordonia*

Much less has been reported on the degradation abilities of the genus *Gordonia*, possibly because the taxon was established much later than the genera *Mycobacterium* or *Rhodococcus*. In recent years several reports were published on *Gordonia* strains that degrade xenobiotic compounds. *Gordonia* isolates utilizes benzene, toluene, xylene, pyrene and phthalate diesters as the sole carbon and energy sources and transformed several sulphur-containing compounds (see references in Table 1.2). Two strains of *Gordonia* were able to metabolize several phthalate diesters. *Gordonia* sp. MTCC 4818 grows on dibutyl-, butyl benzyl- and diphenyl phthalates, and strain P8219 grows on diethyl-, dinonyl-, dihexyl- and di-2-ethylhexyl phthalates (Chatterjee and Dutta, 2003; Nishioka *et al*., 2006). Strain P8219 degraded di-ethylhexyl phthalate to phthalic acid; further metabolism was not investigated. The pathway of degradation of butyl benzyl phthalate by the strain MTCC 4818 was investigated in more detail. The initial reaction was hydroxylation that resulted in monoesters of phthalate, phthalic acid, benzyl alcohol and butanol; phthalic acid was a dead-end product. The strain used benzyl alcohol and 1-butanol for growth. Benzyl alcohol was mineralized through benzaldehyde, benzoic acid and catechol, followed by *ortho*-cleavage of the aromatic ring (Chatterjee *et al*., 2005). Esterase activity involved in the hydrolysis of the ester bonds of butyl benzyl phthalate was induced in the presence of butyl benzyl phthalate and monobenzyl phthalate, indicating a specificity of the enzyme for this xenobiotic. So far there is only one report on a *Gordonia*-like strain growing on a PAH compound (pyrene) as the sole carbon and energy source in a mineral medium where the substrate as crystals was used (Mutnuri *et al*., 2005).

One of the very interesting properties of *Gordonia* is its ability to degrade natural and synthetic isoprene rubber. Several strains of *Gordonia* disintegrated and mineralized natural rubber and latex gloves. Two novel species were described to accommodate the rubber-degrading isolates: *G. polyisoprenivorans* (Linos *et al*., 1999) and *G. westfalica* (Linos *et al*., 2002). The biodegradation mechanism of the *Gordonia* strains involved reduction of the chain length of the polymer through oxidation at the cis-1,4 double bond. These strains were unable to grow or form clear zones on latex overlay plates, but showed adhesive growth on the rubber surface. This type of rubber degradation has been observed only in mycolic acid-containing organisms (Rose and Steinbuchel, 2005).

**Table 1.2** Examples of organic compounds metabolized by corynebacteria

| Compound | Organism | References | Type of degradation[a] |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Monoaromatic hydrocarbons: Benzene, toluene, xylenes, phenols, cresol | *G. alkanivorans* CC-JG39 | Lin *et al*., 2005 | SCE (floating in the interface while growing in diesel oil) |
| | *Mycobacterium* sp. T103, T104 | Tay *et al*., 1998 | SCE |
| | *R. opacus* M213 | Uz *et al*., 2000 | SCE |
| | *R. opacus* R7 | Di Gennaro *et al*., 2001 | SCE |
| | *R. opacus* SAO101 | Kimura and Urushigawa, 2001 | SCE |
| | *R. phenolicus* G2P | Rehfuss and Urban, 2005 | SCE |
| | *R. pyridinivorans* PYJ-1 | Jung and Park, 2004 | SCE |
| | *R. rhodochrous* OFS | Vanderberg *et al*., 2000 | SCE |
| | *R. zopfii* T1 | Stoecker *et al*., 1994 | SCE |
| | *Rhodococcus* sp. 33 | Paje *et al*., 1997 | SCE |
| | *Rhodococcus* sp. DK17 | Kim *et al*., 2002 | SCE |
| | *Rhodococcus* sp. MS11 | Rapp and Gabriel-Jürgens, 2003 | SCE |
| Phthalic acid | *Rhodococcus* sp. RHA1 | Navarro-Llorens *et al*., 2005; Patrauchan *et al*., 2005 | SCE |
| | *Rhodococcus* sp. YU6 | Jang *et al*., 2005 | SCE |
| | *M. vanbaalenii* PYR-1 | Stingley *et al*., 2004 | SCE |
| Phthalic acid esters | *Corynebacterium* sp.O18 | Chang *et al*., 2004; | SCE |
| | *Gordonia sp.* MTCC 4818 | Chatterjee and Dutta, 2003; | SCE (phthalic acid - dead-end product) |
| | *Gordonia* sp. P8219 | Nishioka *et al*., 2006 | SCE |
| | *Mycobacterium* sp. NK0301 | Nakamiya *et al*., 2005 | SCE |
| | *R. erhytropolis* 40F | Aleshchenkova *et al*., 1996; | SCE |

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Phthalic acid esters | *R. rhodochrous* ATCC 21766 | Nalli *et al*., 2002 | Cometabolism with hexadecane |
| | *R. ruber* 1K | Li *et al*., 2006 | SCE |
| Mono- and dichlorinated hydrocarbons: | | | |
| Monochlorobenzene | *R. phenolicus* G2P | Rehfuss and Urban, 2005 | SCE |
| | *R. opacus* SAO101 | Kimura and Urushigawa, 2001 | SCE |
| Dichlorobenzene | *R. phenolicus* G2P | Rehfuss and Urban, 2005 | SCE |
| | *Rhodococcus* sp. MS11 | Rapp and Gabriel-Jürgens, 2003 | SCE |
| Mono- and dichlorophenols | *R. erythropolis* M1, | Goswami *et al*., 2002 | SCE |
| | *R. opacus* 1CP | Gurtler *et al*., 2004 | SCE |
| Polychlorinated benzenes and phenols | | | |
| Tri- and tetrachlorophenol | *R. percolatus* MBS1 | Briglia *et al*., 1996 | SCE |
| | *Rhodococcus* CG-1, | Häggblom *et al*., 1988 | O-methylation |
| | *M. fortuitum* CG-2 | | |
| Pentachlorophenol | *M. chlorophenolicum* PCP-1 | Apajalahti and Salkinoja-Salonen, | |
| | (formerly *R. chlorophenolicus* PCP-1) | 1987 | |
| | *M. chlorophenolicum* CP-2 (formerly | Häggblom *et al*., 1988; | Mineralization in the presence of |
| | *Rhodococcus* sp. CP-2) | Nohynek *et al*., 1993 | 0.1% glucose |
| | *M. fortuitum* CG-2 | Häggblom *et al*., 1988; | Mineralization in the presence of |
| | (formerly *Mycobacterium* sp. CG-2 | Nohynek *et al*., 1993 | 0.1% glucose |
| Tri- and tetrachlorobenzenes | *Rhodococcus* sp. MS11 | Rapp and Gabriel-Jürgens, 2003 | SCE |
| Nitrophenols | *R. opacus* SAO101 | Kitagawa *et al*., 2004 | |
| | *R. opacus* spp. | Heiss *et al*., 2003 | Nitrogen source |
| | *R. koreensis* DNP505 | Yoon *et al*., 2000 | SCE |

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Alkyl ethers | | | |
| Ethyl tert-butyl ether | *G. terrae* IFP 2001 | Hernandez-Perez *et al*., 2001 | SCE |
| | *R. ruber* IFP 2001 | Chauvaux *et al*., 2001 | SCE |
| Methyl tert-butyl ether | *M. austroafricanum* IFP2015 | Ferreira *et al*. 2006 | SCE |
| | G. terrae IFP 2001 | Hernandez-Perez *et al*., 2001 | Cometabolism with ethanol |
| Isoprene rubber | *G. polyisoprenivorans* Kd2 | Linos *et al*., 1999 | SCE |
| | *G. westfalica* Kb2 | Linos *et al*., 2002 | SCE |
| Biphenyl | *Rhodococcus* sp. RHA1 | Masai *et al*., 1995 | SCE |
| | *R. opacus* SAO101 | Kitagawa *et al*., 2004 | SCE |
| Polychlorinated biphenyls | *R. globerulus* P6 | Asturias *et al*., 1994 | SCE |
| | *Rhodococcus* sp. RHA1 | Masai *et al*., 1995 | SCE |
| | *Rhodococcus* spp. *(14 strains)* | Leigh *et al*., 2006 | Degradation by the resting cells |
| | *Williamsia* sp. *OUCZ63* | Leigh *et al*., 2006 | Degradation by the resting cells |
| Dibenzofuran, dibenzo-p-dioxin, chlorinated dibenzo-p-dioxin | *R. opacus* SAO101 | Kimura and Urushigawa, 2001 | SCE |
| Dibenzothiophene | *G. alkanivorans* 1B | Alves *et al*., 2005 | Desulphurization |
| | *G. amicalis* IEGM | Kim *et al*., 2000 | Desulphurization |
| | *Gordonia* sp. CYKS1 | Rhee *et al*., 1998 | Desulphurization |
| | *Mycobacterium* sp. X7B | Li *et al*., 2003 | Desulphurization |
| | *Rhodococcus* sp. IGTS8 | Oldfield *et al*., 1997 | Desulphurization |
| Polyaromatic hydrocarbons (PAHs) | *Gordonia*-like strain BP9 | Mutnuri *et al*., 2005 | SCE |
| | *M. frederiksbergense* LB501T | Van Herwijnen *et al*., 2003 | SCE |
| | *M. frederiksbergense* Fan9 | Willumsen *et al*., 2001 | Mineralization in the presence of small amounts of complex organic nutrients |

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| PAHs | *M. gilvum* BB1 (formerly *Mycobacterium* sp. BB1) | Mrozik *et al*., 2003; Boldrin *et al*., 1993. | SCE |
| | *M. gilvum* VF1 | Mutnuri *et al*., 2005 | SCE |
| | *M. hodleri* EM12 | Kleespies *et al*. ,1996 | SCE |
| | *M. vanbaalenii* PYR-1 (formerly *Mycobacterium* sp. PYR-1) | Heitkamp and Cerniglia, 1988; Heitkamp *et al*., 1988; Moody *et al*., 2005 | Mineralization in the presence of small amounts of complex organic nutrients |
| | *Mycobacterium* sp. CP1, CP2, AP1 | Lopez *et al*., 2005, 2006 | SCE |
| | *Mycobacterium* sp. KR2, KR20 | Rehmann *et al*., 1998, 2001 | SCE |
| | *Mycobacterium* sp. RJGII-135 | Grosser *et al*.,1991; Schneider *et al*., 1996 | Mineralization in the presence of small amounts of complex organic nutrients |
| | *Rhodococcus* sp. UW1 | Walter *et al*.,1991 | SCE |

[a] *SCE - sole carbon and energy source*
*CN- carbon and nitrogen source*

### 1.4.3 Degradation power of the genus *Mycobacterium*

A relatively small range of genera, considering the prevalence of PAHs in the environment, is capable of degrading PAHs: *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus* and *Sphingomonas* (Kanaly and Harayama, 2000, Mrozik *et al*., 2003). However, most of the isolates especially those capable of growth on four-ring PAHs, such as fluoranthene and pyrene, belong to the genus *Mycobacterium* (Mrozik *et al*., 2003**).** Members of the genera *Mycobacterium* as well as *Rhodococcus* and *Gordonia* were reported to be a major part of the soil microflora able to mineralize PAH (Kastner et *al*., 1994; Uyttebroek *et al*., 2006). Although a majority of the mycobacterial degraders of PAHs are isolated from polluted environments, there are only fragmented data available on the diversity of mycobacterial populations in heavily contaminated sites. For instance, contamination of soil with PAH decreased the diversity of mycobacteria (Cheung and Kinkle, 2001). Mycobacteria are present in higher numbers in PAH-contaminated soils containing low concentrations of mainly high-molecular-weight PAHs than in soils containing higher concentrations of PAHs (Leys *et al*., 2005).

Several strains of the species *M. chlorophenolicum* (formerly *R. chlorophenolicus*) degraded tri-, tetra- and pentachlorophenols (Apajalahti and Salkinoja-Salonen, 1987; Häggblom *et al*., 1988). Rapidly growing mycobacteria that degraded toluene were isolated from rock surface biomass in a freshwater stream contaminated with toluene (Tay *et al*., 1998). The authors suggested that these organisms were enriched by toluene, since any attempt to isolate mycobacteria from a pristine stream failed (Tay *et al*., 1998). In another study the same authors highlighted the important role of mycobacteria in the toluene-degrading community, mostly due to their persistence in the environment (Tay *et al*., 2001).

## 1.5 Pathogenic potentials of the corynebacteria

Despite the fact that most members of the suborder *Corynebacterineae* are soil saprophytes, there has been in the last decade increasing evidence of infections caused by coryneform bacteria. This occurs most often in immunocompromised persons.

### 1.5.1 Pathogenicity of environmental mycobacteria

More than 40 out of about 130 validly described species of environmental mycobacteria are classified as Hazard Group 2 or 3 in the European Union classification and the hazard status of more than eight species is uncertain. There are a number of reviews that cover almost all species of environmental mycobacteria recognized as being of clinical importance (Falkinham III, 1996; Wallace *et al*., 1998; Phillips and von Reyn, 2001; Brown-Elliott and Wallace Jr., 2002; Primm *et al*., 2004).

A few species of mycobacteria are frequently isolated from human specimens but are not considered to be clinically significant. Examples of such are the *M. terrae* complex and *M. gordonae*. These species are found in many different environments, including water (fresh, tap, bottled, swimming pools) and soil (Le Dantec *et al.* 2002; Primm *et al*. 2004; Vaerewijck *et al*., 2005), and thus may be incidental lung contaminants e.g. from dust or water aerosols. It is not always clear whether such bacteria are only contaminants or are active pathogens; e.g. mycobacteria are frequently isolated from the sputum of patients with cystic fibrosis. Some individuals tolerate the presence of the nontuberculous mycobacteria (NTM) without obvious disease, while others develop complications (Kilby *et al*. 1992, Saiman and Siegel 2004). Despite the information available on health-associated environmental mycobacteria, their effects on humans are not fully understood. Mycobacteria that are not yet considered as pathogens and are believed to be contaminants in clinical specimens may prove to affect human health.

Recently, concern was raised over hypersensitivity pneumonitis caused by environmental mycobacteria in places where persons are exposed to water aerosols (Falkinham III, 2003). Mycobacterial cell envelope components such as LAM, trehalose mycolates, as well as some secondary metabolites and HSP can trigger inflammatory responses (Clark-Curtiss, 1998; Huttunen *et al*. 2000; Garton *et al.*, 2002; Rha *et al*. 2002).

### 1.5.2 Pathogenicity in the genus *Gordonia*

Although bacteria of the genus *Gordonia* were only rarely isolated from clinical samples, there is increasing concern regarding *Gordonia* species that cause infections in immunocompromised persons or individuals with background diseases (Pham *et al*., 2003; Arenskötter *et al*., 2004; Bakker *et al*., 2004; Kempf, *et al*., 2004; Sng *et al*., 2004; Zardawi *et al*., 2004; Gil- Iida *et al*., 2005; Werno *et al*., 2005; Sande *et al*., 2006; Verma *et al*., 2006). There is some evidence that blood infections related to *Gordonia* species are associated with intravenous catheters (Riegel *et al*., 1996; Lesens *et al*., 2000; Pham *et al*., 2003; Kempf *et al*., 2004; Sng *et al*., 2004; Verma, P. *et al*., 2006). The number of clinical cases resulting from *Gordonia* infection may currently be underestimated due to the confusing taxonomic history of the genus and difficulties reported in correct identification of the members of this genus in clinical laboratories (Sng *et al*., 2004; Werno *et al*., 2005; Verma *et al*., 2006)

### 1.5.3 Pathogenicity in the genus *Rhodococcus*

Members of the genus *Rhodococcus* are mostly soil saprophytes; however, *R. equi* is known as a respiratory pathogen of foals. *R. equi* is becoming increasingly reported as an important pathogen for humans, mainly immunocompromised persons (Scott *et al*., 1995). Recently, *R. equi* was found to be a source of infections in immunocompetent individuals (Kedlaya *et al*., 2001). There is also a report of an eye infection by *R. globerulus*

associated with surgery (Cuello *et al*., 2002). The problem in assessing the importance of *Rhodococcus* as hazardous organisms is similar to that in *Gordonia* and mostly relates to its taxonomy.

## 1.6 Organic solvent toxicity toward microorganisms

### 1.6.1 Effects on membrane functioning

The toxic effects of aromatic and aliphatic hydrocarbons, phenols and alcohols due to interaction of these compounds with the membrane and membrane constituents have been studied for many years. Small hydrophobic molecules are highly toxic for microorganisms due to their partition into the cytoplasmic membrane (Sikkema *et al*., 1994, 1995). They interrupt the protein-lipid and lipid-lipid connections in the membrane and, as a result, cause functional disturbances and increase membrane fluidity and passive diffusion of the hydrophobic compounds into the cell (Sikkema *et al*, 1994, 1995). Pentachlorophenol (PCP), for instance, easily penetrates into the hydrocarbon core of the membrane and affects the phase of the lipid bilayer, converting it from a liquid crystalline phase to one that is more gel-like (Mukhopadhyay *et al.*, 2004).

The partition coefficient of lipophilic compounds in an octanol:water mixture (expressed as log $K_{ow}$) has been used to predict the effect of different compounds on intact cells. The correlation between hydrophobicity of the compound and its toxicity toward microorganisms was observed in various studies and is summarized in the reviews (Sikkema *et al*., 1995; Ramos *et al*., 2002, Sardessai and Bhosle, 2002). Solvents with log $K_{ow}$ values between 1.5 and 3 are as a rule more toxic for microorganisms than those with high log $K_{ow}$ values ( > 4-5) (Aono and Inoue, 1998). The transition from toxic to nontoxic organic solvents usually occurs between log $K_{ow}$ 3 and 5 and is dependent on the homolog series (Vermuë *et al*, 1993). DeYoung and Dill (1988, 1990) showed, however, that partitioning of benzene and hexane into lipid bilayers differed from that into bulk hydrocarbons and was a function of the surface density of the bilayer. The specific lipid composition of a lipid bilayer also influences the solubility of compounds in the membrane (Antunes-Madeira and Madeira, 1987, 1989).

### 1.6.2 Solvent adaptations in microorganisms: regulation of membrane fluidity

Most of the studies on mechanisms of bacterial adaptation to toxic hydrophobic compounds have been done on Gram-negative bacteria, mainly *Pseudomonas* spp. (Ramos *et al*., 2002). There are a number of mechanisms of adaptation of bacterial cells to toxic hydrophobic solvents. Among them are changes in the structure of lipopolysaccharides, in the composition of the fatty acids of lipids, alteration in cell wall structure and active

export of hydrophobic compounds from the cell (Sikkema *et al*., 1995; Ramos *et al*., 2002; Sardessai and Bhosle, 2002). An increase in the amount of lipid in the cell membrane was observed in several cases; e.g. cells of *Sphingomonas* sp. altered their phospholipid composition by increasing the cardiolipin content in responce to PCP (Sikkema *et al*., 1995).

One of the best studied mechanisms of adaptation to the presence of hydrophobic compounds is an increase in the level of saturated fatty acids in the lipids of the cytoplasmic membrane (Ramos *et al*., 2002; Sardessai and Bhosle, 2002). Increase in the level of saturation of the fatty acids as a mechanism for decreasing the fluidity of the cell membrane to tolerate benzene was also shown for the corynebacterium, *Rhodococcus* sp. 33 (Gutierrez *et al*., 1999). Another mechanism involved in adaptation and protection of the bacterial cell against the toxic effects of hydrophobic compounds is isomerization of the *cis* form of unsaturated fatty acids to their *trans* isomers (Heipieper and deBont, 1994; Weber *et al*., 1994; Pinkart *et al*., 1996). This phenomenon has been described only for the members of the species *Pseudomonas.* The *trans* isomers of unsaturated fatty acids have a configuration similar to that of saturated acids (Cevc, 1991). Thus, replacement of the *cis* form with the *trans* form of fatty acids leads to tighter packing of the lipid layer and decreases the fluidity of the membrane. *Staphylococcus haemolyticus,* which may tolerate saturated levels of toluene, benzene and *p*-xylene in liquid cultures, uses a different strategy to tolerate hydrophobic toxic compounds, i.e. it increases the relative amounts of anteiso-branched fatty acids (Nielsen *et al*., 2005).

## 2. Aims of this study

The aims of the study were

1. Isolate from polluted environments bacteria capable of degrading and/or tolerating recalcitrant hydrophobic substrates and characterize these abilities

2. Clarify the taxonomic position of selected bacteria with the capacities mentioned in 1

3. Isolate and characterize mycobacteria with potential health risk from biofilms in drinking water distribution systems and water-damaged buildings

4. Assess the suitability of an automated DNA typing method for identifying environmental mycobacterial isolates

5. Increase the level of understanding of the mechanisms of adaptation of selected actinobacterial cells for tolerating or degrading hydrophobic toxic substances

# 3. Materials and methods

## 3.1 Methods used in this thesis

The reference strains used in this study were supplied by culture collections as indicated by a collection code: National Collection of Type Cultures (NCTC), HPA Centre for Infection, London, UK; Deutsche Sammlung von Mikroorganismen und Zellkulture GmbH (DSM), Braunschweig, Germany; Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden; American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA.

The experimental procedures used in this study are described in the original publications as listed in Table 3.1 or in Chapter 3.2. References to published methods can be found in the articles.

## 3.2 Methods other than those described in I-V

### 3.2.1 Measurement of toluene and benzene in the aqueous phase

To determine the concentrations of toluene and benzene in the aqueous phase of cultures, a 0.1-2-ml sample was placed in a headspace vial, made up with water to a final volume of 2 ml and NaCl added to a final concentration of 30%. Equilibrium headspace analysis was performed with an HS 40XL Headspace sampler (Perkin Elmer, Uberlingen, Germany), connected to a gas chromatograph HP 6890A with mass-selective detector HP 5972A (Hewlett Packard, Palo Alto, CA, USA) and an HP-volatile organic compound/mass spectrometry (VOC/MS) capillary column (60 m, 0.32-mm inner diameter; 1.8-µm film thickness). The headspace sampler parameters were as follows: equilibration 30 min at 60 °C; pressurization 5 min; injection 0.2 min; needle temperature 70 °C. The transfer line from the sampler to the chromatograph was set at 120 °C and the oven temperature was programmed as follows: 5-min hold at 40 °C, followed by an increase to 100 °C at 25 °C min-1, then to 160 °C at 5 °C min-1. The mass detector was operated at electron ionization energy of 70 eV. The peaks (total ion current chromatogram) were quantified by comparing injections with external standards that were prepared by adding defined amounts of toluene or benzene to headspace vials.

**Table 3.1** Experimental procedures used in this study.

| Experimental procedures | Article |
|---|---|
| Isolation of bacteria by enrichment culture | I |
| Decontamination of biofilm specimens for isolation of mycobacteria | III |
| Identification and characterization of bacteria | |
| Oxidation of organic compounds (microtitre plate technique) | IV |
| Utilization of carbon sources by bacteria in batch culture | I |
| Analysis of catalase and hydrolytic activities | I |
| Analysis of whole cell fatty acids | I, IV, V, this thesis |
| Analysis of menaquinones | I, IV |
| Analysis of mycolic acids | I, IV, this thesis |
| Analysis of polar lipids | IV |
| G+C content of DNA | I, IV |
| Antibiotic susceptibility testing | III |
| Automated riboprinting | III, IV |
| 16S rRNA gene sequence analysis | I, III, IV |
| DNA:DNA hybridization | IV |
| Clustering analyses by Bionumerics 4.1 (Applied Math BVBA, Kortijk, Belgium) | III, this thesis |
| Degradation of aromatic compounds | I, II, IV |
| Microscopic methods | |
| Negative staining electron microscopy | I |
| Epifluorescence microscopy | IV |
| Transmission electron microscopy | II, this thesis |
| Analytical methods | |
| Gas chromatography-mass spectrometry | I, IV, V |
| Halide ion concentration | I |
| Esterase activity using fluorogenic substrates | IV |
| Protein concentration | IV |

### 3.2.2 Conditions for assaying the tolerance of *Rhodococcus* to organic solvents

Experiments on the tolerance of bacteria to organic solvents were conducted in 500-ml flasks containing 10 ml of the medium. The inoculum was grown overnight in acetate-malt-yeast extract (AMYE) medium, then diluted with the same medium to an initial optical density (OD) of 0.1 ($OD_{540}$). The solvents were added directly to the flasks, which were then sealed tightly with Teflon stoppers to prevent evaporation and incubated at 22 °C for 48 h on a gyratory shaker (150 rpm). Growth of the cultures was monitored by determining the increase in $OD_{540}$. When the solvent concentrations exceeded the solubility and a two-phase system developed, growth of the strains was recorded by measuring ATP. Growth was recorded as positive when the concentration of ATP in the

cultures with solvent was equal to or higher than in cultures with no solvent at $OD_{540}$ = 0.2.

The AMYE medium contained 1.0 g sodium acetate, 1.0 g malt extract and 0.5 g yeast extract per liter of KSN mineral medium. The composition of the KSN medium is described elsewhere (I).

### 3.2.3 Assay for growth in liquid culture by ATP content measurement

ATP was extracted by adding one volume of ice-cold 10% (wt/vol) trichloroacetic acid containing 4 mM ethylenediaminetetraacetic acid to the culture flask. The aqueous phase was harvested and the trichloroacetic acid removed by extraction with diethyl ether. The water phase of the resulting two-phase system was collected and bubbled with nitrogen gas to remove the dissolved diethyl ether. The ATP content was measured using a luciferin-luciferase assay with a 1243-102 ATP monitoring kit (BioOrbit, Turku, Finland). The bioluminescence was read using a BioOrbit 1253 luminometer (BioOrbit).

### 3.2.4 Hydrophobicities of cells and solvents

The cell-surface hydrophobicity was measured with a microbial adhesion to hydrocarbon (MATH) method, using hexadecane as the hydrophobic phase (Rosenberg *et al.*, 1980). The difference between the ODs of the aqueous phase before and after mixing with n-hexadecane was used to calculate the hydrophobicity as a percentage: $100 \times [1-(OD_{600}$ after mixing/$OD_{600}$ before mixing)]. Each experiment was performed in triplicate with independently growing cultures.

Log $K_{ow}$ values of the organic solvents tested as substrates were taken from the literature (Verschueren, 1983; Mackay *et al.*, 1992) or calculated using the KowWin program, version 1.66 (Syracuse Research Corporation, Environmental Science Center, North Syracuse, NY, USA).

### 3.2.5 Scanning electron microscopy

For scanning electron microscopy (SEM) and field-emission scanning electron microscopy (FESEM), 10-µl samples were taken from the aqueous phase and the organic-aqueous interface of the culture, suspended in 0.1 M phosphate buffer (pH 7.2), filtered onto Nuclepore® filters (13 mm in diameter; pore size 0.2 µm) and fixed on the filters with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. The filters were then washed three times with the same buffer, dehydrated in a graded series of ethanol (20%, 40%, 60%, 80% and 100%) and dried in hexamethyldisilazane (Fluka, Buchs, Switzerland). The filters were coated with gold-palladium and analysed with SEM using a

Zeiss DSM 962 (Jena, Germany) microscope at operating voltages of 10-20 kV and with FESEM using a Jeol JSM-6335F at an operating voltage of 15 kV.

# 4. Results and discussion

## 4.1 Isolation history of the actinobacteria studied in this thesis

The bacteria that degraded the recalcitrant compounds, strains GM-14 and GM-29, were isolated using enrichment cultures with chlorobenzene (CB) and toluene, respectively (I, V; Zaitsev *et al*., 1993). The strains originated from rice field soil that had been treated with organochlorine pesticides for several years. Strain B293 was isolated from oil-contaminated soil, using paraffin as the substrate for enrichment (Eroshina and Golovlev, 1979).

The initial identification of the isolates GM-14 and B293 was done based on morphological and limited physiological characteristics. Isolate GM-14 was originally placed in the species *Rhodococcus rhodochrous* (Zaitsev *et al*., 1993). Strain B293 was published by several authors under different names: *`Nocardia erythropolis´ (*Eroshina and Golovlev, 1979), *`Nocardia minima´* (Baryshnikova *et al.*, 1979) and *`Rhodococcus minimus´* (Baryshnikova *et al*., 1988).

Biofilms retrieved from water meters in the cities of Tampere and Helsinki (Finland) were analysed for the presence of mycobacteria (III). For isolation of slowly growing mycobacteria from environmental samples, decontamination of the specimen is necessary to reduce contamination of the mycobacterial culture by fast-growing organisms. In this study 5% $H_2SO_4$ was used for decontaminating the biofilm samples before culturing (III).

## 4.2 Characterization of the actinobacteria selected for this study

The taxonomic positions of strains GM-14, GM-29 and B293 were evaluated in a polyphasic study including the examination of phenotypic properties, nutritional characteristics, the cell wall, composition of whole cell fatty acids, menaquinones and polar lipids, determination of the G+C content of DNA and sequence analysis of the 16S rRNA gene (I, IV).

The cell morphology of strain B293 is shown in Fig. 4.1 and that of strain GM-14 in Fig. 1 (I). Strain GM-14 showed cyclic changes in morphology from cocci to branched filaments, followed by fragmentation into cocci with aging (Fig.1 in I). The rod-shaped cells of strain B293 were from 0.3 to 0.5 µm wide and 1.6 to 3.0 µm long, slightly curved and sometimes with rounded ends (Fig. 4.1).

The results of polyphasic analysis (I) showed that strain GM-14 belonged to the species *R. opacus*. Chemotaxonomic analysis and 16S rRNA gene sequencing indicated that strain B293 belonged to the genus *Gordonia* (IV). DNA:DNA hybridization showed 84% similarity to *G. polyisoprenivorans* DSM44302$^T$. Therefore, strain B293 was reassigned to the species *G. polyisoprenivorans* (IV). Strain GM-29 was identified as a member of the species *R. opacus* based on the 16S rRNA gene sequence and chemotaxonomic characteristics as follows. The strain possessed mycolic acids of the same size as the type strain of *R. opacus*. Whole cell fatty acid analysis showed that the main fatty acids of strain GM-29 were pentadecanoic acid (15:0) 8%, hexadecanoic acid (16:0) 28%, hexadecenoic acid (C16:1ω7) 18%, heptadecanoic acid (17:0) 6%, heptadecenoic acid (17:1ω8) 13%, octadecenoic acid (18:1ω9) 15%, and TBSA (10-methyl-18:0) 2%. This fatty acid profile is very close to those of the type strain of *R. opacus* and strain GM-14 (I). At the time of publication in 1999, the strain closest to GM-29 was *R. opacus* DSM 43205$^T$ with a 16S rRNA gene similarity of 99.6%. In 2002 the new species *R. wratislaviensis* was described that differed from *R. opacus* only by a DNA:DNA relatedness value of 69% and by combination of carbon-source utilization and enzymatic testing (Goodfellow *et al.*, 2002). No chemotaxonomic marker was described as discriminating between the species *R. wratislaviensis* and *R. opacus*. It remains unclear whether strain GM-29 should be named *R. opacus* or *R. wratislaviensis.*

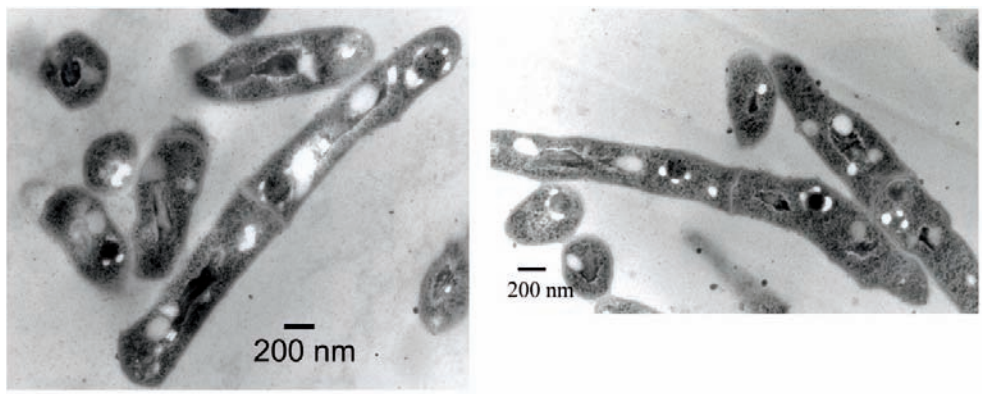



**Figure 4.1** Electron micrographs of thin sections of strain B293 grown on tryptic soy agar at 28°C for 2 days.

Strains obtained from biofilms growing in public drinking water distribution systems were subjected to sequencing of the 16S rRNA gene (from 448 to 1525 bp in length). The sequence of one isolate was identical with that of the type strain of *M. lentiflavum* ATCC 51985$^T$, those of four strains had 100% similarity to *M. lentiflavum* ATCC 51988; and one was 100% identical to *M. gordonae* ATCC 14470$^T$. One sequence (strain MH1) showed 100% identity to *Mycobacterium* sp. (AJ550515) isolated by another research group from deposits in a drinking water distribution system elsewhere in Finland (Torvinen *et al.*,

2004). The closest (99.3% similarity) sequence was that of a slowly growing pathogen of moray eels, *M. montefiorense,* related to *M. triplex* (Levi *et al*., 2003).

We evaluated automated riboprinting by the RiboPrinter® microbial characterization system as a tool for rapid identification of environmental mycobacteria. Previously, automated riboprinting was shown by us and other teams to be a useful means of identifying several genera and species (Busse *et al*., 2000; Suihko *et al*., 2006). In our laboratory a limited set of NTMs were earlier analysed (Vuorio *et al*., 1999).

The RiboPrinter system has so far no commercial database for mycobacteria. In fact, the lack of adequate databases has also hampered its use for other types of hazardous bacteria (Grif *et al*., 2003). We constructed a database of ribopatterns of selected environmental mycobacteria. As a first step the type strains of 30 mycobacterial species were analysed with two restriction enzymes *Eco*RI and *Pvu*II. All strains were successfully typed with satisfactory reproducibility of the ribopatterns with fragments ranging between 1.1 kb and 25 kb in size. Bands larger than 25 kb in size were poorly reproducible, which may be attributed to the method of biomass preparation (III).

All strains, except *M. septicum* DSM 44393$^T$, yielded ribopatterns with one or two bands after digestion with *Pvu*II (Fig. 1 in III). The type strains of *M. aichiense, M. aurum* and *M. terrae* had identical *Pvu*II ribopatterns and thus could not be separated by this enzyme. This was also found for the type strains of *M. celatum* and *M. obuense* as well as *M. abcessus* and *M. parafortuitum.* When the combined *Eco*RI and *Pvu*II ribopatterns were considered, all type strains of the 30 mycobacterial species analysed had unique patterns and were easily distinguished (Fig. 4.2), although the clustering based on ribopatterns differed from that of 16S rRNA gene sequences (Fig. 4.3).

Restriction fragments that hybridize with the ribosomal operon probe in the RiboPrinter ranged in size from 1 to 50 kb. This means that the fragments obtained represented genetic information not only within the rRNA operons, but also of flanking areas upstream or downstream of the ribosomal operons. This may explain the differences between clustering based on the 16S rRNA gene sequences and that based on the ribopatterns.

The database of 30 environmental mycobacterial species was used to analyse isolates from biofilms in the drinking water distribution systems and the clinical strains identified as *M. lentiflavum* in the Finnish Mycobacterial Reference Laboratory, National Public Health Institute, Turku, Finland. *M. chlorophenolicum* CP-2 and CG-1 earlier isolated and studied in our laboratory (Häggblom *et al*., 1988) and three strains of the species *M. murale* (Vuorio *et al*., 1999) were also included in the ribopattern analysis. A total of 60 isolates representing 30 species of slowly and rapidly growing NTMs were analysed and the ribopatterns obtained were included in our database (Fig. 1 in III).

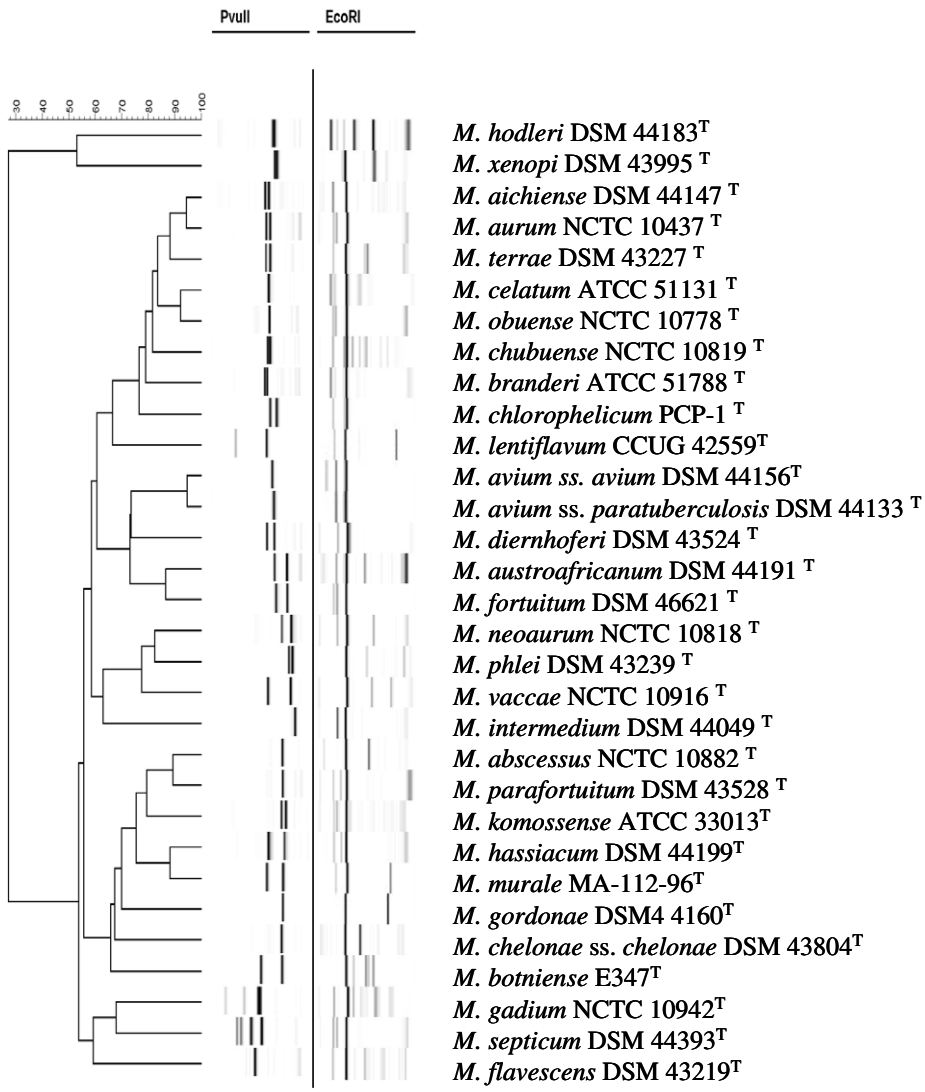



**Figure 4.2** Dendrogram generated from combined *Eco*RI and *Pvu*II ribopatterns of the type strains of 30 mycobacterial species. The percentage of similarity among strains was determined using the Pearson correlation coefficient and clustering was performed with UPGMA. The scale bar indicates the percentage of similarity.

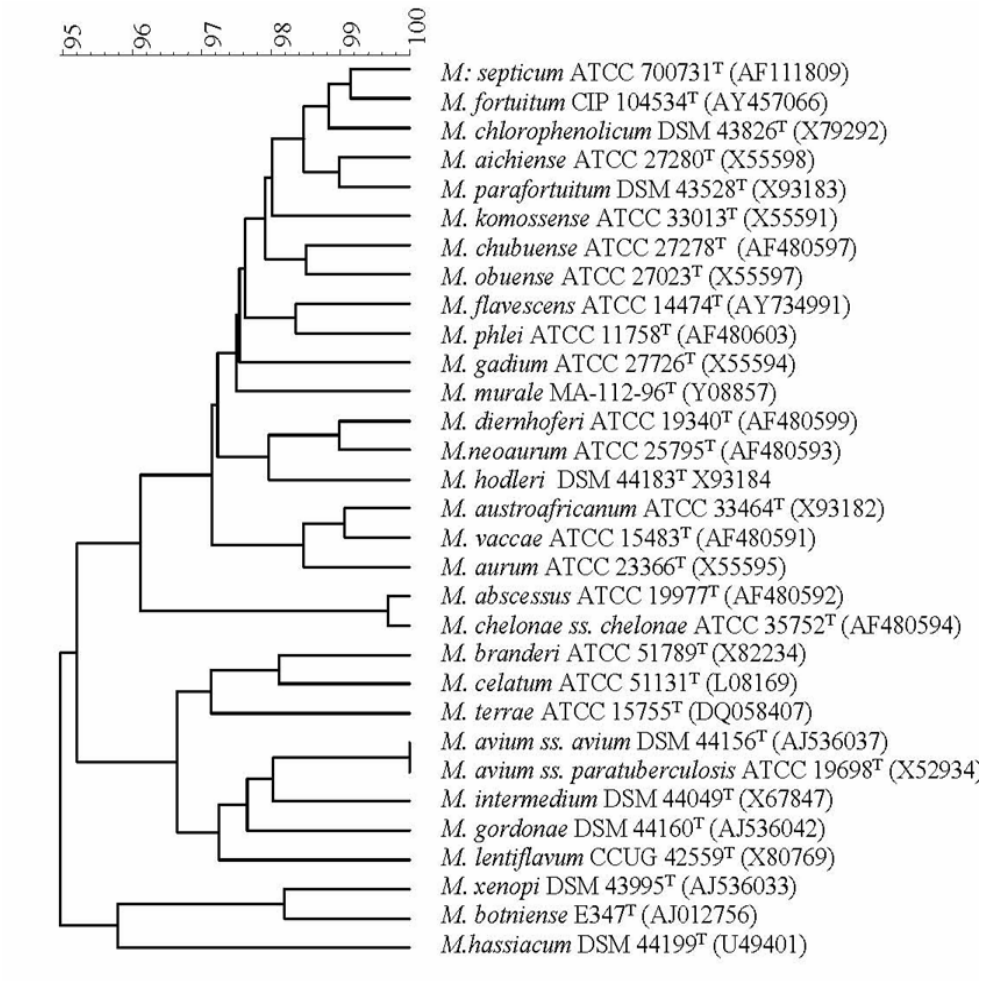



**Figure 4.3** Dendrogram comparing 16S rRNA gene sequences (1200 bp). Genebank accession numbers of the deposited sequences are given in parentheses. Clustering was performed with UPGMA.

Three strains published as *M. chlorophenolicum* (formerly *R. chlorophenolicus*) PCP-1, CP-2 and CG-1 differed in the ribopatterns. Of these strains only the 16S rRNA gene sequence of strain PCP-1$^T$ (Briglia *et al*., 1994) was available when the novel species *M. chlorophenolicum* was described (Häggblom *et al*., 1994). We found that the nearly full-length 16S rRNA gene sequences of strains CP-2 and CG-1 were only distantly related to type strain PCP-1 (in III). The strain closest to CG-1, with a similarity of 99.6%, was *Mycobacterium* sp. SM7.6.1 (AF247497), a degrader of PAHs (Friedrich *et al*., 2000). Strain CP-2 showed highest similarity (99.9%) to the mycobacterial isolate TA27 (AB028482) a degrader of 1,1,1-trichloroethane, oil and PAHs (Yagi *et al*., 1999). Thus,

the results form a framework for the further taxonomic characterization of strains CP-2 and CG-1.

Ribopattern analysis of clinical isolates named *M. lentiflavum* and deposited at the Finnish Mycobacterial Reference Laboratory showed that two strains HO184/97 and HO850/95 differed from the entire *M. lentiflavum* group. The initial identification was based on the sequencing of hypervariable region B (*E. coli* positions 430-500) of the 16S rRNA gene. However, the species *M. lentiflavum* has 100% sequence homology with hypervariable region B of the 16S rRNA gene in seven other species, including *M. simiae*, *M. palustre* and *M. triplex* (Torkko *et al*., 2002; Tortoli, 2003). As a result of the nearly full-length sequencing of the 16S rRNA genes, the strains HO184/97 and HO850/95 were assigned to the species *M. palustre* and *M. simiae*, respectively (Table 2 in III).

The clustering of the combined *Eco*RI and *Pvu*II ribopatterns of seven species that are represented by at least two strains (30 in all) is shown in Fig. 2 (III). All strains are clustered according to the identification based on 16S rRNA gene analysis. Table 4.1 contains the strains whose taxonomic position was evaluated in this study.

**Table 4.1** Strains with identification executed or clarified in this study. The nucleotide sequences of the 16S rRNA genes were compared using Blast Search on August 8, 2006 with sequences published in the NCBI.

| Strain | New and former identifications | Methods used in this study for identification | | | Source of isolation | Physiological characteristics | Described in |
|---|---|---|---|---|---|---|---|
| | | Analyses performed | Length of the 16S rRNA gene sequenced fragment | 16S rRNA gene with closest match (EMBL number, similarity %) | | | |
| 1 | *2* | 3 | 4 | 5 | 6 | 7 | 8 |
| GM-14 | *R. opacus* GM-14 (formerly *R. rhodochrous* GM-14) | Morphology, physiology, G+C mol% of DNA, mycolic acids, menaquinones, whole cell fatty acids | 1460 | DSM 43205$^T$ (X80630, 100%) | Contaminated soil | Degrader of benzene, phenol, chlorobenzene, chlorophenol | I |
| GM-29 | *R. opacus* | Mycolic acids, menaquinones, whole cell fatty acids | 1485 | DSM 43205$^T$ (X80630, 99.6%); DSM 44107$^T$ (Z37138, 99.6%)[a] | Contaminated soil | Degrader of toluene, benzene | V |
| B293 | *G. polyisoprenivorans* B293 (formerly *`Nocardia erythropolis´*, *`Nocardia minima´*, *`Rhodococcus minimus´*) | Morphology, physiology, mycolic acids, whole cell fatty acids, polar lipids, polyamines; riboprinting, DNA:DNA hybridization | 600 | DSM 44302$^T$ (Y18310, 100%) | Soil contaminated with oil | Degrader of phthalic acid esters | V |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| M2 | *M. lentiflavum* | Acid fastness, riboprinting | 488 | ATCC 51988 (X93995, 100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| M3 | *M. lentiflavum* | Acid fastness, riboprinting | 483 | ATCC 51988 (X93995, 100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| M4 | *M. lentiflavum* | Acid fastness, riboprinting | 1385 | ATCC 51988 (X93995, 100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| M6 | *M. lentiflavum* | Acid fastness, riboprinting | 1385 | ATCC 51988 (X93995, 100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| AHGA13 | *M. lentiflavum* | Acid fastness, riboprinting | 448 | ATCC 51985$^T$ (X80769, 100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| MH1 | *Mycobacterium* sp. | Acid fastness, riboprinting | 1360 | *Mycobacterium* sp. SA394 (AJ550515, 100%), *M. montefiorense* ATCC BAA-256$^T$ (AF330038, 99.3%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| AGHA3 | *M. gordonae* | Acid fastness, riboprinting | 1525 | ATCC 14470$^{T}$ (100%) | Biofilm in drinking water distribution system | Resistance to multiple antibiotics | III |
| H0184/97 | *M. palustre (M. lentiflavum* | Riboprinting | 1260 | E846$^{T}$ (AJ308603, 100%) | Clinical specimen | Resistance to multiple antibiotics | III |
| H0850/95 | *M. simiae (M. lentiflavum)* | Riboprinting | 1350 | ATCC 25275 $^{T}$ (100%) | Clinical specimen | Resistance to multiple antibiotics | III |
| CG-1 | *Mycobacterium sp. (M. chlorophenolicum)* | Acid fastness, riboprinting | 1400 | *Mycobacterium* sp. SM7.6.1 (AF247497, 99.6%) | | Degrader of PCP | III |
| CP-2 | *M. chlorophenolicum* | Acid fastness, riboprinting | 1400 | *Mycobacterium* sp. TA27 B028482, 99.9%) | | Degrader of PCP | III |

a- the species *R. wratislaviensis* was described in 2002 to accommodate the strain of *Tsukamurella wratislaviensis*.

## 4.3 Conclusions and comments on the isolation and identification of the selected actinobacteria

Many species of the genera *Rhodococcus*, *Gordonia* and *Mycobacterium* play important roles in the biotechnology of bioremediation and synthesis of various organic compounds. At the same time many species are opportunistic pathogens in humans and animals. Before these organisms are used in any biotechnical applications, risk assessment for these strains should be applied. Biohazard legislation is based on genus and species names; therefore, reliable identification is essential to assess the safety status. The polyphasic approach used in the present study, which was based on chemotaxonomic analysis, 16S rRNA gene sequencing and DNA:DNA hybridization, helped to clarify the taxonomic positions for many strains isolated from polluted soils or biofilms in water. Strains GM-14 and GM-29, degraders of monoaromatic compounds, belong to the species *R. opacus*. This species is included in Hazard Group 1 in the European Union classification and to our knowledge has not yet been reported from clinical specimens. A third degrader (strain B 293) used for many years under different names, was identified as *G. polyisoprenivorans.* This species is listed under Hazard Group 1. Nevertheless, two cases of bacteraemia caused by *G. polyisoprenivorans* were recently reported (Kempf *et al*., 2004; Verma *et al* 2006). In accounting the novelty of this species and the difficulties in identifying *Gordonia* in clinical laboratories, the number of *G. polyisoprenivorans*-associated infections may currently be underestimated. The present study provides examples of the importance of correctly identifying bacteria with biotechnological potential.

The numbers of NTM patient isolates in Finland in the National Mycobacterial Reference Laboratory during 1996-2003 ranged from 288 to 379 per year (Table 1 in III). Although water was suggested as a source of potentially pathogenic environmental mycobacteria, especially *M. avium* (Vaerewijck *et al*., 2005), the reservoirs of most NTMs are still unclear. In the present study we isolated several strains of *M. lentiflavum* from biofilms growing in water meters of public drinking water distribution systems. Decontamination of environmental samples during the isolation of pure cultures aiming at removing most of the contaminants may have resulted in loss in numbers and species diversity of mycobacteria as well (Buijtels and Petit, 2005; Vaerewjik *et al*., 2005). The isolation procedure and cultivation parameters also select for certain organisms. Sulphuric acid decontamination increased the amount of *M. lentiflavum* recovered from clinical specimens in comparison to treatment with NaOH-N-acetyl cystein (Buijtels and Petit, 2005). Thus, other species of slowly growing mycobacteria in biofilm samples may have been lost due to the decontamination protocol used here.

The *M. lentiflavum* found in this study in biofilms formed in water meters, as well as the recent report by Torvinen with coauthors (2004), point out that *M. lentiflavum* is deposited in drinking water networks in Finland. Thus, drinking water calls for attention as a potential source of opportunistic pathogens.

Accurate identification of mycobacteria from environmental sources is one of the steps necessary for tracing their environmental reservoir. The rapid and reliable identification of mycobacterial isolates poses a challenge, mostly due to the slow growth of the organisms. There are a number of identification methods useful for NTMs, including 16S rRNA gene sequencing and several commercial systems based on DNA hybridization with specific probes (Wagner and Young, 2004). However, none of the currently available methods alone can distinguish between all mycobacterial species. The other approach for identifying bacteria in environmental samples is direct DNA amplification followed by cloning and sequencing. This approach requires time-consuming sequence analysis. Moreover, some species of NTMs cannot be distinguished by sequencing 16S rRNA, or the *hsp*65 or *sod*A genes (Tortoli, 2003; Adekambi and Drancourt, 2004).

In the present study we showed that automated riboprinting, when using two restriction enzymes, was a useful means for identifying environmental and clinical isolates of *M. lentiflavum*. Moreover, the ribopattern analysis of several other environmental NTMs showed the necessity for clarifying the taxonomic position of several *M. chlorophenolicum* strains. Thus, automated riboprinting may constitute a simple and rapid procedure for the differentiation of environmental mycobacteria.

## 4.4 Degradation and surviving properties of selected strains of *Rhodococcus, Mycobacterium* and *Gordonia*

### 4.4.1 Degradation of aromatic compounds by strains of *R. opacus*, *G. polyisoprenivorans* and *M. murale*

The degradation capacities of *R. opacus* GM-14 and GM-29 isolated from contaminated soil, *G. polyisoprenivorans* B293 isolated from an oil field and two strains of *M. murale* isolated from a children's day care centre (Andersson *et al*., 1997; Vuorio *et al*., 1999) were assessed (I, II, IV). *R. opacus* GM-14 used a wide range of aromatic substrates for growth, including benzene, 15 different halobenzenes, 18 phenols and 7 benzoates (Table 3 in I). Toluene, aniline and the four PAH compounds checked (biphenyl, anthracene, phenanthrene and pyrene) were not utilized as substrates for growth. The suitability of various halogenated substrates for growth was dependent on the substituents in the aromatic ring. Of the five dichlorophenol (diCP) isomers, only 2,4-diCP supported growth of strain GM-14. Of the dichlorobenzenes (diCBs) two isomers (1,3- and 1,4-diCB) but not 1,2-diCB, were utilized by the strain. Strain GM-14 grew on all three isomers of monoCPs; the highest concentration of 2-CP and of 4-CP supporting growth of strain GM-14 was 0.25 g $l^{-1}$. 3-CP was more toxic and the organism grew only when this substrate was added to a concentration not higher than 0.1 g $l^{-1}$ (Fig. 9 and Table 4 in I). The amount of chlorine ions released into the medium during growth on all isomers of monoCPs was 100% of the theoretical level (Fig. 10 in I), indicating mineralization of the organically bound chlorine.

Strain *R. opacus* GM-14 grew at high concentrations of benzene and CB. As shown in Fig. 4 of (I) and in Table 4 (I), strain GM-14 grew well in mineral medium in which benzene or CB were added in concentrations of 3 g $l^{-1}$ or 0.5 g $l^{-1}$, respectively, when the air-to-liquid ratio in the flask was 4:1. When the air-to-liquid ratio was increased up to 94:1 the concentrations of benzene and CB tolerated by the strain were as high as 13 and 3 g $l^{-1}$, respectively (Figs. 5 and 6 in I).

The other strain of *R. opacus* studied for its ability to utilize toxic aromatic solvents, GM-29, grew after a lag period of 4 days on toluene or benzene added as the sole carbon sources to mineral salts medium KSN in amounts of up to 7 g $l^{-1}$. In the batch culture experiments (50 ml of KSN medium in 2-l gastight flasks closed with Teflon-lined rubber stoppers), addition of 7 g $l^{-1}$ of toluene or benzene resulted in initial aqueous concentrations of 470 and 650 mg $l^{-1}$, respectively. The solubilities of toluene and benzene in the KSN medium, as checked by GC, were 517 mg $l^{-1}$ and 1560 mg $l^{-1}$, respectively. These data show that *R. opacus* GM-29 grew in KSN media that were >90 % saturated with toluene and 41% saturated with benzene.

A number of bacteria, mainly *Pseudomonas* spp., capable of growing in high concentrations of toluene (50-90%) were reported (Ramos *et al*., 2002). Members of the genera *Rhodococcus* and *Mycobacterium* can grow on toluene as the carbon source, when provided at low concentrations or via a gas phase (Burback *et al*., 1994; Duetz *et al*., 2001; Malachowsky *et al*., 1994; Tay *et al*., 1998). Recently rhodococcal strains that can grow in high concentrations of benzene or toluene were described (Paje *et al*., 1997; Na *et al*., 2005). *Rhodococcus* sp. 33 grew in medium saturated with benzene (Paje *et al*., 1997). *R. opacus* strain B-4 utilized many aromatic and aliphatic substrates: benzene, toluene, styrene, xylene, propylbenzene, n-octanol and n-decane. It was reported to grow when liquid benzene was added to the basal medium at 10-90% (Na *et al*., 2005). However, when the strain was cultivated in liquid medium with 10% benzene, the increase in OD from less than 0.1 up to 1.5 corresponded to only a 4-fold increase in the cell number (from $1 \times 10^7$ to $4 \times 10^7$ cfu $ml^{-1}$), probably from emulsification of the solvent.

*G. polyisoprenivorans* B293 was studied for its ability to utilize phthalic acid esters for growth (IV). Strain B293 grew on benzyl-butyl phthalate, di-butyl phthalate, di-benzyl phthalate and di-n-octyl phthalate, but not on the branched phthalate esters bis-(2-ethyl)-hexyl phthalate, di-isoheptyl phthalate or di-isononyl phthalate. The degradation proceeded through hydrolysis of the ester bonds. The organism grew using the carbon chains of the alcohols octanol, butanol and benzyl alcohol released by hydrolysis. The phthalic acid not utilized for growth accumulated as a dead-end product (IV).

The species *G. polyisoprenivorans* has so far only been described as degrading rubber. The ability to degrade phthalate esters is an additional property that we showed here for the species. A clinical isolate of *G. polyisoprenivorans* was reported capable of degrading latex and synthetic cis-1,4-polyisoprene (Kempf *et al*., 2004). These authors associated the ability of the strain to degrade the natural rubber with the ability to colonize catheters. Our isolate degraded the phthalate diesters used as softeners in polyvinyl chloride (PVC)

plastics, including those used for medical purposes. The ability to utilize phthalate esters for growth, together with high level of hydrophobicity of the cell surface, may promote colonization of medical plastic devices resulting in increased infection risk in the patient.

The strains of *M. murale* were isolated from the water-damaged wall material in a children's day care centre it amounts of $10^6$ cfu $g^{-1}$ (II). We hypothesized that the ability to use toluene, or other components of the cleansing formula, used at that site, for growth may explain the abundance of this organism. Growth of two strains of *M. murale* in toluene as a sole carbon source was checked in two different systems: toluene was added directly to the KSN medium or was supplied as a vapour. We found that strain MA-112 was able to grow when toluene was directly added in to KSN medium (II). At the same time, another strain (MA-168) isolated from the same water-damaged wall did not grow on toluene. Thus, factors other than simple utilization of toluene for growth must exist to explain the high level of colonization of the water-damaged wall by *M. murale*. It is possible that *M. murale* may utilize another compound used in the cleansing formulas.

### 4.4.2 Tolerance of *R. opacus* strains to water-miscible and water-immiscible organic solvents

The ability of *R. opacus* GM-14 and GM-29 to grow in rich media in the presence of organic solvents was investigated. Growth of the strains in the presence of 1-10 vol% of the organic solvents was tested in AMYE medium (Table 4.2). The hydrophobicities of the tested solvents, expressed as log $K_{ow}$ values, ranged from -0.66 to 9.44. Strains GM-29 and GM-14 grew in the presence of 18 and 19 (1 vol%) of the 39 organic solvents tested , respectively. The solvents tolerated included those commonly used in cleansing formulas and disinfectants (trichloroethane, ethanol, acetone, 2-propanol, ethanol and methanol) (Russell *et al*., 1999). Both strains grew in AMYE medium in which 10 vol% of n-pentane, ethanol, methanol, hexadecane, 1-chlorotetradecane or 1-chlorooctadecane were added. Several authors have suggested that the tolerance of microorganisms to solvents correlates with the log $K_{ow}$ values of the solvents. For instance, all solvents tested with log $K_{ow}$ values < 2.3-2.4 were reported as toxic for *Pseudomonas* strains in liquid culture (Cruden *et al*., 1992; Inoue and Horikoshi, 1989, 1991; Ogino *et al*., 1995). Some *Rhodococcus* strains did not grow on solid medium in the presence of solvents with log $K_{ow}$ values < 6.0 (Inoue and Horikoshi, 1991). In our study no correlation between the log $K_{ow}$ values of the solvents and the level of tolerance of *R. opacus* strains was seen. Our results support the conclusions of other authors that in a two-phase liquid system, bacterial growth inhibition caused by solvents is not always correlated with the log $K_{ow}$ value (Vermuë *et al*, 1993; Aono *et al*., 2001).

**Table 4.2** Growth of *R. opacus* GM-29 and GM-14 in AMYE medium in the presence of organic solvents.

| Solvent | Log $K_{ow}$ | Density (g $ml^{-1}$) | Solubility in water (mg $l^{-1}$) | Growth of the strain with solvents (vol%): | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Strain GM-14 | | | Strain GM-29 | | |
| | | | | 1.0 | 5.0 | 10.0 | 1.0 | 5.0 | 10.0 |
| 1-Chlorooctadecane | 9.44[c] | 0.86 | na[d] | + | + | + | + | + | + |
| 1-Chlorotetradecane | 7.47[c] | 0.87 | na[d] | + | + | + | + | + | +* |
| n-Heptane | 5.0[a] | 0.68 | 2.9[a] | + | - | - | + | + | - |
| n-Hexane | 4.11[a] | 0.66 | 9.5[a] | + | + | - | + | + | - |
| n-Pentane | 3.45[a] | 0.63 | 38.5[a] | + | + | + | + | + | + |
| Cyclohexane | 3.44[a] | 0.78 | 5.5[a] | + | - | - | + | - | - |
| 1-Hexene | 3.39[a] | 0.67 | 50.0[a] | + | + | - | + | + | - |
| 1,1,1-Trichloroethane | 2.49[a] | 1.34 | 1,494[a] | + | - | - | + | - | - |
| Chloroform | 1.97[a] | 1.49 | 8,200[a] | + | - | - | - | - | - |
| Nitrobenzene | 1.85[b] | 1.2037 | 1,900[b] | + | - | - | + | - | - |
| Dichloromethane | 1.25[a] | 1.33 | 13,200[a] | + | - | - | + | - | - |
| MTBE | 0.94[a] | 0.74 | 42,000[a] | + | - | - | + | - | - |
| n-Butanol | 0.88[b] | 0.81 | 77,000[b] | + | - | - | + | - | - |
| Ethylacetate | 0.66[b] | 0.90 | 79,000[b] | + | - | - | + | - | - |
| n-Propanol | 0.34[b] | 0.80 | ∞ | + | - | - | + | - | - |
| 2-Propanol | -0.16[b] | 0.80 | ∞ | + | - | - | + | + | - |
| Acetone | -0.24[b] | 0.79 | ∞ | + | + | - | + | + | - |
| Ethanol | -0.32[b] | 0.79 | ∞ | + | + | + | + | + | + |
| Methanol | -0.66[b] | 0.79 | ∞ | + | + | + | + | + | + |

[a] Data taken from Mackay et al., 1992

[b] Data taken from Verschueren, 1983

[c] log $K_{ow}$ calculated using KowWin program (version 1.66)

[d] na; not available

No growth in the presence of 1 vol% of nonylphenol (5.76[c]), n-nonane (5.65[a]), 1-chlorodecane (5.51[a]), n-octane (5.15[a]), o-, m- or p-xylene (3.15, 3.20 or 3.18[a]), n-octanol (3.15[b]), ethylbenzene (3.13[a]), tetrachloroethene (2.88[a]), chlorobenzene ( 2.80[a]), toluene (2.69[a]), trichloroethene ( 2.53[a]), n-heptanol (2.41[b]), benzene (2.13[a]), n-hexanol (2.03[b]), 1,2-dichloroethane (1.48[b]), n-pentanol (1.40[b]), aniline (0.90[b]), and morpholine (-1.08[b])

## 4.5 Growth of *R. opacus* and *G. polyisoprenivorans* in biphasic liquids

We observed that strains GM-14 and GM-29 formed clumps of cells when grown in liquid media in the presence of hydrophobic organic solvents with a two-phase system (water-organic solvent). When strain GM-29 was cultivated in AMYE medium in the presence of 10 vol% of chlorotetradecane (log $K_{ow}$ 7.47), practically all cells accumulated in the organic-aqueous interface. Clump development was the reason for using the ATP content for measuring growth (Table 4.2). The cell surface of both strains GM-14 and GM-29 was highly hydrophobic (86-88%). The cell hydrophobicity was not dependent on culture conditions and remained the same regardless of whether the cultures were pregrown in AMYE medium with no solvent added or in KSN medium supplied with benzene, phenol or toluene (only for GM-29). We propose that the clumpy growth may be associated with the high level of cell wall hydrophobicity that resulted in migration of the cells from the aqueous to the organic phase.

SEM was performed to assess whether the observed clumps were the result of accumulation of dead debris or represented living cell agglomerates. The SEM revealed that the cells were in flocks tightly adhered to one another (Fig. 4.4 A, B). The flocculated cells had smooth surfaces (Fig. 4.4 C) and were morphologically similar to those grown in the same medium in the absence of solvent (Fig. 4.4 D).

When strain GM-29 was grown in mineral KSN medium with toluene as the sole carbon source in amounts of 7 g $l^{-1}$, the biomass mostly floated on the surface of the culture medium. There were more cells per 10-µl sample taken from the film floating on the aqueous medium surface (Fig. 4.5 A) than in that taken from the submerged aqueous phase (Fig. 4.5 C). When viewed at a magnification of 50,000×, the cells from the aqueous phase containing 470 mg $l^{-1}$ of dissolved toluene appeared wrinkled (Fig. 4.5 D). The cells harvested from the toluene-water interface had bleb-like structures visible on the surface (Fig. 4.5 B). Several authors observed similar appearances of blebs on the surface of *M. tuberculosis* and *M. smegmatis* treated with membrane-active antimicrobial peptides (Miyakawa *et al*., 1996; Stenger *et al*., 1998), or isoniazid (Vilcheze *et al*., 2000) or as the results of aging and/or nutrient depletion (Dahl, 2004). Similar change in morphology of *R. opacus* was observed under conditions of water stress (Alvarez *et al*., 2004).

When toluene was added in an amount of 1.5 vol% exceeding the aqueous solubility in KSN medium, the culture fragmented into short forms (Fig. 4.6 A). The cell surfaces were rough and the cells appeared to be collapsing (Fig. 4.6 B). The cells appeared extensively covered with blebs that may present fragments from damaged cells, indicating that toluene at high concentrations caused alteration of the cell envelope.

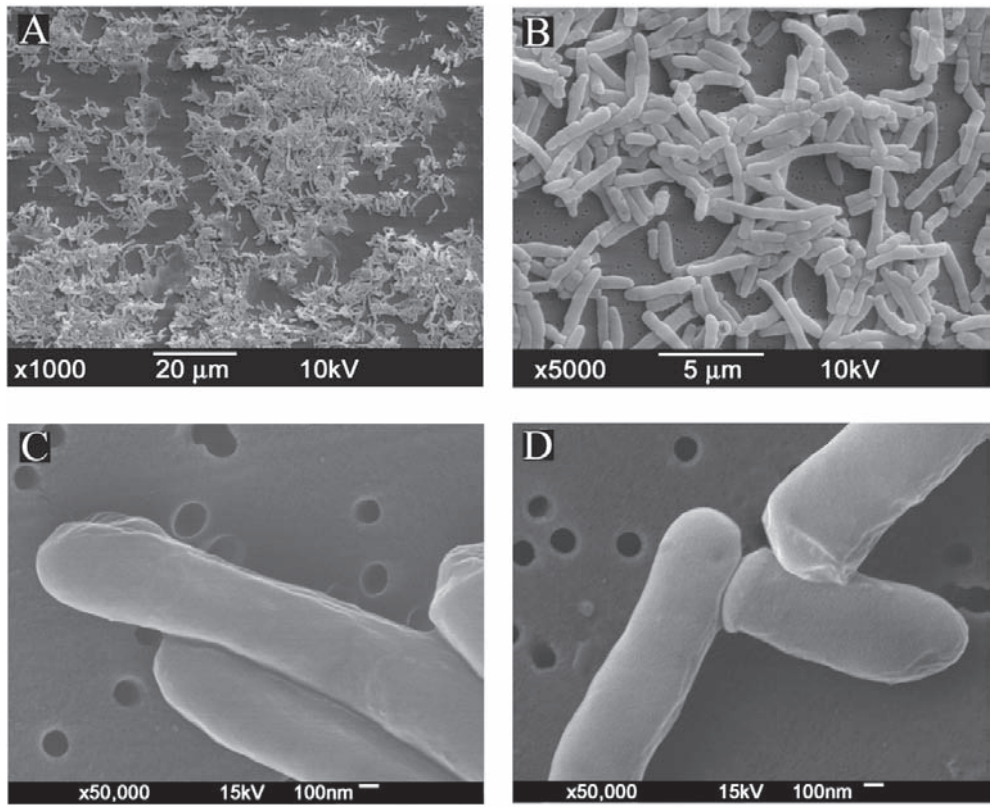



**Figure 4.4** SEM and FESEM micrographs of *R. opacus* GM-29 grown in AMYE medium in the presence of 10 vol% of chlorotetradecane.
Micrographs at magnifications of 1,000× and 5,000× (A, B) were taken with SEM; those at magnification of 50,000× (C, D) with FESEM. Panels A, B, and C show samples taken from organic solvent-water interface. Panel D shows cells from AMYE medium with no organic solvent. The FESEM pictures were taken by Dr. K. Lounatmaa.

We observed clumpy growth of *G. polyisoprenivorans* B293 on 1 g $l^{-1}$ of benzyl-butyl phthalate (log $K_{ow}$ 4.84 ) or di-n-octyl phthalate (log $K_{ow}$ 8.54). In 5 days of cultivation almost all cells were in tight agglomerates (Fig. 1 in IV). Epifluorescence microscopy showed that the cells of B293 grew attached to droplets of the phthalate ester (Fig. 2 in IV).

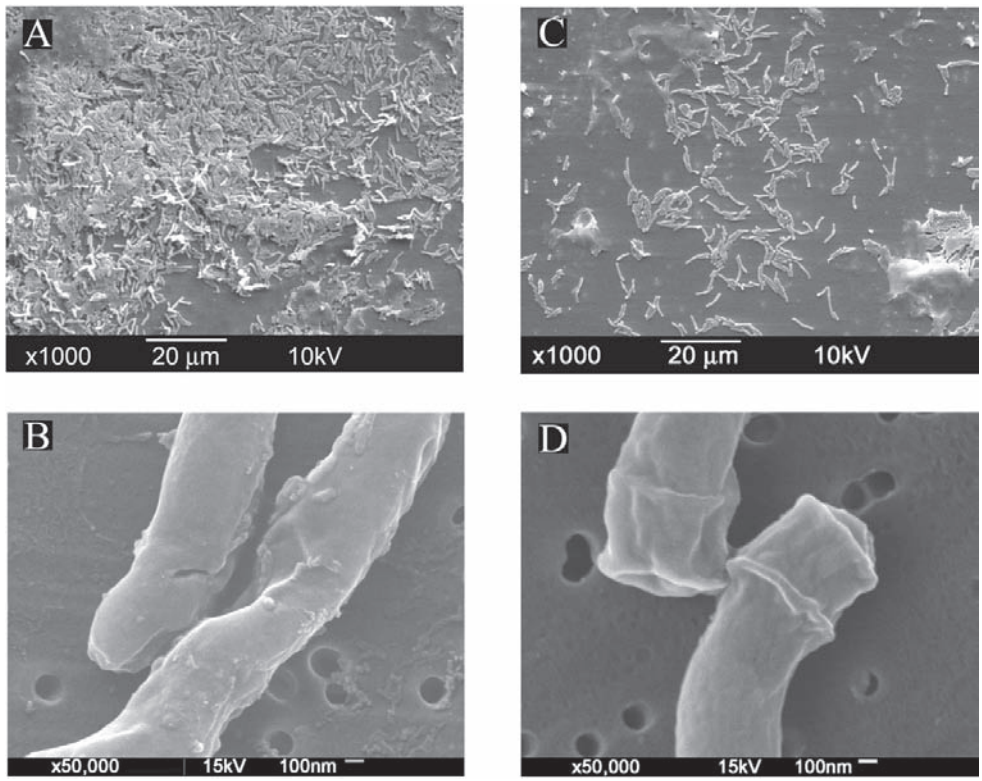



**Figure 4.5** SEM and FESEM micrographs of *R. opacus* GM-29 grown in KSN medium with toluene as the sole carbon source.
Micrographs at 1,000× and 5,000× magnifications (A, C) were taken with SEM; those at magnification of 50,000× (B, D) with FESEM. Panels A and B show samples taken from the toluene-aqueous interface of a culture with 7 g $l^{-1}$ of toluene (the concentration of toluene in the aqueous phase was 470 mg $l^{-1}$). Panels C and D show samples taken from the aqueous phase of the same culture. The FESEM pictures were taken by Dr. K. Lounatmaa.

From the results presented above we draw the following conclusions. *R. opacus* and *R. polyisoprenivorans* strains were able to degrade resistant aromatic compounds at high concentrations. *R. opacus* was able to grow on a wide range of aromatic, including halogenated, compounds. The strains of *R. opacus* and *G. polyisoprenivorans* tended to grow on hydrophobic substrates by direct attachment to the liquid-liquid interphase. The ability of *R. opacus* GM-14 and GM-29 to grow in the presence of high concentrations of organic solvents and to form aggregates indicates that *R. opacus* is potentially able to degrade solvent-containing technical products and to biofoul instruments and surfaces.

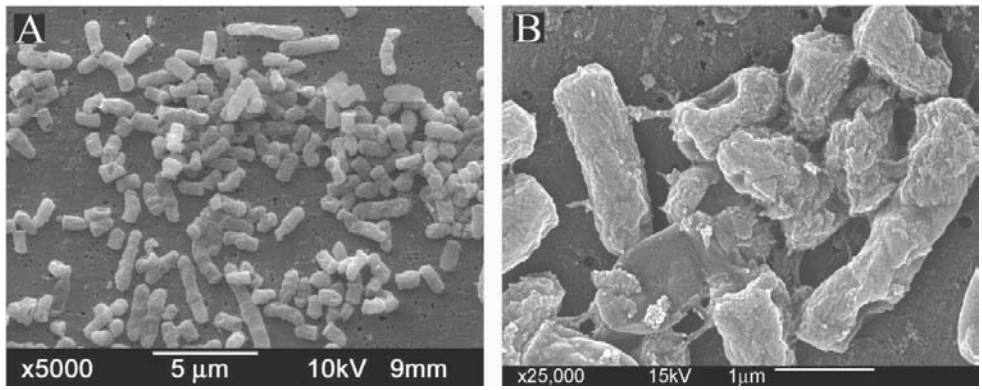



**Figure 4.6** SEM and FESEM micrographs of *R. opacus* strain GM-29 cells adhered to toluene-water interface from the culture where aqueous (mineral medium) phase was saturated with toluene (1.5 vol% toluene added). Micrographs were taken with SEM with 5,000× magnification (A) and with FESEM (B) using 25,000× magnification. The FESEM pictures were taken by Dr. K. Lounatmaa.

## 4.6 Toxic compound-induced changes in whole cell fatty acids of *R. opacus* GM-14 and GM-29

We attempted to determine the mechanisms of adaptation of *R. opacus* to toxic aromatic substances (V). The experiments were designed to observe any changes in the cells of *R. opacus* strains GM-14 and GM-29 in response to a challenge with phenol, CP, benzene or toluene (V). The relative proportion of saturated fatty acids in the whole cell fatty acids of *R. opacus* GM-14 and GM-29 increased when the bacteria grew on the aromatic compounds, as compared with cells grown on fructose (Figs. 4.7 and 4.8; Table 1 in V). The increase in relative amounts of saturated fatty acids observed in cells of strain GM-14 grown on phenol or 4-CP, compared with those grown on fructose, was mainly due to the increased abundance of 10-methyl-branched fatty acids (from 2.4% to 15.4% or 24.1%). The increase in contribution of 10-methyl-branched fatty acids from 2.4% (fructose) to 24.1% (4-CP) was mainly by TBSA (10-methyl-octadecanoic) (Table 1 in V). The relative amount of straight-chain saturated fatty acids remained constant: 48.8%, 49.9% or 48.5%, in cells grown on fructose, phenol or 4-CP, respectively. When the cells of strain GM-14 were grown on benzene or CB, the relative amounts of both straight-chain and of 10-methyl-branched saturated acids increased compared with cells grown on fructose (Fig. 4.7, Table 1 in V). Unlike the cells of strain GM-14 there was an increase in the amount of straight-chain-saturated acids in the cells of GM-29 grown on phenol compared with those grown on fructose (from 42.9% to 51.1%). When GM-14 was cultivated in various concentrations of phenol, there was a dose-related increase in abundance of saturated fatty acids (Fig. I A in V). This increase was mostly due to TBSA.

Similar dose-related increases in TSBA were observed in the cells of strain GM-14 grown in the presence of toluene (Fig.1 B in V).

These results show that fatty acid alteration, particularly the increase in TBSA, is associated with the adaptation of *R. opacus* strains GM-14 and GM-29 to the presence of hydrophobic toxic compounds. Gutierrez *et al*. (1999) proposed that increase in the abundance of saturated fatty acids in cells of *Rhodococcus* sp. 33 grown in the presence of benzene was a possible mechanism for tolerating benzene. These authors found hexadecenoic acid (16:1ω6*cis*), witch is uncommon in *Rhodococcus,* but known to occur in some methanotrophs (Bowman *et al*., 1993), *M. phlei* (Suutari and Laakso, 1993) and *Bacillus* spp. (Moss and Daneshvar, 1992). We observed an increase in 16:1ω7*trans* in the cells of *R. opacus* GM-14 grown on phenol (8.3%) and 4-CP (10.8%) compared with those grown on fructose (4.8%). This fatty acid was analysed with GC-MS and identified as hexadecenoic acid, based on its mass spectrum. It was identified as the *trans* form of hexadecenoic acid according to its retention time, using the *cis* and *trans* isomers as standard compounds.

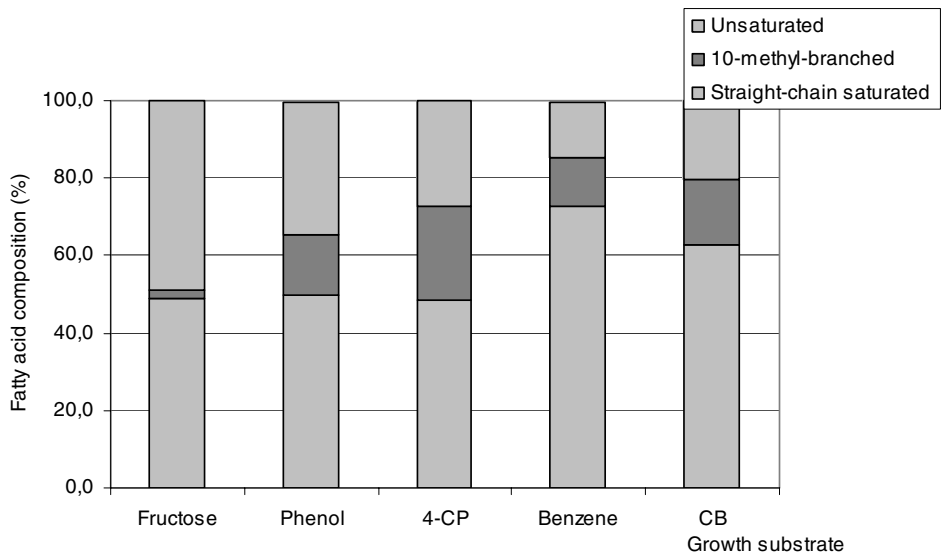



**Figure 4.7** Histogram showing the relative amounts of the three major classes of fatty acids (straight-chain saturated, 10-methyl-branched saturated and unsaturated) in whole cell fatty acid composition of *R. opacus* GM-14 grown in KSN medium supplemented with fructose (1 g $l^{-1}$), phenol (0.5 g $l^{-1}$), 4-CP (0.1 g $l^{-1}$), benzene (1 g $l^{-1}$), and CB (0.5 g $l^{-1}$) as carbon sources.

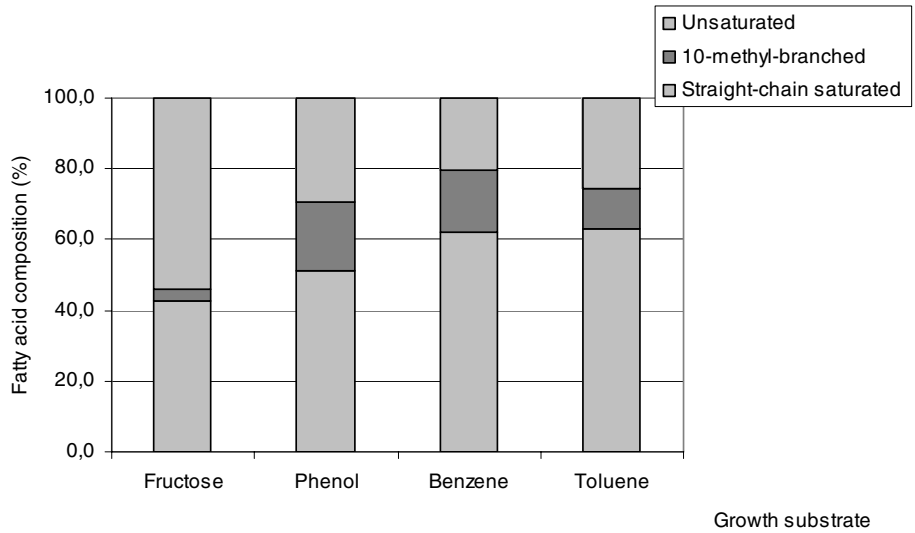



**Figure 4.8** Histogram showing the relative amounts of the three major classes of fatty acids (straight-chain saturated, 10-methyl-branched saturated and unsaturated) in whole cell fatty acid composition of *R. opacus* GM-29 grown in KSN medium supplemented with fructose (1 g $l^{-1}$), phenol (0.5 g $l^{-1}$), benzene (1 g $l^{-1}$), and toluene (1 g $l^{-1}$) as carbon sources.

The results of this study indicate a role played by fatty acids, particularly 10-methyl-branched TSBA, in adaptation of the cells of *R. opacus* to toxic aromatic compounds.
An increase in 10-methyl-branched fatty acids was also observed as a response of *M. phlei* and *M. murale* to increase in growth temperature (Suutari and Laakso, 1993; Vuorio *et al*., 1999). Since hydrophobic solvents cause an increase in membrane fluidity, adjusting the degree of saturation of the cellular fatty acids plays a role in reduction of fluidity that in term result in lower permeability of toxic compounds into the cell (Sikkema *et al*., 1995; Ramos *et al*., 2002). The role of TBSA in such adaptation is presently unclear. Methylation of *cis*-unsaturated fatty acids to the corresponding, more stable 10-methyl-branched acids, may help in maintaining a functional membrane under stressful conditions, such as ageing (Dhariwal *et al*., 1976; Kroppenstedt and Kutzner, 1976; Hallas and Vestal, 1978). An increase in the total amount of phospholipids and lipoarabinomannan, which contain TBSA, may also be one of the explanations.

# 5. Conclusions

The following conclusions summarize the main findings of this work:

1. The taxonomic positions of strains isolated for their ability to grow on monoaromatic compounds and previously published strains were clarified in a polyphasic approach. Strains GM-14 and GM-29, isolated from contaminated soil as degraders of benzene and toluene, were identified as *Rhodococcus opacus*. The position of strain B 293, published earlier under different names, was clarified as a member of the species *Gordonia polyisoprenivorans*.

2. Potentially hazardous bacteria were shown as significant parts of biofilms in man-made environments. Several species of slowly growing mycobacteria were isolated from biofilms growing in water meters of public drinking water distribution systems, including *Mycobacterium gordonae* and *M. lentiflavum,* a species that is recognized as an important emerging pathogen. *M. murale* colonized the indoor walls of water-damaged buildings. *G. polyisoprenivorans* B293 from contaminated soil utilized the phthalate esters used as PVC softeners, showing its potential to form biofilm on medical plastics.

3. Automated riboprinting with two restriction enzymes, *Eco*RI and *Pvu*II, was a useful means for rapid characterization of several species of environmental mycobacteria, particularly *M. lentiflavum*. The database containing the *Eco*RI and *Pvu*II ribopatterns of 60 strains representing 32 species of slowly and rapidly growing environmental mycobacteria was constructed.

4. *R. opacus* strains GM-14 and GM-29, *G. polyisoprenivorans* B293 and *M. murale* degraded a number of toxic organic solvents and other resistant compounds. Strain GM-14 used as the sole source of carbon and energy a wide range (48 compounds) of aromatic substrates, including 15 different mono- and dihalogenated benzenes and 11 halogenated phenols. During growth of strain GM-14 on CB, 1,3-diCB, and all isomers of monoCP, stoichiometric amounts of chloride were released. *R. opacus* GM-29 and one strain of *M. murale* degraded toluene. *G. polyisoprenivorans* B293 used four phthalic acid esters (benzyl-butyl phthalate, di-butyl phthalate, di-benzyl phthalate and di-n-octyl phthalate) as the sole carbon sources. The organism grew at the expense of the alcoholic side chains, accumulating phthalic acid as the dead-end product.

5. This study revealed interesting characteristics of *R. opacus* GM-14 and GM-29 with respect to their tolerance to high concentrations of aromatic compounds. The strains grew in liquid medium in which organic solvents were added in excess of their solubility in

water. Strain GM-14 grew in aqueous solutions saturated with benzene or CB, 13 and 3 g $l^{-1}$, respectively. Strain GM-29 grew in KSN medium supplemented with 7 g $l^{-1}$ of liquid toluene or benzene as the sole carbon source, corresponding to aqueous concentrations of 470 and 650 mg $l^{-1}$, respectively. *R. opacus* GM-14 and GM-29 tolerated the presence in rich media of 19 different organic solvents at concentrations of 1%. Both strains grew when 10 vol% of n-pentane, ethanol, methanol, hexadecane, 1-chlorotetradecane, or 1-chlorooctadecane were added to the liquid medium. In biphasic water/organic solvent systems, *R. opacus* GM-14, GM-29 and *G. polyisoprenivorans* B23 grew in water-solvent interfaces.

6. *R. opacus* GM-14 and GM-29 adapted to the presence of hydrophobic compounds in their growth environment by changing the lipid structure of their cell envelope. The most remarkable was change in the amount of TBSA from 2.4% in GM-14 grown on fructose to 24.1% when grown on 4-CP. The content of this 10-methyl-branched fatty acid in GM-14 increased in a dose-related manner in response to phenol or toluene in the medium even though toluene was not utilized for growth. The results suggest that the cell envelope lipids that contain 10-methyl branched-fatty acids are involved in the adaptation of *R. opacus* to membrane-damaging compounds.

# 6. Acknowledgements

This thesis work was carried out in the Department of Applied Chemistry and Microbiology, Division of Microbiology of University of Helsinki. The study was funded by the SUNARE project Academy of Finland 52798, the centre of Excellence project (Academy of Finland 53305) and the Finnish Graduate School in Environmental Science and Technology (EnSTe).

I wish to express my gratitude to my supervisor Prof. Mirja Salkinoja-Salonen. The years under her supervision have been a priceless learning experience.

Special thanks go to Dr. Gennadi Zaitsev who introduced me to the fascinating world of rhodococci.

I wish to acknowledge reviewers Dr. Anja Veijanen and Doc. Merja Kontro for their valuable comments on my thesis.

I am grateful to my co-authors, Dr. Terhi Ali-Vehmas, Dr. Maria Andersson, Prof. Georg Auling, Prof. Hans-Juergen Busse, Prof. Eugenii L. Golovlev, Prof. Rainer M. Kroppenstedt, Prof. Anatolii G. Lobanok, Dr. Outi Priha, Riitta Rahkila, Prof. Fred A. Rainey, Prof. Salkinoja-Salonen, Dr. Hanna Soini, Dr. Zewdu Terefework, Dr. Jussi Uotila, Dr. Riitta Vuorio, and Dr. Gennadi Zaitsev for sharing their expertise.

I would like to thank the present and past colleagues in MSS group and the Microbiology Division for an enjoyable working atmosphere. Special thanks goes to Camelia C., Douwe H., Hannele T., Irmgard S, Jouni J., Katri M., Leena S., Liisa N., Maria A., Marko K., Minna P., Raimo M., Riitta B., Tuija P., Tuula S., and Zewdu T. for their help. I would especially like to mention Katri Mattila because of her enduring support not only at work but also outside the lab. Thanks to Maria Andersson for many philosophical discussions and psychological observations. My thanks also go to Dr. Assi Weber for her encouragement during the final stages of this thesis.

I owe my thanks to Jurki Juhanoja for his patient teaching in electron microscopy; to Dr. Kari Lounatmaa for the excellent FESEM pictures.

The Instrument Centre of Faculty of Agriculture and Forestry, the Electron Microscopy Unit at Helsinki University and Viikki Science Library are warmly acknowledged for their excellent service.

My greatest debt of gratitude is owed to my husband Raimo who has always stood by me, our daughter Anne for joy and love she has brought to my life and to my dear mother Ariadna for her help during the writing of this thesis.

Irina Vallinoja, December 2006

# 7. References


Adekambi T, Drancourt M. 2004. Dissection of phylogenetic relationships among 19 rapidly growing Mycobacterium species by 16S rRNA, *hsp*65, sodA, *rec*A and *rpo*B gene sequencing. *Int. J. Syst. Evol. Microbiol.* 54: 2095-2105.

Aislabie JM, Balks MR, Foght JM, Waterhouse EJ. 2004. Hydrocarbon spills on Antarctic soil: Effect and management. *Env. Sci. Technol*. 38: 1265–1274.

Aleshchenkova ZM, Samsonova AS, Baikova SV, Kukulianskaia TA. 1996. The degradation of plasticizers by Rhodococcus erythropolis 40F. *Mikrobiol. Z.* 58: 34-38 (in Russian).

Altenburger P, Kampfer P, Akimov VN, Lubitz W, Busse HJ. 1997. Polyamine distribution in actinomycetes with group B peptidoglycan and species of the genera *Brevibacterium*, *Corynebacterium*, and *Tsukamurella. Int. J. Syst. Bacteriol.* 47: 270-277.

Alvarez H, Silva RA, Cesari AC, Zamit AL, Peressutti SR, Reichelt R, Keller U, Malkus U, Rasch C, Maskow T, Mayer F, Steinbüchel A. 2004. Physiological and morphological responses of the soil bacterium *Rhodococcus opacus* strain PD630 to water stress. *FEMS Microbiol. Ecol.* 50: 75 -86.

Alves L, Salgueiro R, Rodrigues C, Mesquita E, Matos J, Girio FM. 2005. Desulfurization of dibenzothiophene, benzothiophene, and other thiophene analogs by a newly isolated bacterium, *Gordonia alkanivorans* strain 1B. *Appl. Biochem. Biotechnol*. 120: 199-208.

Andersson MA, Nikulin M, Koljalg U, Andersson MC, Rainey F, Reijula K, Hintikka EL, Salkinoja-Salonen M. 1997. Bacteria, molds, and toxins in water-damaged building materials. *Appl. Environ. Microbiol.* 63: 387-393.

Antunes-Madeira MC, Madeira VM. 1987. Partition of malathion in synthetic and native membranes *Biochim. Biophys. Acta*. 901: 61-6.

Antunes-Madeira MC, Madeira VM. 1989. Membrane fluidity as affected by the insecticide lindane. *Biochim Biophys Acta* 982: 161-166.

Aono R, Inoue A. 1998. Organic solvent tolerance in microorganisms, pp.287-310. *In* Horikoshi and rant (ed.), *Extrimophiles. Microbial life in extreme environments.* Wiley, New York.

Aono R, Tsukagoshi, N, Miyamoto, T. 2001. Evaluation of the growth inhibition strength of hydrocarbon solvents against *Escherichia coli* and *Pseudomonas putida* grown in a two-liquid phase culture system consisting of a medium and organic solvent. *Extremophiles.* 5: 11-15.

Apajalahti JH, Salkinoja-Salonen MS. 1987. Dechlorination and para-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus. J. Bacteriol.* 169: 675–681.

Arenskötter M, Broker D, Steinbuchel A. 2004. Biology of the metabolically diverse genus *Gordonia. Appl. Environ. Microbiol.* 70: 3195-3204.

Asturias JA, Moore E, Yakimov MM, Klatte S, Timmis KN. 1994. Reclassification of the polychlorinated biphenyl-degraders *Acinetobacter* sp. strain P6 and

*Corynebacterium* sp. strain MB1 as *Rhodococcus globerulus. Syst. Appl. Microbiol*. 17: 226-231.

Bakker XR, Spauwen PH, Dolmans WM. 2004. Mycetoma of the hand caused by *Gordona terrae*: a case report. *J. Hand Surg. Brit-Eur.* 29: 188-190.

Baryshnikova LM, Reznik GI, Golovlev EL. 1979. Enzymes of intermediary metabolism in coryneform bacteria *Mikrobiologiya.* 48: 187-193 (in Russian).

Baryshnikova LM, Aminov RI,. Sokolova LM, Okorokov LA, Golovlev EL. 1988. Glucose transport and *Rhodococcus minimus* growth at high glucose concentration in the medium, *Mikrobiologiya* 57: 544-549 (In Russian).

Bej AK, Saul D, Aislabie J. 2000. Cold tolerant alkane-degrading *Rhodococcus* species from Antarctica. *Polar Biol.* 23: 100–105.

Bell KS, Philp JC, Aw DWJ, Christofi N. 1998. The genus *Rhodococcus*. *J. Appl. Microbiol.* 85: 195–210.

Böddinghaus B, Rogall T, Flohr T, Blöcker H, Böttger EC. 1990. Detection and identification of mycobacteria by amplification of rRNA. *J. Clin. Microbiol*. 28: 1751-1759.

Boldrin B, Tiehm A, Fritzsche C. 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. *Appl. Environ. Microbiol.* 59: 1927–1930.

Bosshard PP, Abels S, Zbinden R, Bottger EC, Altwegg M. 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). *J. Clin. Microbiol.* 41: 4134–4140.

Bowman JP. 1993. Revised taxonomy of the methanotrophs – description of *Methylobacter* ge-nov-, emendation of *Methylococus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only group-I methanotrophs. Int. J. Syst. Bacteriol. 43: 735 1993.

Brandao PF, Maldonado LA, Ward AC, Bull AT, Goodfellow M. 2001. *Gordonia namibiensis* sp. nov, a novel nitrile metabolising actinomycete recovered from an African sand. *Syst. Appl. Microbiol*. 24: 510-515.

Briglia M, Eggen RI, Van Elsas DJ, De Vos WM. 1994. Phylogenetic evidence for transfer of pentachlorophenol-mineralizing *Rhodococcus chlorophenolicus* PCP-I$^T$ to the genus *Mycobacterium. Int. J. Syst. Bacteriol.* 44: 494-498.

Briglia M, Rainey FA, Stackebrandt E, Schraa G, Salkinoja-Salonen MS. 1996. *Rhodococcus percolatus* sp. nov., a bacterium degrading 2,4,6-trichlorophenol. *Int. J. Syst. Bacteriol.* 46: 23-30.

Brown-Elliott BA, Wallace Jr. RJ. 2002. Clinical and taxonomical status of pathogenic nonpigmented or late-pigmented rapidly growing mycobacteria. *Clin. Microbiol. Rev.* 15: 716-746.

Buijtels PCAM, Petit PLC. 2005. Comparison of NaOH-N-acetyl cystein and sulfuric acid decontamination methods for recovery of mycobacteria from clinical specimens. *J. Microbiol. Meth.* 62: 83-88.

Burback BL, Perry JJ, Rudd LE. 1994. Effect of environmental pollutants and their metabolites on a soil mycobacterium. *Appl. Microbiol. Biotechnol.* 41: 134-136.

Busse HJ, Kainz A, Tsitko IV, Salkinoja-Salonen MS. 2000. Riboprints as a tool for rapid preliminary identification of sphingomonads. *Syst. Appl. Microbiol.* 23: 115-123.

Butler WR, Floyd MM, Brown J, Toney SR, Daneshvar M, Cooksey RS, Carr J, Steigerwalt A, Charles N. 2005. Novel mycolic acid-containing bacteria in the family *Segniliparaceae* fam. nov., including the genus *Segniliparus* gen. nov., with descriptions of *Segniliparus rotundus* sp. nov. and *Segniliparus rugosus* sp. nov. *Int. J. Syst. Evol. Microbiol.* 55: 1615–1624.

Cevc G. 1991. How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of unsaturation: an effective chain-length model? *Biochemistry*. 30: 7186-7193.

Chang BV, Yang CM, Cheng CH, Yuan SY. 2004. Biodegradation of phthalate esters by two bacteria strains. *Chemosphere.* 55: 533-538.

Chatterjee S, Dutta TK. 2003. Metabolism of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818. *Biochem. Biophys. Res. Commun.* 309: 36-43.

Chatterjee S, Mallick S, Dutta TK. 2005. Pathways in the degradation of hydrolyzed alcohols of butyl benzyl phthalate in metabolically diverse *Gordonia* sp. strain MTCC 4818. J. *Mol. Microbiol. Biotechnol.* 9: 110-120.

Chauvaux S, Chevalier F, Le Dantec C, Fayolle F, Miras I, Kunst F, Beguin P. 2001. Cloning of a genetically unstable cytochrome P-450 gene cluster involved in degradation of the pollutant ethyl *tert*-butyl ether by *Rhodococcus ruber. J. Bacteriol.* 183: 6551-6557.

Cheung PY, Kinkle BK. 2001. Mycobacterium diversity and pyrene mineralization in petroleum-contaminated soils. *Appl. Environ. Microbiol.* 67: 2222-2229.

Clark-Curtiss JE. 1998. Identification of virulence determinants in pathogenic mycobacteria. *Cur. Topics Microbiol. Immunol.* 225: 57-121.

Colwell RR. 1970. Polyphasic taxonomy of the genus vibrio: numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. *J. Bacteriol.* 104: 410-433.

Cruden DL, Wolfram JH, Rogers RD, Gibson DT. 1992. Physiological properties of a *Pseudomonas* strain which grows with p-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* 58: 2723-2729.

Cuello OH, Caorlin MJ, Reviglio VE, Carvajal L, Juarez CP, de Guerra EP, Luna JD. 2002. *Rhodococcus globerulus* keratitis after laser *in situ* keratomileusis. *J Cataract Refract. Surg.* 28: 2235–2237.

Daffé M, Draper P. 1998. The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol*. 39: 131-203.

Dahl JL. 2004. Electron microscopy analysis of *Mycobacterium tuberculosis* cell division. *FEMS Microbiol. Lett.* 240: 15-20.

De Young LR, Dill KA. 1988. Solute partitioning into lipid bilayer membranes. *Biochemistry.* 27: 5281 -5289.

De Young LR, Dill KA. 1990. Partitioning of nonpolar solutes into bilayers and amorphous n-alkanes Source: *J. Phys. Chem.* 94: 801-809.

Dhariwal KR, Chander A, Venkitasubramanian TA. 1976. Alterations in lipid constituents during growth of *Mycobacterium smegmatis* CDC 46 and *Mycobacterium phlei* ATCC 354. *Microbios.* 16: 169-182.

Di Gennaro P, Rescalli E; Galli E, Sello G, Bestetti G. 2001. Characterization of *Rhodococcus opacus* R7, a strain able to degrade naphthalene and o-xylene isolated from a polycyclic aromatic hydrocarbon-contaminated soil. *Res. Microbiol.* 152: 641-651.

Duetz WA, Fjallman AH, Ren S, Jourdat C, Witholt B. 2001. Biotransformation of D-limonene to (+) trans-carveol by toluene-grown *Rhodococcus opacus* PWD4 cells. *Appl. Environ. Microbiol.* 67: 2829-32.

Eroshina NV, Golovlev EL. 1979. Characteristics of the tricarboxylic acid cycle in *Nocardia erythropolis*. *Mikrobiologiya.* 48: 23-27 (in Russian).

Falkinham III JO. 1996. Epidemiology of infection by nontuberculous mycobacteria. *Clin. Microbiol. Rev*. 9:1 77–215.

Falkinham III JO. 2003. Mycobacterial aerosols and respiratory disease. *Emerging Infect. Dis*. 9: 763-767.

Ferreira NL, Maciel1 H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW. 2006. Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTB. *Appl. Microbiol. Biotechnol.* 70: 358-365

Flaherty C, Sutcliffe IC. 1999. Identification of a lipoarabinomannan-like lipoglycan in *Gordonia rubropertincta*. *Syst. Appl. Microbiol.* 22: 530–533

Fox GE, Wisotzkey JD, Jurtshuk Jr P. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol*. 42: 166–170.

Friedrich M, Grosser RJ, Kern EA, Inskeep WP, Ward DM. 2000. Effect of model sorptive phases on phenanthrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Appl. Environ. Microbiol.* 66: 2703-2710

Funke G, Stubbs S, Altwegg M, Carlotti A, Collins MD. 1994. *Turicella otitidis* gen. nov., sp. nov., a coryneform bacterium isolated from patients with otitis media. *Int. J. Syst. Bacteriol.* 44: 270-273.

Garrity GH, Holt JG. The road map to the manual. In: Bergey's Manual of Systematic Bacteriology, The Archaea and the deeply branching and phototrophic bacteria. Garrity G.M. (ed.-in-chief). 2001. 2 ed., Vol. 1. SpringerVerlag.

Garton NJ, Sutcliffe IC. 2006. Identification of a lipoarabinomannan-like lipoglycan in the actinomycete *Gordonia bronchialis. Arch. Microbiol.* 184: 425–427.

Garton NJ, Gilleron M, Brando T, Dan HH, Giguere S, Puzo G, Prescott JF, Sutcliffe IC. 2002. A novel lipoarabinomannan from the equine pathogen *Rhodococcus equi.* Structure and effect on macrophage cytokine production. *J. Biol. Chem.* 277: 31722-31733.

Gibson KJC., Gilleron M, Constant P, Puzo G, Nigou J, Besra GS. 2003. Structural and functional features of *Rhodococcus ruber* lipoarabinomannan. *Microbiology*. 149: 1437–1445.

Gibson KJ, Gilleron M, Constant P, Brando T, Puzo G, Besra GS., Nigou JJ. 2004. *Tsukamurella paurometabola* lipoglycan, a new lipoarabinomannan variant with pro-inflammatory activity. *Biol Chem.* 279: 22973-82.

Gil-Sande E, Brun-OteroM, Campo-Cerecedo F, Esteban E, Aguilar L, Garcia-de-Lomas J. 2006. Etiological misidentification by routine biochemical tests of bacteremia caused by *Gordonia terrae* infection in the course of an episode of acute cholecystitis. *J.Clin.Microbiol.* 44: 2645-2647.

Goodfellow M, Alderson G. 1977. The actinomycete genus *Rhodococcus*: a home for the rhodochrous complex. *J. Gen. Microbiol*. 100: 99-122.

Goodfellow M, Chun J, Stackebrandt E, Kroppenstedt RM. 2002. Transfer of T*sukamurella wratislaviensis* Goodfellow et a. 1995 to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov. *Int J Syst Evol Microbiol*. 52: 749-755.

Goren MB. 1972. Mycobacterial Lipids: Selected Topics. *Bacteriol. Rev.* 36: 33-64.

Goswami M, Shivaraman N, Singh RP. 2002. Kinetics of chlorophenol degradation by benzoate-induced culture of *Rhodococcus erythropolis* M1. *World J. Microbiol. Biotechnol.* 18: 779-2002.

Grif K, Dierich MP, Much P, Hofer E., Allerberger F. 2003. Identifying and subtyping species of dangerous pathogens by automated ribotyping. *Diagn. Micr. Infec. Dis.* 47: 313-320

Grosser RJ, Warshawsky D, Vestal JR. 1991. Indigenous and enhanced mineralization of pyrene, benzo[a]pyrene, and carbazole in soils. *Appl. Environ. Microbiol*. 57: 3462–3469.

Gurtler V, Mayall BC, Seviour R. 2004. Can whole genome analysis refine the taxonomy of the genus *Rhodococcus*? *FEMS Microbiol. Rev.* 28: 377-403.

Gutierrez JA, Nichols P, Couperwhite I. 1999. Changes in whole cell-derived fatty acids induced by benzene and occurrence of the unusual 16:1 omega 6c in *Rhodococcus* sp 33. *FEMS Microbiol Lett.* 176: 213-218.

Häggblom MM, Nohynek LJ, Salkinoja-Salonen MS. 1988. Degradation and O-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. *Appl. Environ. Microbiol.* 54: 3043-3052.

Häggblom MM, Nohynek LJ, Palleroni NJ, Kronqvist K, Nurmiahola-Lassila EL, Salkinoja-Salonen MS, Klatte S, Kroppenstedt R.M.1994. Transfer of polychlorophenol-degrading *Rhodococcus chlorophenolicus* (Apajalahti *et al*., 1986) to the genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb nov. *Int. J. Syst. Bacteriol.* 44: 485-493.

Hallas LE, Vestal JR. 1978. The growth of *Mycobacterium convolutum* on solid n-alkane substrates: effect on cellular lipid composition. *Can. J. Microbiol.* 24: 1197-1203.

Heipieper HJ, de Bont JAM. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. *Appl. Environ. Microbiol.* 60: 4440–44.

Heiss G, Trachtmann N, Abe Y, Takeo M, Knackmuss HJ. 2003. Homologous npdGI genes in 2,4-dinitrophenol- and 4-nitrophenol-degrading *Rhodococcus* spp. *Appl. Environ. Microbiol.* 69: 2748–2754.

Heitkamp M, Franklin W, Cerniglia C. 1988. Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.* 54: 2549–2555.

Heitkamp MA, Cerniglia CE. 1988. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. *Appl. Environ. Microbiol*. 54: 1612–1614.

Hernandez-Perez G, Fayolle F, Vandecasteele JP. 2001. Biodegradation of ethyl t-butyl ether (ETBE), methyl t-butyl ether (MTBE) and t-amyl methyl ether (TAME) by *Gordonia terrae*. *J. Appl. Microbiol. Biotechnol.* 55: 117-121.

Huttunen K, Ruotsalainen M, Iivanainen E, Torkko P, Katila ML, Hirvonen MR. 2000. Inflamatory responses in RAW264.7 macrophages caused by mycobacteria isolated from moldy house. *Environ. Toxicol. Pharmacol.* 8: 237-244.

Iida S, Taniguchi H, Kageyama A, Yazawa K, Chibana H, Murata S, Nomura F, Kroppenstedt RM, Mikami Y. 2005. *Gordonia otitidis* sp. nov., isolated from a patient with external otitis. *Int. J. Syst. Evol. Microbiol.* 55: 1871-1876.

Inoue A, Horikoshi K. 1989. A *Pseudomonas* thrives in high concentrations of toluene. *Nature.* 338: 264-266.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter LogP. *J. Ferment. Bioeng.* 71: 194-196.

Jang JY, Kim D, Bae HW, Choi KY, Chae JC, Zylstra GJ, Kim YM, Kim E. 2005. Isolation and characterization of a *Rhodococcus* species strain able to grow on *ortho*- and *para*-xylene. *Journal of Microbiology (Seoul, Korea).* 43: 325-30.

Jung IG, Park CH. 2004. Characteristics of *Rhodococcus pyridinivorans* PYJ-1 for the biodegradation of benzene, toluene, m-xylene (BTX), and their mixtures. *J. Biosc. Bioengin.* 97: 429-431.

Kageyama A, Iida S, Yazawa K, Kudo T, Suzuki S, Koga T, Saito H, Inagawa H, Wada A, Kroppenstedt RM, Mikami Y. 2006. *Gordonia araii* sp. nov. and *Gordonia effusa* sp. nov., isolated from patients in Japan. *Int. J. Syst. Evol. Microbiol*. 56: 1817-1821.

Kämpfer P, Andersson MA, Rainey FA, Kroppenstedt RM, Salkinoja-Salonen M. 1999. *Williamsia muralis* gen. nov., sp. nov., isolated from the indoor environment of a children's day care centre. *Int. J. Syst. Bacteriol.* 49: 681-687

Kanaly RA, Harayama S. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.* 182: 2059–2067

Kasai H, Ezaki T, Harayama S. 2000. Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J. Clin. Microbiol.* 38: 301–308.

Kastner M, Breuerjammali M, Mahro B. 1994. Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). *Appl. Microbiol. Biotechnol.* 41: 267–273.

Kedlaya I, Ing MB, Wong SS. 2001. *Rhodococcus equi* infections in immunocompetent hosts: case report and review. *Clin. Infect. Dis.* 32: 39–47.

Kempf VA, Schmalzing M, Yassin AF, Schaal KP, Baumeister D, Arenskotter M, Steinbuchel A, Autenrieth IB. 2004. *Gordonia polyisoprenivorans* septicemia in a bone marrow transplant patient. *Eur. J. Clin. Microbiol. Infect. Dis*. 23: 226-228.

Kilby JM, Gilligan PH, Yankaskas JR, Highsmith Jr WE, Edwards LJ, Knowles MR. 1992. Nontuberculous mycobacteria in adult patients with cystic fibrosis. *Chest.* 102: 70-75.

Kim D, Kim YS, Kim SK, Kim, SW, Zylstra GJ, Kim YM, Kim E. 2002. Monocyclic Aromatic Hydrocarbon Degradation by *Rhodococcus* sp. Strain DK17 *Appl. Environ. Microbiol.* 68: 3270-3278.

Kim H, Kim SH, Shim TS, Kim M, Bai GH, Park YG, Lee SH, Chae GT, Cha CY, Kook YH, Kim BJ. 2005. Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene (*hsp65*). *Int. J. Syst. Evol. Microbiol*. 55: 1649-1656.

Kim KK, Lee CS, Kroppenstedt RM, Stackebrandt E, Lee ST. 2003. *Gordonia sihwensis* sp. nov., a novel nitrate-reducing bacterium isolated from a wastewater-treatment bioreactor. *Int. J. Syst. Evol. Microbiol.* 53: 1427-1433.

Kim SB, Brown R, Oldfield C, Gilbert SC, Iliarionov S, Goodfellow M. 2000. *Gordonia amicalis* sp. nov., a novel dibenzothiophene-desulphurizing actinomycete. *Int. J. Syst. Evol. Microbiol.* 50 Pt 6: 2031-2036.

Kimura N, Urushigawa Y. 2001. Metabolism of dibenzo-p-dioxin and chlorinated dibenzo-p-dioxin by a gram-positive bacterium, *Rhodococcus opacus* SAO 101. *J. Biosci. Bioeng*. 92: 138-143.

Kitagawa W, Kimura N, Kamagata Y. 2004. A novel p-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. *J. Bacteriol*. 186: 4894-4902.

Kitagawa W, Suzuki A, Hoaki T, Masai E, Fukuda M. 2001. Multiplicity of aromatic ring hydroxylation dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1 demonstrated by denaturing gradient gel electrophoresis. *Biosci. Biotechnol. Biochem*. 65: 1907-1911.

Kleespies M, Kroppenstedt RM, Rainey FA, Webb LE, Stackebrandt E. 1996. *Mycobacterium hodleri* sp. nov., a new member of the fast-growing mycobacteria capable of degrading polycyclic aromatic hydrocarbons *Int. J. Syst. Bacteriol.* 46: 683 - 687.

Konig C, Eulberg D, Groning J, Lakner S, Seibert V, Kaschabek SR, Schlomann M. 2004. A linear megaplasmid, p1CP, carrying the genes for chlorocatechol catabolism of *Rhodococcus opacus* 1CP. *Microbiology.* 150: 3075-87.

Kroppenstedt RM, Kutzner HJ. 1976. Biochemical makers in the taxonomy of the *Actinomycetales. Experientia.* 32: 318-319.

Kulikova T, Aldebert P, Althorpe N, Baker W, Bates K, Browne P, van den Broek A, Cochrane G, Duggan K, Eberhardt R, Faruque N, Garcia-Pastor M, Harte N, Kanz C, Leinonen R, Lin Q, Lombard V, Lopez R, Mancuso R, McHale M, Nardone F, Silventoinen V, Stoehr P, Stoesser G, Tuli MA, Tzouvara K, Vaughan R, Wu D, Zhu W, Apweiler R. 2004. The EMBL Nucleotide Sequence Database. *Nucleic Acids Res.* 32: 27–30.

Larkin MJ, Kulakov LA, Allen CC. 2005. Biodegradation and *Rhodococcus*--masters of catabolic versatility. *Curr. Opin. Biotechnol*. 16: 282-290.

Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, Vincent V. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Appl. Environ. Microbiol.* 68: 5318-5325.

Lechevalier MP, Lechevalier HA. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int. J. Syst. Bacteriol.* 20: 435–443.

Leigh MB, Prouzova P, Mackova M, Macek T, Nagle DP, Fletcher JS. 2006. Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. *Appl. Environ. Microbiol.* 72: 2331-2342.

Lesens O, Hansmann Y, Riegel P, Heller R, Benaissa-Djellouli M, Martinot M, Petit H, Christmann D. 2000. Bacteremia and endocarditis caused by a *Gordonia* species in a patient with a central venous catheter. *Emerg. Infect. Dis.* 6: 382-385.

Levi MH, Bartell J, Gandolfo L, Smole SC, Costa SF, Weiss LM, Johnson LK, Osterhout G, Herbst LH. 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic *Mycobacterium* from moray eels that is related to *Mycobacterium triplex*. *J. Clin. Microbiol.* 41: 2147-2152.

Leys NM, Ryngaert A, Bastiaens L, Wattiau P, Top EM, Verstraete W, Springael D. 2005. Occurrence and community composition of fast-growing Mycobacterium in soils contaminated with polycyclic aromatic hydrocarbons. *FEMS Microbiol. Ecol.* 51: 375-388.

Li FL, Xu P, Ma CQ, Luo LL, Wang XS. 2003. Deep desulfurization of hydrodesulfurization-treated diesel oil by a facultative thermophilic bacterium *Mycobacterium* sp. X7B. *FEMS Microbiol. Lett.* 223: 301–307.

Li J, Chen JA, Zhao Q, Li X, Shu W. 2006. Bioremediation of environmental endocrine disruptor di-n-butyl phthalate ester by *Rhodococcus ruber*. *Chemosphere*. 65: 1627-33.

Lin TC, Young CC, Ho MJ, Yeh MS, Chou CL, Wei YH, Chang JS. 2005. Characterization of floating activity of indigenous diesel-assimilating bacterial isolates. *J. Biosci. Bioengin.* 99: 466-472.

Linos A, Berekaa MM, Steinbüchel A, Kim KK, Spröer C, Kroppenstedt RM. 2002. *Gordonia westfalica* sp. nov., a novel rubber-degrading actinomycete. *Int. J. Syst. Evol. Microbiol.* 52: 1133-1139.

Linos A, Steinbuchel A, Sproer C, Kroppenstedt RM. 1999. *Gordonia polyisoprenivorans* sp. nov., a rubber-degrading actinomycete isolated from an automobile tyre. *Int. J. Syst. Bacteriol.* 49: 1785-1791.

Lopez Z, Vila J, Grifoll M. 2005. Metabolism of fluoranthene by mycobacterial strains isolated by their ability to grow in fluoranthere or pyrene. *J. Ind. Micorbiol. Biotechnol*. 32: 455-464.

Lopez Z, Vila J, Minguillon C, Grifoll M. 2006. Metabolism of fluoranthene by *Mycobacterium* strain AP1. *Appl. Microbiol. Biotechnol.* 70: 747-756.

Mackay D, Shiu WY, Ma KC. 1992. Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals. Vol 1-3. Lewis Publishers Inc., Chelsea, Michigan.

Malachowsky KJ, Phelps TJ, Teboli AB, Minnikin DE, White DC. 1994. Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. *Appl. Environ. Microbiol.* 60: 542-548.

Maldonado LA, Stainsby FM, Ward AC, Goodfellow M. 2003. *Gordonia sinesedis* sp. nov., a novel soil isolate. *Anton. Leeuw. Int. J. G.* 83: 75-80.

Masai E, Yamada A, Healy M, Hatta T, Kimbara K, Fukuda M, Yano K. 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. RHA1. *Appl. Environ. Microbiol*. 61: 2079-2085.

McMinn EJ, Alderson G, Dodson HI, Goodfellow M, Ward AC. 2000. Genomic and phenomic differentiation of *Rhodococcus equi* and related strains. *Anton. Leeuw. Int. J. G.* 78: 331–340.

Minnikin DE. 1991. Chemical principles in the organization of lipid components in the mycobacterial cell envelope. *Res. Microbiol*. 142: 423–427

Miyakawa Y, Ratnakar P, Rao AG, Costello ML, Mathieu-Costello O, Lehrer RI, Catanzaro A. 1996. In vitro activity of the antimicrobial peptides human and rabbit defensins and porcine leukocyte protegrin against *Mycobacterium tuberculosis. Infect. Immun*. 64: 926-932.

Moody JD, Freeman JP, Cerniglia CE. 2005. Degradation of benz[a] anthracene by *Mycobacterium vanbaalenii* strain PYR-1. *Biodegradation*. 16: 513-526.

Moss CW, Daneshvar MI. 1992. Identification of some uncommon monounsaturated fatty acids of bacteria. *J. Clin. Microbiol.* 30: 2511-2512.

Mrozik A, Piotrowska-Seget Z, Labuzek S. 2003. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. *Pol. J. Environ. Stud.* 12: 15-25

Mukhopadhyay P, Vogel HJ, Tieleman DP. 2004. Distribution of pentachlorophenol in phospholipid bilayers: A molecular dynamics study. *Biophys. J.* 86: 337-345.

Mutnuri S, Vasudevan N, Kaestner M. 2005. Degradation of anthracene and pyrene supplied by microcrystals and non-aqueous-phase liquids. *Appl. Microbiol. Biotechnol.* 67: 569-576.

Na KS, Kuroda A, Takiguchi N, Ikeda T, Ohtake H, Kato J. 2005. Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. *J. Biosci. Bioeng.* 99: 378-382.

Nakamiya K, Hashimoto S, Ito H, Edmonds JS, Yasuhara A, Morita M. 2005. Microbial treatment of bis (2-ethylhexyl) phthalate in polyvinyl chloride with isolated bacteria *J. Biosci. Bioeng.* 99: 115-119.

Nalli S, Cooper DG, Nicell JA. 2002. Biodegradation of plasticizers *by Rhodococcus rhodochrous*. *Biodegradation*. 13: 343-352.

Navarro-Llorens JM, Patrauchan MA, Stewart GR, Davies JE, Eltis LD, Mohn WW. 2005. Phenylacetate catabolism in *Rhodococcus* sp. strain RHA1: a central pathway for degradation of aromatic compounds. *J. Bacteriol.* 187: 4498-4504.

Nielsen LE, Kadavy DR, Rajagopal S, Drijber R, Nickerson KW. 2005. Survey of extreme solvent tolerance in gram-positive cocci: membrane fatty acid changes in Staphylococcus haemolyticus grown in toluene. *Appl. Environ. Microbiol.* 71: 5171-5176.

Nigou J, Gilleron M, Puzo G. 2003. Lipoarabinomannans: from structure to biosynthesis. *Biochimie*. 85: 153-166.

Nishioka T, Iwata M, Imaoka T, Mutoh M, Egashira Y, Nishiyama T, Shin T, Fujii T. 2006. A mono-2-ethylhexyl phthalate hydrolase from a *Gordonia* sp. that is able to dissimilate di-2-ethylhexyl phthalate. *Appl. Environ. Microbiol.* 72: 2394-2399.

Nohynek LJ, Häggblom MM, Palleroni NJ, Kronqvist K, Nurmiaho-Lassila EL, Salkinoja-Salonen MS. 1993. Characterization of *Mycobacterium fortuitum* strain capable of degrading polychlorinated phenolic compounds. Sy*stem. Appl. Microbiol.* 16: 126- 134.

Ogino H, Yasui K, Shiotani T, Ishihara T, Ishikawa H. 1995. Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme *Appl. Environ. Microbiol.* 61:4258-4262.

Oldfield C, Pogrebinsky O, Simmonds J, Olson ES, Kulpa CF. 1997. Elucidation of the metabolic pathway for dibenzothiophene desulphurization by *Rhodococcus* sp. strain IGTS8 (ATCC 53 968). *Microbiology*.143: 2961–2973.

Paje MLF, Neilan BA, Couperwhite I. 1997. A *Rhodococcus* species that thrives on medium saturated with liquid benzene. *Microbiology.* 143: 2975-2981.

Patrauchan MA, Florizone C, Dosanjh M, Mohn WW, Davies J, Eltis LD. 2005. Catabolism of benzoate and phthalate in *Rhodococcus* sp. strain RHA1: redundancies and convergence. *J. Bacteriol*. 187: 4050-4063.

Pham AS, De I, Rolston KV, Tarrand JJ, Han XY. 2003. Catheter-related bacteremia caused by the nocardioform actinomycete *Gordonia terrae*. *Clin. Infect. Dis.* 36: 524-527

Phillips MS, von Reyn CF. 2001. Nosocomial infections due to nontuberculous mycobacteria. *Clin. Infect. Dis.* 33: 1363–1374.

Pinkart HC, Wolfram JW, Rogers R, White DC. 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to o-xylene. *Appl. Environ. Microbiol.* 62: 1129–32

Primm TP, Lucero CA, Falkinham III JO. 2004. Health impacts of environmental mycobacteria. *Clin. Microbiol. Rev.* 17: 98-106.

Rahmana MT, Herron LL, Kapur V, Meijer WG, Byrne BA, Rena J, Nicholson VM, Prescott JF. 2003. Partial genome sequencing of *Rhodococcus equi* ATCC 33701. *Vet. Microbiol.* 94: 143–158.

Rainey FA, Klatte S, Kroppenstedt RM, Stackebrandt E. 1995. *Dietzia*, a new genus including *Dietzia maris* comb. nov., formerly *Rhodococcus maris*. *Int. J. Syst. Bacteriol.* 45: 32-36.

Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A. 2002. Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* 56: 743-768.

Rapp P, Gabriel-Jurgens LHE. 2003. Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase. *Microbiology*. 149: 2879-2890.

Rehfuss M, Urban J. 2005. *Rhodococcus phenolicus* sp. nov., a novel bioprocessor isolated actinomycete with the ability to degrade chlorobenzene, dichlorobenzene

and phenol as sole carbon sources. *Syst. Appl. Microbiol.* 28: 695-701.

Rehmann K, Hertkorn N, Kettrup A. 2001. Fluoranthene metabolism in *Mycobacterium* sp. strain KR20: identity of pathway intermediates during degradation and growth. *Microbiology.* 147: 2783-2794.

Rehmann K, Noll HP, Steinberg CE, Kettrup AA. 1998. Pyrene degradation by *Mycobacterium* sp. strain KR2. *Chemosphere.* 36: 2977–2992.

Rha YH, Taube C, Haczku A, Joetham A, Takeda K, Duez C, Siegel M, Aydintug MK, Born WK, Dakhama A, Gelfand EW. 2002. Effect of microbial heat shock proteins on airway inflammation and hyperresponsiveness. *J. Immunol.* 169: 5300-5307.

Rhee SK, Chang JH, Chang YK, Chang HN. 1998. Desulfurization of dibenzothiophene and diesel oils by a newly isolated *Gordona* strain, CYKS1. *Appl. Environ. Microbiol*. 64: 2327-2331.

Riegel P, Ruimy R, de Briel D, Eichler F, Bergerat JP, Christen R, Monteil H. 1996. Bacteremia due to *Gordona sputi* in an immunocompromised patient. *J. Clin. Microbiol.* 34: 2045-2047.

Ringuet H, Akoua-Koffi C, Honore S, Varnerot A, Vincent V, Berche P, Gaillard JL, Pierre-Audigier C. 1999. *hsp65* sequencing for identification of rapidly growing mycobacteria. *J. Clin. Microbiol*. 37: 852–857.

Rose K, Steinbuchel A. 2005. Biodegradation of natural rubber and related compounds: Recent insights into a hardly understood catabolic capability of microorganisms. *Appl. Environ. Microbiol.* 71: 2803-2812.

Rosenberg M, Grutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9: 29-33.

Ruberto LAM, Vazquez SC, Lobalbo A, Mac Cormack WP. 2005. Psychrotolerant hydrocarbon-degrading *Rhodococcus* strains isolated from polluted Antarctic soils. *Antarctic Sci.* 17: 47–56.

Runyon EH. 1959. Anonymous mycobacteria in pulmonary disease. *Med.Clin. North Am.* 43: 273–290.

Russell AD, Hugo WB, Ayliffe GAJ. (ed.) 1999. *Pincipals and practice of disinfection, preservation and sterilization*. 3-d ed. Blackwell Scientific Publications Ltd, Oxford, England.

Saiman L, Siegel J. 2004. Infection control in cystic fibrosis. Clin. Microbiol. Rev. 17: 57-71.

Sardessai Y, Bhosle S. 2002. Tolerance of bacteria to organic solvents. *Res. Microbiol.* 153: 263–268.

Saul DJ, Aislabie J, Brown CE, Harris L, Foght JM. 2005. Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. *FEMS Microbiol. Ecol.* 53: 141–155.

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D. 1996. Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp strain RJGII-135, isolated from a former coal gasification site. *Appl. Environ. Microbiol.* 62: 13–19.

Scott MA, Graham BS, Verrall R, Dixon R, Schaffner W, Tham KT. 1995. *Rhodococcus equi*-an increasingly recognized opportunistic pathogen. Report of 12 cases and review of 65 cases in the literature. *Am. J. Clin. Pathol.* 103: 649-55.

Shen FT, Lu HL, Lin JL, Huang WS, Arun AB, Young CC. 2006. Phylogenetic analysis of members of the metabolically diverse genus *Gordonia* based on proteins encoding the gyrB gene. *Res. Microbiol.* 157: 367-375

Sikkema J, de Bont JAM, Poolman B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* 269: 8022-8028.

Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons *Microbiol. Rev.* 59: 201-222.

Sng LH, Koh TH, Toney SR, Floyd M, Butler WR, Tan BH. 2004. Bacteremia caused by *Gordonia bronchialis* in a patient with sequestrated lung. *J. Clin. Microbiol.* 42: 2870-2871.

Stackebrandt E, Frederiksen W, Garrity GM, Grimont P.A, Kampfer P, Maiden MC, Nesme X, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitman WB. 2002. Report of the Ad Hoc Committee for the Re-Evaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 52: 1043-1047.

Stackebrandt E, Rainey FA, Ward-Rainey NL. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int. J. Syst. Bacteriol.* 47: 479-491.

Stackebrandt E, Smida J, Collins MD. 1988. Evidence of phylogenetic heterogeneity within the genus *Rhodococcus*: revival of the genus *Gordona* (*Tsukamura*). *J. Gen. Appl. Microbiol.* 34: 341-348.

Stackebrandt E, Goebel BM. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

Stahl DA, Urbance JW. 1990. The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J. Bacteriol.* 172: 116-24.

Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM, Modlin RL. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science*. 282: 121-125.

Stingley RL, Brenza B, Khan AA, Cerniglia CEN. 2004. Novel organization of genes in a phthalate degradation operon of *Mycobacterium vanbaalenii* PYR-1. *Microbiology.* 150: 3749-3761.

Stoecker MA, Herwig RP, Staley JT. 1994. *Rhodococcus zopfii* sp. nov. a toxicant degrading bacterium. *Int. J. Syst. Bacteriol*. 44: 106-110.

Suihko ML, Kroppenstedt RM, Stackebrandt E. 2006. Occurrence and characterization of actinobacteria and thermoactinomycetes isolated from pulp and board samples containing recycled fibres. *J. Ind. Microbiol. Biotechnol.* 33: 183-191.

Sutcliffe IC. 2000. Characterization of a lipomannan lipoglycan from the mycolic acid containing actinomycete *Dietzia maris*. *Anton. Leeuw. Int. J. G.* 78: 195–201

Sutcliffe IC. 1998. Cell envelope composition and organisation in the genus *Rhodococcus*. *Anton. Leeuw. Int. J. G.* 74: 49-58.

Suutari M, Laakso S. 1993. Effect of growth temperature on the fatty acid composition of *Mycobacterium phlei*. *Arch. Microbiol.* 159: 119-123.

Tay STL, Hemond FH, Krumholz LR, Cavanaugh CM, Polz MF. 2001. Population dynamics of two toluene degrading bacterial species in a contaminated stream. *Microb. Ecol.* 41: 124-131.

Tay STL, Hemond HF, Polz MF, Cavanaugh CM, Dejesus I, Krumholz LR. 1998. Two new *Mycobacterium* strains and their role in toluene degradation in a contaminated stream. *Appl. Environ. Microbiol.* 64: 1715–1720.

Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* 31: 175–178.

Torkko P, Suomalainen S, IIvanainen E, Tortoli E, Suutari M, Seppänen J, Paulin L, Katila ML. 2002. *Mycobacterium palustre* sp. Nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters. *Int. J. Syst. Evol. Microbiol.* 52: 1519-1525.

Torkko P, Katila ML, Kontro M. 2003. Gas-chromatographic lipid profiles in identification of currently known slowly growing environmental mycobacteria. *J. Med. Microbiol.* 52: 315-323.

Tortoli E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin. Microbiol. Rev.* 16: 319–354.

Torvinen E, Suomalainen S, Lehtola MJ, Miettinen IT, Zacheus O, Paulin L, Katila ML, Martikainen PJ. 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Appl. Environ. Microbiol.* 70: 1973-1981.

Tsukamura M. 1971. Proposal of a new genus, *Gordona*, for slightly acid-fast organisms occurring in sputa of patients with pulmonary disease and in soil. *J. Gen. Microbiol*. 68: 15-26.

Uyttebroek M, Breugelmans P, Janssen M, Wattiau P, Joffe B, Karlson U, Ortega-Calvo JJ, Bastiaens L, Ryngaert A, Hausner M, Springael D. 2006. Distribution of the mycobacterium community and polycyclic aromatic hydrocarbons (PAHs) among different size fractions of a long-term PAH-contaminated soil. *Environ. Microbiol.* 8: 836 -847.

Uz I, Duan YP, Ogram A. 2000. Characterization of the naphthalene-degrading bacterium, *Rhodococcus opacus* M213. *FEMS Microbiol. Lett*. 185: 231-238.

Vaerewijck MJ, Huys G, Palomino JC, Swings J, Portaels F. 2005. Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol. Rev*. 29: 911-934.

Van der Geize R, Dijkhuizen L. 2004. Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications. *Curr. Opin. Microbiol.* 7: 255-261.

Van Herwijnen R, Springael D, Slot P, Govers HAJ, Parsons JR. 2003. Degradation of anthracene by *Mycobacterium* sp. Strain LB501T proceeds via a novel pathway, through o-phthalic acid. *Appl. Environ. Microbiol.* 69: 186-190.

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* 60: 407-438.

Vanderberg LA, Krieger-Grumbine R, Taylor MN. 2000. Evidence for diverse oxidations in the catabolism of toluene by *Rhodococcus rhodochrous* strain OF5. *Appl. Microbiol. Biotechnol.* 53: 447-452.

Verma P, Brown JM, Nunez VH, Morey RE, Steigerwalt AG, Pellegrini GJ, Kessler HA. 2006. Native valve endocarditis due to *Gordonia polyisoprenivorans*: case report and review of literature of bloodstream infections caused by *Gordonia* species. *J. Clin. Microbiol.* 44: 1905-1908.

Vermuë M, Sikkema J, Verheul A, Bakker R, Tramper J. 1993. Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* sp and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. *Biotechnol. Bioeng.* 42: 747-758.

Verschueren K. 1983. *Handbook of environmental data on organic chemicals*, 2nd ed. Van Nostrand Reinhold, New York, NY.

Vilcheze C, Morbidoni HR, Weisbrod TR, Iwamoto H, Kuo M, Sacchettini JC, Jacobs Jr WR. 2000. Inactivation of the inhA-encoded fatty acid synthase II (FASII) enoyl-acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis. J. Bacteriol.* 182: 4059-4067.

Vuorio R, Andersson MA, Rainey FA, Kroppenstedt RM, Kampfer P, Busse HJ, Viljanen M, Salkinoja-Salonen M. 1999. A new rapidly growing mycobacterial species, *Mycobacterium murale* sp. nov., isolated from the indoor walls of a children's day care centre. *Int. J. System Bacteriol.* 49: 25-35.

Wagner D, Young LS. 2004. Nontuberculous mycobacterial infections: a clinical review. Infections. Infection. 32: 257–270.

Wallace Jr RJ, Brown BA, Griffith DE. 1998. Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. *Annu. Rev. Microbiol.* 52: 453–490.

Walter U, Beyer M, Klein J, Rehm HJ. 1991. Degradation of pyrene by *Rhodococcus* sp UW1. *Appl. Microbiol. Biotechnol.* 34: 671–676.

Weber FJ, Isken S, de Bont JAM. 1994 *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. *Microbiology* 173: 3017-3020.

Werno AM, Anderson TP, Chambers ST, Laird HM, Murdoch DR. 2005. Recurrent breast abscess caused by *Gordonia bronchialis* in an immunocompetent patient. *J. Clin. Microbiol.* 43: 3009-3010.

Willumsen P, Karlson U, Stackebrandt E, Kroppenstedt RM. 2001. *Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. *Int. J. Syst. Evol. Microbiol*. 51: 1715-1722.

Xue Y, Sun X, Zhou P, Liu R, Liang F, Ma Y. 2003. *Gordonia paraffinivorans* sp. nov., a hydrocarbon-degrading actinomycete isolated from an oil-producing well. *Int. J. Syst. Evol. Microbiol.* 53: 1643-1646.

Yagi O, Hashimoto A, Iwasaki K, Nakajima M. 1999. Aerobic degradation of 1,1,1-trichloroethane by *Mycobacterium* spp. isolated from soil. *Appl. Environ. Microbiol*. 65: 4693-4696.

Yakimov MM, Giuliano L, Bruni V, Scarfi S, Golyshin PN. 1999. Characterization of Antarctic hydrocarbon-degrading bacteria capable of producing bioemulsifiers. *Microbiologic*a. 22: 249-256.

Yamamoto S, Harayama S. 1995. PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl. Environ. Microbiol.* 61: 1104–1109.

Yoon JH, Cho YG, Kang SS, Kim SB, Lee ST, Park YH. 2000. *Rhodococcus koreensis* sp nov., a 2,4-dinitrophenol-degrading bacterium. *Int. J. Syst. Evol. Microbiol*. 50: 1193-1201.

Zaitsev GM, Tsitko IV, Lobanok AG, Vazhinskaja IS, Chechenin VP. 1993. Utilization of halogenated benzenes by *Rhodococcus rhodochrous*. *Dokl. Akad. Nauk Beloruss*. SSR. 37:65–68. (in Russian).

Zardawi IM, Jones F, Clark DA, Holland J. 2004. *Gordonia terrae*-induced suppurative granulomatous mastitis following nipple piercing. *Pathology.* 36: 275-278.