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Biology of Rhodococcus

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Preface

Rhodococci are metabolically versatile actinobacteria frequently found in the environment with potential applications in bioremediation, biotransformations, and biocatalysis among other biotechnological processes. These microorganisms are currently the subject of research in many countries of the world. The number of publications and patents on rhodococci has increased significantly during the last several years. In this context, the knowledge acquired during the last decade on basic aspects of *Rhodococcus* biology is significant and reveals promising future prospects. Several public and private genomic projects involving *Rhodococcus* members are now in progress due to the increasing interest in their biotechnological applications. The large *Rhodococcus* genomes, which contain a multiplicity of catabolic genes, a high genetic redundancy of biosynthetic pathways and a sophisticated regulatory network, reflect the complexity of *Rhodococcus* biology. The combination of functional genomic studies with biochemical and physiological knowledge is providing new insights that will make it possible to put rhodococci to biotechnological use.

This Microbiology Monographs volume provides a thorough review of many aspects of the biochemistry, physiology, and genetics of *Rhodococcus* in the context of new genomic information. Expert international scientists have contributed reviews on the extraordinary capabilities of the *Rhodococcus* genus with regard to the biodegradation of diverse compounds, biosynthesis of lipids and biosurfactants, and adaptation and tolerance to solvents. Chapters dealing with its taxonomy, the structural aspects of rhodococcal cellular envelope, genomes and plasmids, and central metabolism are also included in this volume. Moreover, the book examines the basic aspects of the unique pathogenic *Rhodococcus* member (*R. equi*) and the phytopathogenic *R. fascians*.

I would like to express my thanks to all of the authors, who contributed high-quality reviews of each topic, to the series editor, Alexander Steinbüchel, and to the staff at Springer, especially Jutta Lindenborn, for supporting this book.

I hope that this volume will serve as a guidebook for researchers and students and will open new avenues for future research.

Comodoro Rivadavia,
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Héctor M. Alvarez

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Systematics of Members of the Genus *Rhodococcus* (Zopf 1891) Emend Goodfellow et al. 1998

The Past, Present and Future

Volker Gűrtler and Robert J. Seviour

Contents

Abstract The genus *Rhodococcus* in the sub-order *Corynebacteriniae* has had a chequered taxonomic history, but many of the early uncertainties and confusions have been resolved satisfactorily through the application of chemotaxonomic and phylogenetic characters. Such information has allowed the creation and formal recognition of the closely related genera *Gordonia*, *Tsukamurella* and *Dietzia* to include isolates once placed unconvincingly in the genus *Rhodococcus*, and the re-assignment of former members of *Nocardia* and *Tsukamurella* to it. However, several taxonomic difficulties remain. Where to place *Rhodococcus equi* is still problematic, and many *Rhodococcus* isolates, some probably representing new species, have never been formally described. This chapter discusses the history of the evolution of this genus and its current status and suggests what systematic issues need to be resolved in the future.

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1 Introduction

It is now clear, as other sections of this book amply demonstrate, that the rhodococci are very important organisms, especially with their remarkably versatile metabolic powers. This is often due to their possessing mobile, large and usually linear plasmids carrying genes encoding enzymes capable of degrading an impressive range of xenobiotic and naturally occurring organic compounds, some of which are likely to pose long-term environmental risks (Bell et al. 1998; Larkin et al. 2006). Thus, their potential in bioremediation is immense (Van der Geize and Dijkhuizen 2004). They are also known to produce metabolites of industrial potential including carotenoids, bio-surfactants and bio-flocculation agents and especially acrylamide (Jones and Goodfellow 2010). These attributes alone emphasise the importance of being able to identify such populations rapidly and unequivocally, which requires that they are classified appropriately. Like most other bacteria, the genus *Rhodococcus* has experienced an often tortuous taxonomic history, consistently gaining and losing members (Table 1a, b) as the characters

Table 1 (a, b) Examples of the reclassification of earlier designated members of the genus *Rhodococcus* to other closely related genera and (b) vice versa

Previous Classification	Current Classification	Reference
(a)		
Rhodococcus aechiensis	*Gordonia aechiensis*	Klatte et al. (1994c)
Rhodococcus aurantiacus	*Tsukamurella paurometabola*	Collins et al. (1988)
Rhodococcus bronchialis	*Gordonia bronchialis*	Stackebrandt et al. (1988)
Rhodococcus chlorophenolicus	*Mycobacterium chlorophenolicum*	Häggblom et al. (1994)
Rhodococcus chubuensis	*Gordonia sputi*	Stackebrandt et al. (1988)
Rhodococcus corallinus	*Rhodococcus rubropertincta*	Mordarski et al. (1980)
Rhodococcus luteus	*Rhodococcus fascians*	Klatte et al. (1994a)
Rhodococcus maris	*Dietzia maris*	Rainey et al. (1995b)
Rhodococcus obuensis	*Rhodococcus sputi*	Zakrzewska-Czerwinska et al. (1988)
Rhodococcus roseus	*Rhodococcus rhodochrous*	Rainey et al. (1995a)
Rhodococcus rubropertincta	*Gordonia rubripertincta*	Stackebrandt et al. (1988)
Rhodococcus sputi	*Gordonia sputi*	Riegel et al. (1994), Stackebrandt et al. (1988)
Rhodococcus terrae	*Gordonia terrae*	Stackebrandt et al. (1988)
"*Rhodococcus australis*"	*Gordonia malaquae*	Stainsby et al. (unpublished)
(b)		
Nocardia corynebacteroides	*Rhodococcus corynebacteroides*	Yassin and Schaal (2005)
Nocardioides simplex	*Rhodococcus erythropolis*	Yoon et al. (1997)
Nocardia calcarea	*Rhodococcus erythropolis*	Rainey et al. (1995a)
Nocardia restricta	*Rhodococcus equi*	Goodfellow and Alderson (1977)
Brevibacterium sterolicum	*Rhodococcus equi*	Ladrón et al. (2003)
Tsukamurella wratislaviensis	*Rhodococcus wratislaviensis*	Goodfellow et al. (2002)

applied to them have increased in their powers of discrimination (Goodfellow et al. 1998; Jones and Goodfellow 2010). Now with molecular techniques, especially 16S rRNA gene sequence analyses, much of this anarchy and confusion is becoming resolved, and *Rhodococcus* taxonomy is looking more stable. Yet several important unresolved problems remain, as this chapter will point out.

Furthermore, as Goodfellow et al. discussed in 1998, many of the new environmental isolates showing novel metabolic activities were never characterised adequately after original isolation to allow their valid speciation. Consequently, it is impossible to know whether each *Rhodococcus* environmental isolate represents a novel population, or is the same as or closely related to those used already in previous studies. Clearly, this is an unsatisfactory situation which must compromise effective communication between people working with members of this genus. In fact, despite considerable advances in our understanding of their systematics in the past two decades, the same unwillingness to meet this essential requirement persists, and in some respects has worsened in its scale, as discussed later.

The aim of this chapter is to look at the present state of the taxonomy of members of the genus *Rhodococcus*, identifying some of the problems that exist with it, and to suggest how these might be addressed and possibly resolved by taking advantage of some of the more powerful molecular techniques now available and by the application of a wider range of phylogenetic markers (Gürtler et al. 2004). But first we need to consider how the genus *Rhodococcus* came to be.

2 The Past: A Brief History of the Genus *Rhodococcus*: A Red Coccus

The reviews of Goodfellow et al. (1998) and Jones and Goodfellow (2010) provide highly readable accounts of the history of this troubled genus and the impacts chemotaxonomic and molecular approaches have had on its emergence into what is now a more clearly defined taxon. Consequently, only some of the major events will be discussed here.

Zopf (1891) first proposed forming the genus *Rhodococcus* to include two red bacteria named then by Overbeck (1891) as *Micrococcus erythromyxa* and *M. rhodochrous*. This genus name, also suggested by Winslow and Rogers (1906) and Molisch (1907) for other red cocci, and with *R. rhodochrous* as type strain, was maintained in the first four editions of Bergey's *Manual of Determinative Bacteriology*. These and several other strains were then reassimilated into the genus *Micrococcus* for the fifth edition of Bergey's *Manual*, an arrangement which persisted into the sixth, and which reflected the existing lack of suitable characters for their description.

Gordon (Gordon and Mihm 1957, 1959) was the first to realise fully the inadequacies of relying totally on cell morphology and staining responses to sort out the taxonomy of a large number of the available strains that had been placed,

often reluctantly, into several different genera including *Rhodococcus*. Consequently, she undertook a series of defining studies applying a more polyphasic approach (e.g. Gordon and Mihm 1961). Initially, she placed all these into the genus *Mycobacterium* as *M. rhodochrous*, based mainly on similarities in their colony morphology and the acid fastness of some cultures (Gordon 1966). It took numerical taxonomy to reveal the true taxonomic rank of *M. rhodochrous*, which emerged from these studies as a grouping worthy of a separate genus status, and one clearly distinct from other members of the genus *Mycobacterium* as well as *Nocardia* and *Corynebacterium* (Goodfellow et al. 1998).

However, it was not until the seventh edition of Bergey's *Manual of Determinative Bacteriology* that some formal action was taken, where these strains were removed from the genus *Mycobacterium* and re-assigned tentatively to a number of *Nocardia* species (McClung 1974). The genus *Gordona* (later *Gordonia*) proposed by Tsukamura (1971), to whose members some *M. rhodochrous* strains were very similar phenotypically, was at that time considered a genus of uncertain status (*incertae sedis*), and was not mentioned in this edition of the *Manual*. Its formation and eventual formal recognition play an important role in the *Rhodococcus* story.

Among the possible taxonomic fates considered by Bousefield and Goodfellow (1976) for these *M. rhodochrous* strains included leaving them where they were, placing them in another pre-existing genus or constructing a new genus to incorporate them. The genus *Rhodococcus* already existed, but had fallen into disuse. It had been resurrected by Tsukamura (1974) to contain six species which had been placed as members of the genus *Gordona*, namely *R. aurantiacus*, *R. bronchialis*, *R. rhodochrous*, *R. roseus*, *R. rubropertinctus* and *R. terrae*. This species list was amended by Goodfellow and Alderson (1977) after their extensive numerical taxonomic study, which led to the addition of four other species *R. coprophilus*, *R. corallinus*, *R. equi* and *R. ruber*. The DNA:DNA hybridisation data generally supported these placements (Mordarski et al. 1976, 1977).

The genus *Rhodococcus* was recognised in the *Approved List of Bacterial Names* (Skerman et al. 1980) and in the first edition of Bergey's *Manual of Systematic Bacteriology* (Goodfellow 1989), where 14 species (some now no longer recognised as *Rhodococcus* species – see below) were listed. Again, the genus *Gordona* was not considered deserving of a mention in the *Manual*.

Now, in the second edition of Bergey's *Manual of Systematic Bacteriology*, 30 *Rhodococcus* species are recognised and described (Jones and Goodfellow 2010). However, as mentioned above, few of the original *Rhodococcus* species of Tsukamura (1974) have survived (Table 1a). The decisions to remove them to other genera have been based largely on comparisons of their chemotaxonomic characters. These include (Table 2) their peptidoglycan structure, wall sugar chemistry, mycolic acid chain lengths, polar lipids and fatty acid compositions and menaquinone chemistry (Goodfellow et al. 1998), supported by 16S rRNA gene sequence comparisons. Such data verified that members of the genera *Nocardia* and *Rhodococcus* were both phylogenetically heterogenous groupings. Consequently, considerable movement of their members has occurred since with the transfer of several members from *Rhodococcus* to *Gordona*/*Gordonia* (a genus re-established by

Table 2 Major chemotaxonomic differences between the genera in the family *Corynebacteriniae*

Character	*Rhodococcus*	*Nocardia*	*Gordonia*	*Skermania*	*Millisia*	*William-sia*	*Tsuka-murella*	*Dietzia*	*Corynebacterium*	*Turicella*	*Mycobacterium*	*Segniliparus*	*Smaragdicoccus*
DNA G+C mol%	63–73	64–72	63–69	68	65	64–65	67–74	66–73	51–67	65–72	62–70	68–72	64
Mycolic acid chain length	30–54	40–64	46–66	58–64	44–52	50–56	64–78	34–39	22–38	Absent	60–90	>90? α^+, α, α' mycolates	43–49
Cell wall sugars	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	Arabinose galactose	nd	Arabinose galactose
Peptido-glycan type	A1-γ	A1-γ	A1-γ	A1-γ	A1-γ	A1-γ	A1-γ	A1-γ	A1-γ	*A1-γ	A1-γ	*A1-γ	A1-γ
Muramic acid substitution	Glycolated	Glycolated	Glycolated	Glycolated	Glycolated	Glycolated	Glycolated	Acetylated	Acetylated	nd	Glycolated	nd	Glycolated
Mena-Quinone type	MK-8(H_2)	MK-8 (H_4.ω-cycl)	MK-9(H_2)	MK-8(H_4.ω-cycl)	MK-8(H_2)		MK-9	MK-8(H_2)	MK-8(H_2) or MK9(H_2)	MK-(10) or MK-(11)	MK9(H_2)	nd	SQA-8(H_4.ω-cycl) SQB-8(H_4.ω-cycl)
Tuberculo-stearic acid?	+	+	+	+ (trace)	+	+	+	−	(+) some	+	±	+	−
Phosphatidyl-ethanolamine	+	+	+	+	+	+	+	+	−	nd	+	nd	+
Current number of species	31	>50	16	1	1	5	5	7	>50	1	>60	2	1

Stackebrandt et al. 1988 to accommodate these strains), *Corynebacterium* and *Nocardia* and from *Nocardia* and *Tsukamurella* to *Rhodococcus* (see below). Furthermore, some of the re-classified rhodococci provided the foundation for establishing new mycolic acid producing genera, i.e. *Dietzia* (Rainey et al. 1995b) and *Tsukamurella* (Collins et al. 1988). Since then, many new species have been described to include isolates from quite different habitats (Table 3), and more new species almost certainly await description, as discussed later.

3 Current Systematics

Our present understanding of the properties that delineate members of this genus is described, and the outstanding unresolved taxonomic problems are discussed.

3.1 The Genus Rhodococcus

Rho.do.coc'cus. Gr.n. *rhodon*, the rose; Gr.n. coccus, a grain, M.L. neut.n.

On the basis of extensive polyphasic taxonomic data, members of the genus *Rhodococcus* are currently placed in the mycolic-acid-forming sub-order *Corynebacterineae* in the family *Nocardiaceae* within in the phylum *Actinobacteria*, which contains the Gram-positive, high mol% G+C bacteria. It has been suggested that the *Nocardiaceae* should be emended on the basis of 16S rRNA signature nucleotides (Zhi et al. 2009) to incorporate all the genera previously placed in the family *Gordoniaceae*. Therefore, in addition to *Rhodococcus* (Goodfellow et al. 1998; Zopf 1891), *Nocardia* (Trevisan 1889) and *Smaragdicoccus* (Adachi et al. 2007), this family would now include *Gordonia* (Stackebrandt et al. 1988; Tsukamura 1971), *Skermania* (Chun et al. 1997), *Williamsia* (Kämpfer et al. 1999) and *Millisia* (Soddell et al. 2006). The major chemotaxonomic features of these and the other genera in the *Corynebacterineae* are given in Table 2.

The genus *Rhodococcus* embraces organisms with the following characteristics (Goodfellow et al. 1998; Goodfellow and Maldanado 2006; Jones and Goodfellow 2010): Members are Gram positive to Gram variable, non-motile, aerobic chemoorganotrophic organisms with an oxidative metabolism, able generally to use a wide range of organic compounds as sole carbon and energy sources. They demonstrate a life cycle whose complexity varies among the different members. Depending on the strain, rods and cocci may undergo a series of morphological changes, with the cocci in some converting to rods and filaments. Some of these forms branch and may develop into extensively branched filaments or hyphae, which can become aerially organised. These various morphological forms then fragment, eventually reverting again to the cocci and short rods. In most but not all species some morphological forms generated during these simple life cycle stages stain partially

Table 3 List of all the currently recognised species of *Rhodococcus*, and their original sites of isolation

Validly Named *Rhodococcus* Species	Original Habitat of Isolated Strain	Reference
Rhodococcus aetherivorans	Sludge from bioreactor treating chemical waste	Goodfellow et al. (2004)
Rhodococcus baikonurensis	Air in Russian space laboratory	Li et al. (2004)
Rhodococcus cercidiphylli	Leaf sample	Gaitanis Li et al. (2008)
Rhodococcus coprophilus	Herbivorous dung/activated sludge foam	Rowbotham and Cross (1977)
Rhodococcus corynebacteroides	Air-contaminated culture medium	Yassin and Schaal (2005)
Rhodococcus equi	Widely distributed in soil	Goodfellow and Alderson (1977, 1979)
Rhodococcus erythropolis	Soil/activated sludge foam	Goodfellow and Alderson (1977, 1979)
Rhodococcus fascians	*Chrysanthemum morifolium*	Goodfellow (1984); Klatte et al. (1994a)
Rhodococcus globerulus	Soil	Goodfellow et al. (1982)
Rhodococcus gordoniae	Blood culture from patient suffering fatal pneumonia	Jones et al. (2004)
Rhodococcus imtechensis	Pesticide-contaminated land site	Ghosh et al. (2006)
Rhodococcus jostii	Skeletal remains in mediaeval grave	Takeuchi et al. (2002)
Rhodococcus koreensis	Industrial waste water	Yoon et al. (2000a)
Rhodococcus kroppenstedtii	Cold Himalayan desert	Mayilraj et al. (2006)
Rhodococcus kunmingensis	Plant rhizosphere	Wang et al. (2008)
Rhodococcus kyotonensis	Rhizosphere soil	Li et al. (2007)
Rhodococcus maanshanensis	Soil	Zhang et al. (2002)
Rhodococcus marinonenascens	Marine sediments	Helmke and Weyland (1984)
Rhodococcus opacus	Soil	Alvarez et al. (1996), Klatte et al. (1994b)
Rhodococcus percolatus	Culture enriched with 2,4,6-trichlorophenol	Briglia et al. (1996)
Rhodococcus phenolicus	Space centre bioprocessor	Rehfuss and Urban (2005)
Rhodococcus pyridinivorans	Industrial waste water	Yoon et al. (2000b)
Rhodococcus qingshengii	Contaminated land site	Xu et al. (2007)
Rhodococcus rhodnii	Intestine of bug	Goodfellow and Alderson (1977, 1979)
Rhodococcus rhodochrous^{T}	Soil/activated sludge foam	Tsukamura (1974), Zopf (1891)
Rhodococcus ruber	Soil/activated sludge foam	Goodfellow and Alderson (1977), Kruse (1896)
Rhodococcus triatomae	Blood-sucking bug	Yassin (2005)
Rhodococcus tukisamuensis	Soil	Matsuyama et al. (2003)
	Soil	Goodfellow et al. (2002)

(*continued*)

Table 3 (continued)

Validly Named *Rhodococcus* Species	Original Habitat of Isolated Strain	Reference
Rhodococcus wratislaviensis		
Rhodococcus yunnanensis	Forest soil	Zhang et al. (2005)
Rhodococcus zopfii	Bioreactor treating toluene/phenol	Stoecker et al. (1994)

acid fast. Not all are red, and colony colour can vary with strain from colourless to buff, cream, yellow, orange and red.

The *Rhodococcus* peptidoglycan contains glycolated muramic acid residues in the glycan chain and *meso*-2,6-diaminopimelic acid as the dibasic amino acid in the tetrapeptide. Thus, the peptidoglycan is of the A1γ type. Arabinose and galactose are major wall sugars. Mycolic acids are 30–54 carbon atoms in length and contain as many as four double bonds. Pyrolysis gas chromatography of their mycolic acid esters releases fatty acids containing 12–16 carbon atoms. The predominant isoprenologue consists of dehydrogenated menaquinones containing eight isoprene units, i.e. MK 8(H_2). Major phospholipids are diphosphatidylglycerol, phosphoethanolamine and phosphatidyly inositol mannosides, and fatty acids are straight-chain saturated and monosaturated fatty acids and 10-methyloctadecanoic branched fatty acids (tuberculostearic acid). The G+C of the DNA is 63–73 mol%.

Type species is *R. rhodochrous* (Zopf 1889) Tsukamura 1974, 43^{VP}.

3.2 Current Species of Rhodococcus

At the time of writing this article, 31 species of *Rhodococcus* had been validly named and recognised (Euzeby, http://bacterio.cict.fr/qr/rhodococcus.html). These are listed in Table 3, together with their original sites of isolation. As mentioned earlier and shown in Table 1a, b, a considerable shuffling among members of this and other genera in the *Corynebacterineae* has taken place, after additional characters have clarified their phylogeny. For example, some species originally included in the genus *Rhodococcus* have been re-assigned subsequently into several different genera (Table 1a) [e.g. *Rhodococcus maris* is now *Dietzia maris* (Rainey et al. 1995b)], while others originally placed elsewhere are recognised now as *Rhodococcus* spp. (Table 1b) [e.g. *Rhodococcus corynebacteroides* was previously *Nocardia corynebacteroides* (Yassin and Schaal 2005)]. In a few, they emerge as later synonyms of earlier described species [e.g. *R. roseus* is a synonym of *R. rhodochrous* (Rainey et al. 1995a)].

Their phylogenetic relationships, based on 16S rRNA sequence data, are illustrated in Fig. 1. The species appear to cluster into three clear groups, which McMinn et al. (2000) have referred to as the *R. equi*, *R. erythropolis* and

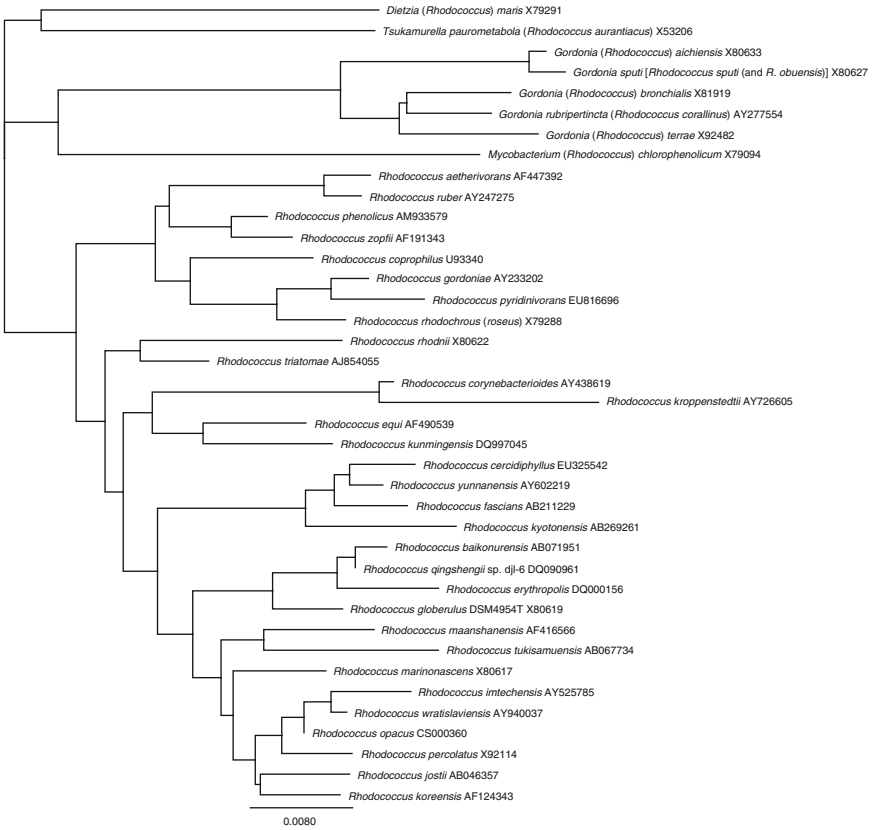

Fig. 1 Phylogenetic tree of earlier designated (Table 1a, b) and all the currently recognised species of *Rhodococcus* (Table 2) constructed by Neighbour-Joining within Geneious 3.8.5 (Biomatters)

R. rhodochrous sub-clades. The taxonomic implications of these groupings are unclear, and although it was suggested that they might represent separate genera (Goodfellow et al. 1998; McMinn et al. 2000), this proposal has not been pursued.

Still to be settled is the true taxonomic position of *Rhodococcus equi*, a serious pathogen in horses and humans, which until recently was the sole member of one such clade (McMinn et al. 2000). It was moved into the genus *Rhodococcus* from the genus *Corynebacterium* because its muramic acid residues in the peptidoglycan were acetylated not glycolated (Goodfellow and Alderson 1977). It now seems that the 16S rRNA sequences of members of another genus *Segniliparus*, clusters into the *R. equi* clade as a near neighbour, being 94.5–94.8% similar. This might support the possible exclusion of *R. equi* from the genus *Rhodococcus*, although DNA:DNA hybridisation values (2%) between *R. equi* and *Segnliparaus* species would suggest they are not closely related. In fact *R. kunmingensis* (Wang et al. 2008) is much more closely related to *R. equi* and is a member of the same clade (Fig. 1), with

98.2% shared similarity in their 16S rRNA gene sequences. However, these too share only a 38.4% DNA:DNA hybridisation level, and *R. kunmingensis* differs markedly in its phenotypic and ecological properties from *R. equi* (Wang et al. 2008). Thus, whether *R. equi* should continue to be placed in the genus *Rhodococcus* or moved to a separate or alternative existing genus still awaits resolution. Its whole genome sequence data, not available at the time of writing, might assist in deciding its taxonomic fate.

4 Ecology of *Rhodococcus* spp.

As Table 3 shows, rhodococci have been isolated from many different habitats, although they mostly arise initially from faecally contaminated soil and aquatic sediments (Jones and Goodfellow 2010), where often they have then become enriched from persistent contamination with xenobiotic and other often complex organic compounds that they can use as sole carbon and energy sources. It seems unlikely that our current understanding of their global distribution is complete, since they have also been isolated frequently from foaming activated sludge reactors (De los Reyes 2009), from skeletal remains in graves (*R. jostii*) and from the atmosphere of a Russian space laboratory (*R. baikonurensis*), as shown in Table 3. Some like *R. equi* are serious animal and human pathogens, especially in immuno-compromised patients, while others are members of the plant rhizosphere community, gall-forming plant pathogens or symbionts in the gastrointestinal tract of blood-sucking arthropods. As discussed elsewhere in this book, their role in degrading xenobiotic compounds in a range of natural habitats is considerable (Larkin et al. 2006; McLeod et al. 2006).

5 Identifying New *Rhodococcus* Species

The problems encountered in attempting to classify and identify strains of *Rhodococcus* when only cell morphology data were available to early taxonomists have been discussed above. Only with a combination of morphological, chemotaxonomic (Table 2) and phylogenetic characters can members of this genus now be identified more reliably (Goodfellow et al. 1998; Goodfellow and Maldanado 2006; Jones and Goodfellow 2010). Detailed descriptions of all the currently validly described species of *Rhodococcus* listing their individual attributes are given in Jones and Goodfellow (2010), and so are not repeated here. The application of rRNA-targeted, fluorescently tagged oligonucleotide probes using fluorescence in situ hybridisation or FISH (Nielsen et al. 2009) has so far been restricted to the rhodococci found in activated sludge foams (Davenport et al. 2000), but this and other rapid polymerase chain reaction (PCR) molecular methods for their identification (Gürtler et al. 2004; Ladrón et al. 2003) will almost certainly increase

in application, and provide a clearer view of their global distribution. Restriction fragment length patterns (RFLP) of the 16S rRNA, *hsp60* and *choE* genes have been developed for typing some members of this genus (Gürtler et al. 2004), and profiling the 16S rRNA–23S rRNA inter-spacer region, which was productively used for many other bacteria (Gürtler and Stanisich 1996), needs to be explored for its value for *Rhodococcus* species identification.

6 Tidying Up *Rhodococcus* Systematics

The literature is full of biodegradation/bioremediation studies employing *Rhodococcus* isolates, for which no formal taxonomic descriptions are available but which have been extensively characterised for their biochemical attributes. The communication problems associated with persisting with this state of affairs were alluded to earlier. However, in many cases the 16S rRNA genes of these isolates have been sequenced, and so using these data, it is possible, with due caution, to ascribe many of these to existing *Rhodococcus* species. Thus, the *Rhodococcus* isolates listed in Table 4 represent isolated strains placed by the original authors into existing validly named species using only 16S rRNA sequence data. Table 5 lists isolates for which 16S rRNA sequence data have been generated but where no attempt was made by the original authors to assign them to existing species of *Rhodococcus*. However, based on their 16S rRNA similarities, it is possible to suggest, with due caution, to suggest to what species each of these might belong, as shown.

These placements require confirmation since the problems associated with relying solely on 16S rRNA sequence comparison data to make decisions of this kind are well documented (Rossello-Mora and Amann 2001; Rossello-Mora and Kampfer 2004). Its highly conserved nature means that other information (DNA: DNA hybridisation values) is required to support such conclusions. Even so, these 16S rRNA sequence comparison data strongly support the view that some of these cultured isolates may belong to novel species, and particular effort should be directed at resolving their taxonomy. Thus, for some strains listed in Table 6 (KT1110, DFA3, TO9), their 16S rRNA sequences had very low similarities to all other known rhodococcal sequences. However, again these decisions need to be confirmed by applying other phylogenetic marker genes and adopting a polyphasic approach (Gillis et al. 2005; Vandamme et al. 1996).

Gürtler et al. (2004) have discussed critically the value of applying alternative genes to help clarify the systematics of *Rhodococcus*. These included those encoding for outer membrane porin proteins, virulence genes only found in *R. equi* and *R. fascians* and, in particular, genes involved in reactions in the biodegradation of complex natural and xenobiotic organic compounds. Examples of the latter and their occurrence in cultured strains are listed in Table 7. Some such as fossil fuel desulfurisation genes seem to be restricted to *Rhodococcus* strains (Gürtler et al. 2004). With others, the particular gene is found in several *Rhodococcus* species and even other genera (Gürtler et al. 2004), while in a few the gene has been found so far in a single

Table 4 Other cultured strains that have been designated provisionally to an existing species of *Rhodococcus* by the authors, based only on the 16S rRNA sequence data listed by GenBank accession number

Species	Strain Name	Reference	Sequence Accession Number
R. aetherivorans AF447392	F (BCP1)	Frascari et al. (2006)	DQ001072
R. baikonurensis AB071951	11_(MB)_6mbsf; 7E_(MB)_50.2mbsf	Biddle et al. (2005)	DQ344825; DQ344821
	EN3	Lee et al. (2006)	DQ842491
R. coprophilus U93340	Bt 14	Mohr and Tebbe (2007)	AJ971866
R. equi AF490539	871-AN029;871-AN030	Brandão et al. (2002)	AF420418;AF420420
	JL-S2	Du et al. (2006)	AY745838
	MI-11a	Ikner et al. (2007)	DQ180958
	KUA-6	Iwaki et al. (2008)	AB376627
	PO-1	Siragusa et al. (2007)	DQ869048
R. erythropolis DQ000156	F22	Aspray et al. (2005)	AY496587
	TM14_1	Barbieri et al. (2007)	DQ279384
	7/1	Bej et al. (2000)	AF181691
	870-AN019; 871-AN053; ANT-AN007; ARG-AN024; ARG-AN025; 122-AN065	Brandão et al. (2002); Heald et al. (2001)	AF420415; AF420422; AF420412; AF420416; AF420417; AY044095
	Pi71; Rs73	Fahy et al. (2006)	AM110074; AM110077
	KS1; NSA5-1; NSA6	Futamata et al. (2004)	AB177888; AB177889; AB177885
	Lact1	Groudieva et al. (2004)	AF513396
	KUA-2; KUA-4	Iwaki et al. (2008)	AB376623; AB376625
	MSB3003	Lagacé et al. (2004)	AY275517
	E38	Lo Giudice et al. (2007)	DQ667074
	GIC31w; GIC32; GIC38	Miteva et al. (2004)	AY439242; AY439243; AY439248
	FB6; FB8	Radwan et al. (2007)	EF092424; EF092422
	MOLA 353	Ribalet et al. (2008)	AM945583
	MB1	Williams et al. (1997)	U68710
	S-7	Qi zhao et al. J Environ Sci 19,332-	DQ306923
R. fascians (*luteus*) *X79187*	Bt 11	Mohr and Tebbe (2007)	AJ971863
R. fascians AB211229	5/1; 5/14	Bej et al. (2000)	AF181689; AF181690
	JL-60; NPO-JL-61	Du et al. (2006)	AY745830; AY745831
	4a-1; RG-14	Fredrickson et al. (2004)	AY561527; AY561580
	NSA3-1	Futamata et al. (2004)	AB177884
	iRIV10; iRIV4; iRV10	Idris et al. (2004)	AY358010; AY358008; AY358017
	B11; B15; B7; D13; D4; D51; E56; E57; F30; F32; G3; G31; G33; G76; G77; G9; H24; I14; I21; N44; E60	Lo Giudice et al. (2007); Michaud et al. (2004)	DQ646855; DQ646857; DQ646853; DQ646868; DQ646867; DQ652551; DQ831960; DQ667082; DQ667090; DQ667091; DQ667103; DQ667123; DQ667124; DQ667128; DQ667129; DQ667109; DQ831961; DQ831962; DQ831964; DQ831975; AY316681

(*continued*)

Table 4 (continued)

Species	Strain Name	Reference	Sequence Accession Number
	GIC26; GIC36	Miteva et al. (2004)	AY439237; AY439246
	NF42	Namba et al. (2007)	AB182204
	43/02	Saul et al. (2005)	AY571803
	GWS-BW-H95M	Stevens et al. (2007)	AY370623
	GA-05	Yakimov et al. (2004)	AJ561177
R. globerulus DSM4954T X80619	EJP75	Poole et al. (2001)	AJ302331
R. imtechensis AY525785	RKJ300	Ghosh et al. (2007)	AY525785
R. koreensis AF124343	871-AN040	Brandão et al. (2002)	AF420421
R. kroppenstedtii AY726605	TW53	Peng et al. (2008)	DQ462176
R. opacus CS000360	RD6.2	De Marco et al. (2004)	AY436807
	G-50; G-81	Gavrish et al. (2008)	EF599977; EF599978
	tSp14	Izumi et al. (2006)	AJ971395
	K004	Sfanos et al. (2005)	AY368569
	SW09	Kim and Fuerst (2006)	DQ227674
	TCH14; TCH4; TKN14; TKN45; TKN46	Taki et al. (2007)	AB183440; AB183439; AB183436; AB183437; AB183438
R. pyridinivorans EU816696	HA01; HN2006A	Aly et al. (2008)	EU622789; AM231909
	TUT1024; TUT1025	Hiraishi et al. (2003)	AB098592; AB098593
	MOP100	Kim et al. (2007b)	AY927229
R. rhodochrous AF439261	ARG-BN062	Brandão et al. (2002)	AF420423
R. ruber AY247275	SP2B	Amouric et al. (2006)	AY887067
	USA-AN012	Brandão et al. (2002)	AF420413
	PR-N14	Daane et al. (2001)	AF353688
	KUA-3	Iwaki et al. (2008)	AB376624
	MOB100	Kim et al. (2007b)	AY927228
	SoD	Quatrini et al. (2008)	EU135971
	F786	Sfanos et al. (2005)	AY368570
R. wratislaviensis AY940037	S1-2; S2-2; S5-3; ZC-3	La Rosa et al. (2006)	AM076669; AM076670; AM076671; AM076672

All the gene databases were searched using Geneious 3.8.5 (Biomatters) and only those sequences for which publications were also available are included in the table

strain (Gürtler et al. 2004). With these different marker genes, the systematics of some strains where 16S rRNA sequence data could not place them into putative species (e.g. strains DFA3, IIPS7, LJ2 and TO9) may be more clearly resolved.

7 The Future of *Rhodococcus* Systematics

This chapter has attempted to assess the current state of the systematics of members of the genus *Rhodococcus* and to point out some areas which clearly need attention. All the available evidence would indicate that the number of recognised and validly

Table 5 Other strains that have been placed provisionally into a recognised species of *Rhodococcus* not in the original publications but as part of this review, again based solely on their 16S rRNA sequences from the given GenBank accession number

Species	Strain Name	Reference	Sequence Accession Number
R. aetherivorans AF447392	SoD	Quatrini et al. (2008)	AY496284
	Ellin170; Ellin172	Schoenborn et al. (2004)	AF409012; AF409014
R. baikonurensis AB071951	iMSN24	Rasche et al. (2006)	DQ401249
	IS0205	Brück et al. (2007)	DQ517184
	iZBN12	Wang et al. (1999)	DQ401257
	KSM-B-3	Koike et al. (1999)	AB032365
R. cercidiphyllus EU325542	B1L4; B2L1	Willumsen et al. (2005)	AJ634936; AJ634934
	YK9	Iida et al. (2002)	AB070471
	4A-4 (4N-4)	Belimov et al. (2005)	AY197005
	A83	Kim et al. (2007a); Kim and Fuerst (2006)	AM179867
	B21; D58; E46; F48	Lo Giudice et al. (2007)	DQ646859; DQ652552; DQ667076; DQ667078
	Fp2	Belimov et al. (2001)	AF288731
	iEI10; iEIII14; iRIV6	Idris et al. (2004)	AY364018; AY364028; AY358009
	K4-07B; OS-20	Mendez et al. (2008)	EF612291; EF612316
	RS-75	Adesina et al. (2007)	DQ846830
R. coprophilus U93340	J109	Süss et al. (2004)	AJ630193
R. corynebacterioides AY438619	cryopeg_11	Bakermans et al. (2003)	AY660692
	OS-11	Mendez et al. (2008)	EF612310
R. erythropolis DQ000156	T12	Kunihiro et al. (2005)	AB108558
	IA1XBOX	Hendrickx et al. (2006)	AY512640
	X309	Denis-Larose et al. (1997)	U87968
	1	Uroz and Heinonsalo (2008)	EF571896
	1S2.E8	Axelrood et al. (2002)	AY043563
R. fascians AB211229	YK2	Iida et al. (2002)	AB070458
	iRV15; iRV8	Idris et al. (2004)	AY358018; AY358016
	NAB16	Broderick et al. (2004)	AY395020
	pfB30	Sessitsch et al. (2004)	AY336559
R. jostii AB046357	FJ1117YT	Fujii et al. (2007)	DQ157773
	OUCZ26; OUCZ35; OUCZ44; OUCZ58	Leigh et al. (2006)	AY785734; AY785735; AY785736; AY785741
	B03	Achour et al. (2007)	AM285018
	OUCZ204	Leigh et al. (2006)	AY785749
	PN1	Takeo et al. (2003)	AB044557
	SoF	Quatrini et al. (2008)	AY496287

(*continued*)

Table 5 (continued)

Species	Strain Name	Reference	Sequence Accession Number
R. koreensis AF124343	YU6	Jang et al. (2005)	DQ011232
R. kyotonensis AB269261	g6	Katayama et al. (2007)	AB272767
	H14	Lo Giudice et al. (2007)	DQ667133
	m23; m3	Katayama et al. (2007)	AB272806; AB272808
R. marinonascens X80617	SW09	Kim and Fuerst (2006)	DQ227674
R. pyridinivorans EU816696	AN-22	Matsumura et al. (2004)	AB087282
	PA	Gaja and Knapp (1997)	AJ457068
	LE2	Wang et al. (2008)	EF683121
R. qingshengii sp. djl-6 DQ090961	TMP2	Kunihiro et al. (2005)	AB108557
	TM1	Kim et al. (2006)	AY642534
	RKJ5	Labana et al. (2005)	AY729889
	1awq	Ma et al. (2006)	AY077846
	ADC4	Valle et al. (2006)	DQ272471
	Q15	Whyte et al. (1998)	AF046885
R. rhodnii X80622	1P-5	Belimov et al. (2005)	AY197007
	2P4	Ghosh et al. (2007)	AY757296
R. rhodochrous AF439261	#1	Panikov et al. (2007)	AY773006
R. ruber AY247275	THF100	Kim et al. (2007b)	AY927230
	1P2; 1P4; 2P2	Ghosh et al. (2007)	AY757292; AY757293; AY757294
	IM-43760	Wang et al. (1999)	AF131484
R. wratislaviensis AY940037	OUCZ16	Leigh et al. (2006)	AY785730
	P2	Connon et al. (2005)	AY429711

All the gene databases were searched using Geneious 3.8.5 (Biomatters) and only those sequences for which publications were also available are included in the table

described species will increase from the present 31. This will be brought about by applying a wider range of molecular characterisation methods to existing and new isolates, including multilocus sequence typing of suitable housekeeping genes (Coenye et al. 2005; Maiden 2006) and whole-genome sequence analysis (Ventura et al. 2007). As far as we know, only one such sequence (McLeod et al. 2006) has been published for *Rhodococcus* strain RHA1 (closely related to *R. opacus*), and so it is too early to assess the taxonomic value of such data with members of this genus. However, these analyses are likely to become more feasible on a routine basis with improvements in the speed and cost of DNA sequencing (Hall 2007), and so their impact will be revealed soon.

It is worth repeating here the final comments of Goodfellow et al. (1998) in their review of *Rhodococcus* systematics, which still apply today. "While it is clear that much has been achieved in the field of rhodococcal systematics in recent years, much remains to be done".

Table 6 The biodegradative properties of cultured strains provisionally designated to recognised species of *Rhodococcus* in the original publications using only 16S rRNA sequence data given by the respective GenBank accession number

Substrate Biodegraded	Species	Strain Name	Reference
2,4-D	*R. erythropolis DQ000156.1*	F22	Aspray et al. (2005)
2,6,10,14-Tetramethylpentadecane (pristane)	*R. erythropolis DQ000156.1*	T12	Kunihiro et al. (2005)
	R. qingshengii sp. djl-6 DQ090961.1	TMP2	Kunihiro et al. (2005)
3-Chlorobenzoate	*R. erythropolis DQ000156.1*	S-7	Qi zhao et al. J Environ Sci 19,332-
Acyclic amines (morpholine, peperidine, pyrrolidine)	*R. qingshengii sp. djl-6 DQ090961.1*	TM1	Kim et al. (2006)
Alkane		KT1110	Koike et al. (1999)
	R. ruber AY247275.1	SoD	Quatrini et al. (2008)
	R. aetherivorans AF447392.1	SoD	Quatrini et al. (2008)
	R. koreensis AF124343.1	SoF	Quatrini et al. (2008)
Alkyl ether	*R. ruber AY247275.1*	MOB100	Kim et al. (2007b)
	R. pyridinivorans EU816696.1	MOP100	Kim et al. (2007b)
	R. ruber AY247275.1	THF100	Kim et al. (2007b)
Aniline	*R. pyridinivorans EU816696.1*	AN-22	Matsumura et al. (2004)
Azaarenes (nitrogen-containing heterocyclic aromatic hydrocarbons)	*R. cercidiphyllus EU325542.1*	B1L4	Willumsen et al. (2005)
	R. cercidiphyllus EU325542.1	B2L1	Willumsen et al. (2005)
Benzene	*R. erythropolis DQ000156.1*	Pi71	Fahy et al. (2006)
	R. erythropolis DQ000156.1	Rs73	Fahy et al. (2006)
Benzothiazoles	*R. pyridinivorans EU816696.1*	PA	Gaja and Knapp (1997)
Biphenyl	*R. erythropolis DQ000156.1*	MB1	Williams et al. (1997)
BTEX benzene toluene xylenes ethylbenzene	*R. erythropolis DQ000156.1*	IA1XBOX	Hendrickx et al. (2006)
Butane	*R. aetherivorans AF447392.1*	F (BCP1)	Frascari et al. (2006)
Cyclohexylacetic acid	*R. erythropolis DQ000156.1*	KUA-2	Iwaki et al. (2008)
	R. ruber AY247275.1	KUA-3	Iwaki et al. (2008)
	R. erythropolis DQ000156.1	KUA-4	Iwaki et al. (2008)

(*continued*)

Table 6 (continued)

Substrate Biodegraded	Species	Strain Name	Reference
	R. equi AF490539.1	KUA-6	Iwaki et al. (2008)
Desulfurisation of dibenzothiophene (DBT)	*R. qingshengii sp. djl-6 DQ090961.1*	RKJ5	Labana et al. (2005)
Dibenzofuran		DFA3	Noumura et al. (2004)
	R. pyridinivorans EU816696.1	HA01	Aly et al. (2008)
	R. pyridinivorans EU816696.1	HN2006A	Aly et al. (2008)
	R. erythropolis DQ000156.1	KS1	Futamata et al. (2004)
	R. fascians AB211229.1	NSA3-1	Futamata et al. (2004)
	R. erythropolis DQ000156.1	NSA5-1	Futamata et al. (2004)
	R. erythropolis DQ000156.1	NSA6	Futamata et al. (2004)
	R. fascians AB211229.1	YK2	Iida et al. (2002)
	R. cercidiphyllus EU325542.1	YK9	Iida et al. (2002)
Dibenzothiophene	*R. qingshengii sp. djl-6 DQ090961.1*	1awq	Ma et al. (2006)
		T09	Matsui et al. (2002)
	R. erythropolis DQ000156.1	X309	Denis-Larose et al. (1997)
Hexane	*R. ruber AY247275.1*	SP2B	Amouric et al. (2006)
Hydrocarbons	*R. fascians AB211229.1*	43/02	Saul et al. (2005)
	R. wratislaviensis AY940037.1	S1-2	La Rosa et al. (2006)
	R. wratislaviensis AY940037.1	S2-2	La Rosa et al. (2006)
	R. wratislaviensis AY940037.1	S5-3	La Rosa et al. (2006)
	R. wratislaviensis AY940037.1	ZC-3	La Rosa et al. (2006)
Methyl-*s*-triazines	*R. jostii AB046357.1*	FJ1117YT	Fujii et al. (2007)
Nitrile hydrolising	*R. erythropolis DQ000156.1*	122-AN065	Brandão et al. (2002); Heald et al. (2001)
Nitrophenol	*R. imtechensis AY525785.2*	RKJ300	Ghosh et al. (2007)
PCB (polychlorinated biphenyl)	*R. wratislaviensis AY940037.1*	OUCZ16	Leigh et al. (2006)
	R. jostii AB046357.1	OUCZ26	Leigh et al. (2006)
	R. jostii AB046357.1	OUCZ35	Leigh et al. (2006)

(*continued*)

Table 6 (continued)

Substrate Biodegraded	Species	Strain Name	Reference
	R. jostii AB046357.1	OUCZ44	Leigh et al. (2006)
	R. jostii AB046357.1	OUCZ58	Leigh et al. (2006)
Phenanthrene	*871-AN040*	I7	Bodour et al. (2003)
Polycyclic aromatic hydrocarbon	*R. ruber AY247275.1*	PR-N14	Daane et al. (2001)
Propane	*R. wratislaviensis AY940037.1*	P2	Connon et al. (2005)
Radiation-resistant	*R. fascians AB211229.1*	4a-1	Fredrickson et al. (2004)
	R. fascians AB211229.1	RG-14	Fredrickson et al. (2004)
TCE (trichloroethylene)	*R. opacus CS000360.1*	RD6.2	De Marco et al. (2004)
Toluene		LJ2	Wang et al. (2008)
Xylene	*R. pyridinivorans EU816696.1*	LE2	Wang et al. (2008)
	R. opacus CS000360.1	TCH14	Taki et al. (2007)
	R. opacus CS000360.1	TCH4	Taki et al. (2007)
	R. opacus CS000360.1	TKN14	Taki et al. (2007)
	R. opacus CS000360.1	TKN45	Taki et al. (2007)
	R. opacus CS000360.1	TKN46	Taki et al. (2007)
	R. koreensis AF124343.1	YU6	Jang et al. (2005)

The list is limited by the number of relevant studies that have been performed and not all strains could be accommodated into existing species. Those species shaded in yellow have been assigned by the respective publication, while those unshaded have been assigned by the current review both using 16S rRNA sequence homology data. All the gene databases were searched using Geneious 3.8.5 (Biomatters) and only those sequences for which publications were also available are included in the table

8 Note Added in Proof

A new species has been described as *R. jialingiae* sp. nov., isolated from sludge of a carbendazim wastewater treatment facility with djl-6-2T (= DSM 45257T = CCTCC AB 208292T) as the type strain and DQ185597 the GenBank accession number of the 16S rRNA gene sequence (Wang et al. 2009).

Table 7 Genes encoded in cultured strains provisionally designated by the authors into species of *Rhodococcus* names based only on 16S rRNA sequence data listed by GenBank accession number

Gene Encoding	Strain Name	Reference	Species	Sequence Accession Number	Substrate Biodegraded
Alkb	SoD	Quatrini et al. (2008)	*R. ruber AY247275.1*	EU135971	Alkane
	SoD	Quatrini et al. (2008)	*R. aetherivorans AF447392.1*	AY496284	Alkane
	SoF	Quatrini et al. (2008)	*R. koreensis AF124343.1*	AY496287	Alkane
Catechol 1,2-dioxygenase	AN-22	Matsumura et al. (2004)	*R. pyridinivorans EU816696.1*	AB087282	Aniline
Cytochrome P450 alkane monooxygena	MOB100	Kim et al. (2007b)	*R. ruber AY247275.1*	AY927228	Alkyl ether
dbfA1A2	DFA3	Noumura et al. (2004)		AB180235	Dibenzofuran
dfdA1A2A3A4 oxygenases	HN2006A	Aly et al. (2008)	*R. pyridinivorans EU816696.1*	AM231909	Dibenzofuran
Dibenzothiophene monooxygenase	IIPS7	Gupta biotechnol left 29 1465–8		DQ140354	Dibenzothiophene
Dioxygenase	LE2	Wang et al. (2008)	*R. pyridinivorans EU816696.1*	EF683121	Xylene
	LJ2	Wang et al. (2008)		EF683119	Toluene
	YK2	Iida et al. (2002)	*R. fascians AB211229.1*	AB070458	Dibenzofuran
	YU6	Jang et al. (2005)	*R. koreensis AF124343.1*	DQ011232	Xylene
dsz, dibenzothiophene desulfurizi	T09	Matsui et al. (2002)		AB074048	Dibenzothiophene
Extradiol dioxygenase	YK9	Iida et al. (2002)	*R. cercidiphyllus EU325542.1*	AB070471	Dibenzofuran
Monooxygenase	RD6.2	De Marco et al. (2004)	*R. opacus CS000360.1*	AY436807	TCE (trichloroe-thylene)
polyketide synthase	SW09	Kim et al. (2007b)	*R. marinonascens X80617.1*	DQ227674	
sox	X309	Denis-Larose et al. (1997)	*R. erythropolis DQ000156.1*	U87968	Dibenzothiophene
tmoA/xylM/xylE1	IA1XBOX	Hendrickx et al. (2006)	*R. erythropolis DQ000156.1*	AY512640	BTEX benzene toluene xylenes ethylbenzene

The list is limited by the number of relevant studies performed, and some strains cannot be accommodated into existing species. Those species shaded in yellow have been assigned by the respective publication, while those unshaded have been assigned by the current review both using 16S rRNA sequence homology data. All the gene databases were searched using Geneious 3.8.5 (Biomatters) and only those sequences for which publications were also available were included in the table

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The Rhodococcal Cell Envelope: Composition, Organisation and Biosynthesis

Iain C. Sutcliffe, Alistair K. Brown, and Lynn G. Dover

Contents

Abstract The cell envelopes of rhodococci and their closest relatives are dominated by the presence of large branched chain fatty acids, the mycolic acids. Here we review the structural features underlying the incorporation of the mycolic acids into the rhodococcal cell envelope, notably their covalent anchoring to the peptidoglycan–arabinogalactan complex and their organisation into an outer lipid permeability barrier. Rhodococcal cell envelopes also accommodate diverse non-covalently associated components such as channel-forming porin proteins, free lipids,

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lipoglycans, lipoproteins and capsules or cell envelope polysaccharides. Based on the extensive studies of cell envelope biogenesis in corynebacteria and mycobacteria, we have used a comparative genomics approach to examine the pathways for the biosynthesis of the major cell envelope components of *Rhodococcus jostii* RHA1.

1 Introduction

The genus *Rhodococcus* belongs to the suborder Corynebacterineae, a distinctive lineage within the phylum Actinobacteria (Gürtler et al. 2004; Jones and Goodfellow in press; Zhi et al. 2009). The members of this taxon are characterised by distinctive cell envelopes typically dominated by large branched chain lipids, the mycolic acids, and collectively they are often referred to as the mycolata. In addition to the mycolic acids, these bacteria share a number of other common cell envelope features, most notably an arabinogalactan (AG) cell wall polysaccharide that is covalently attached to the cell wall peptidoglycan and in turn provides a scaffold for the covalent anchoring of mycolic acids. Thus, the chemistry and organisation of these components in a distinctive cell envelope architecture represents one of the defining features of the biology of the mycolata. The mycolate cell envelope has received extensive study in the context of understanding the targets of several crucial antibiotics that are used against the pathogenic mycobacteria, most notably *Mycobacterium tuberculosis* (Dover et al. 2008a). These studies have been vital in providing comparative insights into cell envelope biology in the genus *Rhodococcus* (Sutcliffe 1998; Gürtler et al. 2004). Here we review recent progress in understanding of the composition, architecture and biosynthesis of the mycolate cell envelope, with particular reference to the rhodococci. In particular insights into the biosynthesis of these cell envelope components are now possible using a comparative genomics approach, based on the recently published genome sequence of *Rhodococcus jostii* RHA1 (McLeod et al. 2006).

2 Cell Envelope Composition in the Genus *Rhodococcus*: Covalently Associated Components

The mycolyl–arabinogalactan–peptidoglycan complex represents the defining covalently interlinked structure of the cell envelope of the mycolata. These components and their linkages are reviewed in the following sections.

2.1 Mycolic Acids

Mycolic acids are 2-alkyl branched, 3-hydroxy long chain fatty acids, which vary in size and complexity with the different genera of the mycolata (Fig. 1). Those of the

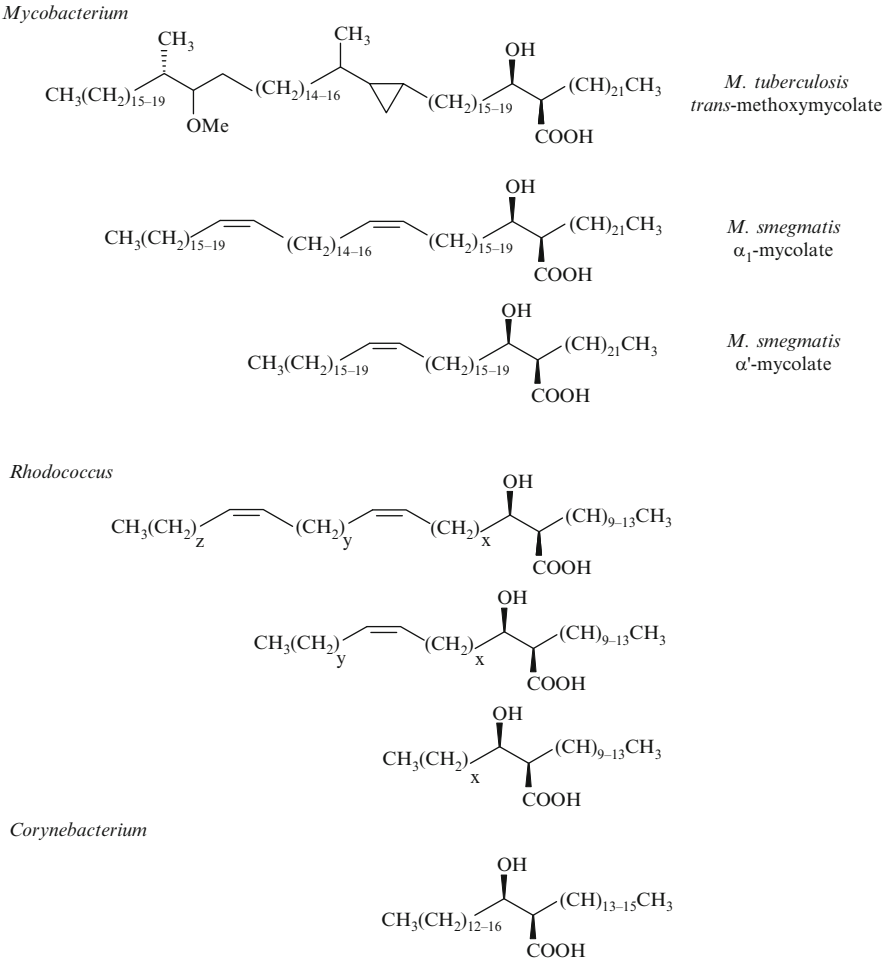

Fig. 1 *Structures of representative mycolic acids from Corynebacterium, Rhodococcus and Mycobacterium species, illustrating their differing complexity*. *M. tuberculosis* methoxymycolate is an example of the most complex mycolic acids. The less complex *M. smegmatis* mycolates encompass either double or single unsaturations whilst retaining the longer chain length. *Rhodococcus* sp. mycolates are of an intermediate size. They present an aliphatic 2-alkyl chain varying from 12 to 16 carbons, whilst the 3-hydroxyl meromycolate typically contains 18–40 carbons (i.e. x, y and z total 18–40). The rhodomeromycolates have relatively simple modifications containing up to four unsaturations at presently undetermined positions. *Corynebacterium* sp. mycolates are the simplest known mycolates. For simplicity, corynemycolates are represented as the typical 32–36 carbon aliphatic mycolic acids but a proportion of the total cell wall mycolates may also contain single or double unsaturations

corynebacteria are typically the smallest (size range 22–38 total carbons) and those of the mycobacteria are the most complex, with a size range of 60–90 carbons and a greater diversity of meromycolate chain functional groups such as cyclopropane, methoxy- and keto- modifications (Dover et al. 2004; Gürtler et al. 2004; Takayama

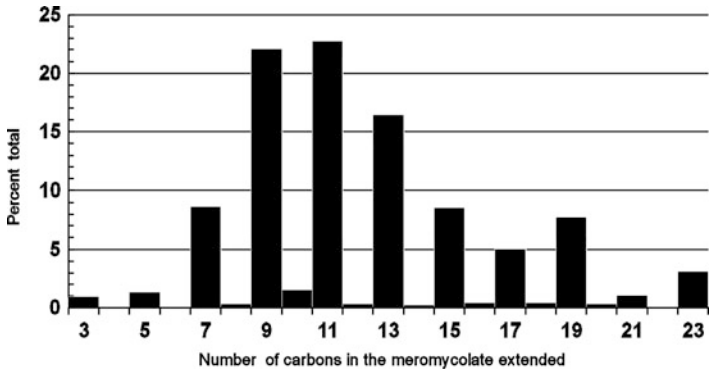

Fig. 2 *Lengths of the meromycolate main chain extensions in the mycolic acids of Rhodococcus rhodochrous*. The data of Stratton et al. (1999) was re-analysed to calculate the number of carbons by which the meromycolate main chain exceeded the length of the alkyl side branch. Data are presented with respect to the proportion of the different mycolic acid types within the total mycolates

et al. 2005). Members of the genus *Rhodococcus* produce mycolic acids of an intermediate size, typically with 28–54 carbons in total (Alshamaony et al. 1976; Klatte et al. 1994; Sutcliffe 1998; Stratton et al. 1999; Nishiuchi et al. 2000). One important feature of mycolic acid structure is the length of the main meromycolate chain compared to the alkyl side branch. In rhodococci, the alkyl side branch is typically a saturated alkyl chain of 10–16 carbons in length, whereas the meromycolate is a longer chain (C20–C42) with up to four carbon–carbon double bonds (Fig. 1). Thus consideration of the detailed profile of the mycolates present in *Rhodococcus rhodochrous* (Stratton et al. 1999) reveals that the lengths of each meromycolate chain will extend beyond that of its alkyl partner (Fig. 2) and similar data are evident for other rhodococci (Nishiuchi et al. 2000). The positions of the unsaturated bonds have not been determined unequivocally but studies of the mycolic acids of *Nocardia asteroides* suggest it is likely that they are localised in the distal regions of the meromycolate chain (Minnikin and O'Donnell 1984; Sutcliffe 1998). This would mean the region of the meromycolate chain proximal to the ester-linked terminus is effectively a saturated chain and would pack closely with the saturated alkyl branch. Thus these features need to be considered when predicting the arrangement of the mycolates esterified to AG within the rhodococcal cell envelope (see below).

2.2 The Peptidoglycan–Arabinogalactan Complex

As in other members of the mycolata, the peptidoglycan structure of members of the genus *Rhodococcus* has been determined to be of the A1γ type, i.e. with the diamino acid meso-diaminopimelic acid forming direct cross-linkages between

the stem peptides (Jones and Goodfellow in press). The muramic acid residues of the glycan strands are N-glycolyated, which is a comparatively unusual peptidoglycan modification (Uchida and Aida 1979; Vollmer 2008; Jones and Goodfellow in press). N-glycolylation is likely carried out during cytoplasmic peptidoglycan precursor biosynthesis, prior to lipid II formation (Raymond et al. 2005; Vollmer 2008; Jones and Goodfellow in press). A clear orthologue of the NamH protein recently identified as the *Mycobacterium smegmatis* oxygen-dependent hydroxylase responsible for N-glycolylation was identified in the *R. jostii* RHA1 genome (Raymond et al. 2005; RHA1_ro04045). Although the physiological function of N-glycolyation remains unclear, it is notable that deletion of *namH* in *M. smegmatis* increased susceptibility to lysozyme and β-lactam antibiotics (Raymond et al. 2005). As an extra hydroxyl group is introduced to the glycan chain, there may be additional hydrogen binding possibilities within the cell envelope, which could contribute to novel aspects of its supramolecular organisation.

The AG of the cell envelope is phosphodiester linked to the peptidoglycan by a well conserved linker unit (LU) of L-rhamnose-D-*N*-acetylglucosamine phosphate (Daffé et al. 1993). Mycobacterial AG has been extensively structurally characterised as the scaffold for the attachment of the mycolic acids (Besra et al. 1995; Bhamidi et al. 2008) and the target for the action of the anti-tubercular ethambutol (EMB; Takayama and Kilburn 1989). The heteropolymer is divided into distinct homopolymer galactan and arabinan domains. A galactan anchored to the peptidoglycan via the LU will typically carry three arabinan domains, the branched termini of which carry the mycolic acids. Comparatively little is known of the fine structure of AG from most mycolic acid containing bacteria, although an important comparative study revealed that the AG of *Rhodococcus equi* and *R. rhodochrous* have a similar domain organisation of a linear homogalactan bearing discrete arabinan domains (Daffé et al. 1993). The galactan of *R. equi* contained both 1→3, 1→5 and 1→6 glycosidic linkages whereas that of *R. rhodochrous* contained 1→2 and 1→5 linkages. Further galactan diversity was revealed in the galactans of *Nocardia* spp. The arabinan domains of the AG also exhibit similar variations, that of mycobacteria typically present pentaarabinosyl branched termini, which can carry four mycolates each (Besra et al. 1995; Bhamidi et al. 2008). In contrast, in *R. equi*, a range of arabinose termini are present including a linear arabinan, a triarabinosyl branched terminus and termini bearing mannose caps (Daffé et al. 1993). As minor variations on the AG core structure have been reported in *Tsukamurella paurometabolum* (Tropis et al. 2005a), it appears that AG is likely to be subject to genus and species-specific variations in fine structure, which may also extend to the presence of substituents such as succinate (Bhamidi et al. 2008). This may have implications regarding the extent to which rhodococcal cells are covered with covalently bound mycolates, the significance of which is discussed later.

In addition to the "secondary" cell wall polymers, Gram-positive bacteria also anchor proteins to their peptidoglycan through the action of sortase transpeptidase enzymes (Marraffini et al. 2006). Sortase substrates have a characteristic LPXTG motif (or variants thereof), of which the threonine residue is targeted for the

transpeptidation reaction. Sortase-mediated anchoring of proteins to the cell wall does not appear to be as prominent in Actinobacteria compared to Firmicute Gram-positive bacteria; important examples include the anchoring of the larger chaplins during production of aerial hyphae by *Streptomyces coelicolor* (Di Berardo et al. 2008) and the polymerisation and anchoring of pili in *Corynebacterium diphtheriae* (Mandlik et al. 2008a, b). Bioinformatic analyses have identified only a single sortase encoded in the *R. jostii* RHA1 genome (see PFAM family PF04203 at http://pfam.sanger.ac.uk//family/pf04203) and no members of the LPXTG family (PF00746) of canonical sortase substrates. In *R. jostii*, the sortase protein RHA1_ro03500 is apparently encoded in an operon with an adjacent coding sequence (RHA1_ro03501), which has the requisite C-terminal features, including an HPETG motif, that suggest RHA1_ro03501 might be the sortase substrate. However, this pairing aside, it is clear that sortase-anchored proteins are not numerically abundant in the predicted proteome of *R. jostii*.

3 Organisation of the Rhodococcal Cell Envelope

Determining how significant quantities of high molecular weight lipids (i.e. the mycolic acids) are organised within the cell envelope presented a significant challenge in earlier studies of the mycolata. However, the landmark studies of Minnikin (1982, 1991) provided a model that, following extensive biochemical, biophysical and structural analyses, has become accepted as the definitive model of the mycobacterial cell envelope (Brennan and Nikaido 1995; Daffé and Draper 1998). Subsequently, this model has been applied to models of the corynebacterial (Puech et al. 2001; Dover et al. 2004) and rhodococcal cell envelopes (Sutcliffe 1997, 1998). Recently, excellent cryo-electron microscopic studies have provided clear visualisations of this structure for both *Corynebacterium* and *Mycobacterium* spp. (Hoffmann et al. 2008; Zuber et al. 2008) and variations on this theme are most likely applicable to all mycolic-acid containing actinomycetes.

The central tenet of the Minnikin model is that the mycolic acids covalently attached to the AG have a perpendicular orientation with respect to the plane of the plasma membrane (Fig. 3). Thus, the mycolates form the basis of a second hydrophobic permeability barrier outside of the plasma membrane. This structure is analogous to the outer membranes of Gram-negative bacteria but is chemically and structurally distinct, most notably in that the defining feature of the permeability barrier is not a bilayer but the monolayer of bound mycolates. Nevertheless, depending on the extent to which bound mycolates are able to provide coverage of the whole bacterial cell surface, additional components may be needed to "plug" potential gaps in the mycolyl layer, a role proposed to be taken most likely by mycolic acid containing-glycolipids (see Sect. 4.3; Sutcliffe 1998; Puech et al. 2001; Zuber et al. 2008). Indeed, trehalose mycolates are likely to act as carriers for incorporation on newly synthesised mycolic acids into the cell envelope (Tropis et al. 2005b).

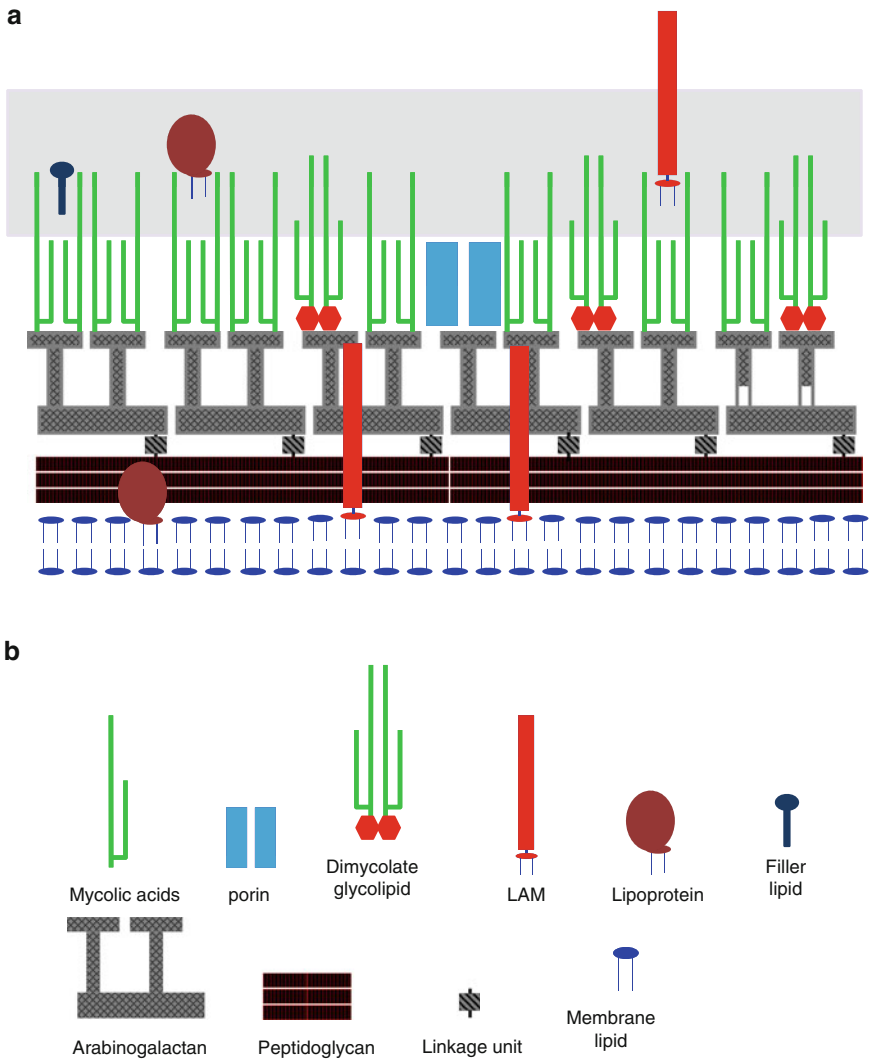

Fig. 3 *Model for the organisation of the rhodococcal cell envelope*. This adaptation of the classic Minnikin model (Minnikin 1991) emphasises that vertically orientated mycolic acids form the basis of the outer lipid permeability barrier. Components of the model in *panel A* are identified in *panel B*. No specific conformation for the peptidoglycan–AG complex is favoured in this schematic representation. Consideration of the sizes and conformations of rhodococcal mycolates suggest that there may be filler lipids (and possibly lipoglycans and lipoproteins) associated with the outer surface of mycolate layer (*grey box*) but this remains hypothetical. Not shown are the outermost layers composed of the cell envelope polysaccharides and/or capsules that are known to be present in many rhodococci

A second consideration is the extent to which the longer meromycolate chains of the mycolic acids project beyond the alkyl branches that are packed alongside them (Sect. 2.1) and, with longer chain mycolates, the extent to which folded conformations can be adopted (Villeneuve et al. 2007). In rhodococci, we propose that as the typical length of these meromycolate extensions corresponds most closely with the lengths of typical fatty acyl lipids (Fig. 2), it is likely that the permeability barrier provided by the mycolate layer is bolstered externally by the interaction of smaller amphiphiles (e.g., acylglycerol-based lipids), which may vary on a species and strain-specific basis, as discussed previously (Sutcliffe 1998; Puech et al. 2001). Moreover, it is notable that rhodococcal mycolates lack both the length and chain modifications necessary to achieve the more complex "Z" and "W" type conformations (e.g., see Villeneuve et al. 2007). Genus, species and strain-specific variations in the length of the mycolic acids (Dover et al. 2004; Gürtler et al. 2004; Takayama et al. 2005) dictate the precise configurations of the mycolates and so the extent to which this necessitates their interactions with "filler" lipids is thus likely to vary in a species-specific manner. Moreover, the mycolate layer is highly unlikely to be a static barrier, and the permeability of this barrier is likely to be regulated, consistent with studies showing that mycolic acid composition can vary with growth conditions (Sutcliffe 1998; Sokolovská et al. 2003; Stratton et al. 2003).

The representation in Fig. 3 is in reasonable agreement with the recently proposed "zippered" version of the classic Minnikin model, wherein free lipids are shown intercalated with the mycolates (Zuber et al. 2008). This model is in reasonable agreement with the measured thicknesses of the outer permeability barrier in corynebacteria (4–5 nm) and mycobacteria (7–8 nm) (Hoffmann et al. 2008; Zuber et al. 2008). However, specific studies are needed to further define the details of the organisation of the rhodococcal cell envelope. Such studies will be of considerable interest given that the length of the rhodococcal mycolates represents an intermediate stage between those of the corynebacterial and mycobacterial species whose cell envelopes have been most extensively studied (Hoffmann et al. 2008; Zuber et al. 2008). Thus the extent to which the projecting meromycolate chains interact with covering amphiphiles and other outermost components (notably capsules and other polysaccharides; see Sect. 4.4) is an important question for future study, particularly as these features will profoundly influence the cell surface hydrophobicity and thus the possible biotechnological applications of rhodococcal strains.

The Minnikin model focuses primarily on the organisation of the mycolates within the cell envelope. Thus, the organisation of the peptidoglycan in the mycolata has traditionally been assumed to be comparable to that of the peptidoglycan in other bacteria, that is, a layered structured wherein the peptidoglycan strands are orientated in parallel with the plane of the plasma membrane (Vollmer and Höltje 2004). Alternatively, it has been proposed that helical glycan strands of the peptidoglycan may have a novel vertical orientation (Dmitriev et al. 2005) and that in mycobacteria this allows for helical galactan chains of the AG to be intercalated within a grid of glycan "pillars" (Dmitriev et al. 2000). As yet this novel "scaffold" hypothesis has not yet received extensive support from studies on other organisms (Gan et al. 2008; Hayhurst et al. 2008) and it remains to be verified in the mycolata.

Finally, the presence of an outer lipid permeability barrier suggests that the location between this layer and the plasma membrane should be considered a "pseudo-periplasm" and also dictates that pathways must exist for both solute uptake (see Sect. 4.1) and secretion (notably of proteins but also of DNA). However, although there is a growing understanding of pathways of protein secretion in mycobacteria (DiGiuseppe Champion and Cox 2007), no specific systems for the export of proteins beyond the pseudoperiplasm have yet been identified.

4 Non-Covalently Associated Cell Envelope Components

As illustrated in Fig. 3, the mycolyl–arabinogalactan–peptidoglycan complex provides a scaffold upon which several crucial classes of cell envelope component can be localised. These non-covalently associated cell envelope components are reviewed in the following sections.

4.1 Channel Forming Porins

The organisation of the covalently linked mycolates and other cell envelope lipids into an outer lipid permeability barrier suggests that channel forming proteins (porins) need to be present to allow the accumulation of hydrophilic solutes. This prediction was confirmed first for *Mycobacterium chelonae* (Trias et al. 1992) and subsequently channel forming proteins have been identified in a considerable range of mycolic acid containing actinomycetes (Nikaido 2003; Ziegler et al. 2008). The channel forming proteins that have been best characterised to date, that is the cation selective MspA channel of *M. smegmatis* (Faller et al. 2004) and the anion selective channel PorB of *Corynebacterium glutamicum* (Ziegler et al. 2008), are both relatively small proteins that oligomerise to form their respective channels. MspA forms a novel octameric 16-stranded β-barrel structure (Faller et al. 2004) whereas PorB forms a putative pentameric structure that is unusual in containing α-helices (Ziegler et al. 2008).

Three studies have confirmed the presence of porins in rhodococci. Cation selective channels have been isolated from *Rhodococcus* (formerly *Nocardia*), *corynebacteroides* (Rieβ and Benz 2000) and *Rhodococcus erythropolis* (Lichtinger et al. 2000), whilst complementary anion and cation selective channels were identified in organic solvent extracts from *R. equi* (Rieβ et al. 2003). The ca. 2-nm wide cation selective channels of *R. equi* and *R. erythropolis* have similar biophysical properties. However, the N-terminal sequence determined for the *R. erythropolis* protein (Lichtinger et al. 2000) does not at present generate any significant homology matches to any known proteins. *M. smegmatis* MspA is the prototype of a porin family (PF09203; http://pfam.sanger.ac.uk//family/PF09203) and three members of this family (RHA1_ro03127; RHA1_ro04074; RHA1_ro08561) with

significant sequence homology to *M. smegmatis* MspA can also be identified in the *R. jostii* genome.

The identity of the anion selective channel of *R. equi* has yet to be determined, and it is notable that the known anion selective channels of corynebacteria appear to be unique to this genus (Ziegler et al. 2008). Thus other novel channel forming proteins are likely to be present in rhodococcal cell envelopes.

In addition to the above porins, Rv1968 from *M. tuberculosis* was recently described as the prototype for a new class of channel forming proteins (Siroy et al. 2008). In vitro, Rv1968 forms channels with a weak selectivity for cations. A single significant homologue of Rv1968 is encoded in the *R. jostii* genome (RHA1_ro00932; 145/308 amino acid sequence identity) suggesting that channels of this type are also likely to be present in rhodococcal cell envelopes.

4.2 Lipoglycans

The cell envelopes of most, but not all, Actinobacteria bacteria are characterised by the presence of membrane-anchored polysaccharides, the lipoglycans (Sutcliffe 1994; Rahman et al. 2009). In all mycolic acid-containing Actinobacteria studied to date, the lipoglycans present belong to the lipoarabinomannan (LAM) family (Nigou et al. 2003, 2008; Gilleron et al. 2005). As with AG, this lipoglycan family is characterised by a conserved core structure that then exhibits considerable species and strain-specific variation in fine structure. The core structure is defined by the presence of a phosphatidylinositolmannoside-based lipid anchor, which is extended into a 1→6 linked mannan domain of variable length (Nigou et al. 2003). In addition to mannose side chains, this mannan core will also carry arabinose or arabinan branches, which in turn may carry a variety of substituent motifs, most notable mannose caps in some strains (Nigou et al. 2003).

Three species of *Rhodococcus* have been investigated as to their lipoglycan structure. The structure of the LAM-like lipoglycan of *R. equi* (ReqLAM) was found to be the first known example of a 'truncated' LAM wherein the typical phosphatidylinositol-anchored lipomannan core is decorated with 1→2 linked mannose branches, some of which bear a single capping by *t*-arabinofuranose residues (Garton et al. 2002). Thus, the substantial arabinan domains of mycobacterial LAM are not present in this structure, which has immunomodulatory properties that may be relevant to the pathogenesis of disease in foals (Garton et al. 2002; Nigou et al. 2008). Likewise, the lipoarabinomannan of *Rhodococcus ruber* (Rru-LAM) is also a truncated LAM structure in which the lipomannan core is directly substituted with *t*-arabinofuranose residues (Gibson et al. 2003b). These truncated LAMs are thus closely structurally related yet distinct from each other and it is apparent that truncated LAMs represent as distinct subfamily within the LAM archetype (Gilleron et al. 2005). A LAM-like lipoglycan has also been identified in *Rhodococcus rhodnii* (Flaherty et al. 1996) but has not yet received full structural

characterisation. However, the arabinose content determined by gas chromatography suggests that this LAM might be more extensively arabinosylated (Flaherty et al. 1996). Notably, in these rhodococci, the LAM-like lipoglycans appear to be the sole membrane-anchored polysaccharides, whereas in mycobacteria, the LAM is accompanied in the membrane by a structurally inter-related lipomannan (Nigou et al. 2003).

The physiological functions of these lipoglycans remain obscure (Sutcliffe 2005) but recent advance in understanding the genetic basis of LAM biosynthesis have led to the generation of mutants abrogated in various stages of the LAM biosynthesis pathway. Mutation of an early stage in LAM biosynthesis (assembly of the mannan core) was achieved in *C. glutamicum*, although at a low frequency of homologous recombination and the mutant obtained exhibited notably poor in vitro growth (Mishra et al. 2008b). This provides the clearest evidence to date that LAM lipoglycans may not be essential for the growth of mycolic acid-containing Actinobacteria but that they are likely to be necessary for optimal growth.

By analogy with both lipoteichoic acids and other lipoglycans (Sutcliffe 1994; Rahman et al. 2009), it has generally been assumed that LAM family lipoglycans are anchored to the outer leaflet of the plasma membrane with the glycan polymer projecting into the 'pseudo-periplasm'. However, it remains possible that a subfraction of lipoglycans is surface exposed through trafficking and intercalation of the lipid anchor into the outer mycolate-based lipid layer (Fig. 3), as recently discussed for mycobacterial LAM (Pitarque et al. 2008). These two subfractions can be usefully distinguished as 'parietal' LAM (associated with the mycolate layer) and 'cellular' LAM (associated with the plasma membrane) (Gilleron et al. 2000; Pitarque et al. 2008).

4.3 Cell Envelope Lipids

The cell envelopes of rhodococci are rich sources of structurally diverse lipids, some of which have pronounced surfactant properties that facilitate the growth of the bacteria on hydrophobic substrates and may be of biotechnological significance (Lang and Philp 1998; Kuyukina, this volume; Sutcliffe 1998). These lipids are typically glycolipids including both acyl- and mycolyl-glycolipids (Table 1). There is also a rich diversity of lipopeptides and glycolipopeptides known to be produced by rhodococci (Table 1). The nature of the associations and the specific functions of these lipids within the rhodococcal cell envelope are largely unknown but it is likely that they can interact/intercalate with the covalently bound mycolic acids (see above). Whether the roles of these lipids are simply structural (i.e. as fillers to complete to the outer lipid permeability barrier) or more dynamic (e.g. in modulating surface physicochemical properties) remain to be determined.

Table 1 Representative cell envelope lipids of rhodococci

Lipid	Species	
Glycerol monomycolates	*R. erythropolis*	Ioneda and Ono (1996)
Glycosyl monomycolates	*R. erythropolis*	Kurane et al. (1995)
	R. rhodochrous	de Almeida and Ioneda (1989)
	R. ruber	Matsunaga et al. (1996)
Trehalose mycolates	*R. corynebacteroides*	Powalla et al. (1989)
	R. erythropolis	Kretschmer et al. (1982); Kurane et al. (1995)
	R. opacus	Niescher et al. (2006)
	R. rhodochrous	Asselineau and Asselineau (1978); de Almeida and Ioneda (1989)
	R. ruber	Matsunaga et al. (1996)
Acylated carotenoid glucosides	*R. rhodochrous*	Takaichi et al. (1997)
Acyl pentaglucoside	*R. corynebacteroides*	Powalla et al. (1989)
Succinylated acyl trehaloses	*R. erythropolis*	Uchida et al. (1989)
	Rhodococcus sp.	Tokumoto et al. (2009)
	R. wratislaviensis	Tuleva et al. (2008)
Peptidolipids (lipopeptides), mycolylpeptidolipids and peptidoglycolipids	*R. erythropolis*	Koronelli (1988)
	Rhodococcus sp.	Chiba et al. (1999); Peng et al. (2008)

4.4 Capsules and Cell Envelope Polysaccharides

The capsular polysaccharides of *Rhodococcus* spp. have received surprisingly little attention. Seven capsular serotypes of *R. equi* were initially defined by Prescott (1981), and the structures of six of these have been extensively characterised by Richards and co-workers (Richards 1994; Severn and Richards 1999). These polysaccharides are structurally diverse acidic heteropolysaccharides, typically characterised by the presence of acetal-linked pyruvate or lactic acid ether substituents. In many cases, the acidic character in part stems from the presence of glucuronic acid in the polymer repeating unit, although the structure of the serotype 4 capsule is notable for containing a 5-amino-3,5-dideoxynonulosonic (rhodaminic) acid (Richards 1994; Severn and Richards 1999). A recent study showed that inactivation of a gene encoding a putative mycolic acid transferase (*fbpA*) resulted in a failure to correctly encapsulate *R. equi* strain 103 (Sydor et al. 2008), possibly due to a failure to correctly incorporate capsule polymer into the cell envelope. Intriguingly, although the capsule has long been considered a potential virulence factor of *R. equi*, it was found that the *fbpA* mutant strain was not attenuated in macrophage or mouse infection models (Sydor et al. 2008).

A cell envelope polysaccharide of *R. jostii* has recently been characterised as having a tetrasaccharide repeating unit containing D-glucuronic acid, D-glucose, 2-acetyl-D-galactose and L-fucose (Perry et al. 2007). Close association of the polysaccharide with the cell envelope was suggested by the need to use hot (60°C) 50% aqueous phenol to extract significant yields of the polymer, an extraction method similar to that used for lipoglycans (Garton et al. 2002; Gilleron et al.

2005). Similarly, an extracellular polysaccharide has also been isolated and characterised from *R. rhodochrous* as having a tetrasaccharide repeating unit containing D-glucuronic acid, D-glucose, D-galactose and D-mannose (Urai et al. 2006b). Small quantities of C16 and C18 fatty acids were found to be esterified to this polymer, suggesting that these might represent a mechanism for anchoring the polysaccharide to the cell envelope. A third polymer, named mucoidan, was identified in *R. erythropolis* PR4 and characterised as having a pentasaccharide repeat unit containing D-glucuronic acid, two D-glucose, *N*-acetylglucosamine and L-fucose (Urai et al. 2007b). The same strain also produces another polysaccharide, named PR4 FACEPS (fatty acid-containing extracellular polysaccharide), with a tetrasaccharide repeating unit containing D-glucuronic acid, D-glucose, D-galactose and pyruvylated D-mannose, which is esterified with small quantities of fatty acids, as in the *R. rhodochrous* polysaccharide (Urai et al. 2007a). The polysaccharide component of PR4 FACEPS is notably identical to the previously described extracellular polysaccharide of *Rhodococcus* sp. 33 (Urai et al. 2006a).

The above rhodococcal cell envelope polysaccharides share some structural features in common with the capsular polysaccharides of *R. equi*. Interestingly, the structural motif of an acetal-linked pyruvic acid (1-carboxyethylidene) substituent, which is present in the *R. equi* serotype 1, 2 and 7 capsules (Richards 1994), was also identified in the polysaccharide from *Rhodococcus* sp. 33 and PR4 FACEPS from *R. erythropolis* (Urai et al. 2006a, 2007a). To date, 27 antigenically distinct capsular types have been defined by serotyping in *R. equi* alone (Nakazawa et al. 1983), so it is likely that the structural diversity of cell envelope and capsular polysaccharides produced by rhodococci is high. Therefore, this remains an interesting area for future study, particularly as these surface polymers may facilitate the ability of the bacteria to utilise hydrophobic substrates; (Urai et al. 2006b; Perry et al. 2007).

4.5 Lipoproteins

Bacteria are capable of covalently modifying proteins by attachment of a lipid group to a cysteine residue, which becomes the N-terminus of the mature protein, that is, synthesising lipoproteins (Hutchings et al. 2009). This provides an important mechanism for localising proteins to bacterial cell membranes. Bacterial lipoproteins are readily identifiable by bioinformatic methods, and analyses of sequenced bacterial genomes have revealed that putative lipoproteins typically represent ca. 2% of the predicted proteomes of Gram-positive bacteria (Sutcliffe and Harrington 2004; Babu et al. 2006; Rahman et al. 2008). As such, bacterial lipoproteins are a functionally diverse and numerically significant class of cell envelope proteins in Actinobacteria. In Gram-positive bacteria, lipoproteins are de facto anchored to the outer leaflet of the plasma membrane, and this is likely to be the major destination of lipoproteins in mycolic-acid containing Actinobacteria, although it remains possible that some lipoproteins are also associated with the mycolate layer (Fig. 3; Sutcliffe and Harrington 2004).

As in *M. tuberculosis* (Sutcliffe and Harrington 2004), bioinformatic analyses of the *R. jostii* genome indicates that ca. 2.0% (>100 proteins) of the predicted proteome are putative lipoproteins (our unpublished observations). As in other Gram-positive bacteria, substrate binding proteins of ABC transport systems for diverse substrates are well represented. Related to this is the recent demonstration that the *mce4* operon of *R. jostii* constitutes a complex ABC transport system variant for cholesterol uptake (Mohn et al. 2008) and it is notable that the Mce4E proteins of both *R. jostii* (RHA1_ro04702) and *M. tuberculosis* are predicted lipoproteins (unpublished observations; Sutcliffe and Harrington 2004). Indeed, the mceE proteins associated with each of the multiple *mce* loci of *M. tuberculosis*, *Nocardia farcinica* and *R. jostii* are putative lipoproteins (Sutcliffe and Harrington 2004; our unpublished observations). Collectively, *mce* operons may encode putative ABC-related transport systems for various (probably hydrophobic) substrates (Casali and Riley 2007). The putative lipoproteins may thus interact with the other membrane-associated/secreted components to form a cell envelope complex involved in substrate scavenging and delivery to the membrane permease.

Consistent with their location in proximity to both the cell membrane and wall, various putative lipoprotein enzymes including cell wall active enzymes can be distinguished. As noted in other Gram-positive bacteria (Hutchings et al. 2009), several putative lipoproteins predicted to be involved in membrane-associated redox processes can be identified (e.g. *R. jostii* RHA1_ro02035, a ResA homologue likely to be involved in cytochrome *c* biogenesis, and RHA1_ro01137, the cytochrome *c* oxidase subunit II CtaC). Moreover, at least two putative lipoproteins (RHA1_ro06090 and RHA1_ro06326) appear to be involved in 'three component systems' involved in cell envelope sensing and signalling processes (Hoskisson and Hutchings 2006; Ortiz de Orué Lucana and Groves 2009). Finally, as in other bacteria, significant numbers of conserved hypothetical proteins of unknown function were identified as putative lipoproteins.

In addition to these canonical lipoproteins, the immunodominant VapA virulence factor of *R. equi* (Jain et al. 2003) has also been reported to be an acylated protein (Tan et al. 1995) , which may explain its association with the rhodococcal cell surface (Sutcliffe 1997). The VapA protein lacks any cysteine and thus cannot be a conventional lipoprotein of the above described type, as a cysteine containing signal peptide is central to the lipid modification pathway (Hutchings et al. 2009). Whether VapA is a unique post-translationally acylated protein or represents the prototype of a novel family of lipid-modified proteins (e.g. in other mycolata) remains an important question for future study. It is notable that the other members of the VapA family are not thought to be lipid-modified but to be surface-associated or secreted proteins (Byrne et al. 2001; Meijer and Prescott 2004).

5 Biosynthesis of Key Cell Envelope Components

As described in Sects. 2 and 4, the mycolate cell envelope is dominated by several distinctive covalently and non-covalently associated components. The biosynthesis and coordinated assembly of these components is reviewed in the following sections.

5.1 Mycolic Acid Biosynthesis

Few studies have directly addressed the production of rhodococcal mycolic acids but the fundamental processes involved in biosynthesis have been extensively investigated in mycobacteria. The synthesis of the component parts of the mycolic acids is, in the main, a straightforward fatty acid biosynthesis, which occurs via the repetition of a cycle of four reactions, where each cycle accomplishes an extension of the alkyl chain by a two-carbon unit.

Two types of fatty acid synthase (FAS) are known. The mammalian-like type FAS-I system is a homo-dimer containing all the necessary functions to achieve de novo fatty acid synthesis (Smith et al. 2003). In contrast, most bacteria utilise a FAS-II system wherein the growing fatty acyl chain is transferred between the active sites of dissociable component enzymes as an acyl thioester of a highly acidic acyl carrier protein (ACP). As with most other mycolata, *R. jostii* is unusual in that both FAS systems are present. FAS-I (*fas*) has been identified as RHA1_ro01426 showing 1965/3100 (63%) amino acid sequence identity to its counterpart in *M. tuberculosis* H37Rv. This FAS-I will be responsible for the de novo fatty acid synthesis, producing fatty acids of C14–C24 carbon chain length. For meromycolic acid biosynthesis, the further extension of the fatty acids produced by FAS-I is performed by a dissociable FAS-II system (Kremer et al. 2001b; Takayama et al. 2005). Like *M. tuberculosis* AcpM, the *R. jostii* AcpM (RHA1_ro01200) that serves FAS-II contains a C-terminal extension relative to other bacterial ACPs (data not shown). The significance of this C-terminal extension is still unknown but a sequence alignment of AcpM from representative mycolata reveals a correlation between the larger mycolates and the size of this extended region (data not shown). Thus it could be speculated that the length of this extension plays a role in the ability of the bacterium to produce longer meromycolates.

The key enzyme that links FAS-I and FAS-II, the β-ketoacyl-ACP synthase III FabH, can be identified as RHA1_ro05206 showing 56% amino acid sequence identity to *M. tuberculosis* H37Rv mtFabH (Choi et al. 2000; Brown et al. 2005). FabH elongates the acyl-CoA primers derived from FAS-I by condensing these with a malonyl-thioester of AcpM to form a β-keto-acyl-AcpM thioester product (Choi et al. 2000; Brown et al. 2005). The malonyl-AcpM substrate is produced by the acyl-CoA/ACP transacylase FabD (RHA1_ro01199), which is encoded within a syntenic gene cluster in *M . tuberculosis* H37Rv (Fig. 4; Kremer et al. 2001b). The β-keto-acyl-AcpM product of FabH is reduced by the β-keto-acyl-reductase, FabG (MabA, RHA1_ro07213) (Banerjee et al. 1998) and its β-hydroxy-acyl-AcpM product is dehydrated by a FabZ-type protein complex. Recently, Rv0635–Rv0637 (FabZ′, FabZ, FabZ″, respectively) in *M. tuberculosis* H37Rv were identified as the three component subunits required to perform the dehydration reaction in this species (Brown et al. 2007; Sacco et al. 2007). Interestingly, *R. jostii* carries only homologues of FabZ′ (RHA1_ro01983) and FabZ (RHA1_ro01984). The core unit of the dehydratase complex, FabZ, associates with the chain length specific subunits FabZ′ and FabZ″ and therefore the absence of FabZ″, which is associated with the later stages of

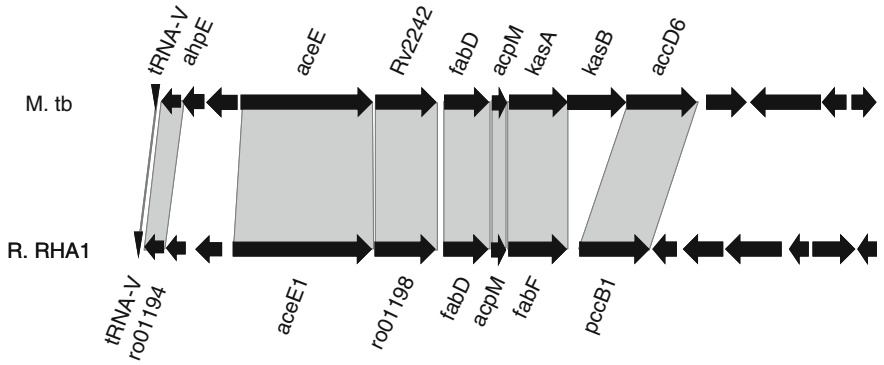

Fig. 4 *Comparative genomic alignment of the KasA loci*. The *M. tuberculosis* protein sequences within the *kasA* locus were BlastP searched against the *R. jostii* proteome. The *R. jostii* protein sequences identified were then reciprocally BlastP searched against the *M. tuberculosis* proteome to confirm the correct selection of the protein. The figure demonstrates the genomic region of the KasA loci where the *grey bars* represent the proteins of significant homology

meromycolate chain extension, is consistent with the shorter mycolate chain lengths observed in *R. jostii* RHA1. The *trans*-2-enoyl-AcpM product of the FabZZ′ complex then participates in the final step of the FAS-II reaction cycle, catalysed by the enoyl-ACP reductase FabI (InhA, RHA_ro07214), which is encoded adjacent to *fabG*, as in mycobacteria (Kikuchi and Kusaka 1984; Banerjee et al. 1994). Completing the cycle thus produces an aliphatic acyl-ACP two carbons longer than its acyl primer (Banerjee et al. 1994).

In *M. tuberculosis*, the subsequent rounds of acyl extension by FAS-II are thought to be initiated by the highly similar β-keto-acyl-AcpM synthases, KasA and KasB (Kremer et al. 2000, 2002a; Schaeffer et al. 2001). These enzymes extend acyl-AcpM thioesters, rather than acyl-CoAs, by condensing them with malonyl-AcpM. Both enzymes require acyl-AcpM primers of at least 16 carbons, consistent with a role of FAS-II in extending FAS-I products towards the biosynthesis of long chain fatty acids (Kremer et al. 2002a). KasA, which is responsible for the extension intermediate chain length meromycolate precursors (Kremer et al. 2000), is present in *R. jostii* (RHA1_ro01201, 67% amino acid sequence identity to *M. tuberculosis* KasA). Bhatt et al. (2007) confirmed that KasB functions predominantly in the extension of long-chain length meromycolate precursors. A Δ*kasB* null mutant in *M. tuberculosis* synthesised shorter mycolic acids compared to the parent strain. Significantly, the only gene missing from the *R. jostii kasA* gene cluster compared to that observed in all mycobacteria (Fig. 4) is a KasB homologue, further supporting the hypothesis that the production of intermediate chain length meromycolates in rhodococci is due to the absence of the requisite machinery to perform further elongation cycles.

Introduction of C=C double bonds into fatty acids and mycolic acids requires fatty acid desaturases. Two putative long-chain fatty acyl ACP desaturases are encoded in the genome of *M. tuberculosis* H37Rv, Rv0824c (DesA1) and Rv1094

(DesA2), respectively (Cole et al. 1998). DesA1 was originally detected as an exported component of an *M. tuberculosis* PhoA fusion library processed in *M. smegmatis* (Lim et al. 1995). The protein contains two copies of the characteristic (D/E)ENXH motif (Jackson et al. 1997) of the class II diiron-oxo proteins to which acyl-ACP desaturases belong (Fox et al. 1994). Two homologues of DesA1 are present in *R. jostii*, RHA1_ro02258 and RHA1_ro04869. Both exhibit 58% amino acid sequence identity to DesA1, and the gene for the latter is situated in a conserved locus comparable with that of *M. tuberculosis* DesA1. *M. tuberculosis* DesA2 does not contain the (D/E)ENXH motifs observed in other acyl-ACP desaturases, although it does possess an EEHXH motif as well as showing a high degree of homology throughout with stearoyl-ACP desaturases. RHA1_ro05863 appears to be an orthologue of DesA2 (37% amino acid sequence identity) in *R. jostii* and like the *M. tuberculosis* DesA2 retains only an EENXH motif. Neither of the *M. tuberculosis* gene products have yet been characterised in terms of desaturase activity and the significance of DesA1 secretion remains unknown. A membrane-associated fatty acyl-CoA desaturase gene is encoded in the genome of *M. tuberculosis* H37Rv (Rv3229c, DesA3; Phetsuksiri et al. 2003). Phetsuksiri et al. (2003) demonstrated that DesA3 was involved in the production of oleate from stearoyl-CoA and therefore it was designated as a Δ9-desaturase. *R. jostii* has six other DesA homologues, five of which (RHA1_ro06336, RHA1_ro03422, RHA1_ro01720, RHA1_ro6335 and RHA1_ro3346) show greater than 55% amino acid sequence homology to *M. tuberculosis* DesA3 and may thus play roles in fatty acid and/or mycolic acid desaturation. The sixth DesA3 homologue, RHA1_ro04464, is noted to contain a significant N-terminal deletion and so may be inactive.

The presence of complex mycolates in mycobacteria can be attributed to the numerous methyltransferases that are involved in functional group formation at proximal and distal modifications sites initially occupied by an unsaturated bond (Dover et al. 2004; Takayama et al. 2005). The absence of modifications in the relatively short mycolic acids of *C. diphtheriae* has been attributed to the absence of similar modification enzymes as well as to the absence of any fatty acyl desaturase DesA homologues that would provide the requisite unsaturation for further modification by the methyltransferases (Dover et al. 2004). Rhodococcal mycolates are intermediate in terms of both length and complexity compared to mycobacterial and corynebacterial mycolates (Fig. 1), containing up to four double bonds (Alshamaony et al. 1976; Barton et al. 1989; Stratton et al. 1999; Nishiuchi et al. 2000) in the distal part of the meromycolate. It is tempting to speculate that the multiple DesA homologues identified above may be involved in the formation of multiply unsaturated mycolates. As in *C. diphtheriae*, the absence of methoxyl mycolic acid synthases and cyclopropane mycolic acid synthases from the genome of *R. jostii* RHA1 is consistent with the simpler mycolate profiles of rhodococci.

The penultimate step in the synthesis of mycolic acids involves the Claisen-type condensation of an acyl-S-CoA (that contributes the alkyl branch) with a meromycolyl-AMP (Takayama et al. 2005; Gokhale et al. 2007). Recently a polyketide synthase (Pks13, Rv3800 in *M. tuberculosis*) has been implicated in this process

(Gande et al. 2004; Portevin et al. 2004; Gokhale et al. 2007). Gene disruption experiments of *Cg-pks* in *C. glutamicum* generated a viable mutant devoid of corynomycolates (Gande et al. 2004). In *R. jostii*, RHA1_ro04065 exhibits 1005/1751 (57%) amino acid sequence identity to Rv3800 in *M. tuberculosis*. The region encompassing *pks13* is highly conserved throughout the mycolata (see Sect. 5; Vissa and Brennan 2001; Dover et al. 2004), including *R. jostii*, due to the essential functions these gene products perform in cell wall biosynthesis (Fig. 5). The *pks13* locus also appears conserved in *R. rhodochrous* (Portevin et al. 2004). As in *M. tuberculosis*, the specific fatty acyl-AMP ligase (FadD32) responsible for the conversion of the meromycolyl-S-AcpM derived from the FAS-II system to meromycolyl-AMP (Trivedi et al. 2004) is present in *R. jostii* adjacent to the *pks13* gene (*RHA1_ro04064*). In mycobacteria, the precursor of the 2-alkyl branch is carboxylated by an acyl-CoA carboxylase composed of AccD4 and AccD5, in complex with an ε-subunit and AccBC, to yield 2-carboxyl-acyl-CoA (Gande et al. 2007). Bioinformatic searches have revealed *R. jostii* RHA1 possesses all the genes required for this function; *accD5* is situated alongside the ε-subunit (*RHA1_ro06292* and *RHA1_ro06291*, respectively). However, it is unclear which of the two possible homologues of AccBC (RHA1_ro06282 and RHA1_ro03742) is most likely to be involved, although the proximity of *RHA1_ro06282* to

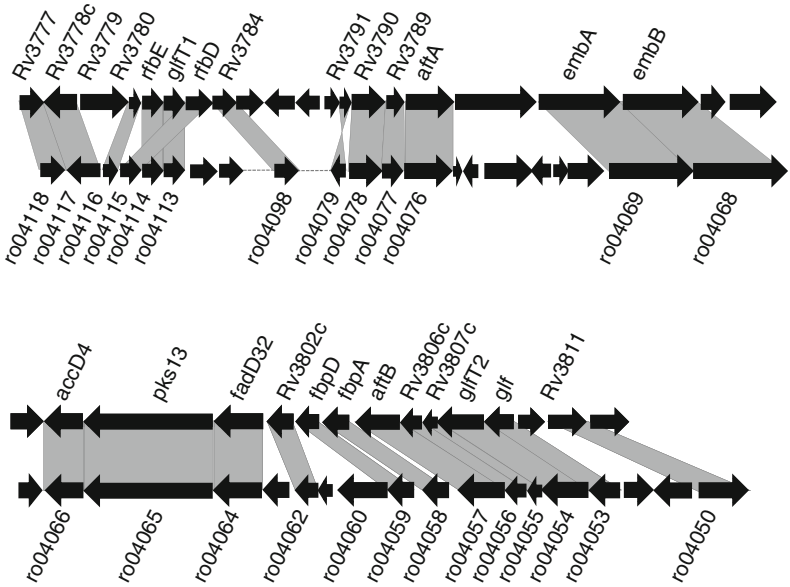

Fig. 5 *Comparative genomic alignment of the pks13-arabinogalactan loci*. The *M. tuberculosis* protein sequences within the pks-arabinogalactan locus were BlastP searched against the *R. jostii* proteome. The *R. jostii* protein sequences identified were then reciprocally BlastP searched against the *M. tuberculosis* proteome to confirm the correct selection of the protein. The figure demonstrates the genomic region of the pks-arabinogalactan locus where the *grey bars* represent the proteins of significant homology

RHA1_ro06291- RHA1_ro06292 is noted. RHA1_ro04066 represents the likely AccD4 candidate showing 67% amino acid sequence identity to the *M. tuberculosis* protein. The meromycolyl-S-AMP and 2-carboxyl-acyl-CoA are transferred to the β-keto-acyl synthase domain of Pks13 for condensation of the two fatty acyl groups (Gande et al. 2004). In most cases, the β-keto-mycolate product of Pks13 is reduced to a (β-hydroxy)mycolate before export for integration into the cell envelope. Recently *M. tuberculosis* Rv2509 was implicated in the catalysis of this final step of mycolate synthesis (Lea-Smith et al. 2007; Bhatt et al. 2008). In *R. jostii*, RHA1_ro01416 exhibits 69% amino acid sequence identity to *M. tuberculosis* Rv2509 and so is the most likely candidate to perform this function.

5.2 *Arabinogalactan Biosynthesis*

The organisation of AG and its interactions with other wall components, such as peptidoglycan, are likely to prove crucial to the formation of a functional outer lipid permeability barrier in the mycolata by defining the relative spacing of the tethered mycolates on which it is based. Accordingly, the biosynthesis of AG appears to be highly conserved across the taxon, although some diversity, most notably in patterns of arabinan branching and the glycosyl linkages of the galactan domain, have been recorded (Daffé et al. 1993; Eggeling et al. 2008). Much of our current understanding of the route to its production is derived from the study of various mycobacteria and, more recently, *C. glutamicum* (Eggeling et al. 2008). The dominant driving force behind this research has been the need to define the mechanisms of action of cell wall inhibitors used in current tuberculosis therapies and, following the emergence of extensively drug resistant *M. tuberculosis*, the need to define new targets in the biosynthesis of the *M. tuberculosis* wall (Dover et al. 2008b).

The first insight into AG biosynthesis was derived from the observation of a series of glycolipids elaborated by plasma membrane fractions of *M. smegmatis* and *M. tuberculosis*. Both preparations catalysed the incorporation of radioactivity from UDP-[^{14}C]-*N*-acetylglucosamine (GlcNAc) into two polyprenyl phosphate (Pol-P)-based glycolipids (GL1 and GL2). The initial step was identified as the formation of GL1, a Pol-P-P-GlcNAc unit (Mikušová et al. 1996). Incorporation of [^{14}C] Rhamnose (Rha) from dTDP-[^{14}C]Rha into GL2 exclusively identified it as Pol-P-P-GlcNAc-Rha (Mikušová et al. 1996). Addition of a cell wall enzyme preparation resulted in the formation of the increasingly polar glycolipids, GL3 and GL4. The inclusion of UDP-[^{14}C]Galactose (Gal) resulted in exclusive labelling of GL3 and GL4 indicating the initiation of a galactan chain on the GL2 primer (Mikušová et al. 1996). Subsequent analysis of the polymerised product resulting from these labelling experiments pointed to the formation of longer chain intermediates, eventually resulting in a polymer containing 35–50 residues (Besra and Brennan 1997; Mikušová et al. 2000). Glycosidic linkage analysis revealed that the bulk of the galactan polymer consisted of alternating 5- and 6-linked linear galactofuran residues, with a small amount of branching.

The incorporation of radiolabel from synthetic Pol-P-[^{14}C]-Arabinofuranose (Ara*f*) (Lee et al. 1995) into this same polymer (Mikušová et al. 2000) suggested that the total synthesis of the AG arabinan domain might occur while it is linked to the Pol-P carrier. This switch to Pol-P-derived sugar donor substrates is likely indicative of a shift in the membrane topology of AG biogenesis. The exclusive use of sugar nucleotides until the incorporation of Ara*f* into the polymer suggests that biosynthesis of galactan occurs at the cytosolic face of the plasma membrane. In contrast, the use of Pol-P-based Ara*f* donors suggests that arabinan deposition occurs at the 'periplasmic' face of the membrane and implies that the Pol-P-P-LU-galactan is translocated across the plasma membrane before further modification.

5.2.1 Linker Unit Synthesis

The LU disaccharide is formed via the addition of first GlcNAc (to form GL1) and then Rha (to form GL2) at the cytoplasmic face of the plasma membrane. The first glycosyltransferase is often purported to be a homologue of *E. coli* Rfe (WecA) (Meier-Dieter et al. 1992) in *M. tuberculosis* (Rfe, Rv1302) though this designation remains presumptive. The RHA1_ro01480 and RHA1_ro01091 proteins are clearly members of the glycosyltransferase family 4 typified by the UDP-GlcNAc/MurNAc:polyprenol-P GlcNAc/MurNAc-1-P transferases (Pfam PF00953, http://pfam.sanger.ac.uk/family?acc=PF00953) (Lehrman 1994). The former displays 67% amino acid identity with *M. tuberculo*sis Rfe, and as *RHA1_ro01480* is located within a highly syntenic locus in *R. jostii*, it is likely to represent an orthologue. *RHA1_ro01091* forms part of an operon that is devoted to the production of peptidoglycan precursors and is clearly the phospho-*N*-acetylmuramoyl-pentapeptide-transferase (MraY).

The complementation of a *wbbL* mutant of *E. coli,* which is deficient in Rha transfer for lipopolysaccharide biosynthesis, with *Rv3265c* (*wbbL1*) of *M. tuberculosis* implicates its product as the probable rhamnosyltransferase involved in GL2 synthesis (McNeil 1999). A WbbL1 homologue (63% amino acid identity) is encoded by *RHA1_ro06306*. Confidence regarding its designation as a rhamnosyltransferase and thus its orthology with *M. tuberculosis* WbbL1 is derived from analysis of its genetic context. The *M. tuberculosis* enzymes providing the dTDP-Rha donor substrate have all been identified and expressed in *E. coli* (Ma et al. 1997, 2001). RmlA to RmlD have been characterised as an α-D-glucose-1-phosphate thymidylyltransferase, dTDP-D-glucose 4,6-dehydratase, dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase and dTDP-Rha synthase, respectively (Ma et al. 2001). Homologues of RmlA (RHA1_ro04097, 73% amino acid identity), RmlB (RHA1_ro04098, 70% amino acid identity), RmlC (RHA1_ro04096, 56% amino acid identity) and RmlD (RHA1_ro06305, 54% amino acyl identity) are apparent within the *R. jostii* genome. In both genomes, *rmlD* and *wbbL1* potentially form an operon supporting their coordinated function and ultimately a role in rhamnosyltransfer to the LU precursor.

5.2.2 Galactan Synthesis

The galactose (Gal) residues of AG occur in the relatively uncommon furanose (*f*) form (McNeil et al. 1987). The requisite UDP-Gal*f* nucleotide sugar donor in *M. tuberculosis* is provided via two sequential reactions from UDP-Glucose (*p,* pyranose form; UDP-Glc*p*). The first is catalysed by UDP-Glc*p* epimerase to form UDP-Gal*p*, which is then converted to UDP-Gal*f* by UDP-Gal*p* mutase. Weston et al. (1997) purified a protein with UDP-glucose 4-epimerase activity from *M. smegmatis*. N-terminal sequence analysis suggested that the protein was related to the product of *M. tuberculosis* Rv3634. A similar strategy was used to identify *M. tuberculosis* Rv3809c (Glf) as an orthologue of *M. smegmatis* UDP-Gal*p* mutase; the designation was confirmed by molecular cloning and analysis of crude extracts containing the recombinant protein (Weston et al. 1997).

Two galactosyltransferases involved in *M. tuberculosis* galactan synthesis have now been identified. The product of *Rv3808c* appeared to be a good candidate in that it occupied the locus adjacent to *glf*. This putative transferase also contained the signature QXXRW motif, which is found only in processive enzymes, i.e. those which carry out multiple sugar transfers (Saxena et al. 1995). Over-expression of Rv3808c in *M. smegmatis* caused an increased yield of a galactofuran polymer in the over-producing strain (Mikušová et al. 2000). Analyses of the incorporation of Gal*f* into artificial Gal*f* disaccharides by membranes of recombinant *E. coli* expressing Rv3808c demonstrated that, consistent with the alternating β(1→5) and β(1→6) linkages of the native galactan, the incoming sugar adopted a (1→6) linkage when using a (1→5) linked disaccharide acceptor and vice versa (Kremer et al. 2001a). Furthermore, larger oligosaccharide products were also formed in these assays confirming that the product of Rv3808c, now designated GlfT2, is a processive enzyme and, consequently, is likely to produce the bulk of the galactan deposited in the *M. tuberculosis* cell wall (Kremer et al. 2001a). The importance of galactan synthesis to mycobacteria was demonstrated by the disruption of *glf* in *M. smegmatis*; growth was only supported when functional copies of both *glf* and *glfT2* were provided on complementing plasmids (Pan et al. 2001).

R. jostii orthologues of Glf (RHA1_ro04053, 82% amino acid identity) and GlfT2 (RHA1_ro04054, 69% amino acid identity) are apparent, and as in *M. tuberculosis*, they are encoded by adjacent genes; *glfT2* lies immediately downstream of and overlaps with *glf* by four nucleotides. Little is known regarding the mechanism by which GlfT2 introduces the distinctive alternating glycosyl linkage pattern that characterises *M. tuberculosis* galactan and thus far the enzyme has proven intractable in structural studies. The genomes of all galactan-producing species sequenced to date contain GlfT2 homologues. A combination of galactan characterisation and a structural genomics survey of GlfT2 homologues or potential alternative galactofuranosyltransferases would provide structural details and illuminate the molecular basis for galactan heterogeneity in the mycolata (Sect. 2.2).

Biophysical analyses of recombinant *M. tuberculosis* GlfT2 confirmed the intuition that, although capable of depositing the bulk of the Gal*f* residues, the enzyme would require a galactosyl primer to extend towards galactan; specifically

GlfT2 bound and donated Gal*f* to both β5- and β6-linked Gal*f*–Gal*f* disaccharides but could not donate Gal*f* to an artificial β-D-Gal-(1→4)-α-L-Rha acceptor, which mimics the reducing terminus of galactan (Alderwick et al. 2008). Bioinformatic analyses led to the identification of a second *M. tuberculosis* galactosyltansferase (Rv3782) that participates in the biogenesis of GL4, the Pol-P-LU-$Galf_2$ (Mikušová et al. 2006; Alderwick et al. 2008). *E. coli* extracts containing recombinant Rv3782 (now designated GlfT1) transferred galactosyl residues to artificial acceptors designed to emulate LU and LU-Gal*f* (Alderwick et al. 2008; Beláňová et al. 2008). These combined data suggest that GlfT1 might represent an even more versatile bifunctional protein than GlfT2, able not only to produce both β-(1→4) and β-(1→5) linkages but also to utilise diverse acceptor groups, that is a rhamnosyl acceptor in the initial reaction.

The *R. jostii* genome encodes a convincing GlfT1 orthologue in RHA1_ro04113 (68% amino acid identity with *M. tuberculosis* GlfT1) and, as in *M. tuberculosis,* the gene is clustered with two others encoding an apparent polysaccharide exporting ABC transporter (*RHA1_ro04114* and *RHA1_ro04115*). This transport complex represents an attractive candidate to facilitate the export of Pol-P-LU-galactan to the periplasm for arabinosylation.

5.2.3 Arabinan Synthesis

The structure of the arabinan portion of *M. tuberculosis* AG is much more complex than that of its galactan partner A series of branches contributes to the formation of the characteristic terminal pentaarabinofuranosyl motif that provides the esterification sites for AG-linked mycolates. Until the recent development of the genetically tractable *C. glutamicum* as a model for AG biosynthesis (Alderwick et al. 2005; Eggeling et al. 2008), much of our insight into arabinan biogenesis emerged from studies related to the mode of action and resistance against the important antitubercular drug EMB (reviewed in Dover et al. 2008a). In vivo pulse-chase labelling experiments in *M. smegmatis* suggested that the Ara*f* residues ultimately deposited in AG derive directly from a Pol-P-Ara*f* sugar donor (Wolucka et al. 1994). EMB, which inhibits biosynthesis of both AG and LAM (Takayama and Kilburn 1989), led to the accumulation of Pol-P-Ara*f* (Wolucka et al. 1994) suggesting the drug caused a lesion in arabinosyltransfer. Application of a synthetic Pol-P-[^{14}C]Ara*f* (Lee et al. 1995) in a cell-free assay system led to deposition of radiolabel in all recognised cell wall arabinan moieties, defining Pol-P-Ara*f* as the major arabinosyl donor in mycobacteria (Xin et al. 1997). However, the possibility of both UDP-Ara (Singh and Hogan 1994) and GDP-Ara (Takayama and Kilburn 1989) in *M. smegmatis*, as well as an undefined soluble Ara*f* donor in *C. glutamicum* (Tatituri et al. 2007), have all been proposed and cannot yet be ruled out as minor cell envelope Ara*f* donors.

Pol-P-Ara*f* appears to arise from 5-phosphoribose pyrophosphate (pRPP) with a 2′ epimerase mediating the ribose→arabinose conversion at an intermediate stage (Scherman et al. 1996). *M. tuberculosis* Rv3806c (UbiA) was identified as the

pRPP/polyprenyl-phosphate 5-phosphoribosyltransferase and represents the first committed step towards Pol-P-Ara*f* synthesis (Huang et al. 2005). Mikušová et al. (2005) hypothesised that Pol-P-β-D-5-phosphoribose is dephosphorylated to form Pol-P-β-D-ribose before epimerisation of the 2′ hydroxyl group is achieved in a two-stage process. First, oxidation of the hydroxyl probably forms Pol-P-2-keto-β-D-erythro-pentofuranose, which is subsequently reduced to generate Pol-P-β-D-Ara*f*. Two candidate gene products were identified in *M. tuberculosis* through their similarity to Noe proteins implicated in the arabinosylation of the *Azorhizobium caulidans* nodulation factor. Rv3790 and Rv3791 were annotated as a putative FAD-dependent oxidoreductase and a probable short-chain dehydrogenase/reductase, respectively, both functions consistent with the reaction schemes hypothesised (Mikušová et al. 2005; Wolucka 2008). Together the purified recombinant proteins were able to catalyse the epimerisation reaction despite neither protein being sufficient to promote the initial oxidation step independently (Mikušová et al. 2005). The enzyme that catalyses the dephosphorylation of Pol-P-β-D-5-phosphoribose that precedes this epimerisation remains unidentified but a candidate is the putative phosphatase encoded by *Rv3807c* i.e. adjacent to *ubiA* (Wolucka 2008). The genome of *R. jostii* encodes proteins that represent likely orthologues for each of these Pol-P-Ara*f* biosynthetic enzymes (Table 2).

The products of the *emb* locus of *Mycobacterium avium* were identified as the targets for EMB. Overexpression of *embA* and *embB* from *M. avium* conferred EMB resistance in *M. smegmatis* (Belanger et al. 1996). Taken together with the immediate inhibition of [^{14}C]Ara incorporation into both AG and LAM on EMB

Table 2 Comparison of the enzymology for arabinogalactan biosynthesis in *R. jostii* and *M. tuberculosis*

Function	*M. tuberculosis* archetype	RHA1 orthologue	% Identity
Pol-P arabinose precursor synthesis			
pRPP: Pol-P 5-phosphoribosyltransferase	Rv3806c	RHA1_ro04056	71
pRPP: Pol-P 5-phosphoribosyl phosphatase	Rv3807c	RHA1_ro04055	62
Pol-P-Ribose 2′ epimerisation			
FAD-dependent oxidoreductase	Rv3790	RHA1_ro04078	77
Short chain dehydrogenase	Rv3791	RHA1_ro04077	78
Arabinosyltransferases (AraT)			
α(1→5) AraT	EmbA (Rv3794)	Absent	
α(1→5) AraT	EmbB (Rv3795)	RHA1_ro04068	51
	EmbC (Rv3793)	RHA1_ro04069	50
	EmbC (Rv3793)	RHA1_ro01774[a]	47
Galactan priming α(1→3) AraT	AftA (Rv3792)	RHA1_ro04076	56
Arabinan branching α(1→3) AraT	AftC (Rv2673)	RHA1_ro06863	54
Arabinan terminating β(1→2) AraT	AftB (Rv3805c)	RHA1_ro04057	50

[a]The *R. jostii* genome contains three clear Emb proteins. This one is located outside of the locus containing the EmbA/EmbB arabinosyl transferases likely to be involved in arabinogalactan biosynthesis (see Sect. 5.2.3)

treatment of *M. smegmatis* (Takayama and Kilburn 1989) and the accumulation of Pol-P-Ara*f* (Wolucka et al. 1994), a hypothesis that explains this resistance phenotype at the molecular level is that Emb proteins are the arabinosyltransferases contributing to AG biosynthesis. However, their possession of glycosyltransferase activities has yet to be demonstrated through their over-production in a heterologous organism.

As in *M. tuberculosis, M. smegmatis* possesses three closely related *emb* genes, clustered *embCAB*, whilst despite possessing only one *emb* gene, *C. glutamicum* produces a similar AG to the mycobacteria (Eggeling et al. 2008). Gene knock out studies in *M. smegmatis* have shed light on the apparent redundancy in its *emb* locus (Escuyer et al. 2001). Individual mutants inactivated in *embC*, *embA* and *embB* were characterised. All three strains were viable but of them, the *embB*$^{-}$ mutant was most profoundly affected. Cell wall integrity seemed to be compromised as morphological changes were evident, and the cells displayed increased sensitivity to hydrophobic drugs and detergents. The arabinose content of the AG was diminished for both the *embA*$^{-}$ and *embB*$^{-}$ strains. Nuclear magnetic resonance studies showed that these mutations resulted in considerable effects upon the formation of the terminal pentaarabinofuranosyl motifs, specifically the addition of the β-D-Ara*f*-(1→2)-β-D-Ara*f* disaccharide to the 3 position of the 3,5-linked Ara*f* residue resulting in a linear terminal motif. However, AG formation in the *embC*$^{-}$ strain seemed unaffected whereas arabinan deposition in LAM was abolished. These data support the hypothesis that Emb proteins are intimately involved in the process of cell envelope arabinan deposition and that EmbA and EmbB are crucial to the formation of the pentaarabinofuranosyl motifs of AG that are crucial for the deposition of mycolic acids.

Construction of a knock out mutant in the single *emb* gene of *C. glutamicum* (Alderwick et al. 2005) heralded a period of rapid progress towards the definition of arabinan biosynthesis. The mutant exhibited a slow growing phenotype and was significantly depleted in arabinan. Residual arabinosylation of galactan at the 3′ positions of its 5-linked 8th, 10th and 12th Gal*f* residues by a single Ara*f* residue was detected. This modification was not present in the galactan of a strain disrupted in *ubiA* that lacks Pol-P-Ara*f* (Alderwick et al. 2005). Deletion of the gene immediately upstream of *C. glutamicum emb*, now designated *aftA*, which encodes a member of the GT-C glycosyl transferase superfamily, resulted in an arabinan deficient strain (Alderwick et al. 2006). Clearly, AftA represents a novel arabinosyltransferase that primes arabinan biosynthesis on galactan by addition of a single Ara*f* residue that is presumably elaborated upon by EmbA/B or possibly another Ara transferase. Systematic deletion of other GT-C transferases that might contribute to the biosynthesis of cell envelope polysaccharides in *C. glutamicum* and mycobacteria has recently revealed two further conserved Ara*f* transferases. AftC represents a α-(1→3)-Ara*f* transferase that is essential for the branching of the arabinan towards its reducing end and may also contribute to the formation of the pentaarabinofuranosyl motif (Birch et al. 2008). AftB is another GT-C enzyme that forms the β-D-Ara*f*-(1→2)-β-D-Ara*f* structure that effectively terminates arabinan and also provides one of the sites for mycolylation (Seidel et al. 2007).

Although the *R. jostii* RHA1 AG has not been characterised, it appears that the bacterium possesses orthologues of all of the enzymes implicated in *M. tuberculosis* and *C. glutamicum* arabinan biosynthesis (Table 2). Two homologues of the Emb proteins, as well as the enzymes that initiate and terminate arabinan biosynthesis, AftA and AftB, respectively, are all encoded in a highly conserved cluster of 31 genes first recognised in *M. tuberculosis* by Belanger and Inamine (2000) incorporating *Rv3779–Rv3809c* and occupying 48.5 kb or ~1% of the chromosome (Fig. 5). Among these genes are *glf* and *glfT* (galactan polymerisation), *embCAB, aftA and aftB* (arabinan deposition), *pks13* and associated enzymes (mycolyl condensation) and *fbpA* (mycolyltransfer). Significantly, the region is well conserved in *M. leprae*, the aetiological agent of leprosy (Vissa and Brennan 2001). This bacterium is an obligate intracellular pathogen and exemplifies an extreme case of reductive evolution as less than half of its genome contains functional genes (Cole et al. 2001). The retention of function over such a large syntenous genomic region in *M. leprae* clearly emphasises the essentiality of the cell wall to the pathogenic mycobacteria. Comparison of the *M. tuberculosis* cell wall locus with the equivalent from the more distantly related bacterium *C. diphtheriae* showed that the overall genetic arrangement remained well conserved but was split into two discontinuous segments resulting in the *emb* homologue of *C. diphtheriae* lying over 460 kB away from the *glfT* homologue (Dover et al. 2004). Likewise in *R. jostii* RHA1, two clusters are apparent, encompassing *RHA1_ro04050* to *RHA1_ro04079* and *RHA1_ro04098* to *RHA1_ro04118* (Fig. 5); each shows evidence of rearrangement and carry additional genes relative to *M. tuberculosis*, although it is not clear whether these represent rhodococcal acquisitions or mycobacterial losses or, indeed, whether they contribute to the construction of the rhodococcal cell envelope.

5.2.4 Macromolecular Ligation

Thus far, we have considered the independent biosyntheses of AG and the mycolic acids but these components must be brought together in the pseudoperiplasm and covalently combined to form the massive mycolyl–arabinogalactan–peptidoglycan complex. This process will require export of each of the structural components as well the enzymes responsible for mycolyl transfer to the terminal Ara*f* residues of AG.

Although their role in galactan export remains to be confirmed, *M. tuberculosis rfbDE* and *R. jostii* RHA1_ro04114 and RHA1_ro04115 appear to represent a conserved polysaccharide-exporting ABC transporter (Content and Peirs 2008) and, as both are clustered with a gene encoding the galactan-priming Gal*f* transferase GlfT1(RHA1_ro04113), their coordinated function in galactan biosynthesis and export is likely. Once translocated, the arabinosylation of galactan can commence with AftA. On completion, AG units must be integrated into the growing murein sacculus; little is known regarding the process other than ligation requires simultaneous synthesis of both AG and peptidoglycan (Hancock et al. 2002). The enzymology of AG ligation remains enigmatic.

An interesting Pol-P-based mycolylated glycolipid, 6-*O*-mycolyl-β-D-mannopyranosyl-1-monophosphoryl-heptaprenol (Myc-PL), was purified from *M. smegmatis* and suggested to be the carrier of newly synthesised mycolic acid during translocation across the plasma membrane (Besra et al. 1994). A similar lipid had also been reported in *C. diphtheriae* (Datta and Takayama 1993) suggesting a conserved means for translocation of mycolates across the membrane for the synthesis of trehalose dimycolates and cell wall mycolates.

While prospecting for genes involved in mycolate biosynthesis and processing, Wang et al. (2006) isolated a slow-growing transposon insertion mutant of *Corynebacterium matruchotii* with an apparent impairment in corynomycolate production. The transposon had inserted within a probable orthologue of *C. diphtheriae* DIP1297, an integral membrane protein encoded by the first of a four gene cluster of which the latter three genes had been annotated as encoding an antibiotic transporter (Braibant et al. 2000). The genes of the equivalent cluster in *C. glutamicum* were apparently cotranscribed on a polycistronic mRNA suggesting coordinated function. Application of comparative genomics techniques demonstrated the conservation of the cluster in *M. tuberculosis* (*rv1459c, rv1458c–rv1456c*), other mycobacteria, corynebacteria and nocardiae and, by supposition, across the *Corynebacterineae* but not in other Actinobacteria (Wang et al. 2006). A similar cluster also occurs in *R. jostii* (*RHA1_ro07191* to *RHA1_ro07194*). Analysis of mycolic acid chain length in the *C. matruchotii* mutant revealed that shorter chain-length corynomycolates (C_{24}–C_{32} rather than C_{34}–C_{36}) were under-represented (Wang et al. 2006) leading the authors to suggest that this represented an export complex for short-chain mycolates (Wang et al. 2006). However, such short-chain mycolates are likely to be, at best, infrequent modifications to the cell wall of mycobacteria. As there was effective export of the larger mycolate subpopulation of corynomycolates in the *C. matruchotii* mutant, suggesting some redundancy in corynomycolate translocation, one might expect that other *Corynebacterineae* producing larger mycolates would possess this alternate system.

Another important factor in the processing of mycolic acids is the requirement for glucose or α-D-glucopyranosyl-containing oligosaccharides such as trehalose, which is essential for the growth of the *M. tuberculosis* but not corynebacteria. Despite *M. tuberculosis* possessing three potential routes to trehalose, inactivation of a component of the OtsAB pathway (OtsB2, Rv3372; trehalose-6-phosphate phosphatase) abrogated growth (Murphy et al. 2005). *R. jostii* possesses a single homologue *of M. tuberculosis* OtsB2 (RHA_ro00045, 56% amino acid identity) and two homologues of *M. tuberculosis* OtsA (trehalose-6-phosphate synthase; RHA_ro04708, 77% amino acid identity; RHA_ro04708, 69% amino acid identity).

In the absence of exogenous α-D-glucopyranosyl-containing oligosaccharides, a multiply-mutated *C. glutamicum* strain incapable of trehalose synthesis exhibited altered surface properties and lacked mycolic acids in its envelope. The mycolyl residues synthesised by the mutant grown with suitable oligosaccharides were transferred both onto the cell wall and free sugar acceptors. Furthermore, as the mutant had shown no capacity for trehalose uptake, radioactive labelling experiments with [^{14}C]trehalose showed that the transfer of mycoloyl residues onto sugars

occurs outside the plasma membrane (Tropis et al. 2005b). Thus trehalose appears to be an important extracytoplasmic carrier for mycolates, allowing their deposition in the cell wall.

A mycolyltransferase capable of exchanging mycolyl residues between mycolyltrehalose and the free disaccharide was purified from *M. smegmatis* and a role in mycolyl deposition suggested (Sathyamoorthy and Takayama 1987). Belisle et al. subsequently demonstrated that three members of the *M. tuberculosis* antigen 85 complex, Ag85A, Ag85B and Ag85C2 (encoded by *fbpA*, *fbpB* and *fbpC2* respectively) were able to catalyse mycolyltransferase reactions (Belisle et al. 1997). In order to shed light upon this apparent redundancy in mycolyltransferases and to ascertain the biological roles of the individual enzymes, *fbpC2*, *fbpA* and *fbpB* have all been disrupted (Jackson et al. 1999; Armitige et al. 2000). The disruption of *fbpC2* in *M. tuberculosis* decreased transfer of mycolates to the cell wall by 40% without affecting the profile of mycolate types esterified to AG or occurring as free glycolipids. Thus FbpC2 is involved, either directly or indirectly, in the transfer of mycolates onto the cell wall and is probably not specific for a given type of mycolate, or at least the remaining mycolyltransferases are able to maintain the balance between the mycolate types through their own broad specificity (Jackson et al. 1999). Although an *fbpA* mutant grew as well as the parent strain in laboratory media and macrophage-like cell lines, the *fbpB* mutant only grew well in laboratory media. In macrophage-like cell lines, the strain grew very poorly, if at all (Armitige et al. 2000; Puech et al. 2002).

Corynebacteria possess genes with significant homology to those encoding the antigen 85 complex (Joliff et al. 1992). Disruption of *csp1* encoding the secreted Fbp-like protein PS1 of *C. glutamicum* led to a 50% decrease in the amount of cell wall-linked corynomycolates and an alteration in the cell wall permeability (Puech et al. 2000). The expression of *fbpA*, *fbpB* and *fbpC2* from *M. tuberculosis* in this *csp1*-deficient strain restored the cell wall-linked mycolate content and the outer permeability barrier of the mutant. The enormous structural differences between corynomycolates and their mycobacterial counterparts (Fig. 1) suggest that these enzymes possess a broad specificity (Puech et al. 2002). All three enzymes are able to transfer mycolates to AG and display no preference for mycolyltransfer to the terminal or 2-linked Araf residues of the pentaarabinosyl motifs of AG (Puech et al. 2002). Redundancy in mycolyltransferase activity is apparently a common theme among the mycolata.

R. jostii RHA1 appears to possess 13 (RHA1_ro04059, ro04058, ro04960, ro04060, ro04126, ro04189, ro05513, ro02206, ro02143, ro05007, ro05217, ro05431, ro03469) potential mycolyltransferases (BLASTP query, *M. tuberculosis* FbpA; cut off, E 10^{-21}) and, consistent with a periplasmic location, signal peptides were predicted for all 13 proteins and all retained a conserved triad of active site residues (Belisle et al. 1997; Kremer et al. 2002c). Similarly, Sydor et al. (2008) suggested that *R. equi* might possess up to 13 FbpA homologues. RHA1_ro04060 is distinct from the mycobacterial mycolyltransferases because of its larger size (640 amino acids, i.e. almost double the size of *M. tuberculosis* FbpA and the other *R. jostii* homologues, which are ca. 330 amino acids) and is likely to represent an orthologue of

PS1/Cop1 of *C. glutamicum* (Joliff et al. 1992; Brand et al. 2003). *RHA1_ro04058–RHA1_ro04060* are situated within one of the large cell wall biosynthetic clusters (Fig. 5) in a position analogous to *fbpAC2*, that is, immediately downstream from AftB which supplies terminal Ara*f* residues to which mycolates are ultimately esterified by mycolyltransferases. Thus RHA1_ro04058 and RHA1_ro04059 are almost certainly mycolyltransferases. A thorough biochemical characterisation of this group of rhodococcal proteins will determine whether these and their *R. equi* counterparts represent astonishing redundancy in mycolyltransfer activity or whether some represent a series of paralogous secreted esterases that have significance in the metabolism of the rhodococci. As noted (Sect. 4.4), mutation of a *fbpA* homologue in *R. equi* affected capsule incorporation, but a detailed mycolate profile of mutant compared to wild type was not reported (Sydor et al. 2008).

Like *C. glutamicum* PS1, the N-terminus of RHA1_ro04060 exhibits significant amino acid identity with *M. tuberculosis fbpA* over its full length (Joliff et al. 1992) with the remaining sequence representing a C-terminal extension that carries three LGFP repeats (Pfam08310) (Adindla et al. 2004). The four LGFP repeats of *C. glutamicum* PS1 are hypothesised to anchor the protein to the wall and may be important for maintaining cell wall integrity (Ramulu et al. 2006). Deletion of *C. glutamicum* PS1 results in a tenfold increase in cell volume and implicates the corresponding proteins in cell shape formation (Brand et al. 2003).

5.3 LAM Biosynthesis

As with the biosynthesis of other cell envelope polymers, understanding of LAM biosynthesis has been greatly advanced by comparative studies on mycobacteria and corynebacteria. Consistent with the structural elements of the lipoglycans, the biosynthetic pathway can be divided into distinct stages, with initial synthesis of phosphatidylinositol mannosides (PIM) at the cytoplasmic face of the plasma membrane preceding 'flipping' of the glycophospholipid prior to mannose chain extension and arabinosylation at the outer face of the plasma membrane. As in many other actinomycete genomes, and consistent with the widespread distribution of PIM glycophospholipids, an operon containing the phosphatidylinositol synthase, an acyltransferase and PimA mannosyltransferase required for the biosynthesis of acylated phosphatidylinositol monomannoside (PIM_1; Korduláková et al. 2002, 2003) is present in the *R. jostii* RHA1 genome (RHA1*_ro06880–RHA1_ro06882*). The mannose in PIM_1 is added to the inositol C2 position. The second mannose, added to the inositol C6 position in PIM_1, is added by the recently defined PimB′ mannosyltransferase, which generates PIM_2 (Lea-Smith et al. 2008; Mishra et al. 2008b, 2009). Both PimA and PimB are cytoplasmic enzymes that utilise GDP-mannose as the mannose donor. In *R. jostii*, RHA1_ro01122 can be clearly identified as PimB′ by its homology with *C. glutamicum* NCgl2106 and *M. tuberculosis* Rv2188c (Lea-Smith et al. 2008; Mishra et al. 2008b, 2009).

The mono- and dimannosylated 'lower' PIMs, notably acylated PIM_2, are readily observed as free lipids in the membranes of rhodococci and other mycolata (Minnikin et al. 1977; Barton et al. 1989). Further to PIM_2 biosynthesis, subsequent mannosyltransferase activities are needed to convert these 'lower' PIMs to the 'higher' PIMs that are found in mycobacteria (notably phosphatidylinositol hexamannoside, PIM_6). Some relevant mannosyltransferases have been identified but the extent to which there is redundancy in this pathway is not yet clear (Kremer et al. 2002c; Morita et al. 2006; Crellin et al. 2008). Moreover, at an as-yet undefined stage, PIMs are 'flipped' from the inner leaflet of the plasma membrane to the outside leaflet such that the final steps of PIM mannosylation are carried out by GT-C family glycosyltransferases, using Pol-P-linked mannose (see below) as the mannose donor (Berg et al. 2007). After PIM translocation, PIM_4 can be either shunted towards lipomannan/LAM biosynthesis by the LpqW lipoprotein (Kovacevic et al. 2006; Marland et al. 2006; Crellin et al. 2008) or, in mycobacteria, mannosylated with $\alpha1\rightarrow2$ linked mannose to generate PIM_6 (Morita et al. 2006; Crellin et al. 2008). The PIM_4 precursor is mannosylated to generate the $\alpha1\rightarrow6$ lipomannan core of LAM by the sequential action of the MptB (Mishra et al. 2008a) and MptA GT-C mannosyltransferases (Kaur et al. 2007; Mishra et al. 2007), each using Pol-P-mannose as mannose donor. Branching $\alpha1\rightarrow2$ mannose units on the mannan core can be introduced by the Rv2181 GT-C mannosyltransferase (Kaur et al. 2008). Further to the generation of the lipomannan core unit, arabinosylation of mycobacterial LAM is carried out by the EmbC arabinosyltransferase (Zhang et al. 2003; Goude et al. 2008). However, as an *embC* mutant of *M. smegmatis* still incorporated two to three arabinosyl units per lipomannan (Zhang et al. 2003), it is likely that the initial 'priming' arabinose units are added by a separate arabinosyltransferase in a manner analogous to the priming by AftA in arabinogalactan synthesis (Alderwick et al. 2006). Thus a nearly complete pathway for mycobacterial LAM biosynthesis has been defined, with the crucial remaining questions including the nature and substrate(s) of the PIM 'flippase' and the mannosyltransferase(s) that convert PIM_2 to PIM_4, and the arabinosyl 'priming' activity.

From the above a near complete pathway for the biosynthesis of rhodococcal LAM can be reconstructed from the *R. jostii* genome. In addition to the above described acyl PIM_1 biosynthethic locus and PimB′, clear homologues of all the key enzymes identified in corynebacteria and/or mycobacteria can be identified (Table 3). In corynebacteria, higher PIMs are apparently not synthesised as free lipids, as indicated by the buildup of PIM_2 in *C. glutamicum* mutants unable to synthesise LAM or the Pol-P-mannose sugar donor (Gibson et al. 2003a; Mishra et al. 2008a). Thus PIM_2 is most likely flipped and elaborated into LAM (Mishra et al. 2008a). Intriguingly, the *R. jostii* genome contains a locus (RHA1_ro05934, RHA1_ro05929) comparable to that in mycobacterial genomes, which contains homologues of both the lipoprotein LpqW required to shunt PIM_4 towards LAM biosynthesis and the PimE mannosyl transferase required to synthesise PIM_6 from PIM_4 (Kovacevic et al. 2006; Marland et al. 2006; Morita et al. 2006; Crellin et al. 2008). Thus, *R. jostii* may be able to synthesise both a LAM-like lipoglycan (as in other rhodococci, see Sect. 4.2) and higher PIMs. PIMs larger than PIM_2 have not

Table 3 Conservation in the pathway for LAM-like lipoglycan biosynthesis in *R. jostii*

Step	*M. tub.*	*C. glut.*	RHA1	Identity
Polyprenyl-P-sugar precursor biosynthesis				
UbiA, polyprenyl phosphoribose 5′phosphate synthase	Rv3806c	NCgI2781	Ro04056	216/302 (71%)
Polyprenyl phosphoribose 5- phosphate phosphatase	Rv3807c	NCgI2782	Ro04055	86/138 (62%)
Polyprenyl phosphoribose 2-epimerase (heterodimer)	Rv3790 Rv3791	NCgI0187 NCgI0186	Ro04078 Ro04077	362/466 (77%) 198/253 (78%)
Ppm1, Polyprenylphosphate mannosyl transferase	Rv2051c	NCgI1423	Ro00145	164/242 (67%)
PIM biosynthesis				
PimA, mannosyl transferase	Rv2610c	NCgI1603	Ro06882	254/367 (69%)
PIM_1 acyltransferase	Rv2611c	NCgI1604	Ro06880	131/209 (62%)
PgsA, phosphatidylinositol synthase	Rv2612c	NCgI1605	Ro06881	192/300 (64%)
PimB′, mannosyl transferase	Rv2188c	NCgl2106	Ro01122	213/287 (74%)
PimC	MT1800[a]	Absent[b]	Ro04052	219/365 (60%)
PIM_n extension to lipomannan				
LpqW, lipoprotein delivering PIM_n to MptB	Rv1166	Absent[b]	Ro05934	304/620 (49%)
MptB, mannosyl transferase	Rv1459c	NCgl1505	Ro07194	315/564 (55%)
MptA, mannosyl transferase	Rv2174	NCgl2093	Ro01108	255/460 (55%)
Branching mannosyl transferase	Rv2181	NCgl2100	Ro01114	175/394 (44%)
Arabinosyltransferases				
Priming arabinosyl transferase	Unidentified	Unidentified	Unidentified	
EmbC	Rv3793	NCgl0184	Ro01774[c]	508/1091 (46%)
Capping mannosyltransferase	Rv1635c	Absent[b]	Ro04110	200/507 (39%)

[a]PimC is a redundant mannosyltransferase capable of synthesising PIM_3. However, this protein is absent from the genome of *M. tuberculosis* H37Rv (Kremer et al. 2002b)

[b]No clear orthologue (cut off, E 10^{-35}) identified

[c]The *R. jostii* genome contains three clear homologues of EmbC. This one is located outside of the locus containing the EmbA/EmbB arabinosyl transferases likely to be involved in arabinogalactan biosynthesis (see Sect. 5.2.3)

been reported previously in rhodococci but have been reported in some mycolata other than mycobacteria (e.g. Khuller 1977; Furneaux et al. 2005). This may reflect the nature of the solvent systems used for extraction and analysis in early studies (Minnikin et al. 1977) and so a re-evaluation of the distribution of higher PIMs in rhodococci and other mycolate bacteria is warranted.

The extracytoplasmic stages of LAM biosynthesis rely on Pol-P-linked mannose and arabinose sugar donors. The requisite genes for the biosynthesis of each are present in the *R. jostii* genome (Sect. 5.2.3; Tables 2 and 3). Intriguingly, a *ubiA* mutant of *C. glutamicum* can still produce a truncated LAM-variant (Tatituri et al. 2007). In conjunction with the above described apparent residual arabinosylation of LAM in an *M. smegmatis embC* mutant (Zhang et al. 2003), it is possible to speculate that an alternative arabinose donor may be needed to prime the core lipomannan during LAM biosynthesis and that this might occur during the

cytoplasmic stages of biosynthesis (Tatituri et al. 2007). This priming activity alone might therefore be sufficient to generate the truncated LAM types observed in *R. equi* and *R. ruber* (Sect. 4.2). In *R. jostii*, the presence of a third Emb family protein (RHA1_ro01774; Table 3) might be related to LAM or AG biosynthesis, although its gene is located outside of the cell wall biosynthetic loci (Fig. 5). Further investigation of both the LAM structure in *R. jostii* and the functional redundancy of the Emb proteins is needed. In this respect, it is intriguing that the *R. jostii* genome contains a clear orthologue (Table 3) of the Rv1635c mannosyltransferase that is involved in adding the mannan caps to the arabinans of mycobacterial LAM (Dinadayala et al. 2006; Appelmelk et al. 2008).

A PIM-anchored lipomannan as well as a second lipomannan most likely anchored by a mannosylglucosyluronic acid glycolipid have recently been identified in *C. glutamicum* (Tatituri et al. 2007; Lea-Smith et al. 2008). Synthesis of the mannosylglucosyluronic acid glycolipid from glucosyluronic acid-diacylglycerol depends on the mannosyltransferase MgtA (NCgl0452). An orthologue of MgtA, ro01995 (64%, 248/382 amino acid sequence identity) is encoded in the *R. jostii* genome raising the possibility that this species also synthesises novel mannosylglucosuronic acid based glycolipid(s). However, it is notable that extracts of *R. equi* and *R. ruber* that contain the truncated LAMs of these species do not contain a separate lipomannan fraction (Garton et al. 2002; Gibson et al. 2003b).

Finally, in addition to providing mannose for lipomannan biosynthesis, Pol-P-linked mannose can also be the sugar donor for protein glycosylation in Actinobacteria (VanderVen et al. 2005; Mahne et al. 2006; Wehmeier et al. 2009). *R. jostii RHA1*_ro05660 encodes a clear homologue of these protein mannosyltransferases, suggesting some cell envelope or secreted proteins are glycosylated.

6 Concluding Comments

The presence of a mycolic acid containing cell envelope is clearly one of the defining features that influences the biology of members of the genus *Rhodococcus*. Significant studies have confirmed the presence of all of the components typical of the cell envelopes of the mycolata, notably a peptidoglycan–arabinogalactan–mycolic acid complex, mycolyl glycolipids, channel-forming porins and LAM-like lipoglycans. As reviewed here and previously (Sutcliffe 1997, 1998), understanding of the general principles underlying the organisation of these components can be drawn from both theoretical models and experimental evidence obtained with other mycolata, notably members of the genera *Corynebacterium* and *Mycobacterium*. However, it is equally clear that there are likely to be genus, species and strain-specific variations in the fine detail of the organisation of these cell envelopes. Further to these models of cell envelope organisation, a comparative genomics approach should allow a rapid growth in knowledge of the pathways leading to the biosynthesis and assembly of cell envelope components, as illustrated herein by our

analyses of the genome of *R. jostii* RHA1. These developments are likely to herald a productive era in defining both the basic biology and the biotechnological potential of members of this fascinating genus.

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Genomes and Plasmids in *Rhodococcus*

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Contents

Abstract Members of the genus *Rhodococcus* are a very diverse group of bacteria that are found in many different niches. They are commonly found in the wider environment, but are also associated with pathogenesis in plants and mammals, including humans. They possess the ability to degrade a large number of organic compounds including some of the most difficult compounds with regard to recalcitrance and toxicity. This ability appears to be based upon the acquisition of a wide and diverse range of catabolic genes by cells that can withstand stressful conditions. Recent completion of genome sequences and analysis has revealed that they have very large genomes (up to 9.7 Mbp) and many possess genes that encode multiple catabolic enzymes and pathways. In addition to smaller circular plasmids, they also harbour many large linear plasmids that contribute to their substrate diversity, and these appear to be vehicles for the "mass storage" of numerous catabolic genes. The presence of multiple catabolic pathways and gene homologues seems to be the basis of their catabolic versatility. However, many of the genes associated with the

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pathways are dispersed around the genome, and it is becoming clear that their co-regulation of gene expression is a feature of how the rhodococci adapt to utilise many substrates.

1 Introduction

Members of the genus *Rhodococcus* are aerobic bacteria that are common in many environmental niches from soils to sea water and also as mammalian and plant pathogens. A notable feature is that they have been discovered in surprisingly diverse environmental niches and can degrade numerous recalcitrant and toxic pollutants. Taxonomically, the rhodococci belong to the wider grouping of Actinomycetes. They differ from other Actinomycetes due to the presence of mycolic acids in their cell walls and belong to the diverse grouping of non-sporulating mycolic-acid-containing bacteria called the *Mycolata*. All of the rhodococci investigated to date appear to have sizeable and complex genomes, which reflect their catabolic diversity. There is increasing evidence from biochemical analyses and genome sequencing that there are multiple pathways and gene homologues in many strains, which further indicates the versatility of *Rhodococcus* spp. in this respect. Many also possess a variety of large linear plasmids and smaller circular plasmids that contribute to and also explain the immense repertoire of catabolic abilities. There also appears to be an ability to adapt readily to degrade many substrates when presented with a new catabolic challenge; however, the molecular genetic mechanisms underlying the flexibility of the *Rhodococcus* genome are still to be elucidated. One striking feature is the presence of very large linear plasmids in many of the strains isolated. Other characteristics include a system that promotes high-frequency illegitimate recombination and the presence of transposons (although a small number of insertion sequences have been identified). Events of illegitimate recombination appear to occur, and these may serve to promote the introgression of DNA in their genomes without the help of mobile genetic elements (de Vries and Wackernagel 2002). Aspects of their overall genetics and metabolic diversity have been covered previously in reviews (Bell et al. 1998; Larkin et al. 2005, 2006; Kulakov and Larkin 2002; O'Brien et al. 2002; Warhurst and Fewson 1994; van der Geize and Dijkhuizen 2004; McLeod and Eltis 2008; Gurtler et al. 2004). This review will focus on aspects of their genome content and organisation: particularly, in the context of their roles in biodegradation in the environment.

2 Historical Context of Studies on *Rhodococcus* Genetics

The wide diversity, niches and the range of metabolic abilities and virulence determinants that the rhodococci possess have attracted attention for some considerable time. However, it is only in recent years that a better appreciation of the

genetics in relation to their complex genome organisation has emerged. There have been a number of technical obstacles to studying these bacteria, which have largely been overcome in recent times. Also, there has been a lack of emphasis on studying a single type strain and efforts have been extended over many different strains from different environments and with widely varying functions. The emergence of several genome sequences has coincidently addressed this, and considerable progress in understanding has been made. *Rhodococcus* genetic diversity is, however, immense and selecting a representative strain on logical grounds is difficult to justify. Other technical obstacles have included the recalcitrance of their cell walls to digestion, thereby rendering it difficult to extract nucleic acids. Coupled with this is their obvious cellular pleomorphism, whereby many strains grow as short rods, cocci or branched multinucleated filaments (Williams et al. 1976; Locci and Sharples 1984). This raises the issue of mutants not being fully segregated within the filaments during replication and selection. Despite these difficulties, the interest in these bacteria, which in earlier times were referred to as *Nocardia* species, extends back to the early days of bacterial genetic studies. At that time, it was noted that these bacteria possessed relatively unstable genetic traits. In 1950, Waksman referred to the genetic instabilities of nocardias in these terms: "In view of these variations, the question was raised: Is it possible that many of the *Nocardia* species represent degenerate forms of *Streptomyces*?" (Waksman 1950). To date, there have been some notable reviews on the subject of *Rhodococcus* genetics (Kulakov and Larkin 2002; Finnerty 1992; Larkin et al. 1998) that provide a more detailed backdrop to earlier discoveries.

From a taxonomic point of view, the rhodococci have presented a further challenge to microbiologists. This is not inconsiderable and holds out a further challenge in relation to their comparative genomics where studies are still in their infancy. The rhocococci occupy many niches from soils, sea basins, plants and animals (Finnerty 1992) and the observed diversity reflects this. Classification has traditionally been based upon their cell-wall structure and the presence of mycolic acids (Goodfellow 1989). Analysis of 16SrDNA sequences has since established a close phylogenetic relationship between the genera *Rhodococcus*, *Corynebacterium*, *Nocardia* and *Mycobacterium* (Rainey et al. 1995; Stackebrandt et al. 1997). As a direct result, the taxonomic position of many actinomycetes has been changed at various times and further comparative genome analyses may lead to other changes.

Research into the genetics of mating and recombination yields some insight into the genetic organisation of various strains, largely due to the pioneering work of Adams in the 1960s. In 1963, a recombination system was demonstrated in *Nocardia erythropolis* and *N. canicruria,* now renamed *Rhodococcus erythropolis* (Adams and Bradley 1963; Adams 1964; Brownell and Adams 1968). In these, and indeed subsequent studies, crosses between mutants of the same strain were found to be self-incompatible, whereas recombinations resulting from crosses of different strains were possible. This suggested the presence of a system of heterothallism in these bacteria, but the mechanism(s) underlying this have yet to be elucidated. Despite this problem, genetic linkage maps have nevertheless been

determined for some strains using non-genomic technology, particularly for *R. erythropolis* (reviewed by Brownell and Denniston 1984). It emerged that the role of extensive DNA homology in such recombinations may be doubtful for *Rhodococcus* and *Nocardia* species. Since it appears that recombination only occurs readily between strains from many different origins, homology may not then be the main determinant for recombination. Also, several different mating types were postulated with more than one mating type locus involved (Brownell and Adams 1968; Brownell and Kelly 1969; Gowan and Dabbs 1994). There is some evidence that the complex system of several mating-type loci may in part be associated with an approximately 8-kb sequence on the 60–80-kb circular plasmid pDA20 (Gowan and Dabbs 1994). A 3-kb region of pDA21 also appeared to be involved in controlling conjugation.

A very early insight into the genomes and genetic makeup of these bacteria also arose from the taxonomic studies involving DNA hybridisation between various strains, which are worth noting here. Although the overall DNA homology between genetically compatible strains of *Rhodococcus* and *Nocardia* can be low, as determined by DNA/DNA re-association (Clark and Brownell 1972), regions of homology, however, do exist. When DNA from the *R. erythropolis* mating type cE2 was hybridised with DNA from the compatible mating type Ce3, there was no more than 60% homology evident. Yet when the reverse DNA/DNA re-association experiment was done, nearly 100% homology was seen. This immediately indicated that the Ce3 mating-type strain had a considerable amount of additional and possibly non-homologous DNA added to its genome. Bearing in mind the number of aberrant segregants also observed, the more recent discovery of large linear and conjugative plasmids in these bacteria provides a ready explanation of these earlier results.

3 Overview of *Rhodococcus* Genomes

Later, molecular genetic studies of rhodococci gave some further insight into their complex and variable genomes. A large linear plasmid of about 1 Mb was reported in 1992 for *Rhodococcus fascians* (Crespi et al. 1992). Around that time, there was also some evidence that the four mega-base chromosome of *R. fascians* D188 may also be linear (Crespi et al. 1992). However, other analyses of several virulent and avirulent strains of *R. fascians* did not detect any linear replicons in the megabase size range, (Pisabarro et al. 1998). Notably, the genome size estimated by the same authors varied for different strains and was in a range of 5.6–8.0 Mb. These reported differences in the genome size appeared to be mainly related to the presence of large plasmids and instabilities in the *Rhodococcus* genomes. These early studies were the first indication of the challenge ahead with regard to genome analysis, and subsequent observations and experiments noted below have generally confirmed the genome size variability initially observed.

3.1 Genome Size and Variability

The most extensively studied genome of *Rhodococcus* confirms the large size of the genome and sets up a challenge with regard to a comparative genomic analysis. However, to this end, the genomes of several strains have now been sequenced and can be compared to the genomes of other important bacteria that fall into the general grouping of the mycolata. A summary of some relevant genome sequences available is presented in Table 1.

Initially, work began in 2001 to determine the complete genome sequence of *Rhodococcus* strain RHA1. This was carried out by Davies and co-workers at the University of British Columbia, Vancouver, as part of the CanadaGenome initiative

Table 1 Summary of *Rhodococcus* genome sequences and those of related mycolata

Bacterial strain	GenBank	Size Mb	%GC	Predicted genes	Reference
Rhodococcus jostii RHA1[a]					McLeod et al. (2006)
Chromosome	CP000431	7.8	67	7,211	
Linear plasmid pRHL1	CP000432	1.12	65	1,146	
Linear plasmid pRHL2	CP000433	0.44	64	454	
Linear plasmid pRHL3	CP000434	0.33	64	334	
Rhodococcus opacus B4[b]					Na et al. (2005)
Chromosome	AP011115	7.9	67	7,246	
Linear plasmid pROB01	AP011116	0.56	65	593	
Linear plasmid pROB02	AP011117	0.24	64	248	
Circular plasmid pKNR	AP011118	0.11	65	102	
Circular plasmid pKNR01	AP011119	0.0044	64	6	
Circular plasmid pKNR02	AP011120	0.0028	63	2	
Rhodococcus erythropolis PR4[b]					Sekine et al. (2006)
Chromosome	AP008957	6.5	62	6,030	
Plasmid pREC1	AP008932	0.1	63	102	
Plasmid pREC2	AP008933	0.0036	62	3	
Plasmid pREL1	AP008931	0.27	61	298	
Rhodococcus equi 103S[c]	Sequencing complete – annotation in progress				
Chromosome		5.0	69		
Circular plasmid		0.081			
Nocardia farcinica IFM 10152[d]					Ishikawa et al. (2004)
Chromosome (circular)	AP006618	6.0	70	5,683	
Circular plasmid pNF1	AP006619	0.18	67	160	
Circular plasmid pNF2	AP006620	0.09	68	93	
Mycobacterium tuberculosis H37Rv[c]					Cole et al. (1998)
Chromosome	AL123456	4.4	65	3,989	

[a]Genome Canada: http://www.rhodococcus.ca/
[b]National Institute of Technology and Evaluation (NITE), Japan: http://www.bio.nite.go.jp/ngac/e/B4-e.html, http://www.bio.nite.go.jp/ngac/e/pr4-e.html
[c]Sanger Centre, Cambridge UK: http://www.sanger.ac.uk/Projects/R_equi/, http://www.sanger.ac.uk/Projects/M_tuberculosis/
[d]National institute of Infectious Diseases, Japan: http://nocardia.nih.go.jp/

(Microbial Envirogenomics: Micro-organisms and their Interaction with the Environment). The outcome was that the catabolic diversity of the rhodococci has been well demonstrated in the genome sequence of this strain, now designated as *Rhodococcus jostii* strain RHA1. It is a polychlorinated biphenyl (PCB) degrading bacterium originally isolated on lindane as a substrate (Seto et al. 1995). It possesses one of the largest bacterial genomes analysed to date, consisting of 9,702,737 bp (G+C 67%) that is shared between three large linear plasmids: pRHL1 (1,100 kb), pRHL2 (450 kb) and pRHL3 (330 kb), and a linear chromosome (McLeod et al. 2006). In the case of each replicon, the telomeric sequences have been clearly identified. The genome sequence data is also available for analysis at the Genome Canada site at http://www.rhodococcus.ca/ and noted in Table 1.

The undoubted economic significance of these bacteria is also exemplified by the completion of the genome sequence of a commercial strain, *Rhodococcus aethorvorans* IG24 (Treadway et al. 1999) employed by Merck Corporation in developing a novel biotransformation route for the production of the anti-HIV drug Crixivan® (Priefert et al. 2004). Sequencing of this strain has been carried out by Integrated Genomics Inc. (Chicago, IL), a part of the Massachusetts Institute of Technology, Cambridge, MA. However, although the genome sequence is not available publicly, the genome has been reported to consist of a single 6.0 Mb chromosome (62 Mbp with approximately 97% sequenced) and plasmids of 300 and 100 Kb.

More recently, the genome sequences of *Rhodococcus opacus* B4 and *R. erythrypolis* PR4 have been released. *R. opacus* B4 was isolated from petroleum-contaminated soil and is especially resistant to solvents (Honda et al. 2008; Grund et al. 1992; Na et al. 2005). As for most environmental rhodococci isolated to date, it also utilises a wide variety of aromatic and aliphatic hydrocarbons. To reflect this ability, it has no less than six replicons: a large linear chromosome (7,913,450 bp) (G+C 67.9%); two linear plasmids pROB01 (558,192 bp) and pROB02 (244,997 bp) and three circular plasmids pKNR (111,160 bp), pKNR01 (4,367 bp) and pKNR02 (2,773 bp). The immediate observation is that there is general overall synteny conservation when compared with the sequenced *R. jostii* RHA1 genome as noted above. Its genome also appears to be arranged similarly to that of *Nocardia farcinica*, which has a circular chromosome (Ishikawa et al. 2004; Wu et al. 2006). The telomeric sequences of the three linear replicons (chromosome and plasmids) again appear to be conserved in relation to those of other Actinomycetes. As expected, its genome encodes many catabolic pathways including those for benzene, benzoate, phenol, 4-nitrophenol, 4-hydroxybenzoate, *p*-cumate, catechol, protocatechuate, phenylacetate, naphthalene, indene, nicotine, the thiocarbamate herbicide (EPTC) and thiocyanate.

In contrast to the terrestrial origins of the isolates above, *Rhodococcus erythropolis* PR4 was isolated from the Pacific Ocean at a depth of about 1 km near Japan. Again, this bacterium was found to utilise a wide variety of hydrocarbons and was solvent-resistant (Sekine et al. 2006; Peng et al. 2006). However, the sizeable genome this time consists of a circular chromosome (6,516,310 bp) (G+C 62.31%); a single, linear plasmid (pREL1: 271,577 bp) and two circular plasmids (pREC1: 104,014 bp; and pREC2: 3,637 bp). The chromosome and linear plasmid

encode most of the genes associated with the ability of this bacterium to degrade a wide variety of alkanes.

The development of the genome-sequencing projects that concentrated on isolates from various environments has been paralleled by the sequencing by the Sanger Centre, Cambridge, UK, of the pathogen *Rhodococcus equi* that causes bronchopneumonia in horses. In this case, the genome consists of 5,043,170 bp (68.82% GC) and a virulence-associated plasmid of 80,609 bp that carries genes associated with the pathogenicity of the bacterium. http://www.sanger.ac.uk/Projects/R_equi/

3.2 Plasmids – Role of Linear and Circular Plasmids

Plasmids are common in *Rhodococcus* species, and the majority of *Rhodococcus* strains analysed to date harbour many different types of plasmids, both linear and circular, in the same cells. Notably, various functions such as hydrogen autotrophy in *R. opacus* (Kalkus et al. 1990, 1993), isopropyl benzene metabolism in *R. erythropolis* (Dabrock et al. 1994), biphenyl metabolism in *Rhodococcus sp.* RHA1 (Masai et al. 1995), the plant virulence/fasciation genes (*fas*) in *R. fascians* (Crespi et al. 1992) and others have been reported to be encoded by linear plasmids. In addition to the genome-sequenced strains noted above, linear plasmids similar to those found in many *Streptomyces* species are a feature of many other *Rhodococcus* strains isolated. The ends of many *Streptomyces* linear replicons have inverted repeats, with proteins covalently bound to the 5′ end of each DNA strand (Hinnebusch and Tilly 1993). Early indications in studies on the telomers of several *R. opacus* linear plasmids showed features typical for linear plasmids of *Streptomyces*. However, plasmid pHG207 (225 kbp) has terminal inverted repeats (TIR) of 583/560 bp that are relatively short (Kalkus et al. 1993). Subsequently, the TIRs of plasmids pHG201, pHG204 and pHG205 were also found to be significantly shorter than those of large linear replicons of *Streptomyces*, and pHG201 was shown to have no defined TIR at all. Here, the homology of the terminal 34/32 bases was only 65% (Kalkus et al. 1998).

For RHA1, it was initially reported that the sequence of the linear plasmid pRHL3 is also a typical actinomycete invertron, containing large terminal inverted repeats associated with a protein (Warren et al. 2004). Twenty-one percent of its 300 putative genes have a probable catabolic function and are organised into three distinct clusters. Interestingly, four distinct regions are likely to have been acquired by horizontal gene transfer involving transposition functions. There are similar observations for pBD2 from the isopropyl benzene utilising strain *R. erythropolis* BD2 (Stecker et al. 2003), where there are 23 of 99 putative catabolic genes. There are also 32 genes that encode possible transposition functions.

The most striking feature of many studies is the presence of many large linear plasmids associated with catabolic genes. Such plasmids have also been shown to encode genes for the catabolism of trichloroethene (Saeki et al. 1999), naphthalene (O'Brien et al. 2002; Kulakov et al. 2005; Uz et al. 2000), toluene (O'Brien et al.

2002; Priefert et al. 2004), alkylbenzene (Kim et al. 2002), biphenyl (Taguchi et al. 2004) and chloroaromatic compounds (Konig et al. 2004). Although not formally tested in many cases, such plasmids often are conjugative and have conjugative *tra* genes and functions (Yang et al. 2007a).

As for most bacteria, circular plasmids are also very common in *Rhodococcus* species, and there are numerous examples noted by many researchers that are too many to cite here. There are many relatively small circular plasmids noted in the rhodococci such as the cryptic plasmid pKA22 (4,969 bp), pRTL1 (100 bp) encoding halolakane degradation genes (Kulakova et al. 1995) and a 150 bp, a 150 kb plasmid in *Rhodococcus* sp. strain IGTS8 encoding genes that are involved in the desulphurisation of organosulphur compounds (Denis-Larose et al. 1997). Interestingly, some of these cryptic plasmids possess mobilisation functions (Yang et al. 2007b). The most extensively characterised plasmids are either associated with pathogenesis determinants of *R. equi* (Tkachuk-Saad and Prescott 1991; Prescott 1991; Takai et al. 1991) or control the degradation of aromatic compounds in various strains (Kulakova et al. 1995). Pathogenicity genes are demonstrably associated in *R. equi* with the presence of large, circular plasmids of between 80 and 90 kb (Takai et al. 1991). These encode *vap* genes that have been clearly associated with genes involved with pathogenicty in horses (*vapA*) or pigs (*vapB*). There is evidence that these genes have evolved in plasmid-encoded pathogenicity islands and the parental *vap* gene was acquired by an ancestral plasmid that subsequently evolved to the currently observed host-adapted plasmids (Letek et al. 2008). A considerable amount of further genetic analysis is reviewed in chapter, "Genetics of the virulence of *Rhodococcus equi*" by Vázquez-Boland. Additionally, small cryptic plasmids have been characterised in various *Rhodococcus* strains. Several cryptic plasmids have been analysed (e.g. Kostichka et al. 2003) and shuttle vectors constructed (e.g. Matsui et al. 2007). Additionally, very useful plasmid vectors that can detect and test promoter activities in *Rhodococc*us have been developed (Knoppova et al. 2007) and these will provide more insight into the identification and versatility of prompter sequences. The sequences of two small circular cryptic plasmids, pKA22 and pFAJ2600 (Kulakov et al. 1997; De Mot et al. 1997), reveal that the putative RepA proteins of these plasmids are related to RepA proteins of the enterobacterial ColE2-type plasmids. Similarly, the replication region of the 100-kbp plasmid (pSOX), encoding desulphurisation from *Rhodococcus* sp X309 has a putative replication (Rep) protein related to the Rep proteins of the pLR7 family of plasmids characterised in *Mycobacteria* (Denis-Larose et al. 1997). Further analysis of the diversity of replication origins and mechanisms may lead to a better understanding of their origins and distribution.

3.3 Mobile Genetic Elements and Genetic Instability

It is likely that multiple recombinations have resulted in many diverse genes being distributed around *Rhodococcus* genomes, which are also made up of many linear plasmids. However, it is notable that very few transposable elements have been

characterised or observed (McLeod et al. 2006; Lessard et al. 1999; Nagy et al. 1997; Kulakov et al. 1999). Earlier research had indicated that the rhodococci possess mechanisms for high-frequency and illegitimate recombination (Kulakov and Larkin 2002) and, as noted above, it may, alongside homologous recombination, lead to the intro-regression of DNA without the need for many of the mobile genetic elements as first suggested for *Acinetobacter* (de Vries and Wackernagel 2002). Indeed, studies of plasmid integration in *Rhodococcus fascians* (Desomer et al. 1991) have shown the involvement of a short palindromic sequence (CCGCGG) and that exogenous DNA, bearing such a sequence, could lead to a non-homologous integration at a recombinational "hot-spot" sequence. Although this phenomenon has not been extensively studied, it is possible that the rhodococci may recombine easily with many heterologous sequences. Direct evidence of transposition has been demonstrated through the use of the *Bacillus subtilis sac*B gene, which encodes the production of levansucrase (Steinmetz et al. 1985; Gay et al. 1985). Its lethal effect in the presence of sucrose is widely used to select the transposition of insertion sequences and has been used to isolate an insertion sequence (IS) (IS-*Rf*) from *R. fascians* (Jager et al. 1995). Additionally, transposon mutagenesis and transposon-based vectors have been shown to function well in various rhodococci. A Tn5-based system has been used to create mutants in rhodococci (Fernandes et al. 2001; Sallam et al. 2007), and transposon-based vectors (pTNR-KA and pTNR-TA) have been used to transfer and express the proteasome complex from *Streptomyces coelicolor* in *R. erythropolis* (Sallam et al. 2006, 2007). Further evidence of effective transposition has been acquired from experiments with artificial constructs. A mini-transposon (Tn*5561*) was constructed by inserting the chloramphenicol resistance gene *cmrA* derived from pDA71 plasmid into IS*1415,* an element of the IS*21* family found upstream of the cobalamin biosynthesis genes (*cobLMK*) in *R. erythropolis* NI86/21 (Nagy et al. 1997). This construct successfully transposed following delivery on a suicide vector to *R. erythropolis* SQ1, and low specificity of the target sequences and 5- or 6-bp duplication of the target sequences was demonstrated.

However, most mobile genetic elements in *Rhodococcus* spp. have been identified when sequencing analysis of DNA regions flanking genes of interest was undertaken. Three of these mobile elements have been IS*1166* located downstream of the sulphur oxidation genes (*soxABC*) in *R. erythropolis* IGTS8 (Denome and Young 1995); IS*1164* found upstream of the structural genes for the high molecular mass-nitrile hydratase (*nhhBA*) in *R. rhodochrous* J1 (Komeda et al. 1996b); and an IS element detected upstream of the hydrogenase subunit genes (*hoxFUYH*) of *R. opacus* MR11 (Grzeszik et al. 1997). All these IS elements belong to the IS*256* family, members of which were identified in a wide range of organisms (Mahillon and Chandler 1998). The obvious fact that there are multiple copies of these elements in some strains indicates their ability to transpose effectively. Of note is the discovery of two copies of IS*2112* on the catabolic plasmid pRTL1 in *Rhodococcus rhodochrous* NCIMB13064 (Kulakov et al. 1999). IS*2112* belongs to the IS*110* family of transposable elements. These elements do not possess typical terminal inverted repeats and are not flanked by directly repeated sequences (Mahillon and Chandler 1998).

The possible involvement of integrons in mediating rearrangements and recombinations in *Rhodococcus* has not been fully established, but they may play a key role in genome evolution. The observation of a 47-bp sequence with some homology with 5′ end of several integrons from Gram-negative bacteria was noted in a region near IS*1415* in *R. erythropolis* NI86/21 (Nagy et al. 1997). However, the essential components of a typical integron, namely an *int* gene (encoding a site-specific recombinase or integrase) for integration of incoming gene cassettes, are not evident.

4 The Genetic Basis of Catabolic Capabilities

The general conclusion from the genome studies to date is that RHA1 and other environmental strains appear to have evolved to simultaneously catabolise a very diverse range of organic compounds. Their obligate aerobic requirement means that this has probably been in an oxygen-rich environment. In this regard, it is particularly notable that there are at least 203 different oxygenases associated with the degradation pathways of aromatic compounds and steroids identified in RHA1. In the case of aromatic compounds, there appears to be at least 26 different "peripheral aromatic" pathways for a very wide range of compounds and eight "central aromatic pathways". In the light of this remarkable potential catabolic ability, it is therefore surprising that the RHA1 genome appears to harbour very few recent gene duplications. Indeed, many genes also do not appear to have been acquired through recent horizontal transfer. However, in other *Rhodococcus* strains there is evidence that gene transfer is a key factor related to the degradation of many xenobiotic compounds as discussed below. For example, there is evidence of transfer of genes associated with the catabolism of haloalkanes (Poelarends et al. 2000a, b), alkenes (Leahy et al. 2003), biphenyl (Taguchi et al. 2004, 2007), naphthalene (Kulakov et al. 2005) and the explosive RDX (Seth-Smith et al. 2008) amongst various independently isolated strains from different global locations. However, in the case of RHA1, it appears to have primarily acquired its large genome through very ancient gene duplications and gene transfers. It is of note, however, that the RHA1 genome possesses only two intact insertion sequences and relatively few transposase genes (McLeod et al. 2006). The large genomes (over 7 Mb each) of two other rhodococci, *Rhodococcus aetherovorans* strain I24 and *Rhodococcus erythropolis* strain PR4, have also indicated the presence of multiple gene homologues and this is probably a feature of most of the biodegradative rhodococci. However, it is interesting that the pathogen *R. equi* has a smaller genome of just over 5 Mb.

The observation of many catabolic genes in RHA1 has in turn led to the discovery of new pathways. Initial analyses showed that putative genes encoding taurine-pyruvate aminotransferase (*Tpa*) and alanine dehydrogenase (*Ald*) for the catabolism and regulation of taurine catabolism were present. Later experiments confirmed growth on taurine and involvement of these genes (Denger et al. 2004). However, it is notable that these genes are separated from the associated

sulfoacetaldehyde acetyltransferase (*xsc*), phosphotransacetylase (*pta*) and possible ABC transporter (*tauBC*) genes. Additionally, proteomic analysis of RHA1, in response to growth on nitriles, has led to the discovery of a new class of acetonitrile hydratase on the linear plasmid pRHL2 (Okamoto and Eltis 2007). Interestingly, transcriptomic analysis of cells grown on cholesterol led to the discovery and confirmation of steroid catabolic genes that are also present in *Mycobacterium tuberculosis* (Van der Geize et al. 2007). Similar transcriptomic studies have concluded that a propane monooxygenase (PrmA) is involved in the degradation of *N*-nitrosodimethylamine in RHA1 (Sharp et al. 2007) and that phthalate is likely to be degraded solely via the protocatechuate pathway. However, terephthalate is degraded via a bifurcated pathway that includes the catechol branch of the protocatechuate pathway (Hara et al. 2007). These studies are tending to lead to the conclusion that there are many hidden capabilities in RHA1 and that this is likely to be the case for many other strains. The initial results also lead to the conclusion that there is a mechanism of co-regulation of these catabolic genes that are expressed from different genomic locations and that this is likely to be a complex arrangement.

5 Gene Regulation and Expression

Not surprisingly, studies on the regulation of *Rhodococcus* biodegradation gene clusters have revealed many examples of both positive gene regulators (Komeda et al. 1996a) and repressors (Barnes et al. 1997; Nga et al. 2004). However, as noted above, recent studies based upon investigating the whole genome indicate that there is co-regulation of genetically unlinked transcriptional units. The most thoroughly investigated examples of this have been the multiple biphenyl/PCB degradation genes of RHA1 (also in *Rhodococcus* strain M5) that were originally noted as distributed in several clusters (Kitagawa et al. 2001; Yamada et al. 1998) on plasmids pRHL1 and pRHL2 (Shimizu et al. 2001). RHA1 expresses two biphenyl dioxygenases (*Bph*A and *EtbA*/*EbdA*) (Iwasaki et al. 2007), and the wide substrate range of the EtbA/EbdA dioxygenase suggested that it may play a role in the degradation of other compounds such as the PCBs (Iwasaki et al. 2006).

Rhodococcus M5 has a two-component regulatory system (*bpdST*) that regulates expression of some of the *bph* genes (Labbe et al. 1997), and this also appears to be the case for RHA1 (Takeda et al. 2004a, b) and for the regulation of expression of the *o*-xylene catabolic genes in *Rhodococcus* sp. strain DK17 (Kim et al. 2005). A putative GntR-like transcriptional regulation mechanism, involving *narR1* and *narR*2, is involved in several naphthalene-degrading *Rhodococcus* strains (Kulakov et al. 2005). Again, there is evidence that the genes are not organised in a single cluster, and different strains have several homologous transcriptional units separated by non-homologous sequences containing direct and inverted repeats. This is complicated by the demonstration of different promoter sequences initiating the expression of the homologous *narAa–narB* gene clusters, and recombination events may be involved in the acquisition and then alignment of the regulatory regions with the catabolic genes.

Further insights arise from studies of *Rhodococcus* RHA1 where the degradation genes for benzoate (*ben*), phthalate (*pad*), uptake of phthalate (*pat*) and the genes for two branches of the β-ketoadipate pathway (*cat* and *pca*) are also dispersed in the genome. Some are contained on a putative "catabolic island" that is duplicated in the linear plasmids pRHL1 and pRHL2. The regulatory interrelationship between the gene clusters is likely to be complex with involvement of *pad*- and *pat*-encoded enzymes in phthalate degradation and *ben* and *cat* gene products in benzoate degradation. Expression of the *pca*-encoded products is also evident after growth on both substrates (Patrauchan et al. 2005).

To further elucidate the complex expression of such gene clusters, both proteomic and gene disruption techniques have been used to investigate the degradation of benzene, styrene, biphenyl and ethylbenzene in RHA1. Cells grown on biphenyl, ethylbenzene or benzene produce enzymes associated with both biphenyl and ethyl benzene catabolism and enzymes from at least two sets of lower biphenyl pathways. Styrene-grown cells do not express the ethylbenzene pathway genes and only one set of lower biphenyl pathway enzymes are produced. Biphenyl dioxygenase is essential for growth on benzene or styrene, but the putative ethylbenzene dioxygenase was not required for growth on any of the substrates (Patrauchan et al. 2008).

These results indicate that co-expression of genes from different loci may be commonplace. Indeed, the utilisation of phenylacetic acid is encoded in part by 13 *paa* genes on the chromosome. A single transcript encodes 11 genes but production of a further 146 proteins was induced by growth on phenylacetic acid (Navarro-Llorens et al. 2005).

Further evidence for such co-expression has also been noted by the analysis of the RHA1 transcriptome when the cells were grown on various substrates. RHA1 encodes multiple isoenzymes for most of the steps in biphenyl catabolism, and co-expression of these is clearly associated with the catabolism of biphenyl, ethylbenzene and PCBs. Transcriptomic analysis of over 8,000 potential catabolic genes indicates that over 320 are up-regulated for growth on biphenyl and ethylbenzene, unlike growth on benzoate that led to up-regulation of 65 genes. There was no difference in the expression of key catabolic genes for ethylbenzene and biphenyl, indicating the likelihood of a common regulation for these (Goncalves et al. 2006).

In terms of regulation and expression of genes in the environment, there has been little research; however, it has been observed that genes associated with biphenyl degradation are expressed in biphenyl-amended soils (Wang et al. 2008). Although it has been acknowledged that rhodococci can withstand environmental stresses such as solvents and desiccation, to date there have been few studies that have addressed this. However, a very interesting transcriptomic study indicated that the genes associated with desiccation were maximally up-regulated upon complete drying of the *Rhodococcus* RHA1 cells. These included genes putatively associated with oxidative stress and biosynthesis of the compatible solute, ectoine and sigma factors, indicating their putative role in regulation (LeBlanc et al. 2008).

6 Concluding Remarks

Analysis of the biochemical diversity, physiology and the genomes of *Rhodococcus* strains is revealing slowly why they can adapt to catabolise many different substrates and how they persist in and adapt to conditions in many different environments. The data confirm many of the earlier genetic observations made in older studies of these bacteria and raise some intriguing questions about the basis of recombination and the acquisition of genes by lateral transfer. Initial whole genome analysis has been based largely upon one strain, *Rhodococcus jostii* RHA1, but the existence of other genome sequences now allows us to embark upon the road to making comparative genome analyses. In relation to catabolic genes, the research to date goes some way to explain how single strains can utilise a remarkably wide range of substrates. The surprise is the extent to which the pathways for biodegradation are dispersed around the genome and apparently the subject of co-regulation. Elucidating how this occurs and how the arrangement works to the benefit of the organism is a major challenge but will enable a better understanding of their relationship with the wider environment and their host–pathogen interactions.

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Central Metabolism of Species of the Genus *Rhodococcus*

Héctor M. Alvarez

Contents

Abstract The metabolism of *Rhodococcus* has evolved to adapt to a wide range of nutritional conditions. This adaptation often involves the flexibility of the central metabolism, which usually provides energy and precursors for the biosynthesis processes, either during growth or during nonreplicative metabolically active periods. The pathways of central metabolism are close to identical across widely divergent organisms, which share essentially the same metabolic network. However, this network possesses species-specific components, which depend on the biology of rhodococci. The central metabolism of the members of the *Rhodococcus* genus in the context of their physiology is the main topic of this chapter. An overview of the main pathways of the central metabolism and their link with other metabolic processes is given. The glycolytic pathways, the gluconeogenesis and the phosphoenolpyruvate–pyruvate–oxalacetate node, the tricarboxylic acid cycle (TCA), the glyoxylate pathway, and some litoautotrophic pathways are included.

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1 Introduction

The members of the genus *Rhodococcus* are aerobic nonsporulating bacteria widely distributed in diverse natural environments. They have been detected in tropical, arctic and arid soils, as well in marine and very deep-sea sediments (Whyte et al. 1999; Heald et al. 2001; Peressutti et al. 2003; Alvarez et al. 2004; Luz et al. 2004; Peng et al. 2008). One striking feature of rhodococci is the metabolic versatility with capabilities for biodegradation and transformation of a wide range of pollutant compounds such as hydrocarbons, pesticides, and xenobiotics (Warhurst and Fewson 1994; Larkin et al. 2005; Martínková et al. 2009). This feature, in addition to their extraordinary environmental persistence and tolerance to stress conditions, makes such microorganisms promising candidates for in situ bioremediation of contaminated soil environments. Moreover, the members of the *Rhodococcus* are able to synthesize diverse compounds, such as surfactants, wax esters, and oils, which are of interest for biotechnological purposes. For these reasons, the studies on rhodococci have intensified significantly within the last years. In this context, most knowledge on metabolism acquired during the last years has been focused principally on catabolism of diverse compounds and on the biosynthesis of lipids. For detailed overviews on these topics, the readers should refer to the recent reviews (Larkin et al. 2005; Alvarez and Steinbüchel 2002; Alvarez 2006; Martínková et al. 2009). Despite the importance of the central metabolism for understanding the biology of rhodococci, this topic has been studied only very poorly, in comparison with the other metabolic processes mentioned above. The pathways of central metabolism are highly conserved among the organisms; however, each species adopts a metabolic configuration specific to its biology. In this context, the pathways of central metabolism in rhodococci must provide energy and metabolic intermediates not only during growth periods, but also in those environmental conditions, where cells are metabolically active despite cessation of cellular growth. Under stress conditions, the physiology of rhodococci seems to depend on the metabolism of diverse storage compounds. The members of the *Rhodococcus* genus possess an extensive capacity to synthesize and metabolize diverse storage compounds, such as triacylglycerols, wax esters, polyhydroxyalkanoates, glycogen, and polyphosphate (Anderson et al. 1995; Alvarez et al. 1997; Alvarez 2003; Hernández et al. 2008).

Several genomic projects of the *Rhodococcus* members are now in progress through public and private efforts due to the increasing interest in their use for biotechnology. Among them, the genome sequence of *Rhodococcus jostii* strain RHA1 has been the first sequence publicly available for screening and identification of genes and metabolic pathways (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). For this reason, *R. jostii* RHA1 is a good model organism for understanding the genetics, physiology, and metabolism of *Rhodococcus* genus. Strain RHA1 possesses one of the largest bacterial genomes sequenced to date, containing 9.7 Mbp arranged in a linear chromosome (7,802,028 bp) and three linear plasmids: pRHL1 (1,123,075 bp), pRHL2 (442,536 bp), and pRHL3 (332,361 bp)

(McLeod et al. 2006). RHA1 is a soil bacterium with the ability to degrade and transform polychlorinated biphenyls and other aromatic compounds (Masai et al. 1995; Van der Geize et al. 2007; Patrauchan et al. 2008). The large RHA1 genome contains a multiplicity of catabolic genes, a high genetic redundancy of biosynthetic pathways, and a sophisticated regulatory network, which reflect the complexity of *Rhodococcus* biology. In addition to the RHA1 genome, databases of the complete genome sequences of *R. opacus* B4 and *R. erythropolis* are now publicly available. *R. erythropolis* PR4 was isolated from the Pacific Ocean, south of Okinawa Island, Japan, at a depth of 1,000 m (Komukai-Nakamura et al. 1996), whereas *R. opacus* B4 has been isolated as an organic solvent-tolerant bacterium from gasoline-contaminated soil (Na et al. 2005).

Based on a genome-wide examination of key genes involved in metabolism in *R. jostii* RHA1 and on biochemical and genetic studies published in peer reviewed journals, this paper summarizes some aspects of the central metabolism of species of the genus *Rhodococcus*, including glycolytic pathways, gluconeogenesis and the phosphoenolpyruvate (PEP)–pyruvate–oxalacetate node, the tricarboxylic acid cycle (TCA), and the glyoxylate shunt. In addition, some new information about the energy pathways will be given.

2 Glycolytic Pathways

Glycolysis (Embden–Meyerhof–Parnas pathway; EMP) is a common pathway for the oxidation of glucose, which is used by eukaryotic cells, and by some aerobic and facultatively anaerobic bacteria, but not by Archaea. In this pathway, glucose is split into two molecules of pyruvic acid with the formation of two ATP through substrate level phosphorylations. Many aerobic bacteria and the Archaea use an alternative glycolytic pathway called the Entner–Duodoroff pathway (ED pathway), which produces pyruvate directly from glucose and only one ATP from each molecule of glucose. This pathway is found only in prokaryotes, such as *Pseudomonas*, *Azotobacter*, *Rhizobium*, and many Gram negatives. ED pathway occurs also in actinomycetes bacteria (Gunnarsson et al. 2004; Borodina et al. 2005).

The genome of *R. jostii* RHA1 was examined for the presence of key genes involved in glycolytic pathways. Strain RHA1 seems to be able to use both glycolytic pathways, the EMP- and ED-pathways, for catabolism of carbohydrates. Figure 1 and Table 1 give an overview on the pathways and enzymes/genes that are implicated in the oxidation of glucose by *R. jostii* RHA1.

The RHA1 genome also contains all necessary genes/enzymes for the pentose phosphate pathway (PP pathway) (Fig. 1 and Table 1). This pathway produces precursors to the ribose and deoxyribose in nucleic acids and provides erythrose for the synthesis of aromatic amino acids. In addition, the PP pathway produces reducing power as NADPH.

In general, all these genes involved in carbohydrate metabolism are widely distributed throughout the RHA1 genome. The genes *tpiA*, *gap2*, and *pgk* involved

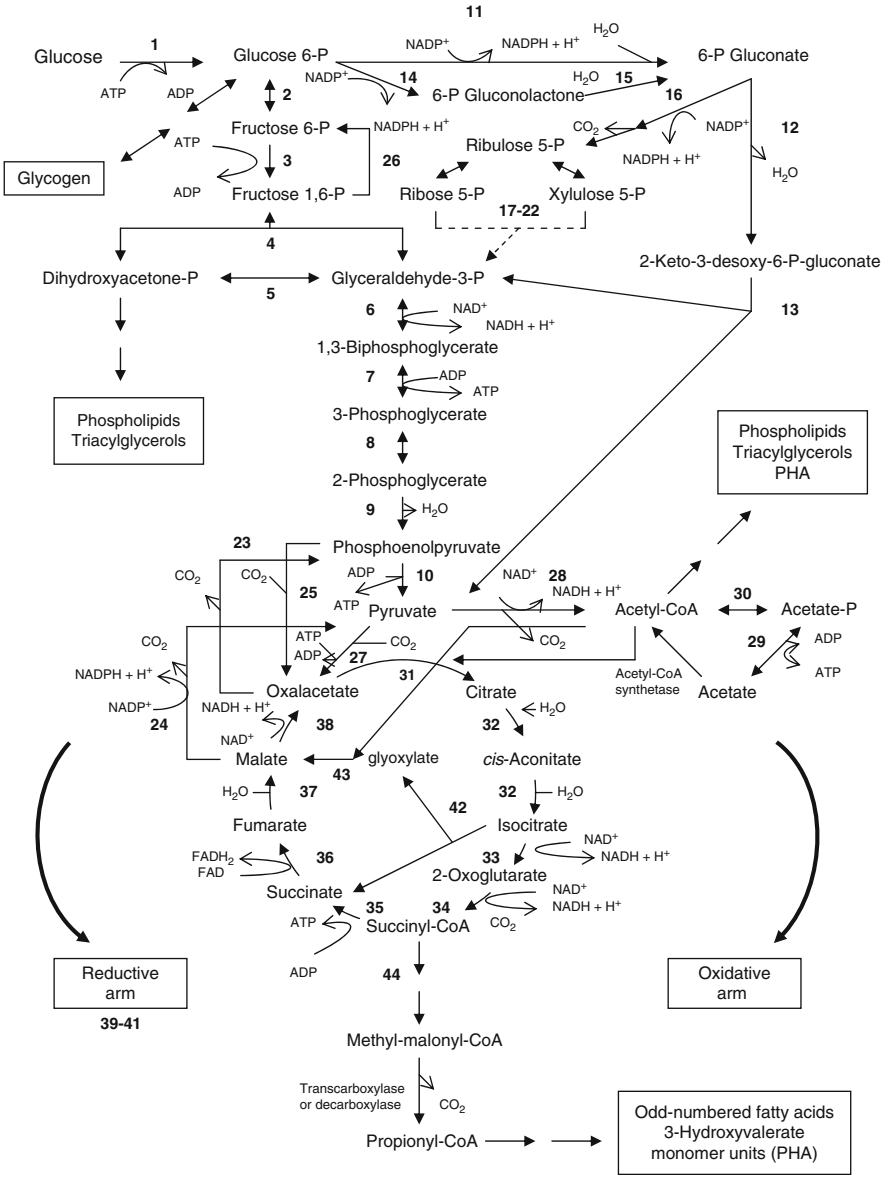

Fig. 1 Pathways involved in central metabolism of *Rhodococcus jostii* RHA1 deduced from the genome database

in the glycolytic pathways are clustered in the genome. In addition, the genes *ro02367* and *ro02368*, coding for phosphogluconate dehydratase and KHG/KDPG aldolase that are specifically involved in the ED pathway, are also clustered in RHA1 genome.

Table 1 Genes involved in the central metabolism of *R. jostii* RHA1

Reaction number[a]	Gene ID	Gene name	Predicted function	Metabolic pathways
1	ro01116		Glucokinase	Glycolysis (EMP–ED), glycogen degradation
1	ro01359		Glucokinase	Glycolysis (EMP–ED), glycogen degradation
1	ro04278		Glucokinase	Glycolysis (EMP–ED), glycogen degradation
1	ro04953		Glucokinase	Glycolysis (EMP–ED), glycogen degradation
1	ro05185		Glucokinase	Glycolysis (EMP–ED), glycogen degradation
2	ro11167	*pgi2*	Glucose-6-phosphate isomerase	Glycolysis (EMP), gluconeogenesis
3	ro00056		1-Phosphofructokinase	Glycolysis (EMP)
3	ro06479	*pfkA*	6-Phosphofructokinase	Glycolysis (EMP)
3	ro06783		1-Phosphofructokinase	Glycolysis (EMP)
4	ro05536	*fba*	Fructose-bisphosphate aldolase	Glycolysis (EMP), gluconeogenesis
5	ro07179	*tpiA*	Triose-phosphate isomerase	Glycolysis (EMP)
6	ro07177	*gap2*	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis (EMP), gluconeogenesis
7	ro07178	*pgk*	Phosphoglycerate kinase	Glycolysis (EMP), gluconeogenesis
8	ro00880		Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis
8	ro01295	*gpmA*	Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis
8	ro06349		Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis
9	ro05777	*eno*	Phosphopyruvate hydratase	Glycolysis (EMP), gluconeogenesis
10	ro01007	*pyk1*	Pyruvate kinase	Glycolysis (EMP–ED)
10	ro02565	*pyk2*	Pyruvate kinase	Glycolysis (EMP–ED)
10	ro03221	*pyk3*	Pyruvate kinase	Glycolysis (EMP–ED)
10	ro04800	*pyk4*	Pyruvate kinase	Glycolysis (EMP–ED)
11	ro02468		Glucose dehydrogenase	Glucose degradation (ED)
11	ro06481		Glucose dehydrogenase	Glucose degradation (ED)
11	ro09067		Glucose dehydrogenase	Glucose degradation (ED)
12	ro02368	*edd*	Phosphogluconate dehydratase	Glucose degradation (ED)
13	ro02367		KHG/KDPG aldolase	Glucose degradation (ED)
14	ro00555	*zwf1*	Glucose-6-phosphate 1-dehydrogenase	PP pathway
14	ro02369	*zwf2*	Glucose-6-phosphate 1-dehydrogenase	PP pathway
14	ro05641	*zwf3*	Glucose-6-phosphate 1-dehydrogenase	PP pathway

(*continued*)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function	Metabolic pathways
14	ro07184	*zwf4*	Glucose-6-phosphate 1-dehydrogenase	PP pathway
14	ro11332	*zwf5*	Glucose-6-phosphate 1-dehydrogenase	PP pathway
15	ro07182	*pgl*	6-Phosphogluconolactonase	PP pathway
16	ro00556	*gnd1*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
16	ro03668	*gnd2*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
16	ro07246	*gnd3*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
16	ro11061	*gnd4*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
16	ro11328	*gnd5*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
17	ro07167	*rpe*	Ribulose-phosphate 3-epimerase	PP pathway
18	ro02899		Ribose-5-phosphate isomerase	PP pathway
18	ro01378		Ribose 5-phosphate isomerase	PP pathway
19	ro07185	*tal*	Transaldolase	PP pathway
19	ro08277		Transaldolase	PP pathway
20	ro00814		Transketolase, C-terminal subunit	PP pathway
20	ro00815		Transketolase, N-terminal subunit	PP pathway
20	ro03843		Transketolase, N-terminal subunit	PP pathway
20	ro03844		Transketolase, C-terminal subunit	PP pathway
20	ro07186		Transketolase	PP pathway
21	ro03376		Sugar phosphate isomerase/epimerase	PP pathway
22	ro04629	*araD1*	Ribulose-5-phosphate 4-epimerase	PP pathway
22	ro07052	*araD2*	Ribulose-5-phosphate 4-epimerase	PP pathway
23	ro05180		Phosphoenolpyruvate carboxykinase	Gluconeogenesis
24	ro00492		Malate dehydrogenase (oxaloacetate-decarboxylating)	Gluconeogenesis
24	ro02571		Malate dehydrogenase (oxaloacetate-decarboxylating)	Gluconeogenesis
24	ro11316		Malate dehydrogenase (oxaloacetate-decarboxylating)	Gluconeogenesis
24	ro08928		Malate dehydrogenase (oxaloacetate-decarboxylating)	Gluconeogenesis
25	ro07181	*ppc*	Phosphoenolpyruvate carboxylase	Gluconeogenesis
26	ro05865	*glpX*	Fructose 1,6-biphosphatase	Gluconeogenesis
27	ro06517	*pycA*	Pyruvate carboxylase	Anaplerotic pathway
28	ro00542		Pyruvate dehydrogenase E1 component	Acetyl-CoA synthesis

28	ro00543		Dihydrolipoyllysine-residue succinyltransferase (PDH)	Acetyl-CoA synthesis
28	ro01066		Pyruvate dehydrogenase (cytochrome)	Acetyl-CoA synthesis
28	ro01197	*aceE1*	Pyruvate dehydrogenase E1 component	Acetyl-CoA synthesis
28	ro01576	*pdhA1*	Pyruvate dehydrogenase E1 component α-subunit	Acetyl-CoA synthesis
28	ro01577		Pyruvate dehydrogenase, β-subunit	Acetyl-CoA synthesis
28	ro01578		Dihydrolipoyllysine-residue succinyltransferase (PDH)	Acetyl-CoA synthesis
28	ro02140	*dldH1*	Dihydrolipoyl dehydrogenanse	Acetyl-CoA synthesis
28	ro02566	*aceE2*	Pyruvate dehydrogenase E1 component	Acetyl-CoA synthesis
28	ro02927	*dldH2*	Dihydrolipoyl dehydrogenase (PDH)	Acetyl-CoA synthesis
28	ro03301		Pyruvate dehydrogenase (acetyl-transferring), β-subunit	Acetyl-CoA synthesis
28	ro03305		Pyruvate dehydrogenase E1 component	Acetyl-CoA synthesis
28	ro03318	*dldH3*	Dihydrolipoyl dehydrogenanse	Acetyl-CoA synthesis
28	ro03319	*pdhC*	Dihydrolipoyllysine-residue acetyltransferase, E2 component of pyruvate dehydrogenase complex	Acetyl-CoA synthesis
28	ro03320	*pdhB1*	Pyruvate dehydrogenase E1 component β-subunit	Acetyl-CoA synthesis
28	ro03321	*pdhA2*	Pyruvate dehydrogenase E1 component α-subunit	Acetyl-CoA synthesis
28	ro03377	*dldH4*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
28	ro03378		Dihydrolipoyllysine-residue acetyltransferase	Acetyl-CoA synthesis
28	ro03379	*pdhB2*	Pyruvate dehydrogenase E1 component β-subunit	Acetyl-CoA synthesis
28	ro03380	*pdhA3*	Pyruvate dehydrogenase E1 component α-subunit	Acetyl-CoA synthesis
28	ro05562	*dldH5*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
28	ro06262	*dldH6*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
28	ro08380		Possible pyruvate dehydrogenase (fragment)	Acetyl-CoA synthesis
28	ro11024	*lpdA*	Dihydrolipoyl dehydrogenanse	Acetyl-CoA synthesis
28	ro11029	*sucB*	Dihydrolipoyllysine-residue succinyltransferase	Acetyl-CoA synthesis
28	ro11030		Pyruvate dehydrogenase, β-subunit	Acetyl-CoA synthesis
28	ro11031		Pyruvate dehydrogenase, α-subunit	Acetyl-CoA synthesis
29	ro02196	*askA*	Acetate kinase	AK-PTA pathway
30	ro02197	*pta*	Phosphotransacetylase	AK-PTA pathway
31	ro00355	*citA1*	Citrate (Si)-synthase	TCA cycle (oxidative)
31	ro01610		Possible citrate synthase, C-terminal	TCA cycle (oxidative)
31	ro01611		Possible citrate synthase, N-terminal	TCA cycle (oxidative)

(*continued*)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function	Metabolic pathways
31	ro04993	*citA2*	Citrate (Si) synthase	TCA cycle (oxidative)
31	ro04998		Citrate (Si)-synthase	TCA cycle (oxidative)
31	ro06955		Citrate synthase	TCA cycle (oxidative)
32	ro02399	*acnA1*	Aconitate hydratase	TCA cycle (oxidative/reductive)
32	ro07207	*acnA2*	Aconitate hydratase	TCA cycle (oxidative/reductive)
32	ro08266	*acnA3*	Aconitate hydratase	TCA cycle (oxidative/reductive)
33	ro00618		Isocitrate dehydrogenase (NADP+)	TCA cycle (oxidative/reductive)
33	ro06238		Isocitrate dehydrogenase (NADP+)	TCA cycle (oxidative/reductive)
34	ro06012	*odhA*	2-Oxoglutarate dehydrogenase, E1 and E2 components	TCA cycle (oxidative)
35	ro05573	*sucC*	Succinate CoA ligase β-subunit	TCA cycle (oxidative)
35	ro05574	*sucD*	Succinate CoA ligase α-subunit	TCA cycle (oxidative)
35	ro03613		Probable succinate CoA ligase	TCA cycle (oxidative)
36	ro01048	*sdhB1*	Succinate dehydrogenase iron–sulfur protein	TCA cycle (oxidative)
36	ro01049	*sdhA1*	Succinate dehydrogenase flavoprotein subunit	TCA cycle (oxidative)
36	ro01050		Possible succinate dehydrogenase	TCA cycle (oxidative)
36	ro04774		Possible succinate dehydrogenase	TCA cycle (oxidative)
36	ro05698		Possible succinate dehydrogenase	TCA cycle (oxidative)
36	ro06245	*sdhB2*	Succinate dehydrogenase Fe–S protein	TCA cycle (oxidative)
36	ro06246	*sdhA2*	Succinate dehydrogenase	TCA cycle (oxidative)
36	ro06247		Probable succinate dehydrogenase hydrophobic membrane anchor protein	TCA cycle (oxidative)
36	ro06248		Probable succinate dehydrogenase (cytochrome)	TCA cycle (oxidative)
36	ro08268		Possible succinate dehydrogenase Fe–S protein	TCA cycle (oxidative)
36	ro08825		Probable succinate dehydrogenase hydrophobic membrane anchor protein	TCA cycle (oxidative)
36	ro08826	*sdhC*	Succinate dehydrogenase cytochrome *b* subunit	TCA cycle (oxidative)
36	ro08827		Succinate dehydrogenase flavoprotein subunit	TCA cycle (oxidative)
36	ro08828	*sdhB3*	Succinate dehydrogenase Fe–S protein subunit	TCA cycle (oxidative)
37	ro05864	*fumC*	Fumarate hydratase class II	TCA cycle (oxidative/reductive)
37	ro05899		Fumarate hydratase class I	TCA cycle (oxidative/reductive)

37	ro08824		Fumarate hydratase, class I	TCA cycle (oxidative/reductive)
38	ro06244	*mdh*	Malate dehydrogenase	TCA cycle (oxidative/reductive)
38	ro06612		Malate dehydrogenase (acceptor)	TCA cycle (oxidative/reductive)
39	ro00185		Citrate lyase	TCA cycle (reductive)
39	ro00306		Citrate (pro-3S)-lyase β-subunit	TCA cycle (reductive)
39	ro00662		Citrate (pro-3S)-lyase	TCA cycle (reductive)
39	ro00775		Citrate lyase	TCA cycle (reductive)
39	ro00778		Citrate (pro-3S)-lyase β-subunit	TCA cycle (reductive)
39	ro02446		Possible citrate lyase β-subunit	TCA cycle (reductive)
39	ro02872		Citrate (pro-3S)-lyase	TCA cycle (reductive)
39	ro02943		Citrate lyase β-subunit	TCA cycle (reductive)
39	ro05986		Citrate (pro-3S)-lyase β-subunit	TCA cycle (reductive)
39	ro06099		Citrate (pro-3S)-lyase (β-subunit)	TCA cycle (reductive)
39	ro06584		Probable citryl-CoA lyase β-subunit	TCA cycle (reductive)
39	ro08641		Possible citrate lyase β-subunit (C-terminal)	TCA cycle (reductive)
39	ro08790		Probable citrate lyase β-subunit	TCA cycle (reductive)
40	ro02389		2-Oxoglutarate synthase β-subunit	TCA cycle (reductive)
40	ro02390		2-Oxoglutarate synthase α-subunit	TCA cycle (reductive)
41	ro02419		Possible fumarate reductase	TCA cycle (reductive)
41	ro09023		Probable fumarate reductase	TCA cycle (reductive)
41	ro09090		Possible fumarate reductase	TCA cycle (reductive)
42	ro02122		Isocitrate lyase	Glyoxylate pathway
43	ro00899	*glcB*	Malate synthase G	Glyoxylate pathway
44	ro07233	*mutA*	Methylmalonyl-CoA mutase small subunit	Methylmalonyl-CoA pathway
44	ro07234	*mutB*	Methylmalonyl-CoA mutase large subunit	Methylmalonyl-CoA pathway

[a]Numbers refer to reactions shown in Fig. 1

3 Glycogen Synthesis and the Link with the Central Metabolism

Recent studies revealed that the members of the *Rhodococcus* genus, such as *R. jostii* (Hernández et al. 2008), *R. opacus*, *R. erythropolis*, *R. ruber*, and *R. fascians* (Hernández and Alvarez, unpublished results), are able to synthesize and accumulate glycogen. In general, the total content of glycogen in those microorganisms amounted up to 2–6% of cellular dry weight. The studied strains accumulated glycogen during exponential growth phase and the content decreased during stationary growth phase. Glycogen accumulation during exponential growth phase has also been observed in other actinomycetes, such as *M. smegmatis* (Belanger and Hatfull 1999) and *Corynebacterium glutamicum* (Seibold et al. 2007). Glycogen may have a role as metabolic intermediate since it is accumulated mainly during the exponential growth phase by cells and is mobilized later in the stationary phase; thus, glycogen has been proposed as a carbon capacitor for glycolysis during exponential growth (Belanger and Hatfull 1999). Glycogen may be a part of a mechanism for controlling sugar excess in *Rhodococcus*, or may act as a part of a sensing/signaling mechanism. Persson et al. (2007) proposed that the expression of some genes involved in the response of *E. coli* to carbon starvation or stationary phase, like that encoding the universal stress protein (*uspA*), is regulated by glycolytic intermediates such as fructose-6-phosphate. Alteration in the pool size of phosphorylated sugars of the upper glycolytic pathway may ensure expression of stress proteins preceding the complete depletion of the external carbon source and growth arrest (Persson et al. 2007). Thus, glycogen formation may act to attenuate phosphorylated sugar signals and to protect cells from sudden increases in fluxes of sugars.

4 Gluconeogenesis and the Phosphoenolpyruvate–Pyruvate–Oxalacetate Node

The metabolic link between glycolysis/gluconeogenesis and the TCA cycle is represented by the PEP–pyruvate–oxalacetate node, also called the anaplerotic node (Sauer and Eikmanns 2005) (Fig. 1). This node comprises a set of reactions that direct the carbon flux into appropriate directions in a flexible manner. The node represents a relevant switch point for carbon flux distribution within the central metabolism (Sauer and Eikmanns 2005).

During growth on acetate, fatty acids, or ethanol, which enter central metabolism via acetyl-CoA or on TCA cycle intermediates, the cycle intermediates malate and oxalacetate must be converted to pyruvate and PEP for the synthesis of sugars. Once PEP is formed, the synthesis of sugar phosphates is accomplished by reversible reactions of glycolysis (gluconeogenesis). Under gluconeogenic conditions, the TCA cycle intermediates, oxalacetate and malate, must be decarboxylated

(C4-decarboxylation) to form pyruvate and PEP, which serve as precursors for sugar phosphate synthesis. The formation of PEP can be achieved either by the PEP carboxykinase enzyme (Pck) directly or oxalacetate decarboxylase and/or malic enzyme in combination with PEP synthetase. As in *C. glutamicum* (Sauer and Eikmanns 2005), PEP carboxykinase (*ro05180*) may be the only enzyme responsible for PEP synthesis from TCA cycle intermediates in strain RHA1, since the gene coding a PEP synthetase is lacking in its genome. The main role of malic enzyme in RHA1 may be the generation of NADPH on those substrates which do not flux through the PP pathway (Fig. 1).

Under glycolytic conditions, the final products of glycolysis, PEP and pyruvate through the pyruvate kinase and pyruvate dehydrogenase complex, feed acetyl-CoA into the TCA cycle (Fig. 1 and Table 1). Anaplerotic reactions (C3-carboxylation) must replenish TCA cycle intermediates that were bled off for anabolic processes. This function is accomplished in most bacteria by PEP carboxylase and/or pyruvate carboxylase, which convert PEP and pyruvate, respectively, to oxalacetate (Sauer and Eikmanns 2005). *R. jostii* RHA1 seems to possess only a PEP carboxylase in its genome as anaplerotic enzyme (Fig. 1 and Table 1). It is known that pyruvate carboxylase plays a major anaplerotic role in mammals and in yeast, and that only few prokaryotes, such as *Rhodopseudomonas spheroids*, *Arthrobacter globiformis*, or *Mycobacterium smegmatis*, possess this enzyme; whereas many bacteria, such as *Pseudomonas citronellolis*, *Azotobacter vinelandii*, or *C. glutamicum*, use both PEP carboxylase and pyruvate carboxylase as anaplerotic enzymes (Sauer and Eikmanns 2005).

As in *C. glutamicum* genome (Sauer and Eikmanns 2005), the putative PEP carboxylase gene (*ppc*) of strain RHA1 is organized in a glycolytic gene cluster together with the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gap*2), phosphoglycerate kinase (*pgk*), and triose-phosphate isomerase (*tpi*A).

Recently, Feisthauer et al. (2008) reported the dependence of externally provided CO_2 for growth in *R. opacus* 1CP in comparison with *Pseudomonas knackmussii* B13, which was able to grow in the absence of external CO_2 under similar culture conditions. Using $^{13}CO_2$, the authors demonstrated that during growth on glucose, *R. opacus* 1CP showed lower C yield than *P. knackmussii* B13. In addition, fatty acids (principally the odd-numbered fraction) and the amino acids (principally the aspartate family) contained in *R. opacus* 1CP were highly enriched in ^{13}C than those in strain B13 (Feisthauer et al. 2008). The authors concluded that the *Rhodococcus* strain used in that study possesses an essential dependence on heterotrophic CO_2 fixation by anaplerotic reactions. The odd-numbered fatty acids are usually produced by *Rhodococcus* species using propionyl-CoA as precursor for biosynthesis, as has been reported previously (Alvarez et al. 1997). Propionyl-CoA is produced through the methyl malonyl-CoA pathway using TCA cycle intermediates as precursors (Fig. 1). These intermediates can be formed by direct carboxylation of PEP or pyruvate through anaplerotic reactions, as has been reported for *R. ruber* (Anderson et al. 1995), *R. opacus* PD630 (Alvarez et al. 1997) and *R. opacus* 1CP (Feisthauer et al. 2008). The use of TCA cycle intermediates for the biosynthesis

of odd-numbered fatty acids, which may account up to 20–30% of the total fatty acids in many *Rhodococcus* strains, is probably in detriment of the energy gain by cells and may explain the lower C yields of *Rhodococcus* in comparison with other Gram-negatives (Feisthauer et al. 2008). Altogether, these results emphasize the role of the PEP carboxylase enzyme in the rhodococci metabolism, which seems to be the unique anaplerotic enzyme occurring in *R. jostii* RHA1 genome; a very close species to *R. opacus*. The occurrence of additional anaplerotic enzymes (C3 carboxylation) in *R. jostii* and *R. opacus* genomes should be investigated in the future.

Pyruvate kinase and the pyruvate dehydrogenase complex, which are responsible for the production of acetyl-CoA from PEP, are also implicated in the PEP–pyruvate–oxalacetate node (Fig. 1 and Table 1). The acetyl-CoA fuels the TCA cycle to produce energy and anabolic precursors. The pyruvate:quinine oxidoreductase enzyme, which is present in *C. glutamicum*, seems not to occur in RHA1 genome; thus, the bypass of the pyruvate dehydrogenase reaction via pyruvate: quinine oxidoreductase, in combination with AskA and Pta enzymes, may not be functional in *R. jostii*. However, the *askA* and *pta* genes encoding a putative acetate kinase and a phosphotransacetylase, respectively, are present in the RHA1 genome. When acetyl-CoA is in excess and the C flux exceeds the amphibolic capability of the central metabolism, acetyl-CoA can be converted to acetate and ATP by the AK-PTA pathway (Yasuda et al. 2007). The acetyl-CoA synthetase enzymes can re-activate acetate to acetyl-CoA when the metabolic situation changes (Fig. 1). The occurrence of the AK-PTA pathway in *R. jostii* RHA1 and probably in other members of the genus may contribute to the control of C fluxes and the maintenance of the intracellular acetyl-CoA pools during fluctuating nutritional conditions, as found in the environment. In this context, *askA* and *pta* genes were down-regulated by strain RHA1 during cultivation of cells under nitrogen-limiting conditions, which promote a high flux of acetyl-CoA toward the fatty acid biosynthesis pathway for the accumulation of triacylglycerols (Alvarez, Miyazawa, Topp, Mohn, unpublished results). The *askA* and *pta* genes are also present in *R. opacus* B4 and *R. erythropolis* PR4 genomes.

In general, the demand of acetyl-CoA in rhodococci is probably high, considering the high content of different lipid species found in their cellular structures, such as the cell envelope and the storage lipids as triacylglycerols. As *Mycobacterium tuberculosis*, the genome of *R. jostii* RHA1 possesses a multiplicity of genes involved in lipid metabolism (Cole et al. 1998; Hernández et al. 2008). The synthesis of acetyl-CoA from PEP via pyruvate kinase and pyruvate dehydrogenase complex may be the main source for acetyl-CoA production in rhodococci. However, other reactions could contribute to the intracellular acetyl-CoA pool, such as the reaction catalyzed by citrate lyase enzyme, which converts citric acid into acetyl-CoA and oxalacetate, and the eventual activation of acetate to acetyl-CoA by an acetyl-CoA synthetase. The significant induction of a gene coding for a putative citrate lyase enzyme by cells of strain RHA1 has been observed during their cultivation under nitrogen starvation conditions (Alvarez, Miyazawa, Topp, Mohn, unpublished results).

5 The Tricarboxylic Acid Cycle

In aerobic bacteria like *Rhodococcus*, the TCA cycle in the oxidative direction oxidizes acetate to CO_2 and provides reducing power (NADH and $FADH_2$) for energy production and metabolic intermediates for use in biosynthesis pathways. *R. jostii* RHA1 seems to be able to drive the entire TCA cycle oxidatively, since it possesses all necessary genes/enzymes (Table 1 and Fig. 1). The reducing power generated under aerobic conditions via the TCA cycle, in addition to the glycolysis or β–oxidation pathway, could be reoxidized through the respiratory electron-transport chain. As mentioned above, the TCA cycle in the *Rhodococcus* members serves as a source for the production of propionyl-CoA, via methyl malonyl-CoA pathway, which is used for the biosynthesis of odd-numbered fatty acids (Anderson et al. 1995; Alvarez et al. 1997; Feisthauer et al. 2008) (Fig. 1).

Interestingly, genomic data revealed the presence of the key enzymes of the reductive TCA cycle in the RHA1 genome, including genes encoding citrate lyase and 2-oxoglutarate synthase, in addition to the rest of the TCA cycle enzymes, which may act reversible (Fig. 1 and Table 1). The occurrence of those enzymes in the RHA1 genome suggests that this strain should be able to drive the entire reductive TCA cycle. Citrate lyase converts citric acid into acetyl-CoA and oxalacetate; whereas the 2-oxoglutarate synthase produces 2-oxoglutarate from succinyl-CoA. This permits the metabolism to incorporate CO_2 and to serve as an engine for synthesis instead of one of energy production (Srinivasan and Morowitz 2006). The switch from oxidative to reductive TCA cycle may both facilitate carbon fixation and restore the balance of oxidative and reductive reactions during environmental fluctuations (Srinivasan and Morowitz 2006). The ability to drive the TCA cycle in both directions, either oxidative or reductive, has also been reported for other actinomycetes, such as *M. tuberculosis* and *Streptomyces coelicolor* (Srinivasan and Morowitz 2006). In addition, the genes coding for key enzymes of the reductive TCA cycle also seems to be present in *R. opacus* B4 and *R. erythropolis* PR4 genomes. A partial or fully functional oxidative or reductive TCA cycle may be used by these microorganisms to balance metabolism and to adapt to diverse environments.

6 The Glyoxylate Pathway

Strain RHA1 possesses both enzymes involved in the glyoxylate pathway; isocitrate lyase and malate synthase (Table 1). This pathway serves as a mechanism for replenishing oxalacetate during growth on acetate and fatty acids (Fig. 1). The glyoxylate pathway is relevant not only for *R. jostii* RHA1 but also for other triacylglycerol-accumulating *Rhodococcus* strains, such as *R. opacus* PD630, because it links the gluconeogenesis with the oxidation of acetyl-residues obtained by the β-oxidation of fatty acids during mobilization of the stored triacylglycerols.

The members of the genus *Rhodococcus* are able to accumulate intracellularly variable amounts of triacylglycerols, which are degraded during carbon starvation (Alvarez et al. 2000; Alvarez 2006). The up-regulation of isocitrate lyase (*aceA*) and malate synthase (*glcB*) by cells of strain RHA1 suggested the activation of the glyoxylate shunt under C-starvation, which correlated with the use of intracellular fatty acids as carbon and energy source (Alvarez, Miyazawa, Topp, Mohn, unpublished results). The synthesis of triacylglycerols seems to be an important metabolic pathway in rhodococci for the maintenance of energy homeostasis. Triacylglycerols are synthesized by these bacteria when a carbon source is available, and then degraded to provide carbon and energy during C starvation via the successive operation of β-oxidation, the glyoxylate cycle, the partial TCA, and gluconeogenesis. Thus, the glyoxylate cycle may be very active in rhodococci due to their dependence on the degradation of stored triacylglycerols under nutritional fluctuating conditions. In this context, the glycoxylate cycle plays a pivotal role in the persistence of *M. tuberculosis* in mice by sustaining intracellular infection in inflammatory macrophages (Sharma et al. 2000; Singh and Ghosh 2006). This pathway enables mycobacteria to utilize carbon sources when TCA cycle is shut down during O_2 and nutrient limitation (Boshoff and Barry 2005; Tang et al. 2009).

7 Litoautotrophic Processes in *Rhodococcus*

Rhodococcus bacteria are considered chemoheterotrophic microorganisms, which use organic compounds as sources of carbon and energy. However, there is evidence from literature and genomic data examination that the members of this genus may be rather facultative chemolitoautotrophs. Lithotrophy is the use of an inorganic compound as a source of energy. Some aerobic bacteria are able to remove electrons from a substrate and put them through an electron transport system that will produce ATP by electron transport phosphorylation. When lithotrophs take their carbon from CO_2, they are autotrophs. In this context, Grzeszik et al. (1997) reported the capability of *R. opacus* strain MR11, which is very close to strain RHA1, for growing on CO_2 and gaseous H_2 as the sole carbon and energy sources. Aragno and Schlegel (1992) previously identified a soluble hydrogenase system in strain MR11, which was localized in the cytoplasm and catalyzed the transfer of electrons directly to NAD. The examination of genomic data revealed that *R. jostii* RHA1 possesses a gene cluster (*ro04601–04*) encoding a putative hydrogenase system, which was highly induced by cells during C- and N-starvation (Alvarez, Miyazawa, Topp, Mohn, unpublished results). Hydrogenase genes also occur in the genome of *R. opacus* B4 and *R. erythropolis* PR4. Altogether, these results indicated that the *Rhodococcus* members are not only able to use a wide range of organic compounds as carbon and energy sources, but also to oxidize H_2 as an additional energy source. Moreover, analysis of the genome database of *R. jostii* RHA1 revealed the occurrence of additional litoautotrophic pathways, including genes coding for putative carbon monoxide dehydrogenases (CODH) and a

thiocyanate hydrolase enzyme. Thiocyanate hydrolase, which usually occurs in obligate as well as in facultative chemolithotrophs, is a cobalt(III)-containing enzyme catalyzing the degradation of thiocyanate to carbonyl sulfide and ammonia (Yamasaki et al. 2002). The genes coding for the three subunits of the enzyme (α, β, and γ) are localized into an operon (*ro04428–30*) in the RHA1 genome. The homologous of these RHA1 genes can be identified in *R. opacus* B4 genome with the same locus organization. Curiously, thiocyanate hydrolase genes are missing in *R. erythropolis* PR4 genome. On the other hand, the genomes of *R. jostii* RHA1 and *R. opacus* B4 contains three clusters including genes coding for putative CO dehydrogenases. Bioinformatics analyses have identified only a single CO dehydrogenase cluster encoded in the *R. erythropolis* PR4 genome. Altogether, comparative genomic of the *Rhodococcus* members suggests that litoautotrophic pathways could be a species-dependent feature. CODH as well as hydrogenase and thiocyanate hydrolase systems may avoid the release of carbon as CO_2 by rhodococcal metabolism and may serve as auxiliary mechanisms for energy metabolism during nutritional starvation conditions.

8 Concluding Remarks

Rhodococcus bacteria are endowed with a robust, flexible, and versatile central metabolism, which is necessary for metabolically adapting to environments with fluctuating nutritional conditions. The central metabolism must provide all necessary intermediates for the biosynthesis of a wide diversity of molecules and complex macromolecules for assembling cellular structures like the cellular envelope or secondary metabolites. These bacteria exert a very efficient management of their nutritional resources, based on the flexibility of their metabolism and the diversity of metabolic reactions. The partitioning of carbon through the central metabolism of the substrates to either energy production or synthesis of compounds depends on the metabolic demands of the cells. Rhodococci seem to posses the ability to conserve useful energy during catabolism, distributing the carbon flux of the substrate between the energy production, the biosynthesis of essential molecules, reserving a part of the carbon into diverse storage compounds, such as triacylglycerols, polyhydroxyalkanoates, and glycogen. Thus, cells may resign energetic productivity and growth yield, which may be compensated to some extent with additional processes providing energy and carbon intermediates from inorganic compounds, such as H_2, CO_2, or thiocyanate, among other possible. This feature may be environmentally favorable for growth and survival in environments with low energy fluxes, such as in soil or marine sediments. The flexibility of the central metabolism is other essential feature of rhodococci. These microorganisms seem to have the potential to use alternatively different glycolytic pathways, such as EMP or ED pathways, the PP pathway, and a partial or fully functional TCA in both oxidative or reductive directions according to the conditions. Moreover, the central metabolism of rhodococci possesses some mechanisms that probably permit cells to

respond rapidly to changes in nutritional state and to balance metabolism. One of them may be the glycogen biosynthesis, which may deal with the sugar excess during exponential growth phase, and probably serve as a pool of sugars for using when necessary. Other point may be the AK-PTA pathway, which may contribute to the control of C fluxes and the maintenance of the intracellular acetyl-CoA pools during fluctuating conditions. The management of acetyl-CoA pool may be a key point for rhodococci metabolism, since they usually synthesize many different lipid species, which perform important function in their interactions with the environment. In this context, the PEP–pyruvate–oxalacetate node may play a key role in the C flux distribution within the overall cell metabolism.

The biochemistry and the molecular biology of the central metabolism of the *Rhodococcus* genus have not been studied in depth. More studies involving the regulation of metabolic genes and enzymes, the kinetic characterization of enzymes, or the analysis of carbon fluxes through the metabolism should help to define a clearer picture of the functionality and regulation of the central metabolism within the cellular context in rhodococci.

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Adaptation of *Rhodococcus* to Organic Solvents

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Contents

Abstract Bacterial tolerance and ability to adapt to organic solvents can be of valuable importance in biocatalytic and bioremediation processes. Strains of *Rhodococcus* have been reported to be particularly solvent tolerant, while presenting a broad array of enzymes with potential for the production of commercially interesting compounds and/or for the metabolism of recalcitrant organic solvents. The adaptability and versatility of *Rhodococcus* cells can further broaden their application scope. In fact, these cells can adapt the cell wall and membrane compositions, as well as the physicochemical properties of the cell surface, can degrade or bioconvert toxic compounds such as benzene and toluene, and can aggregate and produce exopolymeric substances to protect the cell population from stressful environments.

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1 Introduction

Bacterial strains tolerant or adapted to organic solvents are of interest in both biocatalysis and bioremediation processes. These cells may work in the presence of organic solvents effective in overcoming the solubility of hydrophobic substrates and/or products in biocatalytic processes (de Carvalho and da Fonseca 2002a; Daugulis 2001; Heipieper et al. 2007), or biodegrade organic solvents released in the environment (Aislabie et al. 2006; de Carvalho and da Fonseca 2007; Leisinge 1996; Parales and Haddock 2004).

The first report of a bacterium tolerant to organic solvents was by Inoue and Horikoshi (1989), who discovered a *Pseudomonas putida* strain, IH-2000, able to tolerate and grow in the presence of 50% (v/v) toluene. This strain was, nonetheless, unable to metabolize toluene as sole carbon source. Since then, several Gram-positive strains belonging to the genus *Bacillus*, *Arthrobacter*, and *Rhodococcus* have shown high tolerance to organic solvents including benzene, which is much more toxic than toluene. *Rhodococcus* strains have been reported as efficient catalysts in the presence of organic solvents and also as degraders of these compounds because of their ability to metabolize a wide range of organic compounds under a wide set of conditions (de Carvalho and da Fonseca 2005a; Larkin et al. 2006). *Rhodococcus* cells are ubiquitous and are able to thrive in stressful environments, having been found in Antarctica (Bej et al. 2000; Pini et al. 2007), in the Artic (Thomassin-Lacroix et al. 2001; Whyte et al. 2002), at sea level (Bell et al. 1998), in the deep sea (Colquhoun et al. 1998), at high altitude (Margesin et al. 2003), and in semiarid soils (Pucci et al. 2000). The high hydrophobicity of *Rhodococcus* cells and the production of surface active compounds enhance their biocatalytic/degradation abilities.

1.1 Predicting Solvent Toxicity

The use of organic solvents in biocatalysis and bioremediation processes is limited as organic solvents can be extremely toxic to bacterial cells even at low concentrations. The toxicity of water-immiscible solvents may result from its direct contact to the cells (phase toxicity) and/or from the solvent molecules dissolved in the aqueous phase (molecular toxicity). These compounds partition to cell membranes, increasing its fluidity and disrupting the lipid bilayer, and ultimately cause cell death (Heipieper et al. 1991, 1994; Sikkema et al. 1995). In fact, organic solvents such as alcohols and phenols contained within natural oils and balsams have been widely used as antimicrobial agents since antiquity, long before their mode of action and effect was known.

Several authors have tried to present a way to predict solvent toxicity based on its physicochemical properties. In 1985, Brink and Tramper proposed that the suitability of a solvent to be used in a multiphasic biocatalytic system could be predicted by evaluating the solvent polarity and the molecular size of the solvent. The first could be determined by the Hildebrand solubility parameter, δ, while the

latter could be expressed as molecular weight or molar volume. In this system, high biocatalytic rates should be obtained using organic solvents with low polarity ($\delta < \sim 8$) and high molecular weight ($M > \sim 150$).

The antimicrobial action of a solvent was found later to better correlate to its hydrophobicity, measured as the logarithm of the octanol–water partition coefficient, log $P_{O/W}$, (Laane et al. 1987; Osborne et al. 1990; Sikkema et al. 1994). According to this scale, enzymes and microorganisms present a minimum of activity with solvents with log P values of 0–2 and 2–4, respectively, after which the use of solvents with increasing log P values will result in increased biocatalyst stability. However, the actual concentration of the solvent in the bacterial cell membrane will depend both on the solvent concentration in the water phase and on the partitioning of the solvent from the water phase to the membrane. In 1994, Sikkema and coworkers proposed the following equation to correlate the log $P_{O/W}$ value of a solvent and its partitioning value between the membrane and water, log $P_{M/W}$:

$$\log P_{M/W} = 0.97 \times \log P_{O/W} - 0.64. \quad (1)$$

Hydrophobic solvents, with log $P_{O/W} > 4$, accumulate in the membrane but will not reach a high membrane concentration and are not toxic because of their low water solubility. On the contrary, solvents with log $P_{O/W}$ between 1 and 4 present higher water solubility values, while being also able to partition to biological membranes, resulting in relatively high concentrations of these solvents in the membranes and high toxicity to the cells (de Bont 1998). The fact that solvents with high partition coefficient to the membrane, such as *n*-dodecane and *n*-hexadecane, are not toxic to bacterial cells is, apparently, contradictory. However, a "cut-off" in toxic effect around log P 4–5 was observed for microorganisms (Laane et al. 1985; Vermuë et al. 1993), above which the solvents do not present toxicity. The reasons presented involve low solubility of the solvents in the membrane bilayer or absence of a membrane disturbing effect, with significant responses being observed with different organisms and solvents (Sikkema et al. 1994). As pointed out by de Bont (1998), calculating the actual solvent concentration in membranes could be helpful. However, the author notes that several parameters that can also affect the partitioning of solvents, such as the composition of the biological membrane, are disregarded. Furthermore, many of the potentially interesting substrates are not very soluble in apolar solvents (those with higher log P values) and the reaction rates in favorable solvents are often low (Cassells and Halling 1990). Nevertheless, the log P values could be used as an indication of the biocatalyst behavior, even in processes requiring cofactor regeneration by viable cells (Fig. 1).

Recently, Hamada et al. (2008) compared several methods to predict bacterial predilection for organic solvents, namely: bacterial adhesion to hydrocarbon (BATH), contact angle measurement (CAM), hydrophobic interaction chromatography (HIC), and glass adhesion test (GAT). They concluded that CAM could be used to predict the dispersibility of bacteria in anhydrous organic solvents, while the BATH assays were better to predict the behavior of bacterial cells in organic–aqueous two phase systems. The differences in the obtained results using

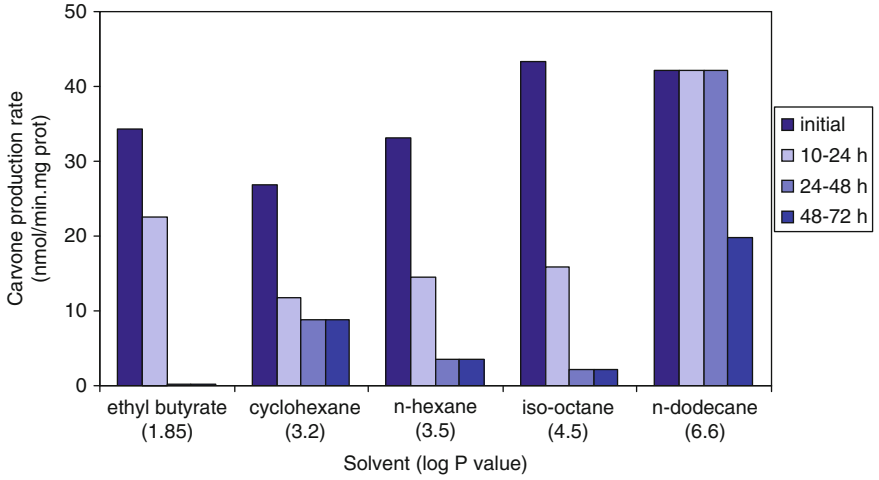

Fig. 1 Effect of the log *P* value of the solvent in *R. erythropolis* DCL14 cells carrying out the biotransformation of carveol into carvone in an organic-aqueous system (adapted from de Carvalho and da Fonseca 2002)

the two techniques were ascribed to the effect of electrostatic interactions between bacteria and oil droplets.

1.2 Effect of Solvents on Bacterial Cells

In a review on the cellular toxicity of lipophilic compounds, Sikkema et al. (1994) discussed the results published until then showing that the accumulation of lipophilic compounds occurs at various depths in the membrane bilayer, depending on the presence of hydroxyl, carboxyl, or phenyl groups. Hydrophobic cyclic hydrocarbons should accumulate in the acyl region of the membrane, while hydrophobic compounds should interact with hydrophobic end of acyl chains and more hydrophilic compounds should affect the hydration of the head groups of the membrane lipids. Small lipophilic compounds should intercalate with the acyl chains, resulting in membrane swelling and in an increased fluidity of the membrane. Since larger hydrophobic molecules affect both the inner and outer leaflet of the lipid bilayer, an increased ordering of the membrane lipids reduces the membrane permeability and fluidity but increases the bilayer width.

The cellular membrane of bacteria acts as a permeability barrier to solutes, regulating the intracellular environment, but is also responsible for the maintenance of the energy status of the cell, turgor pressure, signal transduction, and other energy-transduction processes (Sikkema et al. 1995). When solvents disrupt the membrane, loss of ions, metabolites, lipids and proteins, and impairment of the proton motive force across the membrane may occur, which stops the pH gradient

and the electrical potential and inhibits the function of membrane proteins. The ability of the microorganisms to maintain their biological functions under stressful conditions, such as the presence of organic solvents, results from changes in protein, sterol, hopanoid, and carotenoid content, and mainly changes in membrane lipid composition (Heipieper et al. 1994; Weber and de Bont 1996). The cells try to modify the fatty acid composition of the cellular membrane to maintain the membrane fluidity. The membrane fluidity is kept constant by changes in the degree of saturation of the fatty acids of the membrane phospholipids through a mechanism called "homeoviscous adaptation" (Sinensky 1974). Gram-negative bacteria, such as those of the genera *Pseudomonas* and *Vibrio*, isomerize unsaturated fatty acids from conformation *cis* to *trans* (Heipieper et al. 1992; Weber et al. 1994), which seems to be a special mechanism of adaptation to high concentrations of toxic compounds when de novo synthesis of lipids is not possible (Diefenbach et al. 1992). The studies carried out suggested that *cis* to *trans* conversion increases membrane ordering, decreasing the membrane fluidity. The level of isomerization has been found to correlate with the concentration (Diefenbach et al. 1992; Heipieper et al. 1992) and hydrophobicity (Heipieper et al. 2003) of the toxic compounds. Changes in the saturated–unsaturated and in the long chain–short chain fatty acid ratios can be seen as long-term changes to regulate membrane fluidity as synthesis of fatty acids is required. Other mechanisms involved in the response of Gram-negative bacteria to organic solvents include changes in the phospholipids headgroups, in the outer membrane proteins and lipopolysaccharides, and the action of efflux pumps. These mechanisms have been reviewed by Segura et al. (1999). Several other reviews have been published on solvent-tolerant bacteria (de Bont 1998; Isken and de Bont 1998; Ramos et al. 2002; Sardessai and Bhosle 2002; Sikkema et al. 1994). Although these works report solvent-tolerant *Rhodococcus* and other Gram-positive strains, the authors agree that without an outer membrane, Gram-positive bacteria should be less tolerant to organic solvents and an in-depth study on the mechanisms of solvent tolerance in these bacteria is still missing. The permeable cell wall of Gram-positive bacteria does not usually restrict the penetration of antimicrobial agents, but vancomycin-intermediate resistant *Staphylococcus aureus* strains are resistant due to a significantly thickened cell wall (Lambert 2002). Bacteria containing mycolic acids, such as mycobacteria and *Rhodococcus*, have cell walls with a high lipid content, which may act as a barrier to both hydrophobic and hydrophilic antimicrobials (Brennan and Nikaido 1995). In this chapter, the resistance and the mechanisms conferring adaptation capabilities to *Rhodococcus* strains will be presented and discussed.

2 Intrinsic Resistance to Organic Solvents

Some microorganisms have the ability to resist or tolerate concentrations of a certain compound that would kill or stress others. Intrinsic resistance is defined as an innate genetically controlled property of a bacterial cell that enables it to elude

the action of a biocide (Russell 1995). Resistance genes may reside on the chromosome, on a plasmid, or on a transposon. This natural resistance is the result of penetration barriers, metabolic pathways, or effective efflux pumps, while the acquired resistance results from an increased tolerance gained through a genetic change by which an organism and its progeny will be able to remain viable and/or multiply under the stress conditions. As long as the basal level of the repair systems is not surpassed by the stress damages, the cells will be able to survive.

Gram-negative bacteria are generally considered less susceptible to biocides than Gram-positive bacteria because their outer membrane acts as a permeability barrier. However, mycobacteria can present a high intrinsic tolerance as their cell is highly hydrophobic due to the mycoylarabinogalactan–peptidoglycan skeleton (McDonnell and Russell 1999). In the case of *staphylococci*, the cell wall is mainly composed of peptidoglycan and teichoic acid, which are not effective as penetration barriers. However, the physiological state of the cells can influence the susceptibility of the cells to biocides as the growth rate or growth under limiting nutrient conditions may change the thickness and degree of cross-linking of the peptidoglycan (Gilbert and Brown 1995). Slime producing *S. aureus* strains also present a higher resistance to antimicrobial agents than nonmucoid strains, indicating that exopolymeric substances may act as physical barrier or as absorbent of biocide molecules (McDonnell and Russell 1999). When the physical barriers allow the passage of the toxic compound, intrinsic resistance is given by efflux pumps that may extrude different types of compounds (Piddock 2006; Poole 2008).

In a study comparing the resistance of Gram-negative *P. putida*, and the gram-positive *Mycobacterium* sp. and *R. erythropolis* cells to various water miscible and immiscible solvents, it was found that of the three strains only *R. erythropolis* was able to endure high concentrations of water miscible solvents (de Carvalho et al. 2004). This result showed that *R. erythropolis* was able to maintain viability at higher solvent concentrations than *P. putida*, which should be more tolerant to organic solvents than Gram-positive bacteria because of the protective effect of the outer membrane (Isken and de Bont 1998). Fang et al. (2007) also found the Gram-positive *Bacillus subtilis* to be more tolerant than *P. putida* toward fullerene-based manufactured nanomaterials. *Bacillus*, *Rhodococcus*, and *Arthrobacter* have been found to be organic-solvent-tolerant even to benzene, one of the most toxic organic compounds (Sardessai and Bhosle 2002). The dominance of BTEX degrading Gram-positive bacteria in nearshore surface water and in sediments from the Pacific Ocean also contradicts the general idea that Gram-negative bacteria are the main group of organic pollutant degraders (Wang et al. 2008). Apparently, the more extensively linked peptidoglycan in Gram-positive cells (Sikkema et al. 1995) and the enzymes/substances excreted by these cells to emulsify/solubilize the organic solvents could play an important role in decreasing the solvent toxicity toward them (Abe et al. 1995).

Rhodococcus strains have been found to endure considerably high concentrations of known toxic compounds, for example, phenol, benzene, and toluene (Table 1). The work of Gutiérrez et al. (2003) with *Rhodococcus* sp. showed that

Table 1 Intrinsic resistance of several *Rhodococcus* strains to organic solvents

Strain	Compound	Tolerance	References
R. phenolicus G2P^T	Phenol	0.75%[a]	Rehfuss and Urban (2005)
R. opacus B-4, B-9, B-10	Benzene	90%[b]	Na et al. (2005)
R. opacus B-4	*n*-Tetradecane	100%	Hamada et al. (2008)
Rhodococcus sp. CN6,	*p*-Nitrophenol	100 mg/L[a]	Zhang et al. (2009)
R. rhodochrous S-2	*n*-Hexadecane	10%	Iwabuchi et al. (2000)
R. sp. NO14-1	*n*-Hexadecane	34%[c]	Margesin et al. (2005)
	Diesel oil	27%[c]	
	Phenol	12.5 mM[c]	
Rhodococcus sp. NO20-3	*n*-Hexadecane	54%[c]	Margesin et al. (2005)
	Diesel oil	37%[c]	
	Phenol	12.5 mM[c]	
Rhodococcus sp.	Benzene	200 mg/L	Gutiérrez et al. (2003)
R. equi 85F	Hydrogen peroxide	150 mM	Benoit et al. (2002)
R. erythropolis UPV-1	Phenol	1,000 mg/L	Prieto et al. (2002)
	Ethanol	40%	de Carvalho et al. (2004)
	Butanol	20%	
	Dimethylformamide	50%	
	Dodecane	5%	
	bis(2-Ethylhexyl) phthalate	5%	
	Toluene	1%[b]	
	Toluene	20%[d]	de Carvalho et al. (2007)
R. erythropolis DCL14	Methanol	15%[a]	de Carvalho et al. (2005)
	Ethanol	20%[a]	
	Butanol	2%[a]	
	Cyclohexanol	1%[a]	
	Dodecanol	5%[a]	
	Iso-octane	99.99%	de Carvalho et al. (2000)
	C5–C16 *n*-alkanes	0.25%[a]	de Carvalho and da Fonseca (2005b)
	Motor oils	2%[a]	
	Fuel oil	1.6%[a]	

[a]Organic solvent used as sole carbon source
[b]Growth observed
[c]At 10°C
[d]After 1 h incubation

although benzene caused an increase in membrane fluidity after 0.5 h, the cells did not change the fluidity of the membrane in the 6 h of the assay, during which the cells were alive. The authors ascribed this to a well-adapted inherent mechanism allowing the cells to survive benzene and other solvent "shocks" in the environment. Recently, Gutiérrez et al. (2009) studied the constitutive processes that confer resistance to benzene. Nonadapted *Rhodococcus* sp. 33 cells were able to endure shock concentrations of up to 1,000 mg/L of presolubilized benzene, a concentration usually lethal to most microorganisms. The production of an extracellular polymer and the composition of the cell wall and cell plasma membrane may be responsible for this high tolerance. In some cases, tolerance was related to the

capacity of the cells to degrade the toxic molecule and to use it as sole carbon and energy source (de Carvalho et al. 2005; de Carvalho and da Fonseca 2007). In the case of *R. rhodochrous*, the extracellular polysaccharide produced by the mucoidal strains was responsible for the tolerance of the cells to *n*-hexadecane (Iwabuchi et al. 2000).

In 1942, Withell observed an exponential relation between the duration of a stress episode and bacterial death, which could be explained by cells with different tolerance within the population. According to Booth (2002), survival to a stress agent largely occurs at the level of a single cell (as seen by the ability of a single cell to form a colony on an agar plate). After exposure to stressors that kill the majority of the cells, the heterogeneity of a population determines its survival as a small fraction of the cells may possess the necessary tools to endure the stress. The results obtained by de Carvalho et al. (2007) indicated that cells that are able to remain viable after the first seconds of exposure to high toluene concentrations will also be able to endure this toxic for longer periods. Two explanations could justify this result (1) the existence of toluene tolerant persistent cells within the population and (2) the tolerance is induced by cell exposure to a toxic, and until tolerance is acquired, cell death occurs at a toluene-concentration dependent rate.

3 Adaptation Mechanisms to Organic Solvents

Several reviews have been published on the tolerance of bacterial cells to organic solvents where the mechanisms of cell adaptation are discussed (de Bont 1998; Chapman 2003; Isken and de Bont 1998; Sardessai and Bhosle 2002; Segura et al. 1999; Sikkema et al. 1994). Among the most important mechanisms described are (1) changes in the cell membrane to modulate its fluidity, (2) the metabolism of the toxic compound or its inactivation, (3) increased efflux of the toxic compound. Most of the papers published on this subject dealt with Gram-negative bacteria. Although the interest in using Gram-positive bacteria in biocatalysis and bioremediation processes is increasing, studies on the adaptation of nonpathogenic strains to nonantibiotic compounds are still scarce.

One basic idea observed in adaptation studies is that cells growing at a slow rate acquire general tolerance to the stress agent (Booth 2002 ; Sonnleitner 1998). Furthermore, by growing at slower growth rates, separate but overlapping pathways that confer tolerance of diverse stresses are induced, and the cells become simultaneously resistant to, for example, acid, heat, alkali, and hydrogen peroxide (Booth 2002). This mechanism of tolerance acquisition is particularly important in biofilms. The slow growth observed in the matrix-embedded cells and the limited transport of nutrients, metabolites, and oxygen between the surface and the interior of the biofilm could be responsible for an increased antibiotic and disinfectant resistance of biofilm cells over planktonic cells (Stewart and Costerton 2001; Donlan and Costerton 2002).

3.1 Adaptation of the Cell Wall and of the Cellular Membrane

Several papers have reported that when exposed to toxic organic compounds, tolerant bacterial strains change the fatty acid profile of their membrane (e.g., Heipieper et al. 1994; Isken and de Bont 1998; Sardessai and Bhosle 2002; Sikkema et al. 1995). The existence of an outer membrane allow Gram-negative bacteria to quickly modify and adapt the lipopolyssacharides, efflux pumps, and the fatty acid composition of the cellular membrane (Ramos et al. 2002). Among the modifications described are *cis*/*trans* isomerization of fatty acids, changes in the saturated/unsaturated fatty acid ratio, and changes in the phospholipids headgroups (de Bont 1998; Sardessai and Bhosle 2002; Segura et al. 1999). The *cis*/*trans* isomerization, which results in a lower penetration of the solvents through the inner membrane (Cronan 2002), is a short-term response, which takes place within 1 min after solvent exposure, while the remaining changes are long-term responses (Junker and Ramos 1999). The mechanisms involved in the adaptation of Gram-positive strains are not fully known (Fang et al. 2007; Nielsen et al. 2005), although it has been suggested that the mechanisms should be similar to those presented by Gram-negative bacteria (Ramos et al. 2002). In this case, the mycolic acids of *rhodococci* will act as a permeability barrier for hydrophilic compounds, and water-filled channels are required for their entrance in the cell. Lichtinger et al. (2000) identified and purified a channel-forming protein in *R. erythropolis* with a molecular mass of just 8.4 kDa with no significant homology to known protein sequences. The authors suggested that 2.0 nm channels were formed by protein oligomers in the cell wall, being highly cation selective due to negative charges located at the channel mouths.

n-Alkanes droplets have negative zeta potentials, for example, the value for *n*-hexadecane droplets is -46.0 ± -3.4 mV (de Carvalho et al. 2009). The negative zeta potential of *n*-alkane droplets is the result of selective adsorption of OH^- ions, which causes gathering of the excessive negative charge at the oil–water interface (Stachurski and Michalek 1996). Since *n*-alkanes and the channels at the cell wall have both localized negative charges, the entrance of these compounds could be prevented in the cells. However, several papers published showed that *Rhodococcus* cells are able to adhere directly to organic layers in organic–aqueous two-phase systems (de Carvalho and da Fonseca 2002b, 2003, 2007). Bouchez-Naïtali et al. (2001) also observed a direct uptake of *n*-hexadecane by four *R. equi* strains, which did not produce biosurfactants.

When comparing the effect of low and higher doses of buckminsterfullerene (*n*-C60) on *Bacillus subtilis* with the effect of hyperosmotic conditions, Fang et al. (2007) concluded that Gram-negative bacteria can present different responses to the same type of membrane-active compounds under different conditions, whereas Gram-positive bacteria can have the same response in terms of fatty acid composition under different stress conditions. *Rhodococcus* strains responded to the presence of organic solvents by changing the degree of saturation of the fatty acids of the cellular membrane, by changing the length of the fatty acids and mycolic acids according to the chain length of the carbon source, and by altering the percentage of

Table 2 Mechanisms of solvent tolerance observed at the cellular membrane level in *Rhodococcus* strains

Strain	Compound	Reported mechanism	References
Rhodococcus sp. 33	Benzene	Increased degree of saturation	Gutierrez et al. (1999)
Rhodococcus opacus GM-14, GM-29, and 1CP	Benzene, phenol, 4-chlorophenol, chlorobenzene, or toluene	Increase content of branched (10-methyl) fatty acids	Tsiko et al. (1999)
R. erythropolis DCL14	Short-chain alcohols (methanol, ethanol)	Decrease of degree of saturation	de Carvalho et al. (2005)
	n-alkanes and *n*-alkanols	Increase in degree of saturation	
R. erythropolis DCL14	C5–C16 *n*-alkanes	Saturated fatty acids with chain length corresponding to the substrate used; Net surface charge increased with chain length	de Carvalho et al. (2009)
R. erythropolis DCL14	Carveol and carvone	Lower percentage of long chain fatty acids; decrease of the unsaturation index	de Carvalho and de Fonseca (2007)
R. erythropolis DCL14	Toluene	C14:0 and C16:0 increased while C18:0 decreased; Increased percentage of iso-branched fatty acids when compared to straight-chain	de Carvalho et al. (2007)
R. erythropolis E1	C2–C7 *n*-alkanoic acid salts	MA profile according to the even–odd nature of the carbon chain of substrate	Sokolovská et al. (2003)
	C9–C15 *n*-alkanes	Changed cell wall permeability	
R. erythropolis 17, *Rhodococcus* sp. 20, *R. opacus*	Pentadecane, hexadecane	Fatty acids related to the chain length of the substrate	Alvarez (2003)
Rhodococcus sp. Q15	Alkanes at low T	Decrease of degree of saturation	Whyte et al. (1999)

branched fatty acids (Table 2). Rodgers et al. (2000), by using ^{13}C-enriched C16 and C18 alkanes and electrospray ionization fourier transform ion cyclotron resonance mass spectrometry, showed that complete mineralization was achieved by *R. rhodochrous*, with complete ^{13}C incorporation in the bacterial lipids. The incorporation of saturated fatty acids in the membrane phospholipids with chain length corresponding to the substrate used for cell growth has been found frequently. Studies carried out by Alvarez (2003) showed that most fatty acids in actinomycetes were related to the chain length of the substrate and also to β-oxidation derived fatty acids. Only when *R. erythropolis* DCL14 cells were grown on *n*-tridecane and

n-pentane, were the fatty acids C13:0 and C15:0 found in the cellular membrane, respectively (de Carvalho et al. 2009). Sokolovská et al. (2003) also observed two types of responses in mycolic acid patterns of *R. erythropolis* E1: lack of odd-numbered carbon chains when the cells grew on linear alkanes with even number of carbon atoms; mycolic acids with both even and odd carbon chains in cells grown on branched alkanes, or on mixtures of substrates. Furthermore, hydrocarbons can also be used in the biosynthesis of tryacylglycerols and wax esters under nitrogen starvation (Alvarez et al. 1996; Voss and Steinbüchel 2001).

Gutiérrez et al. (2003) compared a *Rhodococcus* sp. strain, able to tolerate and degrade high concentrations of benzene, with a benzene-sensitive mutant obtained by mutagenesis. The mutant was unable to increase the saturation degree of the fatty acids to the levels achieved by the wild type when exposed to benzene. The fluidity of the membranes increased after only 0.5 h of exposure to benzene, but the wild type was able to respond by changing the saturation:unsaturation ratio of the cellular membrane, especially by changing the proportion of myristic and oleic acids.

R. erythropolis cells adapted to 20–65% toluene concentrations by increasing the percentage of tetradecanoic and hexadecenoic acids while decreasing the percentage of octadecanoic acid (de Carvalho et al. 2007). The proportion of saturated iso-branched fatty acids also increased during toluene adaptation, while the amount of straight-chain fatty acids decreased. By increasing the content of iso-branched fatty acids, the cells decreased the fluidity or flexibility of the cellular membrane.

The content of branched (10-methyl) fatty acids also increased in *R. opacus* GM-14, GM-29, and 1CP, when the cells were grown on benzene, phenol, 4-chlorophenol, chlorobenzene, or toluene as sole carbon sources, as compared to fructose grown cells (Tsiko et al. 1999). A dose-related increase in the percentage of 10-methyl branched fatty acids was also observed as a response to increasing concentrations of phenol and toluene in strain GM-14, which is unable to metabolize toluene. 10-Methyl branched fatty acids are also present in *Rhodococcus koreensis* DNP505^T, which is able to degrade 2,4-dinitrophenol (Yoon et al. 2000). The role and position of 10-methyl branched fatty acids in *Rhodococcus* is still unclear but the results of Tsiko et al. (1999) suggest the cell envelope lipids that contain 10-methyl branched fatty acids should be involved in the adaptation of *Rhodococcus* strains to compounds affecting the cellular membrane, such as aromatics.

Changes in cell hydrophobicity may be promoted by using the cell response to organic compounds. By exposing *R. erythropolis* cells to the terpenes carveol and carvone in organic–aqueous systems, it was possible to demote biofilm formation and even to disrupt established biofilms (de Carvalho and da Fonseca 2007). Strain DCL14 responded to the presence of the solvents tested by decreasing the unsaturation index, which reflects the average number of double bonds per fatty acid chain, with increasing number of carbons in the alkane chain. In the presence of carveol or carvone, the cells increased the unsaturated index, thus counteracting the effect of the solvents. The presence of polyunsaturated fatty acids has been reported to allow the cells to change membrane fluidity (Melchior 1982) and permeability (Russell

1988). The major response to the presence of these two terpenes was, however, a decrease in the percentage of fatty acids with a number of carbons higher than 16 (de Carvalho and da Fonseca 2007). Since a direct relation between these fatty acids and cell hydrophobicity was found, by decreasing the percentage of fatty acids with more than 16 carbon atoms, cell hydrophobicity decreased, which resulted in the dispersion of cells previously aggregated because of the presence of organic solvents.

The cell surface hydrophobicity of *Rhodococcus* sp. Q15, which is able to mineralize alkanes, diesel, and Bunker C crude oil at both 5°C and 24°C, was higher after growth on diesel fuel and hexadecane than when the cells were grown on glucose-acetate (Whyte et al. 1999). The carbon source strongly influenced the fatty acid profile of the cells, with small amounts of C18 fatty acids and greater amounts of C16 and C14:0 fatty acids being observed in cells grown on *n*-hexadecane when compared to those grown on glucose-acetate. The degree of saturation of the fatty acids of the membrane of strain Q15 decreased as response to a lower growth temperature: the membrane contained relatively saturated fatty acids at 24°C and relatively unsaturated fatty acids at 5°C. This happened independently of the substrate used, although the decrease in the degree of saturation occurred at a lesser extent when the cells grew on hydrocarbons than on glucose-acetate. The cells were thus able to modulate the membrane fluidity to respond to both the influences of low temperature and hydrocarbon toxicity.

The most interesting physicochemical surface properties adaptation of *Rhodococcus* cells to organic solvents was observed with *R. erythropolis* growing on C5–C16 *n*-alkanes (de Carvalho et al. 2009). A strong correlation between the *n*-alkane chain length and the zeta potential of the bacterial cells was observed, with the cells even becoming positive when they grew on C14–C16. Most known bacteria only exhibit negative surface charges at circum neutral pH (Jucker et al. 1996) and the fact that *R. erythropolis* DCL14 presents a positive surface charge is quite remarkable. According to the extended Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory of colloidal stability (Van Oss 1995), in which electrostatic repulsion, van der Waals attraction, and acid–base (hydrophobic) interactions are considered, the adhesion capacity of bacterial cells is inversely correlated with the (negative) surface charge of the cell. Since most natural surfaces are negative, adhesion will only take place when the electrostatic repulsion is overcome by attractive forces (e.g., van der Waals, hydrophobic interactions) between the bacteria and the surface. In the case of strain DCL14, the positive surface charge will contribute to the attachment of the cells to negatively charged surfaces such as *n*-hexadecane droplets (zeta potential of -46.0 ± -3.4 mM).

3.2 Biocatalysis and Biodegradation of the Toxic Compound

Bioconversion or mineralization of a toxic compound has been presented as a mechanism for bacterial strains to thrive in its presence. However, although some studies indicate that tolerance to solvents derives from the capacity of the cells

to metabolize them, other works suggest that conversion or metabolism of organic solvents is not essential to tolerance. The degradation may mediate the resistance of some bacterial strains to solvents but it cannot be the main mechanism conferring tolerance to a broad number of solvents (Isken and de Bont 1998).

Rhodococci present a broad catabolic diversity and enzymatic capabilities, increasing their importance in environmental and biotechnological processes (Bell et al. 1998; de Carvalho and da Fonseca 2005a; Martínková et al. 2009; Warhurst and Fewson 1994). Their exceptional ability to resist and degrade hydrophobic compounds and xenobiotics is related to the presence and mobilization of large linear and circular plasmids, while the presence of multiple pathways and gene homologous enhance their versatility (Larkin et al. 2005, 2006; van der Geize and Dijkhuizen 2004). Works reporting the ability of *Rhodococcus* strains to act as whole-cell biocatalyst even in anhydrous organic solvents have also been published (e.g., Yamashita et al. 2007).

In a work carried out to study the effects of organic solvents in organic–aqueous systems on *R. erythropolis*, *Xanthobacter* Py2, *Arthrobacter simplex*, and *Mycobacterium* sp. NRRL B-3805, principal components analysis was used to interpret the data (de Carvalho and da Fonseca 2004). The variables used to construct the data matrix were cell viability, cell morphological parameters (e.g., size, elongation factor, circularity), number of cells in clusters, and the conditions to which the cells were exposed [substrate concentration, carbon source used for growth, adaptation time to the solvent prior to substrate addition, and physical properties of the solvents (e.g., density, molecular weight and log *P*)]. Over a third of the variability of the data related to *R. erythropolis* could be explained by solvent toxicity. When studying the effect of several solvents, present at different organic:aqueous ratios, on whole cells of *R. erthropolis* DCL14 carrying out the biotransformation of (−)-carveol to (−)-carvone, principal components analysis showed that 41.2% of the variance of the data responsible for the cell behavior could be ascribed also to solvent toxicity (de Carvalho et al. 2003).

In two-phase systems, *R. erythropolis* cells migrate toward the organic phase because of their high cell hydrophobicity (de Carvalho and da Fonseca 2002a, b, 2003). When emulsion samples were collected and droplets of solvent were observed by fluorescence microscopy, it was found that the cells partitioned between the organic and the aqueous phase. Part of the cell population was even inside the solvent droplets (Fig. 2a, b). The images with cells on the organic phase were not just the result of a superposition of different planes, as shown by a technique developed by de Carvalho and da Fonseca in 2003 (Fig. 2c), which allows the observation of 3D solvent droplets adsorbed to solid particles, for example, of silica gel. Cells were preferentially inside the organic droplets in the solvents with high log *P* value, being the percentage of cells in droplets lower when the organic phase was more toxic (Table 3). However, in the presence of cyclohexane, which has a log *P* value of 3.2 and should be toxic, most of the cells were also positioned inside the solvent droplets. Cells directly positioned in the organic phase can access the dissolved hydrophobic substrates in biocatalytic processes (de Carvalho et al. 2000; de Carvalho and da Fonseca 2002b), and can degrade organic

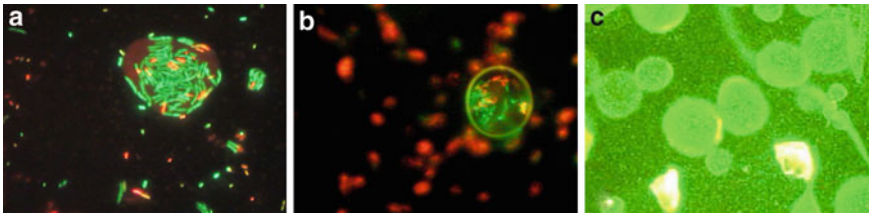

Fig. 2 *R. erythropolis* cells inside *n*-dodecane droplets. (**a**, **b**) solvent droplets trapped between a slide and cover slip (horizontal and vertical field widths equal to 0.16 and 0.12 mm, respectively); (**c**) solvent droplets adsorbed on silica gel particles (horizontal and vertical field widths equal to 0.4 and 0.3 mm, respectively)

Table 3 Percentage of *R. erythropolis* cells inside solvent droplets with different toxicities at different phase ratios

Phase ratio	0.0005					0.0025					0.025		
log *P* (solvent)	1.85	3.2	4.5	6.6	−1.35	1.85	3.2	3.5	9.6	12.88	−1.35	6.6	12.88
Cells in droplets (%)	64.9	93.5	72.5	97.7	73.4	58.5	99.0	57.2	73.0	99.3	68.0	91.7	97.6

The cells were carrying out the conversion of carveol into carvone in the organic–aqueous two-phase systems

solvents by direct uptake in bioremediation systems (Bouchez-Naïtali et al. 2001; de Carvalho and da Fonseca 2007). In these cases, the growth and conversion rates obtained are independent of the interfacial area, due to the strong adsorption of the bacterial cells at the solvent–aqueous interface.

In a paper regarding adaptation of microbes, Sonnleitner (1998) discussed the role of static effectors (such as concentrations of substrates and products) that affect the system instantaneously and independently of time (limiting or inhibiting the cells) and dynamic effectors that are time dependent and associated to changes in the physiological state or protein synthesis or degradation. When in contact with a toxic compound, the cells may express an enzyme(s) or use an alternative pathway to catabolize it and decrease the concentration of the biocide in the microenvironment surrounding the cells. For economical reasons, the cells usually only express the required enzymes after being exposed to the toxic compound.

By slowly increasing the concentration of solvent, substrate, and product, it was possible to adapt *R. erythropolis* DCL14 cells in an air-driven direct contact bioreactor (de Carvalho and da Fonseca 2002b). By recirculating *n*-dodecane through the column reactor at a rate slow enough so that the biotransformation could only take place at a small extent, the cells were able to adapt. When the biotransformation was performed after the adaptation period, the product carvone reached a concentration of 94 mM after 310 h of operation, thus overcoming carvone inhibition (observed at carvone concentrations of 50 mM). By increasing the adaptation period to 136 h, carvone accumulation reached 259 mM. Since the biomass was kept relatively constant during the experiments, the adapted cells were able to produce much more product than the nonadapted cells.

A similar strategy could be used to adapt strain DCL14 cells to toluene to allow its degradation at high concentrations in *n*-dodecane–aqueous systems (de Carvalho et al. 2007). Only 10.5% of the initially nonadapted cells remained viable after 1 h exposure to 20% (v/v) toluene. Cell adaptation was carried out by adding a toluene pulse, whenever its concentration reached ca. 50% of the initial value, to double the previous initial concentration (i.e., if the initial concentration was 1%, a pulse of toluene was added when toluene concentration reached 0.5% so that the concentration would be 2%). Curiously, toluene degradation rate increased with the increasing toluene concentrations added to the reactor. By using this strategy, the cells could endure a maximum toluene concentration of 4.9 M, which corresponds to 52.4% (v/v) in the organic phase, toluene being consumed at 10.7 mg/(h.mg protein). Once more, the amount of biomass was kept nearly constant through out the 35 days of the experiment. The fatty acid composition of the adapted cells presented a higher amount of branched fatty acids and ca. 40% decrease in the amount of straight-chain fatty acids. Interestingly, cells adapted to toluene presented a significant increased resistance to ethanol, silver ions, and iodine when compared to nonadapted cells.

The results obtained with *R. erythropolis* DCL14 suggest that toluene metabolism should be related to toluene tolerance mechanisms, as a correlation between toluene consumption rate and initial toluene concentration was found. The presence and cometabolism of *o*-, *m*-, and *p*-xylene further enhanced the degradation of toluene (de Carvalho et al. 2007). Leneva et al. (2009) could also adapt *R. opacus* 412 and *R. rhodnii* 135 to phenanthrene and anthracene on solid medium. The cells accelerated the metabolism and became able to grow on phenanthrene as sole carbon and energy source in liquid medium. *R. erythropolis* CCM 2595, although able to use phenol, pyrocatechol, resorcinol, *p*-nitrophenol, *p*-chlorophenol, hydroquinone, and hydroxybenzoate, was strongly affected by the substrate and initial concentration, and while some of the monoaromatic compounds suppressed the ability of strain CCM 2595 to use phenol in binary mixtures, others were strong inducers of phenol 2-monooxygenase (Čejková et al. 2005). In the latter case, the mixtures were more rapidly metabolized, if the cells were preexposed to the substrates. Cold-adapted *Rhodococcus* sp. strain NO14-1 and strain NO20-3 were able to fully degrade 12.5 mM phenol after 25 and 28 days, respectively, but when exposed to 15 mM phenol only 4 mM were degraded after 10 days and no further degradation was observed over 36 days (Margesin et al. 2005). Adaptation of *R. rhodochrous* 172 on agar mineral medium with fluorene for 6 months resulted in rapid growth without lag phase of the adapted cells: complete degradation of 12 mg/L fluorene was achieved in liquid medium within 5 days, while the nonadapted cells were unable to grow (Rubashko et al. 2006). The results presented are an indication that in these strains tolerance and degradation capacity may be strongly related.

The initial results obtained by Na et al. (2005) also seemed to indicate a crucial role between solvent tolerance and its utilization or degradation in *R. opacus* B-4. However, a mutant defective in benzene dioxygenase was as tolerant to organic solvents as the wild strain B-4. This suggests that conversion or degradation is

not essential for organic solvent tolerance of *R. opacus* B-4. Two mutants of *Rhodococcus* sp. 33 unable to degrade benzene were still tolerant to 500–800 mg/L of benzene (nonadapted strain 33 cells were able to tolerate ca. 1,000 mg/L), also confirming that benzene-degradation is of minor importance to the tolerance of these variants (Gutiérrez et al. 2009). Mosqueda et al. (1999) also suggested that toluene metabolism is not involved in toluene tolerance in *P. putida* DOT-T1. Independently of the mechanisms used by each strain, solvent tolerance is important as it allows bacterial growth at high organic solvent concentrations, which is of paramount importance in the bioremediation of sites contaminated with compounds such as benzene and toluene (Chen et al. 2009; Na et al. 2005).

Lately, organic solvent tolerant strains have received a further notice because biodesulfurization of petroleum occurs in the presence of high concentrations of hydrocarbons. Many of the described competent bacteria to perform desulfurizations are *R. erythropolis* strains, for example, IGTS8, N1-43, D-1, and KA2-5-1, with strain IGTS8 being the best characterized. The *dszA*, *B*, and *C* genes primarily responsible for DBT metabolism are located in a single operon on a large plasmid in strain IGTS8 and in other related strains (Monticello 2000). Five strains, able to utilize dibenzothiophene (DBT) as sole sulfur source and convert it to hydroxybiphenyl (HBP), isolated from coal storage sites in the north of France and from soils contaminated with heavy crude oil with high sulfur content belonged to the *Rhodococcus/Gordonia* cluster (Abbad-Andaloussi et al. 2003). All strains were able to use DBT in 95% *n*-hexadecane, used as model for diesel oil, although no activity was observed at *n*-hexadecane concentration of 99%. Some of the strains used by Bouchez-Naïtali et al. (2004), namely *Rhodococcus* sp. MK7C1 and MK2.4, exhibited good resistance to solvents, being even more tolerant than nondesulfurizing *Pseudomonas* strains, which could explain why transference of biodesulfurizing genes into Gram-negative strains did not promote biodesulfurization activity. An increased DBT desulfurization activity could be increased in biphasic systems, as reported by Ohshiro et al. (1995) with *R. erythropolis* H-2 in 70% *n*-tetradecane, Patel et al. (1997) with *R. erythropolis* IGTS8 in 50% *n*-hexadecane, and Abbad-Andaloussi et al. (2003) with *Rhodococcus* sp. strain I-2207 in the presence of 50% *n*-hexadecane. Nevertheless, total biodesulfurization of fossil fuel at industrial scale is not expected to occur in the near future (Soleimani et al. 2007).

3.3 Other Mechanisms of Protection

Iwabuchi et al. (2000) reported an association between colony morphotypes and oil tolerance in *R. rhodochrous*. The mucoidal strain was resistant to 10% (v/v) *n*-hexadecane while the rough derivatives were sensitive to this concentration. Furthermore, when the extracellular substance (EPS) produced by the mucoidal strain was added to cultures of the rough strain, the latter acquired resistance to *n*-hexadecane. Rough strains are hydrophobic and mucoidal strains are hydrophilic.

The EPS produced could confer tolerance to organic solvents by lowering the surface hydrophobicity, since, as Kobayashi et al. (1999) showed, low cell surface hydrophobicity could act as a defense mechanism against these compounds. The EPS produced by *R. rhodochrous* S-2 was even effective in stimulating the degradation of aromatic compounds in crude oil by native marine bacteria (Iwabuchi et al. 2002). Urai et al. (2007) also suggested that the large quantity of extracellular polysaccharides produced by *R. erythropolis* PR4 cells (able to degrade several hydrocarbons, including pristine) play an important role in hydrocarbon tolerance.

Cells of *R. erythropolis* DCL14 also present a non-EPS and a EPS producer variant. When exposed to the terpenes carveol and carvone and to organic solvents, the degree of saturation of the membrane phospholipids decreased, while the reverse was observed on the EPS producer counterpart (de Carvalho and da Fonseca 2007). The presence of EPS altered the level of cell exposure to solvents and terpenes. In the presence of organic solvents, part of the initially rough DCL14 population may start producing EPS (de Carvalho and da Fonseca 2002b, 2007). When these cells were under organic solvent stress-induced conditions, the small part of the population that remained viable produced colonies with a different phenotype: they were yellow or white while the nonstressed cells produced pink colonies (de Carvalho et al. 2004). Furthermore, under conditions that allow high cell viability, cells that died presented no significant morphological changes when compared to viable cells. However, under aggressive conditions, nonviable cells were much larger, probably because of an increase in membrane fluidity, with the viable cells succeeding in decreasing their surface area to minimize the area of contact with the toxic agent (de Carvalho and da Fonseca 2004; de Carvalho et al. 2004).

Several rhodococci strains have been reported to produce biosurfactants, usually glycolipids, such as *R. erythropolis* 51 T7 (Marqués et al. 2009), *R. erythropolis* DSM 43215 (Lang and Philp 1998) and *R. equi* Ou2 (Bouchez-Naïtali and Vandecasteele 2008). A relation between the surface tension of the culture medium and the alkane chain length during C5–C16 growth of *R. erythropolis* DCL14 indicated the production of a biosurfactant in the presence of *n*-alkanes with longer carbon chains (de Carvalho et al. 2009). The significant reduction of the surface tension to values lower than 30 mN/m indicates the biosurfactant power of the compound produced.

A common pattern for growth on long-chain alkanes in bacteria not producing biosurfactants is the formation of cellular flocs (Bouchez-Naïtali et al. 2001). In this case, the degradation rates during linear growth do not increase with interfacial area but with the efficiency of stirring, the interfacial uptake being limited by floc formation. Cell clustering was also visible with *R. erythropolis* DCL14 cells growing on *n*-dodecane, *n*-tetradecane, and *n*-hexadecane (de Carvalho and da Fonseca 2005b; de Carvalho et al. 2009). Biofilm formation can also been seen as a form of protection of cells to stress environments as cells inside the biofilm matrix are more protected (Heipieper et al. 1991). For example, no toxic effect of *n*-octane on biofilm growth was observed while the cells in suspension were strongly

inhibited by the accumulation of 1-octanol, a metabolite of the alkane monooxygenase during growth on *n*-octane (de Carvalho et al. 2009).

The action of efflux pumps responsible for the efflux of organic solvents from the inside of cells could also be responsible for adaptation of *Rhodococcus* cells to these compounds. Several antibiotic efflux pumps have been reported in Gram-positive strains, especially in *S. aureus*. However, studies on solvent efflux pumps in these bacteria are nearly inexistent (Fernandes et al. 2003).

4 Application

A large set of enzymes from rhodococci cells have been reported and identified, allowing these cells to carry out a large array of bioconversions and degradations: from oxidations to dehydrogenations, epoxidations, hydrolysis, hydroxylations, dehalogenations, and desulfurizations. Since the cells are able to tolerate and adapt to organic solvents, bioreactions can be done in nonconventional media, using low water soluble substrates, which makes this genus one of the most promising in biotechnology.

The ability of *Rhodococcus* cells to metabolize hydrocarbons (even at significantly high concentrations and under a wide variety of environments), to produce biosurfactants (required to increase the bioavailability of low water soluble organic solvents) and to change the physicochemical properties of the cellular surface (making these cells highly adaptive) make these cells ideal candidates to in situ bioremediation of hydrocarbon contaminated sites.

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Catabolism of Aromatic Compounds and Steroids by *Rhodococcus*

Katherine C. Yam, Robert van der Geize, and Lindsay D. Eltis

Contents

Abstract Aromatic compounds and steroids are among the remarkable variety of organic compounds utilized by rhodococci as growth substrates. This degradation helps maintain the global carbon cycle and has increasing applications ranging from the biodegradation of pollutants to the biocatalytic production of drugs and hormones. The catabolism of aromatic compounds and steroids converge as steroid degradation proceeds via aromatic intermediates. Consistent with the aerobic

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lifestyle of rhodococci, these pathways are rich in oxygenases. Analysis of five rhodococcal genomes confirms the modular nature of the aromatic compound catabolic pathways: peripheral pathways degrade compounds such as biphenyl and phthalate to common intermediates, while central pathways transform these intermediates, such as catechol and phenylacetate, to central metabolites. Studies of *Rhodococcus jostii* RHA1 in particular have revealed a similar modular structure of steroid degradation pathways, which is also conserved in related actinobacteria, such as *Mycobacterium tuberculosis*. Indeed, steroid degradation appears to be a very common, potentially ubiquitous characteristic of rhodococci. Nevertheless, the steroid catabolic pathways appear to be more redundant than the aromatic compound catabolic pathways. Finally, studies in rhodococci have helped elucidate the role of key steroid-degrading proteins including the Mce4 steroid uptake system which define a new class of ABC transporters. The significance of some of these recent discoveries for industrial processes and pathogenesis is discussed.

1 Introduction

Aromatic compounds are widely distributed in the biosphere, being produced by a variety of biological and chemical processes. They range in size from low-molecular-mass compounds such as benzene to the large, insoluble biopolymer lignin. The defining characteristic of aromatic compounds is a planar, fully conjugated, ring-shaped moiety possessing $(4n+2)$ π electrons, where n is a non-negative integer (Hückel's rule) (Fig. 1a) (McMurry 1992). The exceptional stability of these compounds arises from the delocalization of their π orbitals, also called resonance energy. It is this stability that has contributed to the widespread production and use of natural and xenobiotic aromatic compounds for a variety of industrial applications. For example, polychlorinated biphenyls (PCBs) have been used as dielectric fluids and coolants (Field and Sierra-Alvarez 2008), while polybrominated diphenyl ethers (PBDEs) are used as flame retardants (Sjodin et al. 2003). Such compounds are among the most stable and persistent organic pollutants. Finally, polycyclic aromatic hydrocarbons (PAHs) constitute a family of compounds possessing fused aromatic rings. These compounds occur in hydrocarbon deposits and are also produced as byproducts of incomplete combustion of fossil fuels or biomass (Harvey 1991).

Steroids are a class of terpenoid lipids characterized by a carbon skeleton of four fused rings, labeled A to D, and side chains consisting of up to ten carbons. Hundreds of steroids have been identified in plants, animals, and fungi, varying in functional groups attached to the four fused rings. Bacteria contain the structurally related five-ringed hopanoids (Fernandes et al. 2003). The most important physiological roles of steroids are as hormones and in modulating membrane fluidity. In addition, these bioactive compounds have a range of therapeutic applications including as anti-inflammatory agents (Ko et al. 2000), antifungals (Chung et al. 1998), and contraceptives (Tuba et al. 2000). The discovery of the 11α-hydroxylation activity of the

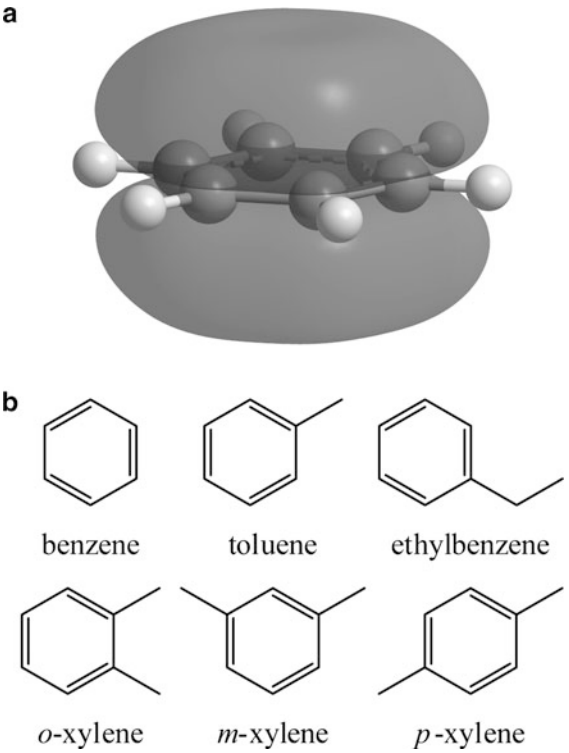


Fig. 1 Aromatic compounds. (**a**) A representation of π-orbital delocalization in a benzene ring. The *orbitals* shown represent the lowest occupied molecular orbital configuration. (**b**) BTEX hydrocarbons

fungus *Rhizopus* in 1949 enabled the transformation of simple sterols to corticosteroids and sparked interest in the synthesis and production of active steroid molecules (Hogg 1992). Cholesterol, obtained from animal fats and oils, and phytosterols, such as stigmasterol, β-sitosterol, and campesterol, are major starting materials for the production of steroid drugs and hormones owing to their low cost and ease of transformation.

In light of the exceptional ability of rhodococci to utilize a wide range of organic compounds as growth substrates, particularly hydrophobic ones, it is hardly surprising that these organisms figure prominently among known degraders of aromatic compounds and steroids (van der Geize and Dijkhuizen 2004). Indeed, *Rhodococcus jostii* RHA1, isolated from lindane-contaminated soil (Seto et al. 1995a), is one of the most potent PCB degraders characterized to date, contains up to four steroid-degrading pathways, and has recently been reported to degrade lignin. The catabolic activities of *Rhodococcus* likely help sustain the biosphere, as these organisms are found in a broad range of environments including various soils, sea water, and eukaryotic cells. Indeed, in at least one study of *o*-xylene-contaminated soils, rhodococci were the most prominent

species (Taki et al. 2007). The exceptional ability of rhodococci to degrade such compounds may be due in part to their mycolic-acid-containing outer membrane (see chapter "The Rhodococcal Cell Envelope: Composition, Organisation and Biosynthesis" by Sutcliffe et al.) as well as their production of surfactants (Iwabuchi et al. 2002; Vogt Singer et al. 1990). Recent genomic, molecular genetic, microbiological, and biochemical studies have increased our understanding of this degradation in rhodococci as well as in related mycolic-acid-producing actinomycetes such as *Corynebacterium*, *Nocardia*, and *Mycobacterium*.

This chapter focuses on the catabolic pathways utilized by rhodococci to degrade aromatic compounds and steroids. We first discuss the overall strategies used by these bacteria to degrade naturally occurring mononuclear aromatic compounds. The underlying principles are illustrated using several pathways. We then discuss the catabolism of more complex compounds, including lignin, PAHs, some halogenated pollutants, and steroids. Differences and similarities of rhodococcal catabolism with that of other bacteria are highlighted by genomic analyses of five rhodococci: *R. opacus* B4, *R. erythropolis* PR4, *R. jostii* RHA1,[1] *R. erythropolis* SK121, and *R. equi* 103S. Particular emphasis is placed on recent discoveries that provide new insights into how this degradation occurs. These advances have important implications for industrial processes, ranging for bioremediation to biocatalysis, as well as for the pathogenesis of *Mycobacterium tuberculosis*, the leading cause of mortality from bacterial infection, and *R. equi*, a horse pathogen that can infect immunocompromised humans (Prescott 1991).

2 Mononuclear Aromatic Compounds

Mononuclear aromatic compounds possess a single aromatic ring within their structure. While these compounds are chemically simpler than the others considered in this chapter, their catabolism illustrates a number of features that are central to the catabolism of all aromatic compounds and steroids in rhodococci, if not aerobic bacteria in general. Mononuclear aromatic compounds are the most prevalent aromatic compounds in the biosphere, being produced by a variety of biological and geochemical processes. Due to their stability, compounds such as benzene and its derivatives are used extensively in the chemical, agriculture, and petroleum industries. For example, gasoline contains a mixture of benzene, toluene, ethylbenzene, and xylene isomers, collectively known as BTEX hydrocarbons (Fig. 1b). BTEX compounds are frequently found as groundwater contaminants as a result of leaking fuel tanks (Cozzarelli et al. 1990).

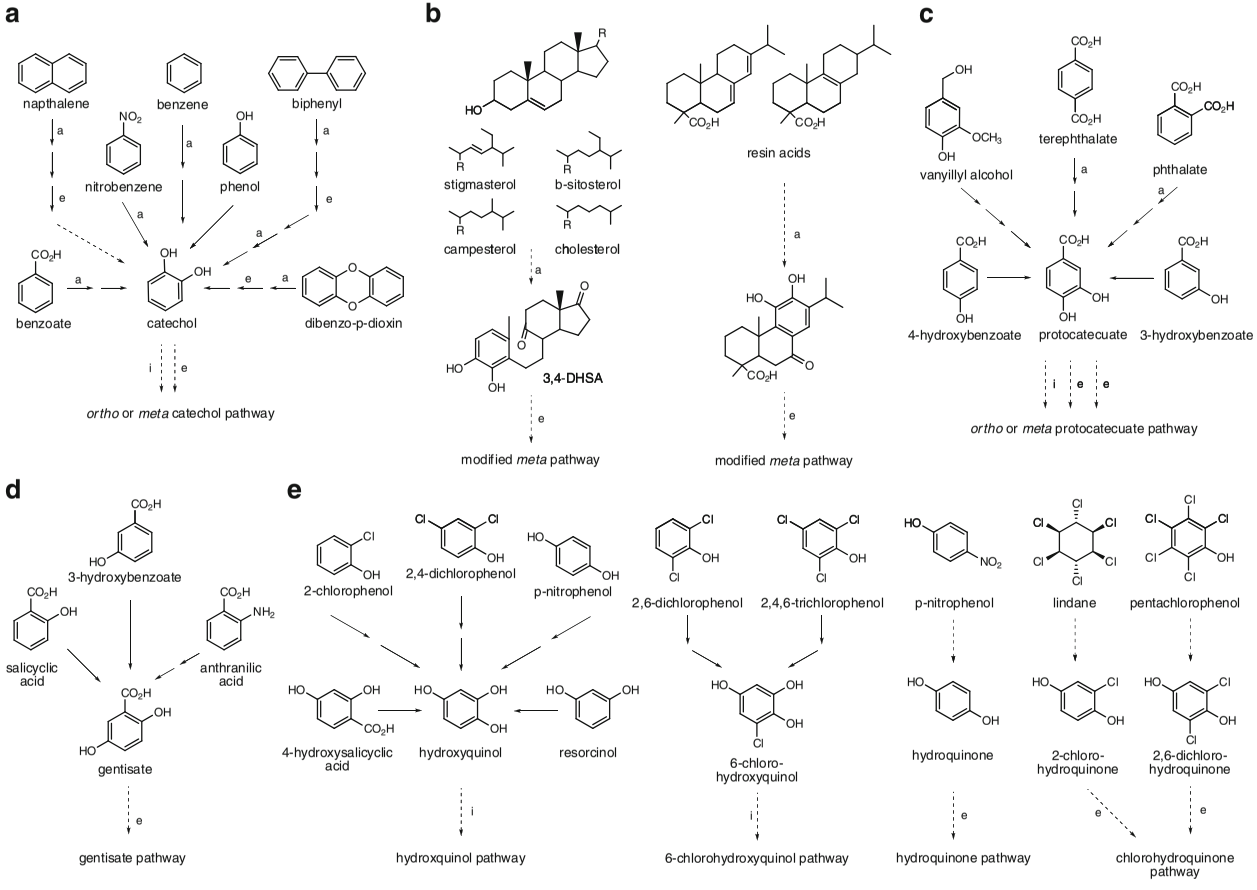
[1]Herein, *R. opacus* B4, *R. erythropolis* PR4, and *R. jostii* RHA1 are referred to by their strain names: B4, PR4 and RHA1, respectively.

2.1 *Underlying Strategies of Aromatic Compound Catabolism in Rhodococci*

The bacterial catabolism of aromatic compounds involves two key steps: the activation of the thermodynamically stable benzene ring, and its subsequent cleavage. While bacteria have evolved diverse anaerobic and aerobic strategies to effect these two steps, rhodococci utilize predominantly the latter strategies, consistent with their aerobic lifestyle. More particularly, rhodococci make extensive use of Rieske non-heme iron oxygenases and other oxygenases to activate the benzene ring by catalyzing the incorporation of hydroxyl groups (Mason and Cammack 1992). Such reactions eventually yield central aromatic metabolites such as catechol, protocatechuate (dihydroxylated at positions 1,2), gentisate (dihydroxylated at positions 1,4), and hydroquinone (dihydroxylated in a *para* position). The critical step of ring fission is then catalyzed by ring-cleaving oxygenases (Vaillancourt et al. 2006). This cleavage can occur either between the hydroxyl groups (intradiol, *ortho*-cleavage) or adjacent to the hydroxyl groups (extradiol, *meta*-cleavage). Each of these four central aromatic metabolites occurs in various catabolic pathways, as summarized in Fig. 2. Steps that are catalyzed by Rieske non-heme iron oxygenases are indicated with "a," whereas extradiol and intradiol dioxygenases are indicated with "e" or "i," respectively.

The pathways summarized in Fig. 2 illustrate an important principle that has been recognized in rhodococci and other bacterial species including the well-studied pseudomonads (Luengo et al. 2001); a wide variety of aromatic compounds are transformed to central metabolites via a relatively limited number of dihydroxylated metabolites. Indeed, the efficiency of this catabolic strategy is such that it has been adapted to degrade polyalicyclic compounds such as steroids (van der Geize et al. 2007) (Fig. 2b). While this figure summarizes our knowledge of the aerobic catabolism of aromatic compounds in all bacteria, all of the intermediates and most of these pathways are known to occur in rhodococci.

A second aerobic catabolic strategy involves the derivatization of aromatic acids by co-enzyme A (CoA) and nonoxygenolytic ring fission (Denef et al. 2006; Navarro-Llorens et al. 2005; Olivera et al. 1998), reminiscent of CoA-dependent reductive pathways responsible for the anaerobic cleavage of aromatic nuclei. However, in the aerobic CoA-dependent pathways, an oxygenase transforms the aromatic acyl-CoA ester prior to ring fission. While, these types of pathways have been called hybrid pathways (Ferrandez et al. 1998), the evolutionary relationship of the aerobic and anaerobic CoA-dependent pathways is unclear. Despite being strict aerobes, rhodococci contain at least one hybrid pathway, the phenylacetate (Paa) pathway described below (Navarro-Llorens et al. 2005). However, they do not appear to contain the Box pathway, which transforms benzoate to β-ketoadipyl-CoA in *Burkholderia*, perhaps under O_2-limiting conditions (Denef et al. 2006).


a
napthalene
benzene
biphenyl
nitrobenzene
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dibenzo-p-dioxin
ortho or meta catechol pathway
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b-sitosterol
campesterol
cholesterol
3,4-DHSA
modified meta pathway
resin acids
modified meta pathway
c
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p-nitrophenol
4-hydroxysalicyclic acid
hydroxyquinol
resorcinol
hydroxquinol pathway
2,6-dichlorophenol
2,4,6-trichlorophenol
6-chloro-hydroxyquinol
6-chlorohydroxyquinol pathway
p-nitrophenol
hydroquinone
hydroquinone pathway
lindane
pentachlorophenol
2-chloro-hydroquinone
2,6-dichloro-hydroquinone
chlorohydroquinone pathway

3 Peripheral Versus Central Aromatic Pathways

Analyses of bacterial genomic sequences has revealed that the catabolism of aromatic compounds is organized such that a large number of "peripheral" aromatic pathways funnel a range of growth substrates into a restricted number of "central" aromatic pathways. The latter complete the transformation of these compounds to tricarboxylic acid (TCA) cycle intermediates. Thus, the organizational logic of the pathways follows the logic of the chemistry outlined in Fig. 2. The term "catabolon" has been used to define each set of peripheral pathways and corresponding central pathway in a given organism (Luengo et al. 2001). Thus, each catabolon is a complex functional unit of integrated catabolic pathways which transform related compounds via common metabolites. This organization was first described in pseudomonads, where analyses of the genomic sequences of four strains together with functional studies have identified at least 38 peripheral pathways, some of which are strain-specific, and five conserved central pathways (Luengo et al. 2001). Subsequent analyses have confirmed that aromatic catabolic pathways are similarly organized in other bacteria, including rhodococci (McLeod et al. 2006).

3.1 Central Pathways

Up to eight central aromatic pathways have been identified in rhodococci to date. The β-ketoadipate pathway is encoded by the *pca* and *cat* genes, and transforms catechol and protocatechuate to acetyl-CoA and succinyl-CoA via the intradiol cleavage of the catecholic intermediate (Harwood and Parales 1996). The Paa pathway is encoded by the *paa* genes and involves the derivatization of aromatic acids by CoA, ring hydroxylation by an oxygenase, and nonoxygenolytic ring fission (Navarro-Llorens et al. 2005). The 2-hydroxypentadienoate (Hpd) pathway, encoded by the *bphIJK* in *Burkholderia xenovorans* LB400 (Erickson and Mondello 1992), the *bphEFG* in RHA1 (Masai et al. 1997) and the similar *hsaEFG* genes in RHA1 (van der Geize et al. 2007), transforms 2-hydroxypentadienoates to acetyl-CoA and pyruvate through the successive actions of a hydratase, an aldolase, and a dehydrogenase. The gentisate pathway, characterized in *R. erythropolis* strain S1 (Suemori et al. 1995), transforms gentisate to pyruvate and fumarate. The homogenisate pathway (Hmg), characterized in *P. putida* (Arias-Barrau et al. 2004) and

Fig. 2 Overall strategy for the aerobic bacterial catabolism of aromatic compounds. Pathways degrade growth substrates to one of four central intermediates: (**a**) catechol; (**b**) substituted catechol in steroid and cryptic aromatic catabolism; (**c**) protocatechuic acid; (**d**) gentisic acid; and (**e**) (chloro) hydroquinol and (chloro) hydroquinone. *Solid arrows* indicate a single enzymatic reaction, while *dotted arrows* represent multiple steps. *Letter labels* designate catalysis via the following enzymes: "a" = Rieske non-heme iron oxygenase; "i" = intradiol dioxygenase; and "e" = extradiol dioxygenase. Adapted from Vaillancourt et al. (2006)

predicted to occur in several rhodococci, involves the extradiol-type cleavage of homogentisate followed by C–C bond hydrolysis to yield fumarate and acetoacetate. The hydroxyquinol pathway, encoded by the *dxn* genes in *Sphingomonas wittichii* RW1 (Armengaud et al. 1999), involves the intradiol cleavage of hydroxyquinol to acetyl-CoA and succinyl-CoA. The homoprotocatechuate (3,4-dihydroxyphenylacetate) pathway, encoded by the *hpc* genes, involves the extradiol-type cleavage of homoprotocatechuate. The resulting product (5-carboxymethyl-2-hydroxymuconic semialdehyde) is transformed to TCA cycle intermediates via a dehydrogenative route. Finally, an eighth pathway comprising a hydroxylase, an extradiol dioxygenase, and hydrolase has been assigned as a central pathway in RHA1 (McLeod et al. 2006) although its substrate has not yet been identified.

Genomic analyses indicate that B4 contains nearly all the same central pathways compared to RHA1. By contrast, the genes encoding the gentisate, homoprotocatechuate, and the above-mentioned unidentified pathways are absent in the two *R. erythropolis* strains, PR4 and SK121. Moreover, these latter two strains have fewer copies of the Hpd pathway. Despite these differences, all of the central aromatic pathways are chromosomally encoded in these rhodococcal strains when present. This is consistent with the notion that these pathways are core to the bacterium's catabolic capabilities. As noted above, the genes, enzymes and regulatory mechanisms of the central pathways are found in a broad range of bacterial species. However, aspects of their organization are unique to *Rhodococcus* as discussed in more detail below for the β-ketoadipate and Paa pathways.

3.1.1 β-Ketoadipate Pathway

The β-ketoadipate pathway, also known as the *ortho*-cleavage pathway, was first identified in *Pseudomonas putida* (Ornston 1966a, b) and was one of first central aromatic pathways to be analyzed in different genera (Harwood and Parales 1996). The pathway has separate branches that catabolize catechol and protocatechuate, which differ by a carboxylate group, to TCA cycle intermediates (Fig. 3, upper panel). Accordingly, the intermediates of the pathway, and therefore the enzymes, are distinct until decarboxylation of the protocatechuate metabolite, catalyzed by PcaC, the third enzyme in this branch of the pathway. Other than this, the steps of the two branches are equivalent, and are catalyzed by homologous enzymes. Briefly, catechol and protocatechuate are cleaved by intradiol dioxygenases (CatA and PcaGH, respectively) to yield muconates which are cyclized by CatB and PcaB to muconolactones. The muconolactone of the catechol branch is isomerized by CatC, whereas that of the protocatechuate branch is decarboxylated by PcaC. These two reactions yield β-ketoadipate enol-lactone, which is hydrolyzed by PcaD to β-ketoadipate and transformed to TCA cycle intermediates in two CoA-dependent steps (PcaIJ and PcaF).

Among rhodococci, the β-ketoadipate pathway has been functionally characterized in each of *R. erythropolis* AN-13 (Aoki et al. 1983), *R. opacus* 1CP (Eulberg et al. 1998a), and RHA1 (Patrauchan et al. 2005). Moreover, genomic analyses

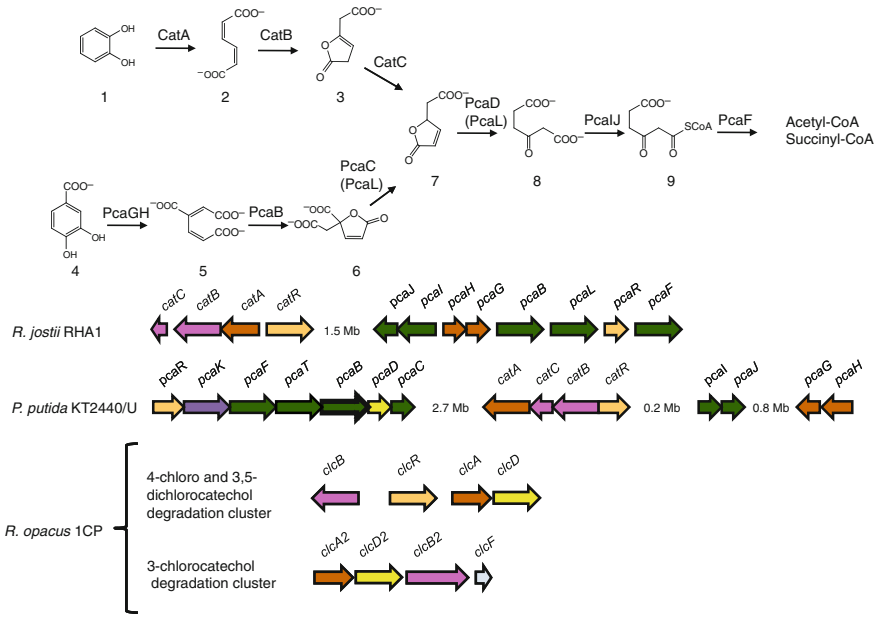

Fig. 3 The β-ketoadipate pathway. The enzymatic steps and the metabolites for the catechol (*upper*) and protocatechuate (*lower*) branches of the pathway are depicted in the *upper panel*. Catabolites are *numbered* as follows: 1, catechol; 2, *cis,cis*-muconate; 3, mucolactone; 4, protocatechuate; 5, γ-carboxymuconate; 6, γ-carboxymucolactone; 7, β-ketoadipate enol-lactone; 8, β-ketoadipate; 9, β-ketoadipyl CoA. The enzymes are: CatA, catechol 1,2 dioyxgenase; CatB, muconate cycloisomerase; CatC, mucolactone isomerase; PcaGH, protocatechuate dioxygenase; PcaB, carboxy-*cis,cis*-mucolactone isomerase; PcaC, 4-carboxymuconolactone decarboxylase; PcaD, β-ketoadipate enol-lactone hydrolase; PcaIJ, β-ketoadipate:succinyl-CoA transferase; and PcaF, β-ketoadipate:succinyl-CoA thiolase. The *lower panel* summarizes the organization of the β-ketoadipate pathway genes in *R. jostii* RHA1 and *P. putida* KT2440 (same as in *P. putida* U) together with the modified β-ketoadipate gene clusters for degrading chlorocatechols in *R. opacus* 1CP. Genes are *colored* by function as follows: *brown*, intradiol dioxygenases; *pink*, catabolism of (chloro) catechol; *dark green*, catabolism of protocatechuate; *yellow*, hydrolase; *pale blue*, dehalogenase; *indigo*, transporter; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from McLeod et al. (2006)

indicate that it occurs in B4 and PR4. The organization of the *pca* gene clusters is identical in three sequenced rhodococcal genomes in which it occurs. Comparison of the gene clusters with those in *Pseudomonas* and *Burkholderia* highlights several features of the rhodococcal pathway. First, rhodococci and other actinomycetes contain a bifunctional enzyme, PcaL, which catalyzes the decarboxylation and hydrolysis of the enol-lactone, the first shared intermediate of the pathway branches. By contrast, these activities are catalyzed by PcaC and PcaD, respectively, in the two *P. putida* strains KT2440 and U (Fig. 3, lower panel). In addition to rhodococci, PcaL has been identified in *Streptomyces* sp. 2065 (Iwagami et al. 2000) and is predicted to occur *S. coelicolor* A3(2) (Bentley et al. 2002) as well as in *Nocardia* and *Corynebacterium* genomes.

An apparent *Rhodococcus*-specific feature of the β-ketoadipate pathway is that the *pca* genes are clustered together in two divergently transcribed operons. More specifically, an analysis of the completed RHA1, B4, and PR4 genomes support our previous analysis that the chromosomal organization of the *pca* and *cat* genes in RHA1 appears to be unique to rhodococci and most similar to that of the closely related corynebacteria (Patrauchan et al. 2005). The *pca* genes are organized in a single cluster in all actinomycetes in which they have been found, as well as in *K. rhizophila* DC2201 (Takarada et al. 2008), *C. crescentus* (Nierman et al. 2001), and *A. baylyi* ADP1 (Brzostowicz et al. 2003), which is a γ-proteobacterium. Non-rhodococcal actinomycetes containing the *pca* genes include *C. glutamicum* ATCC 13032 (Kalinowski et al. 2003), *Streptomyces* sp. 2065 (Iwagami et al. 2000), *S. coelicolor* A3(2) (Bentley et al. 2002), and *S. avermitilis* MA-4680 (Omura et al. 2001). In contrast, the *pca* genes can be arranged in up to three clusters in pseudomonads (Jiménez et al. 2002). Multiple *pca* clusters also occur in β-proteobacteria such as *Burkholderia pseudomallei* and *R. metallidurans* (Jiménez et al. 2002). Nevertheless, the organization of the *pca* genes in a single cluster of two divergently transcribed operons with the gene order of RHA1 appears to be unique to rhodococci; in *C. glutamicum*, the gene order is different, and in streptomycetes, the genes appear to be arranged in a single operon. In all bacteria, the *cat* genes are usually organized in a single cluster (reviewed in Jiménez et al. 2002). In Gram-negative bacteria and *Arthrobacter*, *catR* encodes a LysR-type transcriptional regulator (Murakami et al. 2004), which activates transcription of the adjacent catabolic genes through induction by *cis*,*cis*-muconate. In contrast, the rhodococcal *catR* encodes an IclR-type regulator (Eulberg and Schlömann 1998), which has been shown to function as a repressor in *R. erythropolis* CCM 2595 (Vesely et al. 2007). IclR-type regulators in most cases control the protocatechuate catabolic operons (Eulberg et al. 1998a; Gerischer et al. 1998). However, permutations occur with respect to gene order (e.g., *catRBAC* in the streptomycetes sequenced to date) and the presence of additional genes in the transcriptional unit (e.g., the main *cat* operon in *A. baylyi* ADP1 contains six genes). The order of the genes in RHA1, *catRABC*, is seen in the five sequenced rhodococcal genomes as well as *R. erythropolis* CCM 2595 (Vesely et al. 2007), but not in *Rhodococcus* sp. AN-22 (Matsumura et al. 2006). This aniline-degrading strain was found to constitutively express the *catABC* operon because of a disrupted regulatory *catR* gene. In two *C. glutamicum* isolates (ATCC 13032 and R), *catR* is not adjacent to the other genes.

3.1.2 Modified β-Ketoadipate Pathways

A number of rhodococci possess modified β-ketoadipate pathways to metabolize substituted catechols, thereby expanding the range of aromatic compounds that can be used as a growth substrate. Generally, the modified pathway is incomplete, feeding into the chromosomally encoded classical pathway. Moreover, the modified pathway genes appear to occur on plasmids. The best characterized modified β-ketoadipate pathways are those that degrade chlorocatechols in *R. opacus* 1CP

(formerly *R. erythropolis* 1CP), isolated for its ability to utilize 2,4-dichlorophenol and 4-chlorophenol, which are degraded via 3,5-dichloro and 4-chlorocatechol, respectively. *R. rhodochrous* N75 utilizes a modified pathway to catabolize 4-methylcatechol. This pathway includes a 3-methyl-muconolactone-CoA synthetase (Cha et al. 1998) and a 4-methylmuconolactone isomerase (Bruce et al. 1989).

R. opacus 1CP contains two modified β-ketoadipate pathways, both of which include a chlorocatechol dioxygenase (ClcA), a chloromuconate cycloisomerase (ClcB), and a dienelactone hydrolase (ClcD) (Eulberg et al. 1998b), which correspond to CatA, CatB, and CatD, respectively, of the catechol branch of the β-ketoadipate pathway (Fig. 3, lower panel). In the pathway responsible for the degradation of 3,5-dichloro and 4-chlorocatechols, the *trans*-dienelactone resulting from dehalogenation and cyclization is cleaved by the hydrolase to give maleylacetate, which is then reduced to produce β-ketoadipate. The second pathway, encoded by the *clcA2B2D2F* genes, is specific for the degradation of 2-chlorophenol, 3-chlorophenol, and 3-chlorobenzoate (Moiseeva et al. 2002). The encoded enzymes are only distantly related to the previously known chlorocatechol enzymes and include a dechlorinating enzyme related to mucolactone isomerase (ClcF).

The modified β-ketoadipate pathways for degrading chlorocatechols are distinct from the classical pathway in four respects. Firstly, the enzymes in the modified pathway are highly specific for their chlorinated substrates. Secondly, the modified pathways possess enzymes capable of dehalogenation including cycloisomerases, (ClcB and ClcB2), a maleylacetate reductase (ClcE), and a dehalogenase (ClcF). Thirdly, since the modified pathway lacks the protocatechuate branch of the standard β-ketoadipate pathway, the *clc* and *clc2* clusters encode for dienelactone hydrolases (ClcD and ClcD2) alone, replacing the bifunctional PcaL. Finally, the *clc* and *clc2* operons occur on a 740-kbp plasmid, p1CP (Konig et al. 2004). Among the *Actinobacteria*, chlorocatechol metabolism has been investigated in some detail only for *R. opacus* ICP, and the presence of these chlorocatechol gene clusters has not been reported for other rhodococcal strains that degrade chloroaromatic compounds.

3.1.3 Phenylacetate (Paa) Pathway

Phenylacetate (Paa) arises in the catabolism of a variety of compounds including phenylalkanoates, tropate, and homophthalate. In addition, phenylacetate is the first common intermediate in the degradation of phenyldecane by *R. opacus* PD630 (Alvarez et al. 2002). The chromosomally encoded central pathway of the phenylacetyl-CoA catabolon has been described in both Gram-negative bacteria such as *P. putida* (Olivera et al. 1998) and *E. coli* (Ferrandez et al. 1998; Luengo et al. 2001), as well as Gram-positive bacteria such as RHA1 (Navarro-Llorens et al. 2005). However, the Paa pathway has yet to be functionally characterized. In the representative pathway shown in Fig. 4 (upper panel), phenylacetate is first transformed to phenylacetyl-CoA through the addition of CoA in an ATP-dependent fashion by PaaF, a phenylacetate-CoA ligase, first characterized in *P. putida* (Martinez-Blanco et al. 1990). This suggests that intermediates are processed as CoA-thioesters, an unconventional strategy for aerobic aromatic metabolism. Next, a multicomponent di-iron

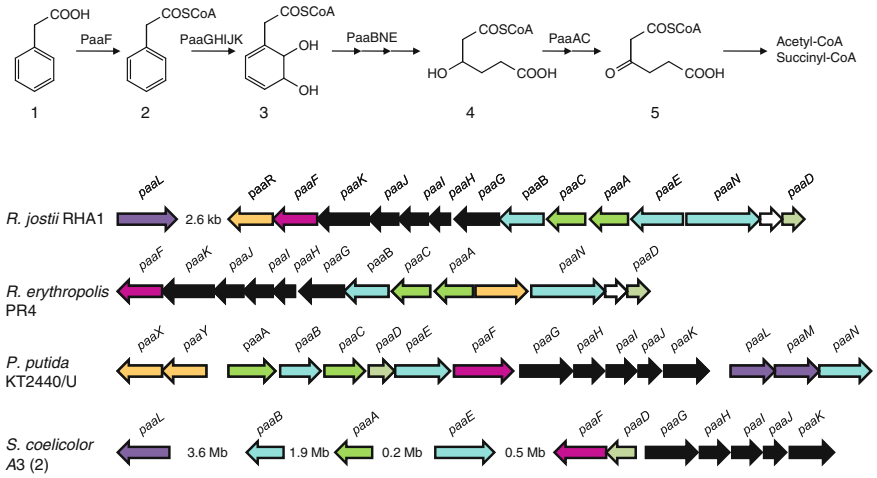

Fig. 4 The phenylacetic acid pathway. The enzymatic steps and the proposed metabolites of the pathway are depicted in the *upper panel*. Presumed catabolites are *numbered* as follows: 1, phenylacetate; 2, phenylacetate-CoA; 3, 2,3-dihydroxy-2,3-dihydrophenylacetate CoA thioester; 4, β-hydroxyadipyl-CoA; and 5, β-ketoadipyl CoA. Other catabolites may occur in this pathway. The enzymes are PaaF, phenylacetate-CoA ligase; PaaGHIJK, a multicomponent oxygenase; PaaN, a non-oxygenolytic ring-cleavage enzyme; PaaB, 2-cyclohexenylcarbonyl CoA isomerase; PaaE, a β-ketoadipyl CoA thiolase.; PaaA, enoyl-CoA hydratase; PaaC, 3-hydroxyl-butyryl-CoA dehydrogenase. The *lower panel* summarizes the organization of the pathway genes in *R. jostii* RHA1, *R. erythropolis* PR4, *P. putida* KT2440 (same as in *P. putida* U), and *S. coelicolor* A3(2). Genes are *colored* by function as follows: *purple*, phenyl-CoA ligase; *black*, ring-hydroxylating oxygenase components; *aqua*, ring-cleavage and associated functions; *lime green*, *paaAC*; *gray*, *paaD*; *indigo*, transporters; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from McLeod et al. (2006)

oxygenase encoded by paaGHIJK is then postulated to catalyze the 2,3-dihydroxylation of the aromatic ring, yielding a cis-dihydrodiol. The ring of this intermediate is non-oxygenolytically cleaved in a reaction that is thought to be catalyzed by PaaN, an aldehyde dehydrogenase. Consistent with its predicted role, a *paaN* knockout strain of RHA1 completely abolished growth on substrates known to be degraded via the Paa pathway and produced tropone, 2-coumaranone, and the methyl ester of 2-methoxyphenylacetate. The latter two metabolites were presumed derivatives of the expected substrate for PaaN (Navarro-Llorens et al. 2005). Together, PaaN, an enol-CoA hydratase (PaaB), and a ketothioesterase (PaaE) are thought to transform the dihydrodiol-CoA thioester to acetyl-CoA and β-hydroxyadipyl-CoA in poorly characterized reactions that utilize water and CoA. Finally, β-hydroxyadipyl-CoA is transformed by an enol-CoA hydratase (PaaA) and a 3-OH-acyl-CoA dehydrogenase (PaaC) to β-ketoadipyl-CoA, which is transformed to succinyl-CoA and a second equivalent of acetyl-CoA. Despite the ubiquitous nature of the Paa pathway, the identity of the metabolites and the function of each gene product are still unknown.

Genomic analyses of the *paa* clusters in three rhodoccocal species (RHA1, B4, and PR4), two pseudomonads (*P. putida* KT2440 and *P. putida* U), and two

non-rhodococcal actinomycetes (*S. coelicolor* A3(2) and *K. rhizophila* DC2201 (Takarada et al. 2008)) reveal several genus-specific features of the pathway (Fig. 4, lower panel). First, genes encoding two core functional units of the pathway are consistently clustered: *paaGHIJK*, encoding a ring-hydroxylating system, and *paaABC*, encoding a β-oxidation system. Other genes commonly occurring in *paa* gene clusters include *paaN* and *paaF*. Some *paa* gene clusters also contain genes encoding a transport system (*paaLM*) and a regulatory system (*paaXY*). In many Gram-positive organisms including *Rhodococcus*, there is no homolog of the *paaM*–encoded porin, consistent with such a protein being unnecessary in organisms lacking an outer cell membrane (Navarro-Llorens et al. 2005). In RHA1, PaaR may function to regulate the *paa* genes, replacing PaaXY found in some Gram-negative bacteria such as *P. putida* (Olivera et al. 1998). Interestingly, the *paa* cluster of PR4 lacks the *paaE*, *paaR*, and *paaL*, which encode a β-ketoadipyl-CoA thiolase, an AraC-type transcriptional regulator, and a transporter, respectively. It is possible that these functions are encoded by different genes. For example, genes encoding an Rrf2 DNA-binding protein or a TetR-type transcriptional regulator are positioned 1.9 and 5.4 kbp, respectively, from *paaF* in PR4. Similarly, a gene encoding a divalent anion-sodium symporter (DASS) is located 9.6 kbp upstream of the cluster in PR4. The PR4 cluster does not include an obvious candidate gene encoding a β-ketoadipyl-CoA thiolase.

The most notable distinguishing feature of the *paa* genes in rhodococci is their organization. In these bacteria, the principal cluster appears to be organized in two divergently transcribed operons despite their different gene contents. The two clusters minimally comprise *paaACBGHIJKF* and *paaN–orfX–paaD*, where *orfX* is a gene of unknown function. By contrast, the *paa* genes in some Gram-positive bacteria, most notably in *Arthrobacter* and *Streptomyces*, are dispersed in the chromosome. For example, in each of three *Arthrobacter* species (*A. oxydans* CECT386, *A. strain* FB24, and *A. aurescens*), soil-dwelling actinomyces, the *paa* genes, are organized in two distinct clusters: *paaDF–tetR–paaN* and *paaGHIJK* (Navarro-Llorens et al. 2008). Moreover, the *paaA, paaC,* and *paaE* genes have not been reported to date in these species. Similarly, the *paa* genes in *S. coelicolor* A3 (2) (Bentley et al. 2002) and *S. avermitilis* (Omura et al. 2001) are distributed throughout the genome with only the *paaGHIJK* genes clustered (Fig. 4, lower panel). Finally, the *paa* genes are also clustered in different ways in Gram-negative organisms, occurring as a single chromosomal cluster in *E. coli* (Ismail et al. 2003) and *P. putida* (Olivera et al. 1998) or multiple clusters separated by over 200 kbp, as in *B. xenovorans* LB400 (Chain et al. 2006).

3.2 Peripheral Pathways

In contrast to the central pathways, the peripheral pathways can be found on both plasmid and chromosome, consistent with the expansion of catabolic capabilities through the exchange of genes on mobile elements (van der Geize and Dijkhuizen 2004). The redundancy of peripheral pathway genes in rhodococci further

contributes to the catabolic diversity of these microorganisms. RHA1 is predicted to contain 26 peripheral pathways. However, only a few have been functionally confirmed. Among the best characterized are the catabolic pathways responsible for the degradation of biphenyl, ethylbenzene (Iwasaki et al. 2006; Sakai et al. 2002, 2003; Seto et al. 1995a), phthalate, and terephthlalate degradation (Hara et al. 2007). These pathways are typical of peripheral aromatic pathways in that oxygenases catalyze the hydroxylation of the aromatic ring, activating it for subsequent cleavage. Accordingly, these pathways are discussed in more detail below as illustrative examples. The degradation of other compounds such as naphthalene, salicylate, and 3-hydroxybenzoate, which are ultimately degraded via the central gentisate pathway (Suemori et al. 1995), will not be discussed in detail.

3.2.1 Biphenyl and Alkylbenzene Pathways

The biphenyl (Bph) pathway has been characterized in a number of rhodococci, including RHA1 (Masai et al. 1995), *R. globerulous* P6 (Asturias et al. 1995), *R. erythropolis* TA421 (Kosono et al. 1997), *Rhodococcus* strain M5 (Labbe et al. 1997), and three *R. rhodochrous* strains: K37, HA99, and TA431 (Taguchi et al. 2007). Much of the interest in this pathway has been driven by its ability to at least partially transform PCBs, discussed below. The Bph pathway in *Rhodococcus* is very similar to that in other bacteria (Furukawa 2000), comprising four enzymes that transform biphenyl into Hpd and benzoate (Fig. 5, upper panel). Degradation is initiated by biphenyl dioxygenase (BPDO), a three-component Rieske-type oxygenase (RO) comprising a reductase, a ferrodoxin, and a catalytic oxygenase. BphB, a member of the short chain dehydrogenases reductases (SDR) superfamily, catalyzes the NAD^+-dependent dehydrogenation of the resulting *cis*-diol to 2,3-dihydroxybiphenyl, a catechol. The latter is cleaved by the BphC extradiol dioxygenase to yield a *meta*-cleavage product (MCP). In the final step, an MCP hydrolase, BphD, adds water across a C–C bond to afford Hpd and benzoate. Hpd is further degraded via a central aromatic pathway, while benzoate is transformed to a catechol by the benzoate (Ben) peripheral pathway before being degraded by the β-ketoadipate pathway (Patrauchan et al. 2008). In RHA1, the expression of the *bph* genes is regulated by a two-component regulatory system: BphS, the sensor kinase, and BphT, the response regulator (Takeda et al. 2004).

The rhodococcal Bph pathway illustrates how rhodococci have developed catabolic versatility and efficiency through genetic redundancy. It also provides a sobering lesson on the challenges of gene annotation. RHA1 carries two copies of an ethylbenzene (Etb) catabolic pathway that is highly similar to the Bph pathway (Fig. 5). This includes two copies of an ethylbenzene dioxygenase (EBDO), two copies of an EtbC extradiol dioxygenase, and two copies of the EtbD MCP hydrolase. Indeed, sequence analyses revealed 54 potential *bph* genes in RHA1 including a total of 13 *bphC* homologs (Goncalves et al. 2006). A variety of studies have revealed that the Etb and Bph pathways are involved in a range of alkylbenzenes in RHA1, including biphenyl, ethylbenzene, styrene, and benzene (Patrauchan et al.

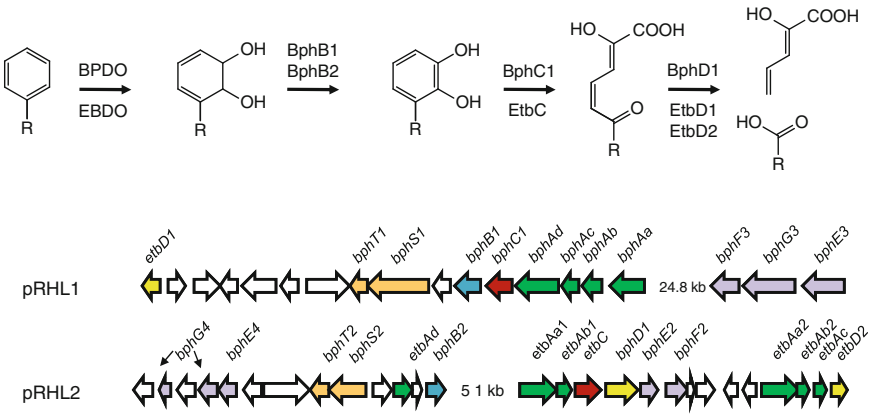

Fig. 5 The proposed catabolic pathways for biphenyl, ethylbenzene, benzene, and styrene. R = phenyl for biphenyl, CH_2CH_3 for ethylbenzene, $CH{=}CH_2$ for styrene, and H for benzene. (In benzene transformation, the resulting catechol is directly degraded by the Cat-PCA branches of the β-ketoadipate pathway.) The enzymatic steps and metabolites of the Bph pathway are depicted in the *upper panel*. The enzymes are: BPDO and EBDO, ring-hydroxylating dioxygenase; BphB1 and BphB2, *cis*-dihydrodiol dehydrogenase; BphC1 and EtbC, extradiol dioyxgenase; BphD1, EtbD1, and EtbD2, *meta*-cleavage product hydrolase. The resulting Hpd is transformed by the Hpd pathway to central metabolites. The *lower panel* summarizes the organization of the pathway genes in clusters located on RHA1 plasmids pRHL1 and pRHL2. Genes are *colored* by function as follows: *green*, Rieske oxygenase components; *blue*, dehydrogenases; *red*, extradiol dioxygenases; *yellow*, MCP hydrolases; *lavender*, Hpd pathway enzymes; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from Patrauchan et al. (2008)

2008; Seto et al. 1995b). Moreover, BPDO of RHA1 shares 98% amino acid sequence identity with isopropylbenzene dioxygenase of *R. erythropolis* BD2 (Stecker et al. 2003), while EBDO of RHA1 shares 100% amino acid sequence identity with *o*-xylene dioxygenase (oXYDO) of *Rhodococcus* sp. DK17 (Kim et al. 2004). oXYDO/EBDO transforms a range of alkylbenzenes (Kim et al. 2007a) and appears to transform larger substrates than BPDO (Iwasaki et al. 2006). Finally, four of the *bphC* homologs of RHA1 are involved in steroid catabolism, as discussed below. In the absence of functional data, it is difficult to know which of the many Bph enzymes that have been identified in rhodococci function primarily in biphenyl catabolism. *R. globerulus* P6, RHA1, and *R. erythropolis* TA421 all clearly contain homologous Bph pathways encoded by similarly organized gene clusters. However, the order and sequences of the *bph* genes in *R. rhodochrous* K37, HA99, and TA431 are clearly different from those in other rhodococci species (Taguchi et al. 2007).

3.2.2 Phthalate and Terephthalate Pathways

Phthalates are widely used as plasticizers to impart flexibility and durability to polyvinyl chloride (PVC) products used in building materials, food packaging,

lubricants, and cosmetics. They are ubiquitous contaminants in food, indoor air, soils, and sediments (Stales et al. 1997). Although toxicity profiles vary according to the phthalate ester, this class of xenobiotic has been implicated in cancer, malformations, and reproductive toxicity in laboratory animals (Gray et al. 1999; Kluwe et al. 1982). The aerobic degradation of phthalate isomers was first reported in pseudomonads in the late 1950s (Ribbons and Evans 1960). Since then, various strains of microorganisms have been found to utilize them as growth substrates (Vamsee-Krishna and Phale 2008). The rhodococcal strains that have been reported to degrade them include *Rhodococcus* sp. DK17 (Choi et al. 2005), *R. erythropolis* S-1 (formerly *Nocardia erythropolis*) (Kurane et al. 1980), and *Rhodococcus* sp. L4 (Lu et al. 2009). *R. rhodochrous* is an interesting case, as it apparently requires hexadecane to degrade various phthalate isomers (Nalli et al. 2002).

RHA1 utilizes both phthalate and terephthalate as growth substrates, degrading them via the Pad and Tpa pathways, respectively (Hara et al. 2007). RHA1 carries two identical sets of *pad* and *tpa* genes on linear plasmids (Fig. 6, lower panel), as does *Rhodococcus* sp. DK17 (Choi et al. 2005). This duplication is required for maximal rates of growth of the latter on phthalate (Choi et al. 2007). The Pad and Tpa pathways are very similar: degradation is initiated by cognate multicomponent RO dioxygenases encoded by *padAaAbAcAd* and *tpaAaAbB*. While both systems comprise large and small oxygenase subunits and a reductase, the phthalate RO system has an additional ferrodoxin component. PadB and TpaC are the respective SDR dihydrodiol dehydrogenases of the pathways (Fig. 6, upper panel). Finally, a decarboxylase, PadC, is required to yield protocatechuate in the Pad pathway. The protocatechuate generated by each of the Pad and Tpa is degraded via the β-ketoadipate pathway. Interestingly, gene knockout and transcriptomic studies indicate that terephthalate can also be transformed to catechol via a bifurcated pathway and can thus feed into the Cat branch of the β-ketoadipate pathway (Hara et al. 2007).

4 Polymeric and Halogenated Aromatic Compounds

The catabolism of lignin, PAHs, and many xenobiotic aromatic compounds is more complex than that described above. Nevertheless, the aromatic nuclei of these compounds are degraded according to the same underlying principles, with some of the resulting metabolites being funneled into peripheral and central aromatic pathways.

4.1 Lignin Degradation

Lignin is the second most abundant polymer in nature after cellulose, comprising 30% of the nonfossil organic carbon (Boerjan et al. 2003) and is arguably the most important aromatic compound in the biosphere (Fig. 7). This polymer is

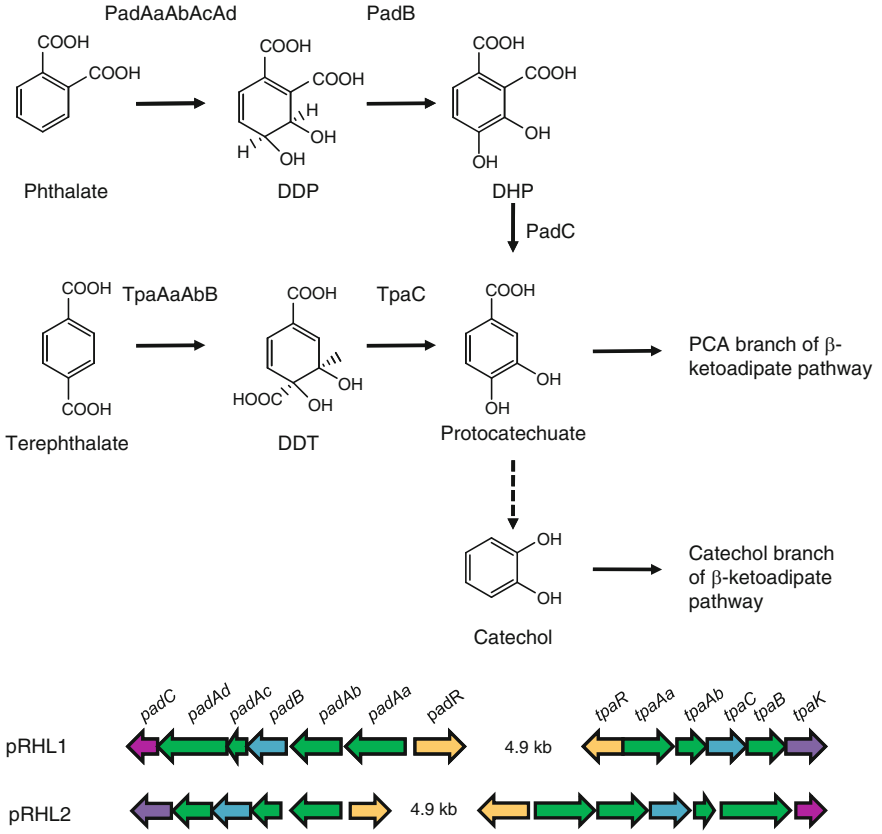

Fig. 6 The degradation pathways for phthalate and terephthalate. The *upper panel* illustrates the degradation of phthalate via the PCA branch of the β-ketoadipate pathway, whereas terephthalate is degraded via a bifurcated pathway that utilizes both PCA and catechol branches. The metabolites are: DDP, *cis*-3,4-dihydroxy-3,4-dihydrophthalate; DHP, 3,4-dihydroxyphthalate; and DTT, *cis*-1,2-dihydroxy-1,2-dihydroterephthalate. The enzymes are: PadAaAbAcAd, a multicomponent ring-hydroxylating 3,4-dioxygenase system; PadB, dihydrodiol dehydrogenase; PadC, decarboxylase; TpaAaAbB, a ring-hydroxylating 1,2-dioxygenase system; and TpaC, dihydrodiol dehydrogenase. The *lower panel* summarizes the organization of the pathway genes in clusters located on RHA1 plasmids pRHL1 and pRHL2. Genes are *colored* by function as follows: *green*, Rieske oxygenase components; *blue*, dehydrogenase; *purple*, decarboxylase; *indigo*, transporters; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from Hara et al. (2007)

synthesized by plants and algae in a radical process from the cinnamyl precursors derived from *p*-hydroxyphenyl, guaiacyl, and syringyl alcohols. The best characterized lignin-degrading organisms are white rot fungi, such as *Phanerochaete chrysosporium* (Gold and Alic 1993), which first break down the polymer into smaller aromatic units using extracellular peroxidases and laccases (Singh and Chen 2008). Lignocellulose is currently of great interest as a feedstock for

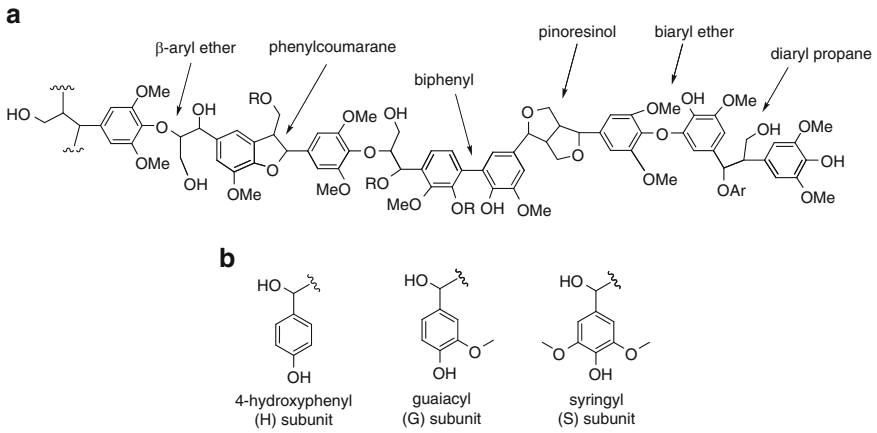

Fig. 7 Structure of lignin complex. (**a**) Schematic of different types of linkages that occur in lignin. (**b**) Three different types of lignin monomers. Adapted from Ahmad et al. (2010)

second-generation biofuel production due to its high energy content, abundance, and renewable status, and represents the most scalable alternative fuel source (Hill et al. 2006). In nature, lignin forms an insoluble, unreactive layer around the energy-rich cellulose, where it plays a role in the vascular system of the plant and in protection from pathogens. Access to the energy stored in the plant material requires breakdown of the lignocellulosic biomass, separation of the cellulose component, and conversion of the fermentable sugars to ethanol (Rubin 2008). Currently, industrial processes for lignin removal are dependent on high heat, pressure, and acid treatments, which tend to be expensive, slow, and relatively inefficient (Ward and Singh 2002). Biological pretreatment for lignocelluloses decomposition is currently being explored.

Bacteria, including rhodococci, have long been recognized to contribute to the mineralization of the lignin break-down products initially generated by fungi (Vicuna et al. 1988; Zimmermann 1990). However, degradation of the lignin polymer by Gram-positive actinomycetes such as *Nocardia*, *Rhodococcus*, and *Streptomyces* has only been observed at a low level, likely due to the heterogeneity of polymeric lignin. Thus, much of the present knowledge of the mechanism of lignin degradation by bacteria has been obtained using lignin model compounds (LMC) as substrates. *R. rhodochrous* (Andreoni et al. 1991) was versatile in utilizing a number of aromatic lignin-related monomers as a sole carbon source. *R. equi* DSM 43349 (Rast and Engelhardt 1980) was able to degrade veratryl-glycerol-β-phenyl ether, a lignin-like synthetic compound. More recently, RHA1 was observed to degrade the lignin polymer (Ahmad et al. 2010). Interestingly, this activity was not dependent on extracellular peroxidases, unlike in other bacterial lignin degraders such as *Streptomyces viridosporus* and *Nocardia autotrophica*.

4.2 Polyaromatic Hydrocarbons

PAHs contain two or more fused benzene rings in linear or cluster arrangements (Fig. 8). The molecular size of PAHs correlates with their lipophilicity, environmental persistence, and toxicity (Jacob et al. 1986). Soil bacterial communities, especially the Nocardioform actinomycetes (e.g., *Rhodococcus, Nocardia,* and *Mycobacterium*), play a crucial role in the mineralization of PAH in contaminated soil (Kästner et al. 1994). However, microbial degradation of PAHs is strongly influenced by a multitude of biotic and abiotic factors, most notably the physical–chemical properties of the PAHs. Lower molecular weight PAHs, such as naphthalene and phenanthrene, are degraded relatively rapidly, whereas higher molecular weight PAHs, such as benz[α]anthracene, chrysene, and benzo[α]pyrene, are more resistant to microbial attack (Cerniglia 1992). While several rhodococcal species can completely mineralize naphthalene, such as B4 and *R. opacus* R7, the metabolic pathway utilized by these Gram-positive organisms differs when compared to the Gram-negatives. In *P. putida* NAH7, naphthalene is degraded to salicylic acid, which is then transformed to central metabolites via catechol. However, in rhodococci, salicylic acid, the common intermediate in naphthalene metabolism, is metabolized to gentisate (Di Gennaro et al. 2001; Grund et al. 1992).

Rhodococcal species are able to metabolize aromatics with up to four rings. For example, *Rhodococcus* sp. UW1 utilizes pyrene, phenanthrene, fluoranthrene, and chrysene as growth substrates and could cometabolize naphthalene, dibenzofuran, fluorine, and dibenzothiophene (Walter et al. 1991). Although complete degradation pathways for four-ringed PAHs have not been described in a *Rhodococcus*, the pyrene and fluorine degradation pathways are likely to resemble the pathways recently described in *Mycobacterium vanbaalenii* PYR-1 (Kim et al. 2007b; Kweon et al. 2007), in which these compounds are eventually transformed to phthalate. Finally, while a small number of bacteria have been reported to degrade PAHs containing more than four rings (Kanaly and Harayama 2000), these do not include rhodococci.

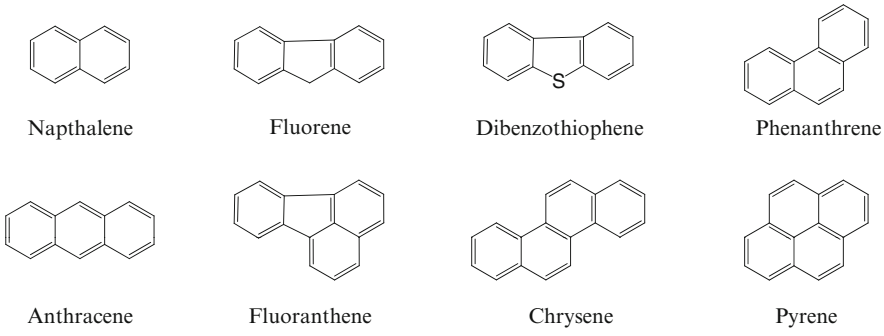

Fig. 8 Structure of various polyaromatic hydrocarbons degraded by rhodococci

4.3 Halogenated Aromatic Compounds

Halogenated aromatic compounds are frequently used in the manufacture of solvents, pesticides, and fire retardants. The substitution of fluorine, chlorine, or bromine on the aromatic ring increases their resistance to microbial degradation. Rhodococci can degrade a wide range of halogenated aromatic compounds, and a number of strains utilize various chlorinated aromatic compounds as sole carbon and energy sources such as *R. percolatus* MBS1 (Briglia et al. 1996), *R. opacus* GM-14 (Zaitsev et al. 1995), *Rhodococcus* strain MS11 (Rapp and Gabriel-Jurgens 2003), and *R. phenolicus* (Rehfuss and Urban 2005). Function studies have established that rhodococci can dechlorinate compounds by either hydroxylation or reduction (Bondar et al. 1999; Haggblom et al. 1988).

R. chlorophenolicus PCP-1 was found to efficiently degrade polychlorinated phenols including penta-, tetra-, and trichloro phenols (Apajalahti and Salkinoja-Salonen 1986, 1987a, b). This strain catalyzes a novel hydroxylation at position 4, regardless of whether a chlorine substituent occupies this position. Dechlorination of penta- and tetrachlorophenols is catalyzed by reductive dehalogenation prior to ring cleavage. Although this strain has been reclassified as a *Mycobacterium* based on mycolic acid analyses (Haggblom et al. 1994), this pathway likely could exist in rhodococci.

4.3.1 PCBs and PBDEs

PCBs and PBDEs are toxic and persistent aromatic compounds that continue to pose an environmental problem. Both compounds usually exist as mixtures of 209 congeners differing in number and position of the halogen substituents. PCBs were once frequently used in the production of plastics and adhesives, but now remain among the most pervasive and recalcitrant of pollutants. They have been linked to cancer, childhood neurological deficits, and endocrine disruption (Cogliano 1998; Walkowiak et al. 2001; Winneke et al. 2002). PBDEs are a class of flame retardants that have been used in a wide variety of manufactured materials (de Wit 2002). PBDEs have varying degrees of chemical and toxicological properties.

Rhodococcal strains capable of degrading PCBs include *R. globerulus* P6 (Asturias et al. 1995), *R. erythropolis* TA421 (Maeda et al. 1995), RHA1 (Masai et al. 1995), *Rhodococcus* sp. M5 (Lau et al. 1996), *Rhodococcus* sp. R04 (Yang et al. 2007b), and three *R. rhodochrous* strains: K37, HA99, and TA431 (Taguchi et al. 2007). Moreover, in a survey of soil microbial populations associated with mature trees growing in a contaminated site, the majority of culturable PCB-degraders were identified as rhodococci (Leigh et al. 2006). As in other aerobic bacteria, PCB degradation often involves their cometabolism by the above-described Bph pathway. Accordingly, the less substituted congeners are subject to dihydroxylation, usually in the 2,3-positions. The extent of further degradation is congener-specific and largely depends on the specificity of the extradiol dioxygenase (Fortin et al. 2005)

and MCP hydrolase (Seah et al. 2001) as has been established in studies of the *R. globerulus* P6 isozymes. The elimination of chlorine or bromine is thought to be a fortuitous event which occurs in later metabolic steps, although the initial dioxygenase can catalyze some dechlorination (Haddock et al. 1995). The potent PCB-degrading properties of RHA1 have been attributed in part to the multiplicity of Bph and Etb isozymes in this strain (Goncalves et al. 2006). Interestingly, EBDO transformed more highly chlorinated, and thus larger, PCB congeners than BPDO (Iwasaki et al. 2006).

The degradation of PBDE has not been as well studied as that of PCBs. However, a recent study established that RHA1 efficiently transforms PBDE congeners containing up to five bromines (Robrock et al. 2009). Analogously to what was observed for the PCB congeners, EBDO transformed more highly brominated congeners than did BPDO.

5 Steroids

Steroids consist of a four-ringed nucleus and a branched alkyl side chain varying in complexity (Fig. 9). Rhodococci have long been known to degrade a range of naturally occurring steroids including cholesterol and phytosterols (van der Geize and Dijkhuizen 2004), although the ubiquity of this ability in this genus is only now becoming clear. In one study, 16 rhodococcal isolates were found to utilize cholesterol as a growth substrate, including strains of *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. fascians*, and *R. rhodnii* (Watanabe et al. 1986). Natural estrogens, including 17β-estradiol, estrone, and estriol, were also found to be degraded by strains of *R. equi* and *R. zopfii* (Yoshimoto et al. 2004). Recent genomic studies have demonstrated that RHA1 contains four clusters of genes potentially encoding distinct catabolic pathways (McLeod et al. 2006). Of these, the cholesterol catabolic pathway (Fig. 10a) is the best characterized (van der Geize et al. 2007). *R. opacus* B4, *R. erythropolis* PR4. erythropolis SK121, and *R. equi* 103S are all predicted to encode this pathway. Indeed, B4 and PR4 carry two additional gene

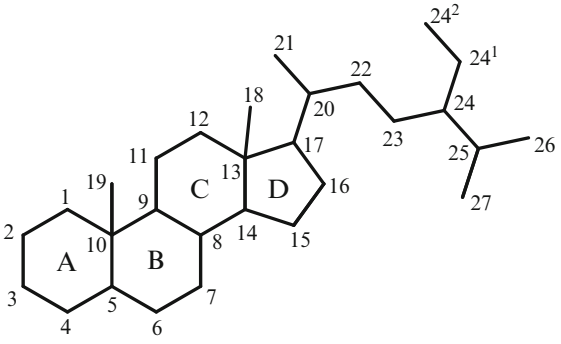

Fig. 9 The steroid skeleton. Rings are labeled A to D. Carbons are numbered according to established nomenclature of steroids

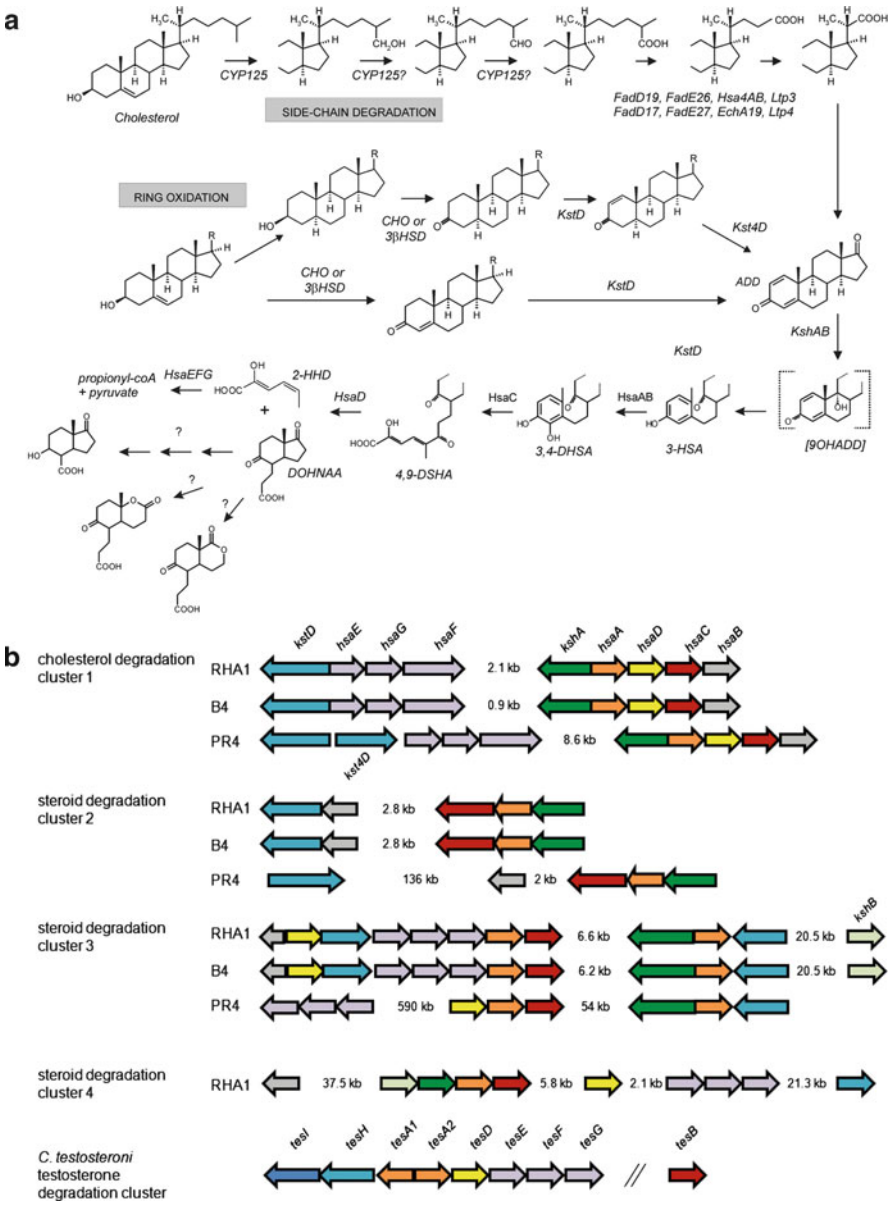

Fig. 10 (**a**) The proposed degradation of the cholesterol side chain and rings A and B. *Brackets* indicate nonenzymatic hydrolysis. CYP125, a steroid 26-monooxygenase, is thought to perform the C26 hydroxylation and oxidations to yield the acid intermediate. The metabolites are: AD, 4-androstene-3-17-dione; ADD, 1,4-androstadiene-3,17-dione; 9-OHADD, 9α-hydroxy-1,4-androstadiene-3-17-dione; 3-HSA, 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 3,4-DHSA, 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 4,9-DSHA, 4,5-9,10-diseco-3-hydroxy-5-9-17-trioxoandrosta-1(10),2-diene-4-oic acid; 2-HHD, 2-hydroxy-hexadieno-ate; DOHNAA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. The enzymes are: CHO,

clusters initially identified in RHA1 (Fig. 10b). Overall, it appears that steroid degradation is a common, perhaps ubiquitous, characteristic of rhodococci.

A common intermediate in bacterial steroid degradation is 4-androstene-3,17-dione (AD) (Fig. 10a). Nevertheless, it is unclear whether side-chain degradation precedes all ring degradation steps or whether these two processes occur concurrently. Recent evidence indicates that the oxidation of the alkyl side chain occurs before transformation of 3-hydroxysterols to 3-oxo sterols (Rosloniec et al. 2009), but it is unknown whether full cleavage of the side chain is necessary before ring degradation can begin. Moreover, cultures of *R. rhodochrous* IFO3338 are capable of selective side-chain cleavage of sterols in the presence of Fe^{2+}-chelating agents, chemically inactivating enzymatic ring degradation resulting in the accumulation of pharmaceutically interesting intermediates such as C-22-oic acid steroid catabolites and 1,4-androstadiene-3,17-dione (ADD) (Arima et al. 1978). In the following text, we summarize first the sterol uptake and then, side-chain degradation and ring degradation.

5.1 Uptake of Sterols

The transport of cholesterol and some other sterols across the cell membrane, periplasm, and thick mycolic-acid-containing cell wall of rhodococci is performed by a multicomponent ATP-dependent uptake system encoded by the *mce4* locus (Mohn et al. 2008). This locus comprises an 11-gene operon consisting of *supAB, mce4ABCDEF, mceHI*, and *ro04706*. SupAB, Mce4ABCDEF, and Mce4HI, all are essential components of the system that transports steroids that have a long, hydrophobic side chain such as cholesterol, 5α-cholestanol, 5α-cholestanone, and β-sitosterol. The SupAB proteins constitute the permease subunits. The roles of Mce4ABCDEF and MceHI in steroid transport remain unknown. However, each of the six Mce4ABCDEF proteins has a signal sequence, suggesting that they are located outside of the cell. Moreover, the homologous Mce1ABECDEF proteins of

Fig. 10 (Continued) cholesterol oxidase; 3βHSD, 3β-hydroxysteroid dehydrogenase; KstD, 3-ketosteroid-Δ1-dehydrogenase; KshAB, 3-ketosteroid-9α-hydroxylase (oxygenase and reductase); HsaAB, 3-HSA hydroxylase (oxygenase and reductase); HsaC, 3,4-DHSA dioyxgenase; HsaD, 4,9-DSHA hydrolase; and HsaEFG, 2-HHD hydratase, aldolase, and dehydrogenase, respectively. Further transformation of DOHNAA is unknown. 2-HHD is degraded by the lower Bph pathway to central metabolites. Adapted from van der Geize et al. (2007). (**b**) The gene organization of the steroid catabolic pathways in *R. jostii* RHA1, *R. opacus* B4, *R. erythropolis* PR4 and *C. testosteroni* TA441. Genes are *colored* by function as follows: *blue*, dehydrogenase; *green*, Rieske oxygenase; *orange*, hydroxylase; *red*, extradiol dioxygenase; *yellow*, MCP hydrolase; *lavender*, lower Bph pathway enzymes; *gray*, reductase; and *pale green*, reductase component of KshA. *Numbers* between genes correspond to the intergenic distance. Identity of the other steroids degraded by the other rhodococcal gene clusters is unknown

M. tuberculosis have been localized to the cell envelope (Shimono et al. 2003). Finally, these six Mce proteins have a shared sequence predicted to fold into five β-strands and eight α-helices, suggestive of a common function (Casali and Riley 2007). The ATPase driving the RHA1 Mce4 transporter may be either Ro01974 or Ro02744, which are orthologs of the ATPase MceG of *M. tuberculosis* H37Rv. The *mce4* locus is conserved in *R. equi* 103S (Meijer and Prescott 2004; van der Geize et al. 2008). Inactivation of the *supAB* genes impaired growth of *R. equi* on cholesterol, but did not affect the intracellular survival of the pathogen (van der Geize et al. 2008). Similarly, cholesterol import by *M. tuberculosis* H37Rv was found not to be required for establishing infection in mice or for growth in resting macrophages, but does appear to be important for persistence of the pathogen (Pandey and Sassetti 2008).

Although Mce4 takes up cholesterol, it appears that not all steroids are taken up by an Mce system and not all Mce systems take up steroids. Thus, while the RHA1 genome encodes two complete Mce systems and four steroid degradation pathways, described further below, the *mce4* cluster is the only one that is proximal to a steroid degradation gene cluster. A similar result is obtained by analyzing the other rhodococcal genome sequences, which revealed the presence of five complete *mceABCDEF* gene clusters in PR4 and six in B4. Overall, it appears that sterols that do not have a long hydrophobic side chain are taken up by other transport systems.

5.2 Side-Chain Degradation

Microbial side-chain degradation was first observed in a strain of *Nocardia*, where cholesterol was poorly transformed to C22-oic acid pathway intermediates, AD and ADD (Whitmarsh 1964). Most notably, the complete pathway by which the cholesterol side chain is removed, resulting in the 17-keto substituent, was elucidated in *Nocardia* (Sih et al. 1968). Subsequently, *Mycobacterium* sp. mutants were isolated that selectively degraded the cholesterol side chain to produce AD and ADD without transforming the steroid rings (Marsheck et al. 1972). The accumulation of AD and ADD suggests that the side-chain degradation occurs prior to sterol ring degradation. In general, microorganisms appear to shorten the side chain (C-21 to C-28) of sterols such as stigmasterol, β-sitosterol, campesterol, and cholesterol (Fig. 10a) by a mechanism similar to that of β-oxidation of fatty acids (Szentirmai 1990).

Recent molecular genetic and spectroscopic data indicate that a cytochrome P450, CYP125, initiates sterol side-chain degradation by catalyzing the oxidation of C-26 (Rosloniec et al. 2009). CYP125 was found to bind tightly to cholesterol and 5α-cholestane-3β-ol, and a *cyp125* knockout in RHA1 was impaired in growth on cholesterol and other 3-hydroxysterols with long aliphatic side chains (Rosloniec et al. 2009). It is unclear whether CYP125 oxidizes C-26 to the carboxylic acid or whether dehydrogenases are involved after an alcohol is formed. Once formed, this acid is activated by an acyl-CoA ligase that is CoA-, ATP-, and magnesium-dependent (Chen 1985). Following CoA activation, dehydrogenation of C-24 and

C-25 occurs, mediated by an acyl-CoA dehydrogenase, followed by hydration of the double bond by an enoyl-CoA hydratase. Subsequent dehydrogenation of the C24-hydroxy moiety, catalyzed by a β-hydroxyacyl-CoA dehydrogenase, and thiolytic cleavage result in shortening of the cholesterol side chain with the release of propionyl-CoA and acetyl-CoA in the first and second cycles of β-oxidation, respectively. The remaining three-carbon side chain of the C-22-oic acid is thought to be released via aldolytic fission. Degradation of the C24-branched side chains of sterols such as β-sitosterol requires cleavage of the C-24 substituent. β-Sitosterol side-chain degradation is initiated by C-28 carboxylation and subsequent CoA-activation (Fujimoto et al. 1982). After dehydrogenation and hydration, the C24-branched chain is released as propionyl-CoA by a reverse-aldol reaction. Further side-chain degradation occurs as in cholesterol. RHA1 has multiple sets of genes that encode the types of enzymes necessary to perform β-oxidation, and many are highly upregulated during growth on cholesterol (van der Geize et al. 2007). Two sets of β-oxidation enzymes are predicted to perform the cycles of β-oxidation, resulting in the formation of the C-24 and C-22-oic acid intermediates (Fig. 10a) (van der Geize et al. 2007).

5.3 Nucleus Degradation

While aspects of steroid degradation have been well documented in several microorganisms, including those of the genera *Nocardia* (Sih et al. 1967), *Pseudomonas* (Owen et al. 1983), and *Mycobacterium* (Marsheck et al. 1972), genes for steroid degradation have only recently been identified. Steroid catabolic genes were first identified in *Comamonas testosteroni* TA441 (Horinouchi et al. 2004). Shortly thereafter, genomic studies of RHA1 led to the identification of four clusters of genes potentially encoding distinct steroid-degrading pathways, one of which is specific for cholesterol (McLeod et al. 2006; van der Geize et al. 2007). These pathways are all predicted to involve aromatization of ring A and are rich in oxygenases (Fig. 10a). Rhodococcal steroid catabolism appears to be analogous to the process in other actinobacteria, and our knowledge of the process is based on studies of several genera.

Degradation of the steroid nucleus is initiated by either an NAD^+-dependent 3β-hydroxysteroid dehydrogenase (3β-HSD) (Yang et al. 2007a) or an O_2-dependent cholesterol oxidase (CHO) (MacLachlan et al. 2000). In either case, the 3β-hydroxy-Δ^5 sterol is oxidized to a 3-keto-Δ^5 intermediate, which then spontaneously isomerizes to the 3-keto-Δ^4 configuration. In the case of cholesterol, 4-cholestene-3-one is produced. Rhodococci displaying moderate cholesterol-degradation activity were found to possess both extracellular and intracellular oxidase activity (Aihara et al. 1986). Further metabolism of AD to 9α–hydroxy-1,4-androstadiene-3,17-dione (9-OHADD) involves two enzymes: 3-ketosteroid-Δ^1-dehydrogenase (KstD), a flavoprotein, catalyzes C-1(2)-dehydrogenation of ring A; and 3-ketosteroid-9α-monooxygenase (KshAB), a two-component RO, catalyzes the 9α-

hydroxylation. The order of these two steps does not appear to be obligate (van der Geize et al. 2000, 2002a, 2002b). The ratio of the respective intermediate metabolites 1,4-androstadiene-3,17-dione (ADD) and 9α-hydroxy-4-androstene-3-17-dione (9-OHAD) produced by KstD or KshAB, respectively, from AD is unknown.

Four phylogenetically distinct types of KstD enzymes have been identified in actinobacterial genomes, three of which were characterized from *R. erythropolis* SQ1 (Knol et al. 2008). Two of these, KstD1 and KstD2, were shown to display broad substrate specificities towards diverse 3-ketosteroids. By contrast, the KstD3-type enzymes are highly specific 3-keto-5α-steroid Δ^1-dehydrogenases, displaying highest activity towards 5α-androstane-3,17-dione and 17β-hydroxy-5α-androstane-3-one (Knol et al. 2008). Interestingly, the cholesterol catabolic gene cluster of RHA1 contains a KstD3, but neither a KstD1 nor a KstD2, suggesting that 5α-H steroids having saturated A-rings may be intermediates in the degradation of sterols (Fig. 10a) (Knol et al. 2008). While it is unclear why such an intermediate would occur during sterol degradation, it is possible that it is formed by a steroid Δ5-reductase to prevent steroid ring degradation from occurring prior to complete side-chain degradation. Located next to *kstD3* on the *R. erythropolis* SQ1 chromosome is a gene encoding a probable 3-ketosteroid-Δ^4-(5α)-dehydrogenase, Kst4D, homologous to TesI of *C. testosteroni* TA441 (Horinouchi et al. 2003a). TesI catalyzes the desaturation of the C4–C5 bond in sterol A ring, a step that is required for the degradation of 5α-H steroids. Thus, Kst4D appears to be involved in cholesterol degradation via a 5α-H steroid intermediate (Fig. 10a) (Knol et al. 2008). As with the 3-ketosteroid Δ^1-dehydrogenases, several phylogenetically distinct groups of 3-ketosteroid Δ^4-dehydrogenases can be distinguished in actinobacterial genomes. TesI and Kst4D belong to one phylogenetic type, whereas the RHA1 genome appears to encode a single Δ^4-dehydrogenase (Ro05698) of a different type (Knol et al. 2008).

KshAB transforms both AD and ADD, as demonstrated by molecular genetic studies in *R. erythropolis* SQ1 (van der Geize et al. 2002b). Purified KshAB of *R. rhodochrous* DSM43269 catalyses the NADH-dependent 9α-hydroxylation of a range of steroids (Capyk et al. 2009; Petrusma et al. 2009), although the *M. tuberculosis* enzyme has higher specificity for ADD than AD, producing 9-OHADD (Capyk et al. 2009). The latter undergoes aromatization and cleavage of ring B via a nonenzymatic reverse-aldol reaction to produce 3-hydroxy-9,10-secondandrost-1,3,5(10)-triene-9,17-dione (3-HSA). Ring A of 3-HSA is hydroxylated by a two-component oxygenase and reductase (HsaAB), requiring molecular oxygen and NADH, to yield a catecholic 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) and subsequently cleaved by an extradiol dioxygenase (HsaC). 3,4-DHSA was deduced as a metabolite in the catabolism of AD by *N. restrictus* ATCC 14887 nearly 50 years ago, based on the limited knowledge of bacterial degradation of aromatic compounds (Sih et al. 1965). An MCP hydrolase then cleaves the C-5:C-6 bond of 4,5-9,10-diseco-3-hydroxy-5-9-17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) through addition of water, resulting in 2-hydroxyhexadienoate (2-HHD) and 9,17-dioxo-1,2,3,4,10,19-

hexanorandrostan-5-oic acid (DOHNAA) as products. 2-HHD is transformed to central metabolites via a homolog of the Hpd pathway involving the successive actions of a hydratase (HsaE), an aldolase (HsaF), and an acetaldehyde dehydrogenase (HsaG). Degradation of the propionate moiety of DOHNAA occurs via a cycle of β-oxidation, first proposed by Lee and Sih (1967) and supported by subsequent studies (Miclo and Germain 1990, 1992). The first step in DOHNAA degradation in *R. equi* is suggested to involve ATP-dependent CoA activation, followed by reduction of the 5′-keto moiety by a DOHNAA-CoA reductase. The CoA activation is required for reduction (Miclo and Germain 1990). The rhodococcal enzymes involved in degradation of the propionate moiety of DOHNAA have yet to be identified. It is predicted that ring D of DOHNAA is degraded by a Baeyer–Villiger monooxygenase and a lactone hydrolase (Ro06698 and Ro06693 in RHA1, respectively) (McLeod et al. 2006). While these genes are upregulated during growth on cholesterol, they are not clustered with the other cholesterol catabolic genes.

Each of the four clusters of steroid degradation genes in RHA1 codes for homologs of each of the four enzymes which together cleave rings B and then A: KstD, KshAB, HsaAB, and HsaC (Fig. 10b), although it is unknown which steroids are degraded by the enzymes encoded in each cluster. The KshA homologs encoded in each gene cluster share at least 52% amino acid sequence identity with KshA of *R. erythropolis* SQ1 (van der Geize et al. 2002b). Indeed, many of these proteins share significant sequence similarity with the Tes proteins that specify growth of *C. testosteroni* TA441 on testosterone (Fig. 10b). For example, each of the four HsaC homologs of RHA1 shares greater sequence identity (at least 37%) with the TesB dioxygenase of *C. testosteroni* TA441 (Horinouchi et al. 2003b) than with any other extradiol dioxygenase.

A notable feature of the rhodococcal steroid degradation pathways is their apparent redundancy. This is perhaps most striking for *hsaEFG*, which encodes a pathway homologous to the Hpd central aromatic pathway involved in the degradation of biphenyl. Three of the four RHA1 clusters encode homologs of HsaEFG to transform 2-HHD to central metabolites. This redundancy is in contrast to the organization of many aromatic pathways. For example, a single β-ketoadipate pathway transforms catechol and protocatechuate generated from a range of aromatic compounds.

6 Conclusion and Prospects

It is clear from the number and range of publications relating to *Rhodococcus* in recent years that the genus is of considerable interest in a wide variety of fields (Fernandes et al. 2003; Martinkova et al. 2009). The metabolic activities of this genus underline the latter's tremendous potential for bioremediation and as biocatalysts in the production of bioactive molecules. The currently sequenced genomes provide important insights into the aerobic degradation of aromatic compounds and pollutants. However, our understanding of how these pathways are regulated and

how we might exploit them for industrial applications is still nascent. The large genome of RHA1 consists of many copies of catabolic genes which are organized in complex pathways (McLeod et al. 2006). Gene redundancy, multiple gene activation, and diversity of metabolic pathways have allowed members of the rhodococcal genus to use mixtures of aromatic compounds as growth substrates. However, this very property has hampered efforts to engineer strains for bioremediation that also survive well in a changing environment (Cases and de Lorenzo 2005). While several transcriptional regulators (Eulberg and Schlömann 1998; Iida et al. 2009; Nga et al. 2004) are associated with rhodococcal catabolic pathways, the mechanisms of gene expression are not fully understood. Regardless, undesirable pathways and genes can be tightly controlled in a number of ways as a result of advances in the tools for rhodococcal genetic engineering. For example, the construction of plasmid vectors for gene transfer in *R. erythropolis* CCM 2595 enabled the study of the P-*catA* and P-*catR* promoters of the β-ketoadipate pathway (Vesely et al. 2003, 2007). Similarly, the development of unmarked gene deletion techniques for constructing multiple gene deletion mutants in *Rhodococcus* should facilitate the further characterization of the catabolic pathways of aromatic compounds and steroids (van der Geize et al. 2000, 2008). Ultimately, greater knowledge of rhodococcal physiology, genetics, and enzymology will contribute to engineering improved transformation of a vast array of aromatic compounds for industrial and environmental purposes.

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Catabolism of Nitriles in *Rhodococcus*

Ludmila Martínková, Miroslav Pátek, Alicja Barbara Veselá, Ondřej Kaplan, Bronislava Uhnáková, and Jan Nešvera

Contents

Abstract The enzymes of nitrile catabolism in *Rhodococcus* include nitrilases and nitrile hydratases/amidase systems. According to their cofactor, nitrile hydratases are classified into Fe-type and Co-type subfamilies, which are typically produced by *Rhodococcus erythropolis* and *Rhodococcus rhodochrous*, respectively. The latter species is also the typical source of nitrilases, most of which strongly prefer

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aromatic substrates. The organization of the nitrilase, nitrile hydratase, amidase and relevant regulatory genes, and mechanisms of their expression control are shown. The unique structural and physico-chemical properties of these enzymes (subunit aggregation; Fe-type nitrile hydratase photoreactivity) are described. The overview of nitrile-converting enzyme applications emphasizes their use in the biodegradation of aliphatic nitriles and benzonitrile herbicides. The significant potential of these enzymes as biocatalysts for the production of bulk and fine chemicals is also presented. The suitability of different preparation methods for whole cells and enzymes is discussed. Finally, analytical methods for monitoring nitrile biotransformations are summarized.

1 Introduction

Nitriles (organic cyanides) occur widely in nature (Legras et al. 1990), mainly as cyanoglycosides, which release cyanohydrins when acted on by hydroxynitrile lyases (oxynitrilases) or spontaneously upon tissue damage. In addition to complex molecules (cyanoglycosides, cyanolipids), simple nitrile molecules are present in organisms (Jallageas et al. 1980), for example, 3-indolylacetonitrile (auxin precursor), 2-phenylacetonitrile, and 3-phenylpropionitrile in plants or cyanohydrins in plants and insects. The intermediates in the biosynthesis of cyanoglycosides seem to be aldoximes, which, in turn, are derived from amino acids. In microorganisms, aldoxime dehydratase (Kato et al. 2005) participates in the formation of nitriles from aldoximes.

Synthetic nitriles are frequently used in the chemical industry and agriculture. They can be prepared via a variety of synthetic routes and are, in turn, converted into amides, amines, acids, and other compounds. As a result, they are popular intermediates for organic syntheses. As a consequence of the common usage of various nitriles in industry and agriculture, a number of nitrile compounds are released into the environment. Nitriles mostly exhibit harmful or toxic effects on humans and the environment, and therefore there is an increasing requirement for the removal of such contaminations. Bioremediation of the polluted soil and water utilizing the degradative capabilities of bacteria has become a generally accepted procedure.

The significant potential of *Rhodococcus* strains to catabolize nitriles has drawn attention to these bacteria. Applications of the *Rhodococcus* nitrile-converting enzymes in biocatalysis and bioremediation are based on the remarkable ability of these bacteria to transform a vast number of man-made nitriles. This enables rhodococci to be used for the synthesis of bulk and fine chemicals or synthons and for the removal of acrylonitrile, acetonitrile, benzonitrile analogs, and other widespread nitrile contaminants from waste materials and the environment.

The major pathway of nitrile catabolism in microorganisms is hydrolysis leading to carboxylic acids as the primary products, which may be further metabolized. The conversion of nitriles to carboxylic acids proceeds via one of the two distinct

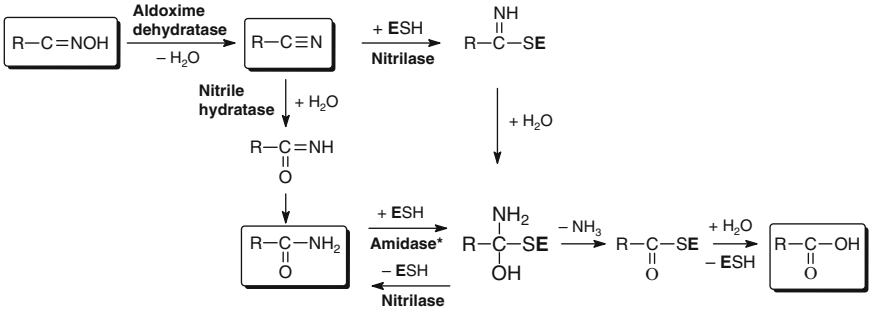

Fig 1 Pathways of nitrile metabolism in rhodococci. Stable compounds are *boxed*. *E* enzyme; *Asterisk* amidases belonging to nitrilase superfamily

mechanisms: direct hydrolysis catalyzed by nitrilases or a cascade pathway catalyzed by nitrile hydratases and amidases (Fig. 1). Despite the fact that nitrilases and nitrile hydratases operate on the same group of substrates, they are genetically unrelated and attack the nitrile group via different mechanisms. Nitrilases belong to the nitrilase superfamily of hydrolases (third EC class), while nitrile hydratases are lyases (fourth EC class) containing a catalytically active metal ion. The major products of nitrilases and nitrile hydratases are also different – carboxylic acids and their amides, respectively, though nitrilase may also form an amide as a side product.

Direct nitrile hydrolysis with a nitrilase was first reported in the 1960s. Nitrilases were first purified and characterized in a bacterium classified as *Pseudomonas* sp. (Hook and Robinson 1964) and in barley (Thimann and Mahadevan 1964). Later, the genus *Rhodococcus* was found to be a promising source of diverse nitrilases with differing substrate specificities (Harper 1976; Kobayashi and Shimizu 1994). Two decades later, nitrile hydratase was first reported in a strain designated *Arthrobacter* sp., which, however, was later reclassified as *Rhodococcus rhodochrous* (see Asano 2002 for a review). Subsequent studies indicated the *Rhodococcus* genus to be probably the best source of different types of this enzyme bearing either a Fe^{3+} or a Co^{3+} cofactor (see Banerjee et al. 2002 for a review).

In the past decade, the extensive literature on nitrile-converting enzymes has been reviewed from a number of perspectives. The work of Sugai et al. (1997) summarized the applications of these enzymes in organic syntheses. This review was later updated by Martínková and Křen (2002) and Martínková and Mylerová (2003). The structural and catalytic properties of these enzymes have been reviewed by Kobayashi and Shimizu (2000) and Banerjee et al. (2002). The reviews by Bunch (1998) and Martínková et al. (2009a) specialized in the genus *Rhodococcus*, focusing on its applications in nitrile biotransformation and bioremediation, respectively. The current state of nitrilase studies was presented by O'Reilly and Turner (2003) and Singh et al. (2006). A survey of the stereoselective biotransformations of nitriles has also been published (Wang 2005). Another recent review provided an overview of nitrile-converting enzyme screens and assays (Martínková et al. 2008).

The aim of this chapter is first to summarize the distribution of nitrile-converting strains in the *Rhodococcus* genus and to describe the organization and regulation of the relevant genes, as well as the biochemical properties of enzymes participating in the nitrile catabolism of these bacteria. The following sections are focused on the applications of rhodococci in biodegradation of the most important nitrile pollutants (acetonitrile, acrylonitrile, and benzonitrile analogs used as herbicides) and in biocatalysis. Finally, a brief overview of methods used for monitoring nitrile biotransformation is provided.

As a result of refining the taxonomy of the genus *Rhodococcus*, a number of nitrile-converting bacteria have been reclassified since their first description. The classification of each strain is given according to the latest available data in this review.

2 Occurrence of Nitrile-Converting *Rhodococcus* Strains

Nitrile-converting enzymes (nitrilases and nitrile hydratases) occur in a number of the *Rhodococcus* strains, primarily in *Rhodococcus rhodochrous* and *Rhodococcus erythropolis* species. The nitrilase-producing rhodococci mostly belong to *R. rhodochrous*. Two major types of nitrile hydratases, namely Fe- and Co-type enzymes, exist in rhodococci. Most of the Fe-type nitrile hydratases have been found in *R. erythropolis*, while Co-type has been isolated from *R. rhodochrous*. Rhodococci harboring both nitrilases and nitrile hydratases (*R. rhodochrous* strains J1 and PA-34) have also been reported.

2.1 Strain Selection

Enrichment processes for nitrile-converting microorganisms consist of repeated subcultivations of mixed microbial populations on a nitrile as the sole nitrogen source and final isolation of the dominant strains. The enrichment substrate and composition of the medium can be chosen according to the parameters of the target biotransformation procedure. It is notable that the strains selected in this way often show high specific activities toward their enrichment substrates (Layh et al. 1997). Nitrile converters belonging to other genera have been selected when different types of enrichment substrates are used, for example, rhodococci on benzonitrile, 2-phenylbutyronitrile, 2-(2-methoxyphenyl)propionitrile or naproxen nitrile (ibid.).

Nevertheless, enrichment techniques are not applicable to substrates that inhibit bacterial growth. Structurally similar substrates can sometimes be used in those cases. For instance, a strain of *R. rhodochrous* catalyzing the hydration of acrylonitrile, which did not serve for growth of the organism, was isolated by enrichment with acetonitrile (Asano 2002). An alternative approach is an "acclimation culture" requiring long-term runs, which is applicable in cases where the desired

microorganisms cannot be easily obtained by an enrichment culture due to the toxicity or unnatural character of the substrate (ibid.). In this way, a strain of *Rhodococcus* sp. harboring the "aldoxime-nitrile pathway" (aldoxime dehydratase – nitrile hydratase – amidase) was obtained after a 4-month acclimation in the presence of *E*-pyridine-3-aldoxime (Kato et al. 1998).

Though some nitrile degraders have been isolated from contaminated environmental sources (Tables 1 and 2), there is no indication that a choice of sites preexposed to nitriles should be necessary for the successful enrichment of these strains.

2.2 Nitrilase-Producing Strains

Most of the known nitrilase producers in the *Rhodococcus* genus have been classified as *R. rhodochrous*, except for a strain classified as *R. ruber* and a few strains not classified into species (designated *Rhodococcus* sp.) (Table 1). Nearly all the enzymes have been unambiguously identified as nitrilases following their purification. As for the nitrilase from *Rhodococcus* sp NDB 1165, the absence of an amide intermediate during nitrile hydrolysis and no activity for amides indicated that this strain also produced a nitrilase but no nitrile hydratase/amidase system (Prasad et al. 2007).

Irrespective of the environmental source, geographic origin, or enrichment substrate (aromatic or aliphatic), seven of the eight enzymes showed similar substrate specificities, preferring aromatic nitriles. Hence, according to the classification of Kobayashi and Shimizu (1994), they could be designated aromatic nitrilases. The aliphatic nitrilase from *R. rhodochrous* K22 differed from all these enzymes not only in substrate specificity but also in its temperature and pH optima (see Sect. 4.1). The aromatic nitrilase from *R. rhodochrous* J1 (Kobayashi et al. 1989) exhibited a 55% amino acid identity with the aliphatic nitrilase from *R. rhodochrous* K22 (Kobayashi et al. 1990) and differing degrees of amino acid identity with a number of other nitrilases and related hydrolases from rhodococci.

2.3 Nitrile Hydratase-Producing Strains

A number of strains of *R. erythropolis* harbor the genes encoding nitrile hydratases bearing the Fe^{3+}-cofactor (Table 2), which all seem to be highly conserved ($\geq$90% identity in most cases). Another strain harboring a highly similar nitrile hydratase has been identified as *R. globerulus*. Nevertheless, differences in the catalytic behavior of Fe-type nitrile hydratases were observed, which could be ascribed either to differing amino acid residues located near the active site (Brandão et al. 2003) or different methods used for the enzyme assays (Kubáč et al. 2008). Some of

Table 1 *Rhodococcus* strains harboring nitrilase

Species	Strain	Environmental source (Geographic origin)	Enrichment substrate	Type of enzyme	Reference(s)
R. rhodochrous	J1	Soil	Acetonitrile	Aromatic	Nagasawa et al. (1988)
	K22	Soil	Crotononitrile	Aliphatic	Kobayashi et al. (1990)
	LL100-21[a]	Barnyard soil	Acetonitrile	Aromatic	Vaughan et al. (1989)
	NCIMB 11215[b]	Soil of a bromoxynil-treated field (County Down, UK)	*p*-Hydroxybenzonitrile	Aromatic	Harper (1985)
	NCIMB 11216[b]	Mud (River Lagan, Belfast, UK)	Benzonitrile	Aromatic	Harper (1977a)
	PA-34	Soil	Propionitrile	Aromatic	Bhalla et al. (1992)
R. ruber	NCIMB 40757	Soil	Acetonitrile[c]		Webster et al. (2001)
Rhodococcus sp.	ATCC 39484			Aromatic	Stevenson et al. (1992)
	NDB 1165	Forest soil (Himachal Pradesh, India)	Propionitrile	Aromatic	Prasad et al. (2007)

[a]Previously classified as *Nocardia rhodochrous* (DiGeronimo and Antoine 1976)
[b]Previously classified as *Nocardia* sp. (*Rhodochrous* group; Harper 1977a)
[c]In the presence of acrylonitrile

Table 2 *Rhodococcus* strains harboring nitrile hydratases

Species	Strain	Environmental source (Geographic origin)	Enrichment substrate	Type of enzyme	Reference(s)
R. erythropolis	67-BEN001[a]	Marine sediment (Japan Trench, JP)	Benzonitrile	Fe-type[b]	Brandão et al. (2003), Heald et al. (2001)
	A4[c]	Contaminated soil (Prague, CZ)	3-Cyanopyridine	Fe-type[b]	Přepechalová et al. (2001), Kubáč et al. (2008)
	AJ270[d,e]	Soil from disused industrial site (Tyne, UK)	Acetonitrile	Fe-type	Blakey et al. (1995), Song et al. (2007)
	ANT-AN007[a]	Lake sediment (Antarctica)	Acetonitrile	Fe-type[b]	Brandão et al. (2003)
	BL1	Marine sediment (DK)	Acetonitrile		Langdahl et al. (1996)
	C3II (DSM 9685)	Soil (Stuttgart, DE)	Naproxen nitrile		Layh et al. (1997)
	MP50 (DSM 9675)	Soil (Stuttgart, DE)	Naproxen nitrile		Layh et al. (1997)
	N-771[d,e]	Soil		Fe-type	Nagamune et al. (1990a, b)
	N-774[d,e]	Soil	Acetonitrile	Fe-type	Watanabe et al. (1987)
	NCIMB11540				Osprian et al. (2003), Vink et al. (2006)
	R-312(CBS 717.73)[d,e]	Soil	Acetonitrile	Fe-type	Arnaud et al. (1976a, b)
R. globerulus	A-4	Soil		Fe-type	Xie et al. (2003)
R. jostii	RHA-1	Lindane-contamintated soil		Fe-type[b]	Masai et al. (1995) Okamoto and Eltis (2007)

(*continued*)

Table 2 (continued)

Species	Strain	Environmental source (Geographic origin)	Enrichment substrate	Type of enzyme	Reference(s)
				Acetonitrile hydratase	
R. pyridinivorans	S85-2		Acetonitrile	Co-type	Kohyama et al. (2006)
R. rhodochrous	J1	Soil	Acetonitrile	Co-type	Nagasawa et al. (2000)
	IFO 15564				Yokoyama et al. (1996)
	LL100-21[f]	Barnyard soil	Acetonitrile	–	DiGeronimo and Antoine (1976), Linton and Knowles (1986)
	PA-34	Soil	Propionitrile		Bhalla et al. (1992)
Rhodococcus sp.	7	Soil	Acetonitrile		Kaakeh et al. (1991)
	OP8E	Soil (Stuttgart, DE)	2-Phenylbutyronitrile		Layh et al. (1997)
	OP5M	Soil (Stuttgart, DE)	2-(2-Methoxyphenyl) propionitrile		Layh et al. (1997)
	YH3-3	Soil		Co-type	Kato et al. (1998)

No entry – no data available

[a]A number of strains harboring highly homologous nitrile hydratases were isolated from terrestrial soils and marine sediments (Brandão et al. 2003)

[b]According to amino acid sequence homology with enzymes whose cofactor was determined

[c]Previously classified as *Rhodococcus equi* (Martínková et al. 1995)

[d]The nitrile hydratases from these strains are identical according to deduced amino acid sequences and differ in the same three amino acids per subunit from the enzymes of *R. globerulus* A4, *R. erythropolis* A4, *R. erythropolis* 67-BEN001, and *R. erythropolis* ANT-AN007

[e]Strains AJ270, N-774, and R-312 were previously identified as *Brevibacterium* sp. (strain R-312) or *Rhodococcus* sp. (others) but reidentified as *Rhodococcus erythropolis*. This probably also applies to the N-771 strain, which is very similar to N-774 (Brandão et al. 2003)

[f]Previously classified as *Nocardia rhodochrous* (DiGeronimo and Antoine 1976)

these enzymes are among the best-characterized nitrile hydratases with unique physico-chemical properties (see Sect. 4.2).

Nitrile hydratases bearing the Co^{3+} cofactor have been reported in *R. rhodochrous* and in a strain designated *Rhodococcus* sp. In *R. rhodochrous* J1 (Table 2), two subtypes of the enzyme occur, low- and high-molecular-mass nitrile hydratase, which also differ in their substrate specificities (Wieser et al. 1998). These enzymes have been characterized biochemically and sequenced (Kobayashi et al. 1991a). Comparing the Co- and Fe-type nitrile hydratases, the α-subunits show a higher degree of identity than the β-subunits. This is not suprising due to the role of the α-subunit in coordination of the metal ion (see Sect. 4.2.1).

A new nitrile hydratase containing three types of metal ions (Co, Cu, Zn) has recently been described in *R. jostii* RHA1 (Okamoto and Eltis 2007). This enzyme (acetonitrile hydratase) acts solely on aliphatic nitriles. There is no homology between this protein and the above nitrile hydratases.

3 Gene Organization and Regulation

Progress in uncovering the genetic basis of nitrile-converting enzymes in microorganisms has accelerated in recent years because of the rapid development of gene- and genome-sequencing techniques. Most of the genes coding for nitrile catabolism enzymes have so far been isolated by the purposeful cloning of chromosomal restriction fragments or PCR-amplified fragments into plasmid vectors using nitrile catabolizing strains as gene sources. The identification of nitrile catabolism genes organized in operons or gene clusters within the complete bacterial genomes and parallel genomics-driven research provide other approaches, which enable discoveries of very different enzymes sharing little sequence similarity with known enzymes.

3.1 Nitrilase Genes

The *nitA* gene coding for isovaleronitrile-induced nitrilase was isolated from *R. rhodochrous* J1. Expression of the modified *nitA* gene cloned in a plasmid under the *lacZ* promoter resulted in the production of an extreme amount of nitrilase (as much as 50% of the total soluble protein) in *E. coli* (Kobayashi et al. 1992a). Another nitrilase gene found in *R. rhodochrous* K22 could also be induced by isovaleronitrile and the *R. rhodochrous* cells produced as much as 20% of their total soluble proteins as nitrilase (Kobayashi et al. 1991b). This gene was cloned in a plasmid and also efficiently expressed in *E. coli* from the *lacZ* promoter. The synthesized *R. rhodochrous* K22 nitrilase (55% identity with *R. rhodochrous* J1 nitrilase) formed 40% of the total soluble proteins in *E. coli* (Kobayashi et al. 1992c). A nitrilase with 56% identity was also found in the related strain *Nocardia* sp. C-14-1 (Acc. No. AAX18182) whereas analysis of the complete genomes of *R. jostii* RHA1 and

R. opacus B4 revealed potential nitrilases with much lower similarity (less than 20%). In *R. rhodochrous* J1, the *nitR* gene, coding for a positive transcriptional regulator of the AraC family essential for *nitA* expression, was found downstream of the *nitA* gene. Cotranscription of the two genes in a single mRNA in the *R. rhodochrous* J1 cells has been induced by isovaleronitrile from the *nitA* promoter (Kobayashi et al. 1992c).

3.2 Nitrile Hydratase Genes

All known nitrile hydratases from rhodococci consist of two subunits (α and β). However, Fe-type and Co-type nitrile hydrolases represent two distinct evolutionary branches of nitrile-hydrolyzing enzymes sharing low level of similarity. Likewise their amino acid sequences, organization of the respective genes coding for the α and β subunits, and location of genes for other enzymes involved in aldoxime → nitrile → amide → carboxylic acid metabolic pathway are different. Completely different genes for nitrile hydratase subunits containing Co, Cu, and Zn ions have recently been described in *R. jostii*.

3.2.1 Fe-Type Nitrile Hydratase

Genes coding for the α- and β-subunits of Fe-type nitrile hydratase were detected in a large number of *Rhodococcus* strains from geographically distinct areas (Brandão et al. 2003; Precigou et al. 2001). In most cases, Fe-type nitrile hydratase genes (*nha1*, *nha2*) are linked to the amidase gene (*ami*) (Hashimoto et al. 1991; Mayaux et al. 1990; O'Mahony et al. 2005). Moreover, the gene coding for aldoxime dehydratase (*oxd*) was also found in proximity to the *ami* and *nha* genes in some rhodococci (Kato et al. 2005). The genes involved in the aldoxime–nitrile–amide catabolic pathway thus form a cluster in these strains. Such gene organization has been found by a sequence inspection in the *R. erythropolis* PR4 genome (Acc. No. AP008957). The *oxd*, *ami*, *nha1*, and *nha2* genes are closely grouped with four regulatory genes, all oriented in the same direction, thus forming an operon-like structure (Fig. 2). The genes *nhr4* (AraC-type transcriptional regulator), *nhr2*, and *nhr1* most probably code for regulators involved in the expression of the *oxd–ami–nha* genes. The *nhr3* gene codes for a nitrile hydratase activator, which is probably involved in the correct folding of the protein and/or incorporation of Fe into nitrile hydratase (Nojiri et al. 1999; Yamada and Kobayashi 1996). Homologous gene clusters with the same structure and overall 95% nucleotide identity were found in *R. erythropolis* A4 (Acc. No. AM946017), which was used as a source of immobilized enantioselective nitrile hydratase (Kubáč et al. 2008; Martínková et al. 2009a; Přepechalová et al. 2001) and in *R. globerulus* A-4 (Acc. No. AB105912) used for *oxd* (alkylaldoxime dehydratase) isolation and overexpression (Xie et al. 2003). The *oxd–ami–nha* clusters from *Rhodococcus* sp. N-771 and N-774 are also closely similar to the clusters from these rhodococci, exhibiting 94% overall nucleotide identity with the

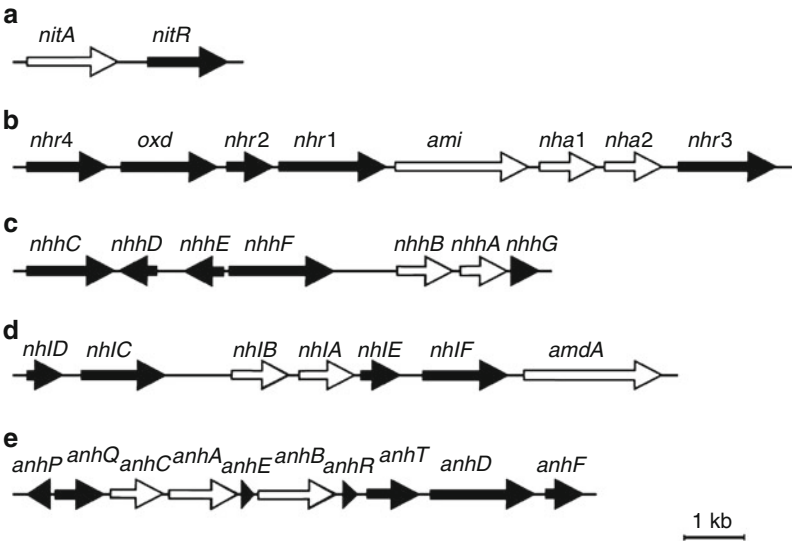

Fig 2 Organization of the genes involved in nitrile catabolism from various *Rhodococcus* strains. Genes encoding nitrilase, amidases, and subunits of nitrile hydratases are shown by *empty arrows*; (**a**) Nitrilase genes (*R. rhodochrous* J1): *nitA* (nitrilase), *nitR* (transcriptional regulator of AraC family) (Komeda et al. 1996a); (**b**) Fe-type nitrile hydratase genes (*R. erythropolis* PR4, A4; *R. globerulus* A-4): *nhr4* (transcriptional regulator of AraC family), *oxd* (aldoxime dehydratase), *nhr2* (regulator), *nhr1* (regulator), *ami* (amidase), *nha1* (α-subunit of nitrile hydratase), *nha2* (β-subunit of nitrile hydratase), *nhr3* (nitrile hydratase activator) (Martínková et al. 2009a; Xie et al. 2003); (**c**) Co-type nitrile hydratase genes, H-NHase (*R. rhodochrous* J1): *nhhC* (regulator), *nhhD* (regulator of MarR family), *nhhE* (unknown function), *nhhF* (transposase), *nhhB* (β-subunit of nitrile hydratase), *nhhA* (α-subunit of nitrile hydratase), *nhhG* (β-subunit of H-NHase homolog) (Komeda et al. 1996b); (**d**) Co-type nitrile hydratase genes, L-NHase (*R. rhodochrous* J1): *nhlD* (repressor), *nhlC* (activator), *nhlB* (β-subunit of nitrile hydratase), *nhlA* (α-subunit of nitrile hydratase), *nhlE* (β-subunit of H-NHase homolog), *nhlF* (cobalt uptake transporter), *amdA* (amidase) (Kobayashi et al. 1992b, 1993; Komeda et al. 1996c, 1997); (**e**) Co–Cu–Zn-type nitrile hydratase genes (*R. jostii* RHA1): *anhP* (transcriptional regulator), *anhQ* (transcriptional regulator), *anhC* (amidase), *anhA* (α-subunit of nitrile hydratase), *anhE* (unknown function), *anhB* (β-subunit of nitrile hydratase), *anhR* (transcriptional regulator), anhT (cobalt transporter), *anhD* (acetyl-CoA hydrolase), *anhF* (transposase) (Okamoto and Eltis 2007)

R. erythropolis PR4 genes (Endo et al. 2001; Kato et al. 2004). A nitrile hydratase/amidase gene cluster was also identified on the chromosome of nitrile metabolizing strains *R. erythropolis* AJ270 and AJ300 and *Microbacterium* sp. AJ115, all isolated from the same industrial site. A copy of insertion sequence IS1166 disrupting the *nhr2* gene was detected upstream of the *nhr1* gene within the sequence of the three strains. The presence of an ORF homologous to the transposase IS666 from *Mycobacterium avium* in this region and the identity of the three clusters suggest that the genes were acquired by horizontal gene transfer. Highly similar linked *ami–nha1–nha2* genes were also described in *Rhodococcus* sp. R312 (Bigey et al. 1999). The function of the putative regulatory genes (*nhr1*, *nhr2*, and *nhr4*) in the *oxd–ami–nha* gene clusters is not apparent, since expression of the nitrile hydratase and

amidase genes is inducible in some cases (e.g., induction by acetonitrile or acetamide in *R. erythropolis* AJ270) whereas it is constitutive in other strains (e.g., in *Rhodococcus* sp. N-771 and *Rhodococcus* sp. R312). The nitrile hydratase from *Rhodococcus* sp. N-771 was only functional in *E. coli* when the downstream *nhr3* activator gene was cloned and expressed (using the T7 expression system) together with *nha1* and *nha2* (Nojiri et al. 1999). The same requirement was found in *Rhodococcus* sp. N-774 (Hashimoto et al. 1994). The genes for the nitrile hydratase from *R. erythropolis* AJ270 and the downstream activator (named P44K and exhibiting 97% identity with the Nhr3 protein) provided the maximal nitrile hydratase activity when cloned separately in two plasmids in *E. coli*.

The enzymes coded by *oxd–ami–nha* genes in the above-mentioned rhodococci share a very high level of similarity (95–98%). The *oxd*, *ami*, and *nha* genes in the chromosome of *R. jostii* RHA1 (McLeod et al. 2006) are also organized in a cluster together with a gene for an AraC-type regulator (in opposite orientation) but the *nhr1* and *nhr2* regulatory genes are missing and the level of amino acid identity in enzymes with those from *R. erythropolis* PR4 is lower (79–90%) (Acc. No. CP000431). No genes coding for nitrile-converting enzymes were annotated in the 7.9-Mb *Rhodococcus opacus* B4 genome (Acc. No. AP011115).

3.2.2 Co-Type Nitrile Hydratase

The best-characterized Co-type nitrile hydratases come from *R. rhodochrous* J1 (Kobayashi et al. 1991a). There are two types of these nitrile hydratases, high-molecular-weight (H-NHase) and low-molecular-weight nitrile hydratase (L-NHase), which were purified from *R. rhodochrous* J1 cultures grown in the presence of urea and cyclohexane carboxamide, respectively (Kobayashi et al. 1992a; Komeda et al. 1996c; Nagasawa et al. 1991; Wieser et al. 1998). The subunits of H-NHase are encoded by the genes *nhhA* and *nhhB*, clustered with the genes *nhhC*, *nhhD*, *nhhE*, *nhhF*, and *nhhG* on the chromosome of *R. rhodochrous* J1 (Fig. 2c) (Komeda et al. 1996b). The *nhhC* (homolog of the *amiC* gene for the regulator of an amidase gene from *Pseudomonas aeruginosa*) and *nhhD* (regulator of the MarR family) code for essential positive regulators involved in the expression of *nhhA* and *nhhB*. The short *nhhG* gene is homologous to the H-NHase β-subunit gene (*nhhB*). The organization of H-NHase genes from *R. rhodochrous* J1 differs from that of the nitrile hydratase genes reported in other strains. The β-subunit gene is located upstream of the α-subunit gene, and these genes are not linked to the amidase gene. Instead, the insertion sequence IS1164 coding for a transposase (*nhhF*) is present upstream of the H-NHase genes. This suggests that events connected to horizontal gene transfer and/or gene rearrangement occurred during its evolution (Komeda et al. 1996b). The genes for H-NHase subunits are transcribed together with the downstream *nhhG* gene. Expression of these genes is induced by urea and various amides (e.g., acetamide and acrylamide).

Genes homologous to *nhhA*, *nhhB*, *nhhC*, *nhhD*, and *nhhG* genes are present in a cluster in *R. rhodochrous* M8 (Acc. No. AAT79339). However, *nhhE* and *nhhF* are

missing in this M8 strain sequence. Homologous H-NHase genes were also found in *R. pyridinovorans* (Kohyama et al. 2006). The *nhhA* and *nhhB* genes from *R. rhodochrous* J1 were cloned in a plasmid and expressed in *E. coli* from the inducible *lac* promoter (Kobayashi et al. 1991a). The H-NHase from *R. rhodochrous* J1 was employed in the industrial production of acrylamide from acrylonitrile (Kobayashi et al. 1992b) and nicotinamide from 3-cyanopyridine (Nagasawa et al. 1988).

Another Co-type of nitrile hydratase, L-NHase, was also discovered in *R. rhodochrous* J1. The *nhlB* and *nhlA* genes coding for the β- and α-subunits of L-NHase are linked to the *amdA* (amidase) gene, similarly to Fe-type nitrile hydratase genes. However, they are arranged in reverse order (*nhlBA*) and are separated from *amdA* by the genes *nhlE* (nitrile hydratase β-subunit homolog) and *nhlF* (transporter involved in cobalt uptake) (Fig. 2d). The genes *nhlD* and *nhlC* are located upstream of the *nhlBA* genes and code for negative and positive regulators, respectively (Komeda et al. 1996c). They are required for the amide-dependent expression of *nhlBA*. NhlD is similar to the MerR, CadC, and ArsR repressors, which regulate the heavy metal resistance systems, whereas NhlC is similar to the AmiC regulator (amide sensor protein) responsible for the expression of amidase in *Pseudomonas aeruginosa*, to the NhhC regulator responsible for H-NHase formation in *R. rhodochrous* J1 as well as to the Nhr1 regulator involved in the expression of genes coding for the Fe-type nitrile hydratase in many *Rhodococcus* strains. The NhlC activator inhibited the action of the NhlD repressor when the growth medium was supplemented with amide, and the *nhlBA* genes were expressed constitutively when *nhlD* was deleted. It was shown that *nhlBA* and *amdA* are regulated by the presence of amide in the same manner (Komeda et al. 1996c).

3.2.3 A Novel Type of Nitrile Hydratase

Genes coding for the α- and β-subunits of a novel nitrile hydratase requiring Co, Cu, and Zn ions for its activity were discovered in the large *R. jostii* RHA1 plasmid pRHL2 (442.5 kb) (Okamoto and Eltis 2007). The *anh* gene cluster (Fig. 2e) codes for amidase (*anhC*), subunits of nitrile hydratase (*anhA* and *anhB*), and acetyl-CoA hydrolase (*anhD*). Other genes of the cluster code for three regulatory proteins and a possible cobalt transporter (*anhT*). There is no homology between AnhA, AnhB, and AnhC and the nitrile hydratases and amidases from other rhodococci. In contrast to other nitrile hydratases, the α- and β-subunits of this novel nitrile hydratase share significant sequence similarity (32%). Expression of three of the genes (*anhB*, *anhC*, and *anhD*) was induced by acetonitrile, as was demonstrated by a proteome analysis of cells grown on this nitrogen source.

3.3 Amidase Genes

Most of the amidase and nitrile hydratase genes are linked, regulated, and transcribed together. A mutant amidase of *Rhodococcus* sp. R312 (strain ACV2)

producing adipic acid from adiponitrile faster than the wild-type strain has been characterized (Azza et al. 1993). Northern blot analysis showed that the amidase and nitrile hydratase genes form an operon transcribed from the *amdA* promoter in the ACV2 strain (Bigey et al. 1995). The *amdA–nhaAB* gene cluster was cloned in the *Rhodococcus* – *E. coli* vector and expressed in *Rhodococcus* sp. A4. A deletion analysis showed that the upstream and downstream regions are required for *amdA* expression. Amidase activity was threefold higher in *E. coli* than in the ACV2 *Rhodococcus* strain when the genes were cloned in an *E. coli* plasmid vector and expressed from the T7 promoter (Bigey et al. 1995). Expression of the amidase gene from *Microbacterium* AJ115 (Doran et al. 2005), identical to the *ami* gene from *Rhodococcus* AJ270 (O'Reilly and Turner 2003) in *E. coli* allowed *E. coli* to grow on acetamide as the sole carbon and/or nitrogen source. The recombinant amidase formed 28% of the total soluble protein in *E. coli* (Doran et al. 2005). The *amdA* gene coding for an enantioselective amidase, which converts aromatic amides, was found on the 40-kb plasmid in *R. erythropolis* MP50 (Trott et al. 2002). The *amdA* gene is not linked to the nitrile hydratase genes and is surrounded by two copies of an insertion element, which suggests that the amidase gene is a part of a transposon structure. The amidase from *R. erythropolis* MP50 has a low similarity to other amidases from other rhodococci (about 30%). The amidase gene was successfully expressed in *E. coli* from an L-rhamnose inducible promoter (Trott et al. 2002). The *ami* gene from another *R. erythropolis* strain was expressed in *E. coli* and *Bacillus subtilis*, the latter organism providing a more efficient expression system controlled by a powerful promotor. The amidase encoded by this gene proved to be useful for the conversion of acrylamide into acrylic acid, a building block of acrylate polymers (Yue et al. 2009).

4 Enzyme Structural and Catalytic Properties

A significant number of the nitrilases and nitrile hydratases encoded by the *Rhodococcus* genomes have been characterized biochemically, and the catalytic potential of these enzymes has been evaluated. The nitrile-converting enzymes widely differ in both structural and catalytic properties. On the one hand, this has practical implications for the use of these enzymes for mild nitrile hydrolysis, since the broad choice of different enzymes increases the chance to find the required biocatalyst. On the other hand, the nitrile-converting enzymes from rhodococci share some disadvantages, such as low activities toward bulky nitriles and rather low temperature stability.

4.1 Nitrilase

The unique properties of nitrilases, primarily their variable quaternary structure and their ability to produce two types of metabolites (carboxylic acids and amides) have attracted attention of both basic and applied research studies. Two types of

these enzymes were detected in rhodococci: aromatic and aliphatic nitrilases, which prefer aromatic and unsaturated aliphatic nitriles as substrates, respectively. The third nitrilase type, namely the arylaliphatic nitrilase, found in other bacterial genera, has not been reported in genus *Rhodococcus*.

4.1.1 Structural Variability

There are significant structural similarities between the proteins of the nitrilase superfamily (Pace and Brenner 2001), some of which have been crystallized, such as the Nit domain of the NitFHit fusion protein from *Caenorhabditis elegans* (Pace et al. 2000), hypothetical CN hydrolase from *Saccharomyces cerevisiae* (Kumaran et al. 2003), *N*-carbamoyl-D-amino acid amidohydrolases from *Agrobacterium radiobacter* (Nakai et al. 2000; Chen et al. 2003), amidase from *Geobacillus pallidus* (Kimani et al. 2007), formamidase from *Helicobacter pylori* (Hung et al. 2007), and the hypothetical protein PH0642 from *Pyrococcus horikoshii* (Sakai et al. 2004). This appears to be helpful in nitrilase modeling, as certain structural motifs seem to preclude the formation of three-dimensional nitrilase crystals (Thuku et al. 2007). Crystallographic analysis of the aforementioned proteins showed the presence of the catalytic Glu–Lys–Cys triad in their active sites.

The nitrilases in rhodococci are multimeric proteins containing a single type of subunit, which is generally an α/β protein with a four-layer αββα sandwich architecture as in other members of the nitrilase superfamily. The native nitrilases from rhodococci are oligomers consisting of 2 to approximately 16 subunits of 40–46 kDa (see Banerjee et al. 2002 for a review), while an active monomer has been reported for *R. rhodochrous* PA-34 (Bhalla et al. 1992). The most detailed structural characterization has recently become available for *R. rhodochrous* J1 (Thuku et al. 2007). Surprisingly, the active nitrilase purified from the native organism was reported to be a dimer (Kobayashi et al. 1989), but what was purified from the heterologous host was a mixture of an inactive dimer and an active dodecamer of about 480 kDa visible as short "c-shaped" particles in micrographs recorded by electron transmission microscopy (Thuku et al. 2007). The formation of a higher multimeric structure could be probably explained by the high salt concentration used, as the effects of various factors such as salts, organic solvents, temperature, or enzyme concentration on enzyme oligomerization have been demonstrated (Nagasawa et al. 2000).

The heterologously expressed protein formed molecular species of up to 1.5 MDa after 1 month of storage at 4°C. These clusters were enzymatically active and observed in electron micrographs as left-handed helical rods of various lengths (Thuku et al. 2007). Similar helical filaments have also been described in nitrilases from filamentous fungi (Vejvoda et al. 2008), cyanide hydratase (Woodward et al. 2008), or cyanide dihydratases (Jandhyala et al. 2003; Sewell et al. 2003). The formation of these extended helices in *R. rhodochrous* J1 was preceded by the removal of 39 amino acids from the C-terminal part of the nitrilase molecule, the cleavage probably catalyzed by the enzyme itself (Thuku et al. 2007).

4.1.2 Chemoselectivity

A unique catalytic property of nitrilases is their ability to form two products, a carboxylic acid and its amide. The hypothetical reaction mechanism (O'Reilly and Turner 2003) involves a nucleophilic attack on the cyano carbon by the cystein sulfhydryl group leading to the covalent enzyme–thioimidate complex that is subsequently hydrolyzed to give the tetrahedral intermediate. The decomposition of this intermediate may occur either via ammonia release, thus leading to acylenzyme, or via an atypical cleavage with the enzyme as the leaving group, yielding the amide (Fig. 1). The latter pathway mainly occurs with substrates bearing electron-withdrawing and bulky substituents, which destabilize the positive charge on the reactant nitrogen atom (Fernandes et al. 2006). Amide formation from nitriles seems to be significantly lower in the enzymes from rhodococci than with some other nitrilases, such as those from *Pseudomonas fluorescens* (Fernandes et al. 2006), *Arabidopsis thaliana* (Oßwald et al. 2002), or *Aspergillus niger* (Kaplan et al. 2006), since it has only been reported in *Rhodococcus* sp. ATCC39484, which produced phenylacetic acid and phenylacetamide at a ratio of about 98:2 from 2-phenylacetonitrile (Stevenson et al. 1992). However, it has to be stressed that this phenomenon has not been examined in detail with rhodococcal enzymes. Enzyme assays based on ammonia detection were used for the most-characterized nitrilases from rhodococci – *R. rhodochrous* strains J1 (Kobayashi et al. 1989), K22 (Kobayashi et al. 1990), PA-34 (Bhalla et al. 1992), NCIMB 11215 (Harper 1985) and NCIMB 11216 (Harper 1977a). As a result, the ability of these enzymes to form the side product amide could not be evaluated except for several substrates where the biotransformation was monitored by HPLC or TLC. In those cases (benzonitrile conversion by strains NCIMB 11215 and NCIMB 11216 or 4-hydroxybenzonitrile conversion by strain NCIMB 11215) no amide formation was observed by TLC (Harper 1977a, 1985) but this method may not have detected low concentrations of amides. The occurrence of this phenomenon was also not mentioned for biotransformations of benzonitrile, 3-cyanopyridine, acrylonitrile, methacrylonitrile, or crotononitrile assayed by HPLC in a study of the *R. rhodochrous* J1 nitrilase. If confirmed, the almost exclusive formation of carboxylic acids would be an advantage of the nitrilases from rhodococci over nitrilases from other sources.

4.1.3 Substrate Specificity Subgroups

Nitrilases with a preference for aromatic and heterocyclic nitriles typically occur in two groups of evolutionarily distant organisms – rhodococci and filamentous fungi (for reviews see O'Reilly and Turner 2003; Martínková et al. 2009a, b). The enzyme from *Rhodococcus* sp. ATCC39484 exhibited a high specific activity (84 U mg^{-1} for benzonitrile), which is similar to those of nitrilases from filamentous fungi, but other nitrilases from rhodococci exhibited specific activities that were more than three times as low. The optimum temperature for activity was between 30 and 45°C

for the aromatic nitrilases and 50°C for the aliphatic nitrilase. The latter enzyme also had a different pH optimum (5.5) while the others were most active at pH 7.5 or 8.0.

The best substrates of the aromatic nitrilases are benzonitrile, its analogs bearing electron-withdrawing substituents at *meta*- and *para*-positions (primarily halobenzonitriles) and 3- and 4-cyanopyridine. *ortho*-Substituted benzonitriles or 2-cyanopyridine are mostly inferior substrates of these enzymes, as are bulky molecules like benzonitrile analogs bearing multiple substituents (benzonitrile herbicides; see Sect. 5.4). In contrast to nitrilases from filamentous fungi, there has been no reports on the activity of aromatic nitrilases from rhodococci for saturated aliphatic nitriles. Of the aromatic nitrilases, only the enzyme from *R. rhodochrous* J1 exhibited a high activity for unsaturated aliphatic nitriles – acrylonitrile and crotononitrile – after the association of its subunits in the presence of ammonium sulfate (Nagasawa et al. 2000). On the other hand, some aliphatic nitriles, especially unsaturated nitriles (acrylonitrile, crotononitrile), and dinitriles (adiponitrile, glutaronitrile, succinonitrile) proved to be good substrates of the aliphatic nitrilase from *R. rhodochrous* K22 (Kobayashi et al. 1990).

4.2 Nitrile Hydratase

The unique structures of nitrile hydratases, primarily their iron (nonheme) or cobalt (noncorrinoid) catalytic centers, have been studied intensively. Similar to nitrilases, these enzymes vary in their quaternary structure, which seems to affect their stability. On the one hand, their broader substrate specificity (in comparison with nitrilases), probably caused by their lower sensitivity to steric hindrances, is their main advantage. On the other hand, their lower stability and low stereoselectivity is their general disadvantage.

4.2.1 Structure and Photoreactivity of Fe-Type Nitrile Hydratase

Fe-type nitrile hydratases have been purified and characterized in a few strains of *R. erythropolis* (Endo and Watanabe 1989; Yamada and Kobayashi 1996; Přepechalová et al. 2001; Song et al. 2007; Table 2), their sequences were all highly similar or even identical in some cases, and the crystal structures of two presumably identical enzymes were determined (Nagamune et al. 1991; Huang et al. 1997). One of the best-characterized Fe-type nitrile hydratases is the enzyme from *Rhodococcus* sp. N-771, whose unique photoactivation mechanism has been elucidated using a variety of spectral methods (for reviews, see Endo and Odaka 2000; Endo et al. 2001). Briefly, the α- and β-subunit form a tight heterodimer with a low-spin nonheme iron located at their interface. The metal ion is coordinated by five ligands contained within the α-subunit, three cysteine sulfure atoms, and two main chain nitrogen atoms. In the inactivated nitrile hydratase, nitric oxide is bound to the sixth coordination site. On light irradiation, it is released making the sixth

coordination center available for binding a hydroxide ion that acts as a nucleophile in nitrile hydration. It has been assumed that the hydroxide ion might attack the nitrile carbon atom either directly or via the activation of a water molecule. The αGln90 residue conserved among all known Fe- and Co-type nitrile hydratases plays a crucial role in forming the catalytically important hydrogen bond networks with the iron center, as demonstrated by mutational studies of this enzyme. The k_{cat} of mutants with glutamic acid or asparagine replacing the glutamin residue decreased to 24 and 5% of the wild-type enzyme, respectively (Takarada et al. 2006).

The Fe-type nitrile hydratase exists in a dimer–tetramer equilibrium, the level of aggregation depending on the concentration of the enzyme. Therefore, the apparent molecular weight of the holoenzyme composed of α- and β-subunits (each about 24 kDa) was typically estimated to be 60–70 kDa (Endo and Watanabe 1989; Yamada and Kobayashi 1996; Přepechalová et al. 2001). X-ray crystallography revealed that the two dimers were connected by 50 hydration water molecules to give the dominant tetramer form (Nakasako et al. 1999).

The iron cofactor in this nitrile hydratase could be replaced by a low-spin Co^{3+}-ion following heterologous expression of the genes encoding the α- and β-subunits in a Co-supplemented medium without coexpression of the nitrile hydratase activator encoded in the flanking regions of the nitrile hydratase gene (Nojiri et al. 2000). The initially low activity of the Co-substituted enzyme was increased by incubation with an oxiding agent, potassium hexacyanoferrate, probably due to oxidizing the Co-atom to a low-spin Co^{3+} state and/or modification of αCys-112 to a cysteine-sulfinic acid. It was suggested that the nitrile hydratase activator acted as a molecular chaperone assisting metal insertion into the catalytic center but also oxidized the iron cofactor.

4.2.2 Structure of Co-Type Nitrile Hydratase

Two subtypes of Co-type nitrile hydratases, high-molecular-weight (H-NHase) and low-molecular-weight nitrile hydratase (L-NHase), were purified from *R. rhodochrous* J1 (Nagasawa et al. 1991; Wieser et al. 1998). The structure around the binding site of the noncorrinoid Co^{3+}-ion is similar to the Fe-center in the Fe-subfamily of nitrile hydratases but serine in the active site sequence is replaced by threonine (Payne et al. 1997). Furthermore, a tyrosine residue, which is likely to be responsible for substrate binding, is replaced by tryptophan (Miyanaga et al. 2001). There is no evidence for photoreactivity in Co-type nitrile hydratases.

The L-NHase from *R. rhodochrous* J1 and another Co-type nitrile hydratase from *Rhodococcus* sp. YH3-3 (Kato et al. 1999) seem to have similar quaternary structures to the Fe-type, as they were shown to consist of four and two subunits, respectively. In contrast, the high molecular-weight enzyme is composed of 20 subunits (Nagasawa et al. 1991), which is probably the reason for its higher thermostability (up to 50°C) in comparison with the Fe-type nitrile hydratase and the low-molecular-weight Co-type nitrile hydratase (all unstable above 30–35°C; for a review, see Banerjee et al. 2002).

4.2.3 Substrate Range

Saturated aliphatic nitriles of medium-chain length (propionitrile, butyronitrile) and acrylonitrile are excellent substrates of nitrile hydratases and are converted by these enzymes with high specific activities, typically several hundred units per mg of purified protein (Nagamune et al. 1990b; Wieser et al. 1998). Chloroacetonitrile is usually converted at an even higher rate (ibid.) probably due to the presence of the electron-withdrawing substituent in its molecule. Considering the high degree of amino acid sequence identity of Fe-type nitrile hydratases, similar substrate specificities could be expected. The primary structures of enzymes from strains N-774, N-771, AJ270, and R312 are identical and differ in six amino acids (three per subunit) from that of the *R. erythropolis* A4 enzyme. Therefore, the same or very similar substrate specificities may be expected in all of them. However, the enzymes from *R. erythropolis* strains A4 (Přepechalová et al. 2001; Kubáč et al. 2008) and AJ270 (Song et al. 2007) accepted a broad range of aromatic and arylaliphatic nitriles as substrates, whereas those from strains N-771 or R312 appeared to be rather specific for aliphatic nitriles (Nagamune et al. 1990b; Nagasawa et al. 1986). It has not yet been clarified if this discrepancy has been due to the use of enzymes of varying degrees of purity, to differing experimental conditions or, in the A4 strain, to the effect of a few amino acid substitutions on its substrate specificity. Another reason for this phenomenon may be the variable quaternary structure (dimer vs. tetramer), which is dependent on the enzyme concentration and could influence the catalytic properties of the enzyme.

The Co-type nitrile hydratases differ from the Fe-type subfamily in substrate specificity, namely by their high activities for aromatic nitriles, which may be caused by the above-mentioned tyrosine to threonine substitution. Benzonitrile and cyanopyridines are good substrates of the Co-type nitrile hydratases, especially of the low-molecular-weight enzyme (Wieser et al. 1998).

4.3 Amidase

A number of different amidases have been described in rhodococci. The aliphatic amidase (also designated wide-spectrum amidase) found in *Rhodococcus* sp. R312 is a member of the nitrilase superfamily and its catalytic residue is cystein. The amidases coded by the genes forming operons with nitrile hydratases genes have been designated enantioselective or GGSS signature amidases. These amidases are supposed to contain asparagine and serine instead of cystein in their active sites.

4.3.1 Enantioselective Amidase

All the amidases encoded as nitrile hydratases by the same nitrile operon in the *R. erythropolis* (Mayaux et al. 1990; Hashimoto et al. 1991; Duran et al. 1993; Doran et al. 2005; O'Mahony et al. 2005), *Rhodococcus* sp. (Mayaux et al. 1991)

and *R. globerulus* (Xie et al. 2003) strains show a high degree of amino acid identity (>96%). However, the amidase genetically coupled with the low-molecular-mass Co-type nitrile hydratase in *R. rhodochrous* J1 (Kobayashi et al. 1993) and the amidase from *Rhodococcus erythropolis* MP50 (Hirrlinger et al. 1996) showed a lower degree of amino acid sequence similarity to the above approx. 61% enzymes.

The enzymes lack any metal cofactor and consist of two identical subunits ranging in size from 48 to 55 kDa (except for *R. erythropolis* MP50 amidase consisting of eight 61-kDa subunits). Good to excellent enantioselectivities toward a broad range of 2- or 3-substituted arylaliphatic amides were demonstrated for a number of them (for a review, see Wang 2005).

The enantioselective amidases were previously classified as sulfhydryl enzymes owing to their sensitivity to sulfhydryl reagents (Ciskanik et al. 1995; Kobayashi et al. 1997). Later on, this hypothesis was questioned, as the respective active amino acid residue was not identified in any of the enzymes. The actual active site residues of the amidase from *R. rhodochrous* J1 were suggested to be Asp-191 and Ser-195, rather than the generally accepted Cys-203 residue (Kobayashi et al. 1997). Since aspartic acid and serine residues of this enzyme were also present in the active site sequences of aspartic proteinases, an evolutionary relationship between amidases and aspartic proteinases was suggested.

4.3.2 Short Chain Aliphatic Amidase

The designation of this group of enzymes as short-chain aliphatic amidases derives from their preference for short chain amides (Hirrlinger et al. 1996). The wide-spectrum amidase from *Rhodococcus* sp. R312 can serve as an example of this type of enzyme, exhibiting maximum activity with propionamide and less than 2% activity with butyramide (Thiery et al. 1986). This amidase, which was overexpressed in *E. coli* and *Rhodococcus* sp. R 312 (Azza et al. 1994), consists of six identical subunits having molecular weights of approximately 35 kDa, and is classified as a sulfhydryl enzyme belonging to the nitrilase superfamily.

5 Applications of Nitrile-Converting Enzymes in Biocatalysis and Bioremediation

Enzymes capable of converting large number of synthetic nitriles into amides or acids have been found, and some of these conversions have been applied in industrial scale. These biotransformations, which proceed at moderate temperatures and near neutral pH, are useful alternatives to chemically catalyzed reactions requiring extreme conditions. Moreover, nitrile-converting enzymes are mostly chemoselective, often regioselective, and some of them also stereoselective toward particular substrates. Efficient use of all these favorable properties of

nitrile-converting enzymes is, to certain extent, hampered by their general instability. This poses special requirements for the biocatalyst forms, which should support the enzyme stability during storage and use.

5.1 Biocatalyst Forms

In laboratory-scale studies, resting cell suspensions have mainly been used as sources of nitrile-converting enzymes. This biocatalyst form is readily available and relatively stable. It has been also demonstrated with *R. erythropolis* R312 that lyophilized cells can be used as a stable and easy-to-use biocatalyst for preparative biotransformations at laboratory scale (Osprian et al. 1999). However, immobilization of the biocatalyst is usually required for process scale-up to make product separation easier and, if possible, to recover the enzyme for repeated use.

Most nitrile-converting enzymes of rhodococci are unstable after being released from the cells. Therefore, in most cases, the immobilized biocatalysts were based on whole cells, and only to a lesser extent on cell-free extracts or purified enzymes. Whole cells were adsorbed on Dowex 1 (Colby et al. 2000) or entrapped in various hydrogels such as alginate, pectate, carrageenan (Martínková et al. 1998), agar (Chand et al. 2004), polyvinyl alcohol (Bauer et al. 1996), polyacrylamide (Hughes et al. 1998), a copolymer of polyvinyl alcohol and polyethylene glycol (LentiKats®; Kubáč et al. 2006) or porous dimethyl silicone rings (Roach et al. 2004). Polyvinylalcohol beads containing entrapped cells of *Rhodococcus* sp. could be dried and reswollen without nitrile hydratase activity loss (Bauer et al. 1996).

The range of suitable methods for the immobilization of a subcellular preparation of nitrile-converting enzymes is not as broad. Primarily, a widely applicable type of biocatalyst are "cross-linked enzyme aggregates" (CLEAs; Cao et al. 2000; van Pelt et al. 2008), which were prepared from a cell-free extract of *R. erythropolis* A4 with nitrile hydratase – amidase activities (Kubáč et al. 2008). This method consists of enzyme precipitation with different additives such as salts or organic solvents and cross-linking the resulting physical aggregates with glutaraldehyde or a milder cross-linking agent, dextran polyaldehyde, the latter being also suitable for the immobilization of labile enzymes such as nitrilases or nitrile hydratases (Mateo et al. 2006; Kubáč et al. 2008; van Pelt et al. 2008). The CLEAs of crude amidase from *R. erythropolis* A4 were used to load a continuous stirred membrane reactor (CSMR). This reactor was then employed in the second step of a cascade reaction consisting of the conversion of 4-cyanopyridine into isonicotinic acid and isonicotinamide (the side product) by nitrilase CLEAs and subsequent conversion of isonicotinamide into isonicotinic acid by amidase CLEAs. Thus the final product contained only approximately 0.1% isonicotinamide (Malandra et al. 2009). A similar cascade process was carried out using the aforementioned nitrilase and amidase immobilized on Butyl Sepharose columns operated in tandem (Vejvoda et al. 2006).

5.2 Applications in Biocatalysis

The potential of nitrile-converting enzymes to produce building blocks for pharmaceuticals with a broad spectrum of biological activities can be used for industrial scale applications. Although the majority of these reactions have so far only been performed at laboratory scale, successful examples of industrial processes such as the manufacture of acrylamide from acrylonitrile and nicotinamide from 3-cyanopyridine have already been demonstrated.

The biotransformation of acrylonitrile was established by the Nitto Chemical Industry Co. in 1985 as the first industrial application of a nitrile-converting enzyme and later scaled-up to over 30,000 tons per year (Yamada and Kobayashi 1996). Currently, the process is performed by the Mitsubishi Rayon Co. using immobilized cells of *Rhodococcus rhodochrous* J1 with a stable Co-type nitrile hydratase. The same biocatalyst has been used for the production of nicotinamide (food and feed additive) at a scale of over 3,500 tons per year by Lonza (Shaw et al. 2003). In addition, a few applications of nitrilases have been used to manufacture fine chemicals on a smaller scale, such as 5-hydroxypyrazine-2-carboxylic acid, 5-chloropyrazine-2-carboxylic acid esters, 6-hydroxypicolinic acid (Lonza AG; Liese et al. 2000), (*R*)-mandelic acid or (*R*)-3-chloromandelic acid (Mitsubishi Rayon Co.; Brady et al. 2004).

Numerous papers concerned with enzymatic nitrile hydrolysis at laboratory scale indicate that its commercial use may increase in the future. The biotransformations studied involved the preparation of many valuable compounds whose chemical synthesis is difficult, for instance α-hydroxy acids such as (*R*)-2-chloromandelic acid, the chiral building block of an antithrombotic agent, or (*R*)-2-hydroxy-4-phenylbutyric acid, one of the key intermediates in the synthesis of ACE-inhibitors (Osprian et al. 2003), β-ketoamides (synthetic intermediates of the antibiotic tirandamycin, alkaloids, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists etc.; Gotor et al. 2004), β-amino acids (antifungal antibiotic cispentacin, building blocks of β-oligopeptides and heterocycles; Winkler et al. 2005), unnatural aromatic amino acids (chiral building blocks; Wang and Lin 2002; Chaplin et al. 2004) or α-arylpropionic acids (nonsteroid antiinflammatory agents; Stolz et al. 1998).

5.3 Biodegradation of Aliphatic Nitrile Pollutants

The most widespread aliphatic nitrile pollutants are acetonitrile and acrylonitrile. The bioremediation applications of nitrile-converting rhodococci were therefore primarily focused on these compounds. Strains capable of removing the aliphatic nitrile pollutants efficiently have been studied in model systems.

5.3.1 Acrylonitrile

Strains harboring nitrile hydratases, which generally show high activities for acrylonitrile, can not only be used for acrylamide production but also for the biodegradation of acrylonitrile in wastewaters and at contaminated sites (Table 3). However, the primary product of acrylonitrile biotransformation is acrylamide, which is classified as a neurotoxic compound (LD_{50} (*p.o.*) of 107–203 mg kg^{-1} in rats; EC 2002) and may not be easily degraded by amidases. In fact, acrylamide was detected as the only reaction product in a study of 20 mM acrylonitrile biodegradation by the indigenous topsoil microflora and by *Rhodococcus* sp. AJ270 (Baxter et al. 2006). The bioaugmentation of sterilized topsoil with this organism resulted in the same level of acrylonitrile degradation as that achieved by the indigenous bacterial flora. However, neither bioaugmentation nor glucose addition increased the rate of acrylonitrile degradation in culture broths containing nonsterilized topsoil samples, though *Rhodococcus* sp. AJ270 became stably established within the soil bacterial community.

In view of the toxicity of biotransformation intermediates, rhodococci with nitrilase activity toward acrylonitrile are more convenient for its degradation than strains harboring the bienzymatic pathway, as the product of nitrilase, acrylic acid

Table 3 Degradation of aliphatic nitriles in *Rhodococcus* genus

Substrate (mM)	Organism	Catalyst	Product detected	Reference
Acetonitrile (30)	*R. erythropolis* BL1	Growing cells	Acid[a]; Ammonia	Langdahl et al. (1996)
Acetonitrile (1,000)	Acetonitrile (30–2,000)	Resting cells[b]	Ammonia[c]	
Acetonitrile (20)	*R. erythropolis* ANT-AN007[d]	Growing cells	Acid[a]; Ammonia	Brandão and Bull (2003)
Acetonitrile (20)	Acetonitrile (1,000–6,000)	Resting cells	Ammonia[c]	
Acetonitrile	*R. pyridinivorans* S85-2 + *Brevundimonas diminuta*[e]	Resting cells	Acid (>90%) + amide	Kohyama et al. (2006)
Acrylonitrile (20)	*R. erythropolis* AJ270	Growing cells[f]	Amide	Baxter et al. (2006)
Acrylonitrile vapor	*R. ruber* NCIMB 40757	Immobilized cells[g] placed in a column bioreactor	Acid	Roach et al. (2004)

[a]Occurred transiently
[b]The resting cells also hydrolyzed a number of other aliphatic nitriles such as propionitrile, acrylonitrile, and butyronitrile (30 mM each)
[c]Not analyzed for other products
[d]A number of genetically related strains gave similar results (Brandão and Bull 2003). Resting cells of all these strains exhibited hydrolytic activity for acetonitrile, propionitrile, butyronitrile, and acrylonitrile (50 mM each)
[e]Tandem reactions [(1) Hydration of acetonitrile and (2) Hydrolysis of acetamide]
[f]Bioaugmentation of topsoil and sterile soil
[g]On ImmobaSil™ support

with LD_{50} (*p.o.*) of 2.59 g kg^{-1} (in rats; O'Neil et al. 2006) is much less toxic than acrylamide.

A process using immobilized *R. ruber* cells with nitrilase activity was designed for bioscrubbing acrylonitrile vapors (Roach et al. 2004; Table 3). Whole cells immobilized in porous dimethyl silicone rings were used to fill a water-jacketed column. Water as the scrubbing liquid was recycled through the biocatalyst bed. More than 90% of the acrylonitrile was removed from the vapor stream passing through this biofilter. The same biocatalyst could also be applied in the construction of a biosensor for the detection and quantitation of acrylonitrile in waste streams (Roach et al. 2003).

5.3.2 Saturated Nitriles

For the degradation of propionitrile and butyronitrile, both Fe- and Co-type nitrile hydratases from various strains of rhodococci seem to be promising enzymes, as they generally show high specific activities for these substrates. On the other hand, acetonitrile is hydrolyzed at a much lower rate by nitrile hydratases, except for the novel acetonitrile hydratase from *R. jostii* RHA1 (Okamoto and Eltis 2007).

The biodegradation of acetonitrile has been mainly studied with the strains *R. erythropolis* BL1 (Langdahl et al. 1996) and *R. pyridinivorans* S85-2 (Kohyama et al. 2006; Table 3). Growing or resting cells of *R. erythropolis* BL1 showed a remarkable ability to degrade acetonitrile at concentrations of up to 1 and 2 M, respectively. In agreement with findings on the substrate specificity of most nitrile hydratases, the enzyme activities of this strain for propionitrile and butyronitrile were still markedly higher. Its resistance and degradation ability for acetonitrile was even more pronounced in resting cell suspensions of *R. pyridinivorans* S85-2, which totally consumed this substrate at up to 6 M concentrations. The gene encoding the nitrile hydratase in this strain was highly similar (99.8%) to the H-NHase gene in *R. rhodochrous* J1. Accordingly, the production of the nitrile hydratase in *R. pyridinivorans* was increased by the addition of $CoCl_2$ (Kohyama et al. 2006).

R. erythropolis BL1 was able to mineralize acetonitrile during its growth on this compound. Acetic acid and trace amounts of acetamide were transiently produced, the former intermediate being later utilized as a source of carbon and energy (Langdahl et al. 1996). Several deep-sea and terrestrial isolates, mostly belonging to *R. erythropolis*, behaved in a similar way during their growth on acetonitrile; the primary products acetamide and acetic acid appeared transiently in the medium but were later fully consumed (Brandão and Bull 2003; Table 3). In contrast, considerable amounts of acetamide (3–11%, depending on substrate concentration) were present as the product of acetonitrile conversion by *R. pyridinivorans* cell suspensions. Acetamide is classified as a carcinogenic compound, while acetate is environmentally more benign and readily biodegradable. Therefore, an effective process to remove acetamide was designed, using an acetamide-hydrolyzing isolate classified as *Brevundimonas diminuta*. Two biodegradation steps coupling the

acetonitrile hydration with the enzymatic hydrolysis of acetamide resulted in >90% conversion of 6 M acetonitrile into acetic acid within 10 h (Kohyama et al. 2006). Another organism applicable in acetamide biotransformation is *Rhodococcus rhodochrous* NMB-2, which also hydrolyzed other toxic amides (acrylamide, propionamide). Whole cells of this organism immobilized in agar were used to remove these contaminants from simulated wastewater in a plug flow reactor (Chand et al. 2004).

5.4 Biodegradation of Benzonitrile Herbicides

Benzonitrile herbicides include bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile), their ammonium, sodium or potassium salts and ester analogs, and dichlobenil (2,6-dichlorobenzonitrile). Dichlobenil is a broad-spectrum contact herbicide suitable for use in gardens, orchards, and forest nurseries and for path treatment. Bromoxynil and ioxynil are selective contact herbicides used mainly for the removal of broad-leaved weeds growing on cereal fields (Holtze et al. 2008).

The positions of the halogen atoms on the benzene ring are important for the herbicidal effect. If the halogen atoms in the molecule of dichlobenil were shifted to the 3,5 positions and in bromoxynil and ioxynil to the 2,6 positions, the effectiveness of all these compounds would be substantially lowered. A similar effect on the herbicide activity was observed if the nitrile group was replaced with a carboxyl group (Carpenter and Heywood 1963).

The degradation of benzonitrile herbicides in a natural environment and by isolated microbial cultures has attracted considerable attention (see Holtze et al. 2008 for a review). These studies have been mostly focused on 2,6-dichlorobenzamide, the major metabolite of dichlobenil, because of its significant persistence and frequent occurrence in groundwater. This metabolite possesses a much higher water solubility than the parent compound.

Using a number of isolated bacteria from different genera, dichlobenil was transformed into 2,6-dichlorobenzamide as the dead-end product (Holtze et al. 2008). Among rhodococci, this biotransformation was performed by *R. erythropolis* strains DSM 9675, DSM 9685 (Holtze et al. 2006), AJ270 (Blakey et al. 1995; Meth-Cohn and Wang) and A4 (unpublished results) (Table 4). The first two strains as well as typical strains of other bacteria (*Rhizobium radiobacter*, *Rhizobium* sp., *Pseudomonas fluorescens*) stoichiometrically degraded 0.05 mM dichlobenil within 1.5–6 days. Both this substrate and benzonitrile were converted to amides, but unlike 2,6-dichlorobenzamide, benzamide was further degraded into benzoic acid (Holtze et al. 2006).

These findings on the difficulties of degrading 2,6-dichlorobenzamide correspond to the frequent occurrence of this metabolite in environmental samples. In Denmark, for instance, this compound, along with other pesticides and their metabolites (atrazine, atrazine metabolites, mechlorprop, and dichlorprop), is one of the

Table 4 Degradation of benzonitrile herbicides in *Rhodococcus* genus

Substrate (mM)	Organism	Catalyst/Method	Product	Reference
Dichlobenil (0.05)	*R. erythropolis* DSM 9675 *R. erythropolis* DSM 9685	Growing cells	Amide	Holtze et al. (2006)
Dichlobenil (60)	*R. erythropolis* AJ270	Resting cells	Amide	Meth-Cohn and Wang (1997)
Dichlobenil (0.5)	*R. erythropolis* A4	Resting cells	Amide	Martínková, unpublished results
Bromoxynil; Ioxynil	*R. rhodochrous* NCIMB 11215	Purified enzyme	Acids	Harper (1985)
Bromoxynil; Chloroxynil; Ioxynil (0.5 each)	*R. rhodochrous* PA-34 *Rhodococcus* sp. NDB 1165	Resting cells	Acids	Veselá et al. (2010)

most commonly detected groundwater pollutants (Jacobsen et al. 2005). Because more than 99% of the drinking water in Denmark is taken from groundwater, which undergoes only simple aeration and sand filtration, the use of dichlobenil was banned there in 1997. Nevertheless, 2,6-dichlorobenzamide was still the most often detected groundwater contaminant during a survey in 2006. The presence of 2,6-dichlorobenzamide in groundwaters was also detected in the Netherlands, Germany, Italy, and Sweden (Holtze et al. 2008).

The ability to transform 3,4-dihalo-4-hydroxybenzonitriles (bromoxynil, ioxynil) seemed to be less widespread among microorganisms and typical for the highly specific nitrilase from *Klebsiella pneumoniae* subsp. *ozaenae* (Hsu and Camper 1976; McBride et al. 1986). The nitrilase purified from *Rhodococcus rhodochrous* NCIMB11215, previously characterized as *Nocardia* sp. (*rhodochrous* group) (Harper 1985), transformed bromoxynil and ioxynil at very low rates (V_{max} 1.0 and 1.6% of V_{max} for benzonitrile, respectively). The K_m-values of this enzyme for bromoxynil and ioxynil were much higher than for benzonitrile (8.18, 11.88, and 0.62 mM, respectively). Harper (1977b) also described another nitrilase produced by a filamentous fungus, *Fusarium solani*, a strain isolated from a bromoxynil-treated field, which was able to degrade ioxynil and bromoxynil, but, once again, with a very low relative activity compared to that for benzonitrile (3.9 and 2.2% of V_{max} for benzonitrile respectively).

Resting cells of *Rhodococcus rhodochrous* PA-34 and *Rhodococcus* sp. NDB 1165, known as nitrilase-producing organisms (Table 4; Bhalla et al. 1992; Prasad et al. 2007), transformed bromoxynil, chloroxynil, and ioxynil (0.5 mM each) into the corresponding substituted benzoic acids as the only products, which were hardly converted further (Veselá et al. 2010). The drop in nitrilase activity of whole cells, which was in the order chloroxynil > bromoxynil > ioxynil, probably reflected both steric and electronic effects in the nitrile molecules (increasing bulkiness and decreasing electron-withdrawing effect of the halogen atoms).

The above examination of several nitrilase- and nitrile hydratase-producing rhodococci enabled preliminary conclusions to be made on the potential of these organisms for the degradation of benzonitrile herbicides. All the examined nitrile hydratase producers were very efficient in the transformation of the 2,6-dihalogenated benzonitrile analog dichlobenil. This suggests the possible role of widespread soil rhodococci as degraders of dichlobenil in the natural environment. Nitrile hydratase producers do not appear to be useful for the bioremediation of dichlobenil-contaminated sites, as their product 2,6-dichlorobenzamide may be more hazardous than dichlobenil owing to its higher mobility in soil (Holtze et al. 2007) and still unknown biological effects. On the one hand, the nitrilase-producing rhodococci did not hydrolyze dichlobenil, as the nitrile group in dichlobenil is probably inaccessible to nitrilases because of the *ortho*-positioned chlorines. On the other hand, these strains readily hydrolyzed 3,5-dihalo-4-hydroxybenzonitriles. Therefore, they can be useful for the bioremediation of sites contaminated with 3,5-dihalo-4-hydroxybenzonitriles, converting benzonitrile analogs into less harmful carboxylic acids, which, moreover, may be prone to further degradation as was shown by the mineralization of these herbicides in soils (Holtze et al. 2008).

5.5 Biotransformation Monitoring

The monitoring of both substrates and products is equally important in biocatalytic and bioremediation processes. Product purity is very important in the former case, while the toxicity of the metabolites must be considered in the latter.

A wide range of chromatographic methods (HPLC, GC) are available for monitoring nitriles, carboxylic acids, and amides. Reversed-phase liquid chromatography with UV detection is usually suitable for the determination of aromatic and unsaturated aliphatic nitriles and their reaction products. In contrast, it can hardly be used for saturated aliphatic nitriles or their corresponding amides and acids, as the absorbance of cyano, amido, and carboxy groups in the near UV region is low. These compounds are mostly analyzed by GC coupled with a flame ionization detector.

The analyses of nitriles and the products of their enzymatic conversion can be significantly accelerated without compromising separation or sensitivity by using monolithic columns. For instance, the run time of analysis of benzonitrile and its biodegradation products (benzamide, benzoic acid) on a monolithic column was <3 min (Martínková et al. 2008) and a similar separation was achieved with benzonitrile herbicides and the corresponding amides and acids (Veselá et al. 2010).

The monitoring of nitrile biotransformations catalyzed by resting cells is usually not too complicated, as the primary products are an amide, acid, or a mixture of both and are mostly not converted further. However, the samples to be analyzed may be more complex when studying nitrile metabolism in growing cultures or even in consortia of organisms. In these cases, GC or HPLC with mass spectrometric detection are useful for identifying the products. In addition, the high

sensitivities and low detection limits of these methods are beneficial for monitoring nitrile biodegradation in environmental samples with a low contaminant content.

For quantitating residual contaminant concentrations, specific enzyme-linked immunosorbent assay (ELISA) is also suitable, as, for example, with 2,6-dichlorobenzamide. This method enabled this intermediate to be detected at concentrations $\geq$0.02 μg L^{-1} in groundwater samples and in samples of culture media after mineralization experiments. There were only negligible cross-reactions with the parent compound dichlobenil or its structural analogs (Bruun et al. 2000; Sørensen et al. 2006).

6 Conclusions and Outlook

In comparison with other nitrile degraders, rhodococci possess a number of advantages owing to their set of unique properties: First, the presence of mycolic acids in their cell envelope, and hence simple uptake of hydrophobic nitriles, and the variability of the fatty acid composition of membrane lipids leading to an increased resistance to toxic compounds. The enrichment of nitrile-degrading rhodococci from soils, sediments, or sea water is usually straigtforward and the nitrile-converting enzymes in these strains often form a high proportion of the total protein. The genome of rhodococci contains a significant number of genes coding nitrilases and nitrile hydratase/amidase enzyme systems with various reaction optima and substrate specificities.

On the other hand, the biocatalytic and biodegradation potential of nitrile-converting enzymes from rhodococci also has certain limits such as their low activity for bulky substrates or their instability. In biocatalytic applications, low enantioselectivity is often seen as a serious drawback of a number of these enzymes. New methods and tools for the genetic engineering of rhodococci are promising for the directed evolution and rational design of enzymes with improved catalytic properties.

To date, few biocatalytic processes using nitrile-converting enzymes have been commercialized but further applications of nitrile biotransformation have the potential for scale-up. There have probably been even fewer applications of nitrile-converting rhodococci in bioremediation (for overview see Tables 3 and 4). The latter are certainly more difficult to develop as the organisms and enzymes have to act under harsh and variable conditions. At contaminated sites or during waste treatment, the organisms may not survive in the presence of the competing microflora or toxic contaminants or at unfavorable pH, temperature, and ionic strength. They also may not produce the desired enzyme activities, as the expression of these genes may be regulated in a different way or the respective enzymes may not function under different conditions. The monitoring of these processes is also more difficult than with biocatalytic reactions operating at high substrate concentrations and yielding a limited number of products. Hence, more

studies are needed to make better use of the nitrile biodegradation potential of rhodococci.

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The Desulfurization Pathway in *Rhodococcus*

Ting Ma

Contents

Abstract The emission of sulfur oxides can have harmful effects on the environment. Biodesulfurization of fossil fuels is attracting more and more attention because such a bioprocess is environmentally friendly. Some bacteria, like *Rhodococcus*, have been used or studied to upgrade the fossil fuels on sulfur content limitation. Recent advances have demonstrated the desulfurization pathway and the molecular mechanism for biodesulfurization. In addition, genetic technology was also used to improve sulfur-removal efficiencies. In this chapter, we summarize the mechanism of biodesulfurization in *Rhodococcus*.

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1 Introduction

Fossil fuels are humanity's most important source of energy. Many of the benefits that we used in our way of life are due to fossil fuel use. There are three major fuels – coal, oil, and natural gas. Oil leads with a proportion to near 40% of the total consumption in the world, followed by coal (25%) and natural gas (22%). Almost all oil is consumed by burning, which causes pollution because of the chemical gasses released.

Sulfur is the third most abundant element in crude oil and can vary from 0.05% to 10% of the composition. In addition to elemental sulfur, sulfate, sulfite, thiosulfate, and sulfide, more than 200 sulfur-containing organic compounds have been identified in crude oils. These include sulfides, thiols, thiophenes, substituted benzo- and dibenzothiophenes, benzonaphthothiophene, and many considerably more complex molecules (Monticello et al. 1985). The condensed thiophenes are the most common form in which sulfur is present (Kropp and Gerber 1998). Dibenzothiophene (DBT), benzothiophene (BT), and their substitutes are the major sulfur-containing aromatic compounds in fuels, accounting for up to 70% of the sulfur content (Fig. 1) (Kertesz 2001) because they have higher boiling points (more than 200°C) and it is difficult to remove them from atmospheric tower outlet streams (e.g., middle distillates) (Kawatra and Eisele 2001; Shennan 1996). Benzothiophene (BT), non-β, single β, and di-β-substituted benzothiophenes (B.P.N219°C) are the typical thiophenic compounds that are found up to 30% in diesel oils (McFarland et al. 1998).

The strict new regulations to lower sulfur content in fossil fuels require new economic and more efficient methods for desulfurization, especially for removing organic sulfur, although the concentrations of BT and DBT are prominently decreased by hydro-desulfurization (HDS) process (Monticello 1998), which has been commercially used for a long time. HDS has several disadvantages (1) For refractory sulfur compounds, it requires higher temperature, pressure, and longer residence time; (2) it removes relatively simple sulfur compounds such as thiols, sulfides, and disulfides effectively. However, some complex aromatic sulfur-containing compounds such as DBTs, BTs, and polyaromatic sulfur heterocycles are resistant to HDS and form the most abundant organ sulfur compounds after HDS (Monticello 1998; Ma et al. 1994); (3) the cost of sulfur removal in industrial factories in HDS process is expensive, although HDS is considered to be a cost-effective method for fossil fuel desulfurization. Atlas et al. (2001) estimated the cost of lowering the sulfur content from 500 to 200 mg/kg to be approximately one cent per gallon. To reduce the sulfur content from 200 to 50 mg/kg, the desulfurization cost would be 4–5 times or higher.

Biodesulfurization (BDS) is a process that is being developed based on naturally occurring bacteria that can remove organically bound sulfur from diesel oil through their metabolism (Borgne and Quintero 2003). Intensive research has been conducted in BDS and has isolated many desulfurizing bacteria from many genera, such as *Rhodococcus* (Kilbane and Jackowski 1992; Izumi et al. 1994; Yu et al. 2006a, b; Ma et al. 2006a, b), *Microbacterium* (Li et al. 2005b), *Gordonia* (Rhee et al. 1998;

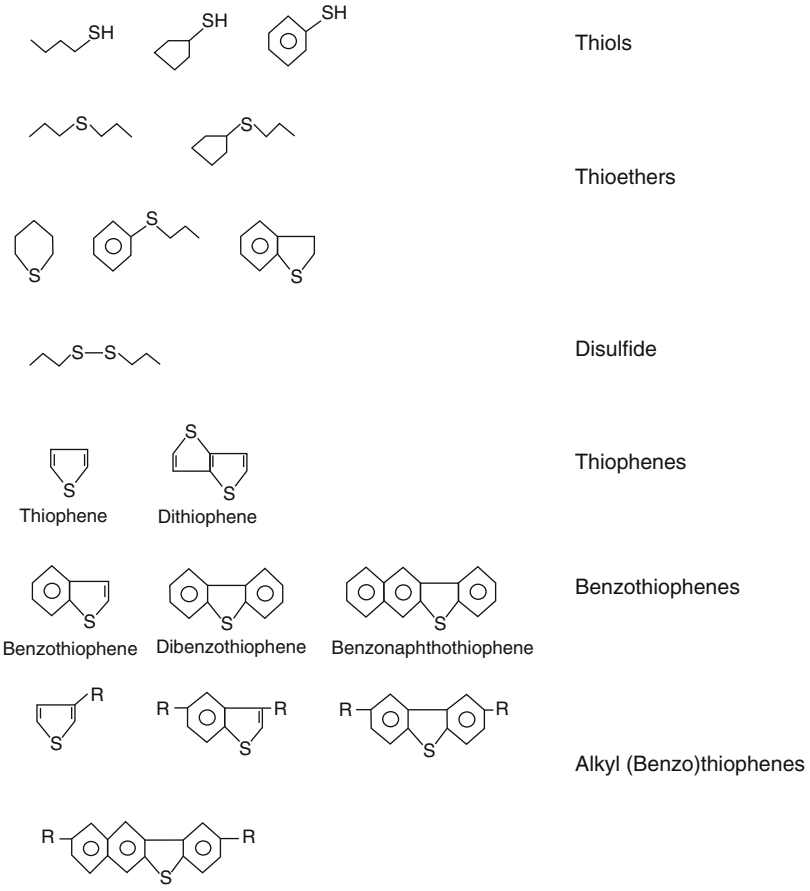

Fig. 1 Chemical structure of typical organic sulfur compounds in fossil fuel. The alkylated DBTs are different in type of substituents, number of substituents and their bond position on benzene ring

Li et al. 2006b), *Mycobacterium* (Li et al. 2003, 2005a, 2007a; Chen et al. 2008), *Pseudomonas* (Gupta et al. 2005; Shan et al. 2003; Luo et al. 2003), and so on, of which *Rhodococcus* sp. is an important desulfurizing bacterium with a wide substrate range and deep desulfurizing activity. In this chapter, we introduce desulfurization by *Rhodococcus*.

2 Biodesulfurization Pathways in *Rhodococcus*

The genus *Rhodococcus* belongs to the phylum and class Actinobacteria, the order Actinomycetales, and the family Nocardiaceae. Rhodococci possess a variety of plasmids, which give them greater capability to remediating environment pollutions (Dosomer et al. 1988; Kayser 2002).

Most sulfur in fossil fuel can be removed easily by HDS. However, there is one type, known as refractory organic sulfur, which is very difficult to remove. The current methods that can remove the refractory part operate under extremely invasive conditions. They are very costly and produce considerable amounts of carbon dioxide. Microbial desulfurization is an environmentally friendly method that can remove sulfur from refractory organic compounds, such as DBTs and BTs, under ambient temperature and pressure without lowering the calorific value of the fuel. These features have been the reason to conduct extensive studies to develop methods by which desulfurization of refractory organic sulfur compounds under mild condition can be viable (Gupta et al. 2005; Soleimani et al. 2007).

2.1 *DBT Biodesulfurization Pathway in* Rhodococcus

Much effort has been put into the investigation of biological desulfurization systems using DBT or alkylated DBTs as model compounds. The pathway specifically cleaving the C–S bond during metabolic desulfurization has been termed the "4S" pathway (Fig. 2) (Gallagher et al. 1993; Kilbane 2006; Gray et al. 2003; Yan et al. 2000), because four different molecules are formed during DBT desulfurization.

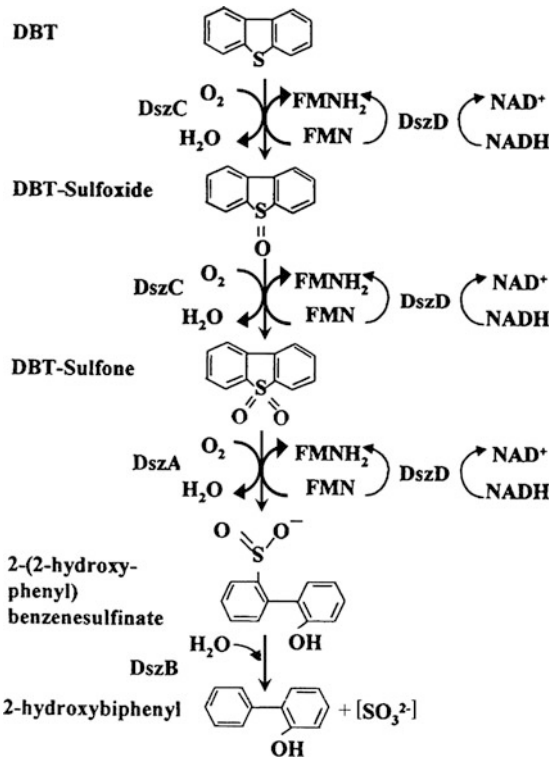

Fig. 2 "4S" Pathway of microbial DBT desulfurization and involvement of relative enzymes and flavin reductase

The "4S" pathway for sulfur removal was first reported for *Rhodococcus erythropolis* IGTS8 by Gallagher et al. (1993). Besides *R. erythropolis* IGTS8 (Kilbane and Jackowski 1992), other *Rhodococcus* that are also reported to follow this 4S pathway are *R. erythropolis* D1 (Izumi et al. 1994; Ohshiro et al. 1994), *R. erythropolis* DS-3 (Li et al. 2006a, b), *Rhodococcus* sp. ECRD1 (Grossman et al. 1999), *Rhodococcus* sp. B1 (Denis-Larose et al. 1997), *Rhodococcus* sp. SY1 (Omori et al. 1992), *Rhodococcus* sp. UM3 (Purdy et al. 1993), *Rhodococcus* sp. KT462 (Tanaka et al. 2002), and *R. erythropolis* KA2-5-1(Kobayashi et al. 2000). Among these, IGTS8 has been studied most extensively. *R. erythropolis* IGTS8 was isolated by Kilbane and Bielaga (1990) and was used by Energy Biosystems Corp. (EBC) for the development of their commercial microbial desulfurization plan. The strain IGTS8 is a Gram-positive rod-shaped bacterium approximately 0.5 μm long.

In the "4S" pathway, DBT is first converted to DBT sulfoxide (DBTO), then DBT sulfone ($DBTO_2$), then 2′-hydroxybiphenyl 2-sulfinic acid (HBPS), and finally 2-hydroxybiphenyl (2-HBP), releasing sulfate into the medium. Isotopic labeling experiments have shown that the oxygen atom of the hydroxyl group of 2-HBP originates from molecular oxygen, implicating a role for an oxygenase or oxygenases in the pathway (Oldfield et al. 1997). BDS processing using this strain will be ideal. In a BDS process the end product, 2-HBP and its derivatives, would partition back into the oil, thus preserving the fuel value.

2.2 BT Biodesulfurization Pathway in Rhodococcus

In contrast to DBT-desulfurizing bacteria, little is known about bacteria that can desulfurize BT. BT predominates in gasoline. Oil contamination may impact the organisms that live in contaminated ecosystems because some of these compounds, such as benzothiophene derivatives, have been reported to be mutagenic and carcinogenic (Kropp and Fedorak 1998). Therefore, the degradation pathway of BTH was also studied.

Based on the mass spectral data, two different pathways of desulfurization of sulfur from benzothiophene were proposed, and each end product identified (Fig. 3). In both the pathways, BT is first converted to BT sulfoxide, then BT sulfone, and followed by 2-(2′-hydroxyphenyl) ethan-1-al. The next desulfurization steps can proceed along two separated pathways. In the first pathway, the sulfinate group is removed with oxygenation of the molecule 2-(2′-hydroxyphenyl) ethan-1-al (Gilbert et al. 1998). This product is recovered as benzofuran due to dehydration under acidic extraction conditions. In the second pathway, the final product is *o*-hydroxystyrene, produced through desulfination of the molecule, which finally may oxygenate the carbon atom to dioxide carbon (Konishi et al. 2000). Most microorganisms can only desulfurize BT with one pathway, but *Rhodococcus* sp. JVH1 and *Rhodococcus* sp. WU-K2R have been reported to produce both end products from the desulfurization of benzothiophene (Kirimura et al. 2002).

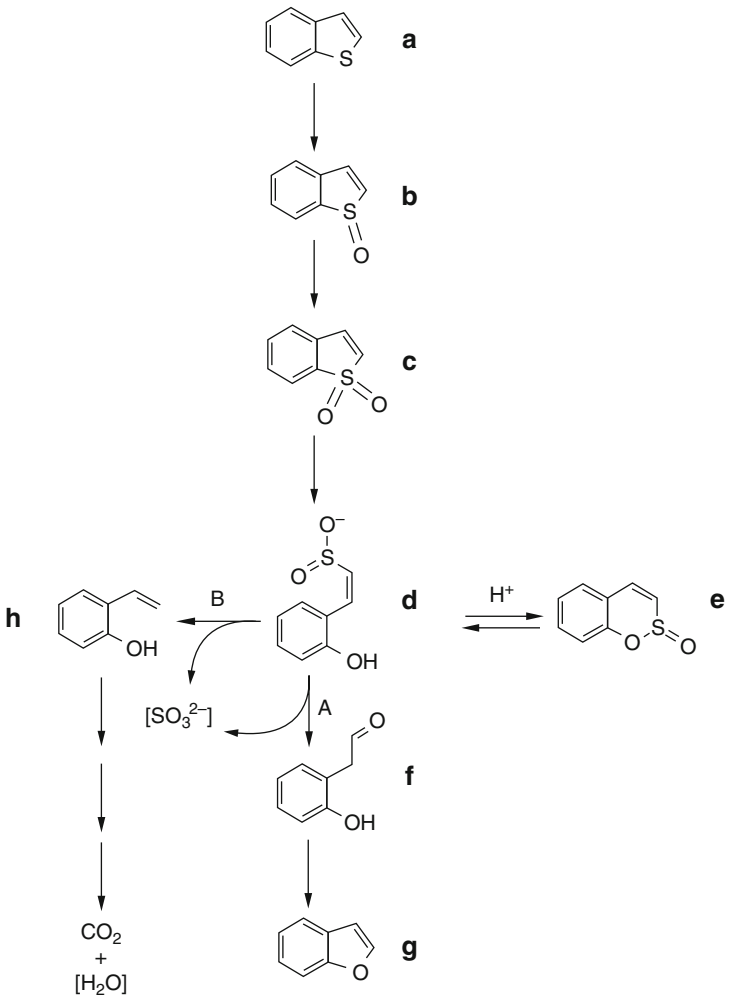

Fig. 3 Two possible degradation pathway of Benzothiophene (BT). (**A**) The first pathway (**B**) The second pathway (**a**) BT; (**b**) BT sulfoxide; (**c**) BT sulfone; (**d**) 2-(2′-hydroxyphenyl)ethen 1-sulfinate; (**e**) benzo[e][1,2]oxathiin S-oxide (sultine); (**f**) 2-(2′-hydroxyphenyl)ethan-1-al; (**g**) Benzofuran; (**h**) *o*-hydroxystyrene

3 Enzymes Involved in Specific Desulfurization

3.1 Enzymes Involved in DBT Desulfurization of the 4S Pathway

The complete removal of sulfur from DBT through the 4S pathway requires four enzymes. DBT monooxygenase (DszC or DBT-MO) catalyzes the stepwise *S*-oxidation of DBT, first to DBTO, then to $DBTO_2$. DBT-sulfone monooxygenase

(DszA or $DBTO_2$-MO) catalyzes the conversion of $DBTO_2$ to HBPS. Both DBT-MO and $DBTO_2$-MO are flavin-dependent and require a third enzyme (the flavin reductase, DszD) for activity. The fourth enzyme, HPBS desulfinase (DszB), catalyzes the desulfurization of HBPS to give HBP and sulfate, completing the reaction sequence (Gray et al. 1996; Ohshiro and Izumi 2000).

3.1.1 DBT-MO

The first enzyme catalyzes the conversion of DBT to DBT sulfone in a two-step process with DBT sulfoxide being the intermediate compound (DBT → DBTO → $DBTO_2$). The presence of DBTO is difficult to detect because it is readily consumed. The first oxidation step (rate constant 0.06 min^{-1}) is one-tenth of the rate of the second step (rate constant 0.5 min^{-1}). Purified DBT-MO is shown to have a peak absorption at 281 nm. This enzyme shows homology to the acyl coenzyme A enzyme and is a homotetramer with a subunit molecular weight of 50 kDa, as reported by Gray et al. (1996). Ohshiro et al. (1994) isolated DBT-MO from *R. erythropolis* D-1 and reported it to be a homohexamer with a subunit molecular weight of 45 kDa. Its activity is maximum at a temperature of 40°C and a pH of 8.0.

DBT-MO can act on the derivatives of DBT such as 4,6-dimethyl DBT, 2,8-dimethyl DBT, and 3,4-benzo-DBT, but it does not show any activity on carbazole, dibenzofuran, and fluorine; i.e., DBT atoms are substituted for sulfur atoms. Isotopic labeling studies indicated that the two oxygen atoms were derived from molecular oxygen. The DBT-MO from *Rhodococcus*, compared with other genera, has been shown to have a higher specific reaction rate for sparsely alkylated DBTs (Arensdorf et al. 2002).

3.1.2 $DBTO_2$-MO

The $DBTO_2$-MO enzyme is widely studied. It is a monooxygenase that oxidizes $DBTO_2$ to HPBS. The enzyme isolated from *R. erythropolis* D-1, a thermophile, (Ohshiro et al. 1999) was found to have a molecular mass of 97 kDa and to consist of two subunits with identical masses of 50 kDa. The N-terminal amino acid sequence of the purified $DBTO_2$-MO completely coincided with the deduced amino acid sequence for $DBTO_2$-MO from *R. erythropolis* IGTS8 except for a methionine residue at the latter N-terminal. The optimal temperature and pH for $DBTO_2$-MO activity are 35°C and about 7.5.

Oldfield et al. (1997) found that $DBTO_2$-MO from *R. erythropolis* IGTS8 catalyzed the conversion of dibenz[c,e][1,2]oxathiin 6,6-dioxide (sultone) to 2,2′-dihydroxybiphenyl (DHBP). Ohshiro and Izumi (2000) demonstrated that, by using $DBTO_2$-MO from *R. erythropolis* D-1, sultone showed 54% activity as a substrate compared with DBT sulfone, and DHBP was formed as a product. In addition, dibenz[c,e][1,2] oxathiin 6-oxide (sultine) showed 23% activity and yielded DHBP as a product. However, $DBTO_2$-MO did not act on DBT and

HBPS. Although sultine was nonenzymatically hydrolyzed to form HBPS, it was also oxidized to sulfomic acid during shaking. It was thought that once sultone was nonenzymatically formed from sultine, it was immediately converted to DHBP by $DBTO_2$-MO. $DBTO_2$-MO may recognize the sulfone moiety within the structure of DBT sulfone and sultone.

Gray et al. (1996) demonstrated that 10 mM EDTA did not inhibit the activity of $DBTO_2$-MO from *R. erythropolis* IGTS8. On the contrary, the activity of $DBTO_2$-MO from *R. erythropolis* D-l was inhibited 50% by 1 mM EDTA. Moreover, 2,2′-bipyridine, 8-quinolinol, and the other metal chelating reagents, such as Mn^{2+} and Ni^{2+}, also inhibited the activity of the enzyme, suggesting that a metal might be involved in its activity. $DBTO_2$-MO acted not only on DBT sulfone but also on dibenz[c,e][l,2]oxathiin 6,6-oxide and dibenz[c,e] (Ohshiro and Izumi 1999; Ohshiro et al. 1995) and oxathiin 6,6-dioxide. Dihydroxybiphenyl was formed from the latter two substrates.

3.1.3 HPBS Desulfinase

HPBS desulfinase is a novel enzyme in that it can specifically cleave the carbon–sulfur bond of HBPSi to give 2-HBP and the sulfite ion without the aid of any other protein components or coenzymes. It has been demonstrated that the activity of HPBS desulfinase is the lowest among enzymes of desulfurization metabolism. It is the rate-limiting enzyme of 4S pathway. It is also the least-studied enzyme since only a very small amount is produced.

It is a monomer with a subunit molecular weight of 40 kDa and shows enzyme activity over a wide temperature range (25–50°C) with the optimum at 35°C (Nakayama et al. 2002; Watkins et al. 2003). The working pH range for this enzyme is 6.0–7.5. Lee et al. (2004, 2006) elucidated the 3D structure of DszB, which was the first X-ray crystallographic study of enzymes involved in DBT desulfurization (Fig. 4). HPBS desulfinase does not require a metal cofactor for catalysis, and the inhibition by Zn^{2+} and Cu^{2+} is likely caused by the interference of substrate binding or catalysis.

A Cys residue must be the catalytic center of DszB because SH reagents inhibited the enzyme activity (Lee et al. 2004). DszB has only one Cys residue, at position 27, and it was found that the C27S mutant enzyme lost its activity completely. Therefore, there is no doubt that this residue is the catalytic center.

Based on the information about the 3D structure of DszB and a comparison of amino acid sequences between DszB and reported thermophilic and thermostable homologs (TdsB and BdsB), two amino acid residues, Tyr63 and Gln65, were selected as targets for mutagenesis to improve DszB. The promising mutant enzymes, replaced with these two residues by other amino acids, were purified and their properties examined. Among the wild-type and mutant enzymes, Y63F had higher catalytic activity but similar thermostability, and Q65H showed higher thermostability but less catalytic activity and affinity for the substrate. Furthermore, the double mutant enzyme Y63F-Q65H was purified and overcame these drawbacks.

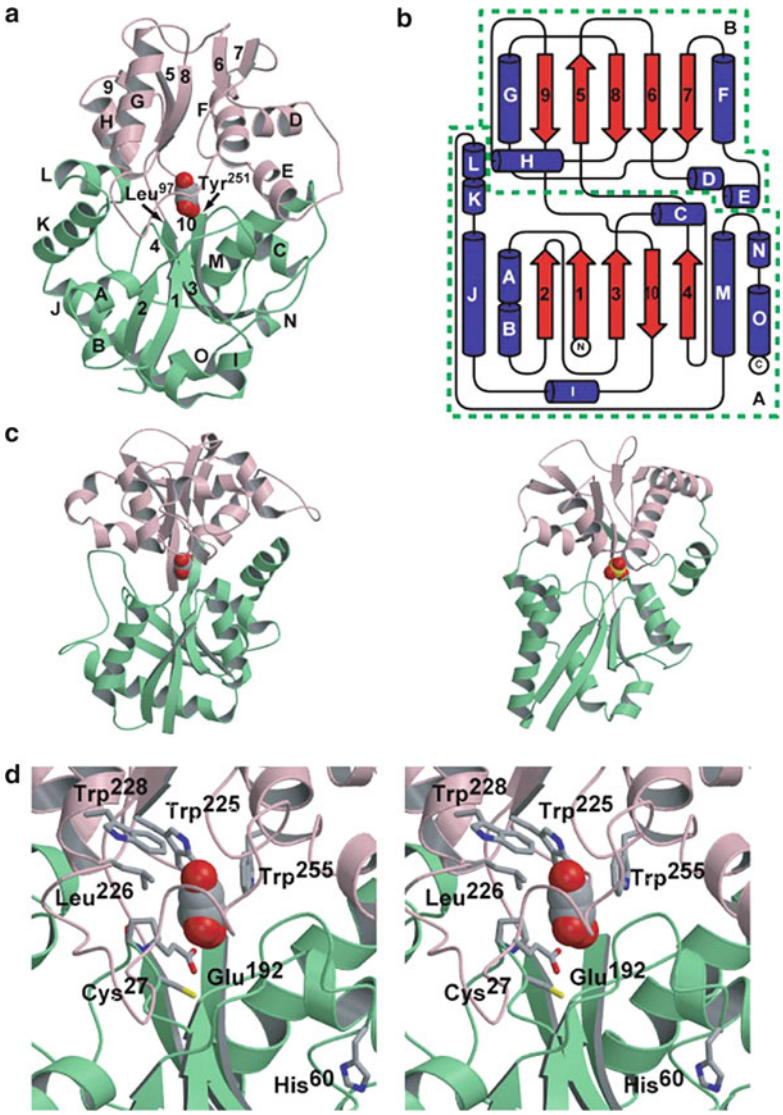

Fig. 4 The overall structure of DszB. (**a**), *ribbon model* of DszB. Domain A and B are *colored* in *light green* and *pink,* respectively. Two crossover residues that define the domains are labeled. (**b**), topology diagram of DszB. *Helices* and *strands* are colored in *blue* and *red,* respectively. *Dotted green lines* designate domain A and domain B. (**c**), *ribbon models* of proteins structurally related to DszB. Ovotransferrin (*left,* PDB code 1NNT) and a sulfate-binding protein (*right,* PDB code 1SBP) are depicted in *ribbon models*. Two domains of each protein are colored in a similar fashion to (**a**). Substrate ferric carbonate and sulfate ions are depicted in *space-filling models*. (**d**), stereo view of the active site. A glycerol molecule is depicted in *space-filling model*. Residues mentioned in the text are depicted as *sticks*

This mutant enzyme had higher thermostability without loss of catalytic activity or affinity for the substrate.

Ohshiro et al. (2007) found that each mutation at positions 63 and 65 of DszB enhanced the maximum activity and thermal stability, respectively, and that the double mutation increased thermostability without losses in maximal activity or affinity for the substrate. For the purpose of developing microbial desulfurization as a practical process, it is necessary to improve DszB further by structural analysis of the mutant enzymes in the near future.

3.1.4 Flavin Reductase

The flavin reductases are associated with monooxygenases since monooxygenases cannot work in the absence of these reductases (DBT-MO and $DBTO_2$-MO). The purified flavin reductase from the thermophilic strain *R. erythropolis* D-l contains no chromogenic cofactors and was found to have a molecular mass of 86 kDa with four identical 22 kDa subunits (Matsubara et al. 2001). The enzyme catalyzed NADH-dependent reduction of flavin mononucleotide (FMN).

For the flavin reductases from *R. erythropolis* D-l, flavin adenine dinucleotide was a poor substrate, and NADPH was inert. The enzyme did not catalyze the reduction of any nitroaromatic compounds. The optimal temperature and optimal pH for enzyme activity were 35°C and 6.0, respectively, and the enzyme retained 30% of its activity after heat treatment at 80°C for 30 min. The N-terminal amino acid sequence of the purified flavin reductase was identical to that of the flavin reductase from *R. erythropolis* IGTS8.

Xi et al. (1997) studied the enhanced desulfurization activity of DszC and DszA under *in vitro* conditions by increasing the concentrations of flavin reductase, suggesting that the two are terminal oxygenases. The reaction rate with 1 unit/ml of flavin reductase was linear for 10–15 min, whereas it was linear for more than 20 min with a lower concentration.

The inhibition experiments revealed that the flavin reductase activity of *R. erythropolis* D-1 was inhibited by 7-hydroxycoumarin but not by other coumarin derivatives, including dicoumarol, which inhibited FRase I activity and was used for the analysis of its crystal structure (Koike et al. 1998). FRase I was a flavoprotein possessing FMN as a prosthetic group. The flavin reductase of *R. erythropolis* D-1 has no flavin cofactor.

3.2 Enzymes Involved in BT-Desulfurizing Pathway

In contrast to DBT-desulfurizing enzymes, little is known about enzymes involved in the BT-desulfurizing pathway. At present, there are no related reports on BT-desulfurizing enzymes at home or abroad. The purified enzymes involved in BTH degradation would provide a detailed explanation for the degradation of BT.

4 Specific Desulfurizing Genes in *Rhodococcus*

In order to obtain better control over the machinery of specific sulfur removal, related research has been conducted on the molecular biology of this and similar strains since the metabolic identification of *R. erythropolis* IGTS8.

The primary genes involved in DBT metabolism, which are called both *dsz* and *sox*, have been cloned and are fairly well characterized. Although the *sox* (sulfur oxidation) designation was used first, the *dsz* (desulfurization) designation has generally been adopted.

The *dsz* genes are arranged in an operon-regulated system in a 4-kb conserved region of a 150-kb mega-plasmid, pSOX, in *R. erythropolis* IGTS8 (Oldfield et al. 1998) and a 100-kb plasmid in other strains. An insertion sequence (IS1166) was found to be associated with the *dsz* gene. It is a cluster of three genes (*dsz*A, *dsz*B, and *dsz*C) transcribed in the same direction, coding for three proteins Dsz A, Dsz B, and Dsz C, respectively (Denome et al. 1994; Piddington et al. 1995). The fourth gene, *dsz*D, was on the chromosome of *R. erythropolis* IGTS8 rather than on pSOX with *dsz*A, *dsz*B, and *dsz*C.

The termination codon of *dsz*A and the initiation codon of *dsz*B overlap (ATGA), indicating that there may be translational coupling of these two genes. Between *dszB* and *dszC*, there was a 13-bp gap. Potential ribosome-binding sites were also present upstream of each putative ATG initiation codon. The spacing and orientation of the three genes suggest that they are expressed as an operon, a suggestion that was also supported by the results of subclone analyses and promoter replacement analyses. Although expressed in the operon, Dsz B is present at concentrations several-fold less in the cytoplasm, as compared with Dsz A and Dsz C (Li et al. 1996).

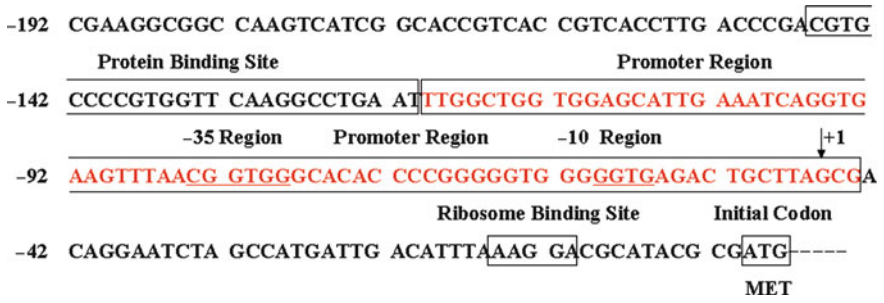

Fig. 5 Sequence of the 385-bp *dsz* promoter-containing fragment that starts immediately after the *Hin*dIII site. Nucleotides *below the line* indicate the positions of mutations, including the deletion of a C at 210. *Boxed* sequences are the protein-binding domain and the promoter region deduced from deletion analysis. The *arrow* indicates the G residue at position 11 in the 5′ end of the mRNA, and the *nucleotides in bold* are the putative −10 and −35 regions of the promoter. The *Hin*dIII site at the 5′ end and the *Spe*I site near the 3′ end were added for cloning purposes. Sequence numbering is for the native fragment, where 21 is the base preceding the A of the ATG initiation codon of *dsz*A

These genes, when cloned into a nondesulfurized strain (called *dsz*$^{-}$), confer the ability to desulfurize DBT to 2-HBP. The *dsz* operon was found on a large, 150-kb plasmid in *R. erythropolis* IGTS8 and on a 100-kb plasmid in other strains. An insertion sequence (IS1166) was found to be associated with the *dsz* gene.

To develop the biodesulfurization process, it was important to know under what conditions the desulfurization genes were expressed or repressed. Li et al. (1996) investigated the effect of various sulfur-containing compounds such as dimethyl sulfoxide (DMSO), cysteine, methionine, and sulfate on *dsz* gene expression. The results showed that desulfurization activity decreased when the concentration of cysteine, methionine, or sulfate in the media increased. In comparison, methionine caused the strongest repression in these substrates. When the concentration of these inhibitors reached more than 375 μM, desulfurization activity was strongly repressed. This repression was found to be due to the binding of a repressor protein next to the *dsz* promoter, which was located within the 385-bp region immediately upstream of *dsz*A (Fig. 5). Deletion analysis showed that the promoter was located to the region between −121 and −44. The S1 nuclease protection assay confirmed that the 5′ end of the *dsz* mRNA was the G at −46. A possible −35 promoter region with the sequence AAGTTTAA and a −10 region of GGGTGA are similar to those of *Bacillus subtilis* promoters that use the sigma factor σ^{B} (Li et al. 1996). The sequence at the transcription initiation site, TAG, is also the same as that of the *dsz* promoter, with two starting at the middle A and one starting at the G, as does the *dsz* promoter. The main difference between the two promoters is that the *Bacillus* promoters have a 14-bp spacer region between the −10 and −35 regions, where the *dsz* promoter is 23 bp.

The promoter region from −75 to −57 could be a potential case of dyad symmetry (Fig. 6). It is a strict inverted repeat sequence and could be part of an operator. An almost identical inverted repeat occurs within *dszB* from 1562 to 1578 and could be part of another operator.

Apart from the promoter, the 385-bp fragment has at least three elements that affect Dsz activity (some overlapping the promoter region). The region from −263 to −244 proved to reduce *dsz* repression. However, deletion of the region did not affect repression or gene expression. The region from −144 to −121 could bind a

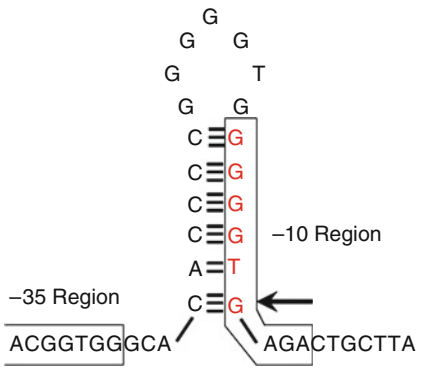

Fig. 6 Potential hairpin structure located in the *dsz* promoter. The hairpin is between −75 and −57 and has a free energy value of −15.4 kcal (1 cal = 4.184 J). Deletion of the G at −57 (indicated by the *arrow*), as in mutant R4, would reduce this to −13.2 kcal

protein such as an activator, and deletion of this region reduced gene expression, but not repression. The region between −98 and −57 may be a repressor-binding site (Li et al. 1996). It is possible that combinations of these mutations could further decrease repression.

5 Enhanced Biodesulfurization by Recombinant Bacteria

These specific bacteria can remove sulfur pollutants from petroleum and will reduce the amount of sulfur oxides released. However, genetic manipulations to improve sulfur-removal efficiencies are necessary before an upgrading process for the removal of harmful sulfur compounds from fossil fuels can be developed. In most times, engineered bacteria are required to remove more sulfur compounds with higher activities. Cultures with improved substrate ranges are also needed to better address the complicated mixture of chemicals present in petroleum.

5.1 Coexpression of Flavin Reductases

Since $FMNH_2$ is essential for the activities of DszC and DszA, the overexpression of flavin reductase in *Rhodococcus* or in recombinant bacteria will enhance the activities of DszC and DszA.

Lei et al. (1997) found enhanced desulfurizing activities of purified DszC and DszA protein from *R. erythropolis* IGTS8 in vitro when activated with flavin reductase from *Vibrio harveyi*. Hirasawa et al. (2001) purified the flavin reductase DszD from *R. erythropolis* IGTS8, and the enzyme was overexpressed in *Escherichia coli*. The specific activity in crude extracts of the overexpressed strain was about 275-fold that of the wild-type strain.

Reichmuth et al. (1999) studied the desulfurization ability of an engineered *E. coli* DH10B strain that contained the plasmids pDSR2 and pDSR3. The plasmid pDSR2 contained a *Vibrio harvey* NADH:FMN oxidoreductase gene, and pDSR3 encoded all of the three enzymes that converted DBT to HBP. In plasmid pDSR3, the native desulfurization control element, located in the promoter, had been removed. Therefore, *E. coli* DH10B/pDSR3 could express its desulfurization trait even in the presence of sulfate ion or rich media such as LB. However, the oxidoreductase level proved to be insufficient for the overexpressed *dsz*ABC. Designing an operon that expresses the proper amount of FMN:NADH reductase to existed *dsz*ABC enzymes is crucial to reach an optimum desulfurization activity. Insufficient FMN:NADH reductase would make NADH the limiting step in DBT oxidation. On the other hand, a high concentration of $FMNH_2$ will give rise to H_2O_2 formation, which would be lethal to cells (Gaudu et al. 1994; Galán et al. 2000).

In the search for the development of a method to provide the required amount of reduced flavin to DBT oxygenation, Galán et al. (2000) used *hpa*C, a flavin

reductase from *E. coli* W, and connected it, in vitro, with a system of *dsz*ABC purified enzymes and an NADH source. They also used catalase in the desulfurization medium to minimize the probability of H_2O_2 formation, which might be produced by nonenzymatic reoxidation of $FMNH_2$ under high oxygen concentrations. The addition of *hpa*C flavin reductase increased DBT desulfurization 7–10 times over 30 min. The enzyme HpaC flavin reductase and the oxidoreductase originated from IGTS8 were from the same subfamily of flavin:NAD(P)H reductases.

All the experiments confirm that the expression of an oxidoreductase with the *dsz* genes caused an increase in the rate of DBT removal.

5.2 Promoter Modification

The expression of *dsz* genes in most desulfurizing bacteria is repressed by sulfate, which is the product of biodesulfurization, through a repressor-binding site that may be in the promoter. So, looking for a new promoter that cannot be repressed by sulfate will be a new pathway to increase the desulfurizing rate.

Gallardo et al. (1997) subcloned the *dsz* cassette into the broad-host-range plasmid pVLT31 under the control of a hybrid promoter, *Ptac*, that has been shown to be functional in a wide range of bacteria. The resulting plasmid was transferred into *Pseudomonas putida*. The recombinant bacteria with *dsz* were shown to keep its desulfurization phenotype even in sulfate-containing media.

Several 16 S ribosomal RNA promoters of mycobacteria have also been studied and found to be functionally constitutive (Ji et al. 1994). Matsui et al. (2002) reported a 16 S ribosomal RNA promoter applied to the expression of *dsz* enzymes in *Rhodococcus* sp. strain T09. The putative *Rhodococcus rrn* promoter region was cloned from the *Rhodococcus* sp. strain T09, and the dibenzothiophene desulfurizing gene, *dsz*, was expressed under the control of the putative *rrn* promoter in strain T09 using a *Rhodococcus–E. coli* shuttle vector. Strain T09 harboring the expression vector could desulfurize dibenzothiophene in the presence of inorganic sulfate, methionine, or cysteine, while the Dsz phenotype was completely repressed in recombinant cells carrying the gene under the control of the native *dsz* promoter under the same conditions.

At the same time, Noda et al. (2002) constructed a promoter–probe transposon using a promoterless red-shifted green fluorescence protein gene (rs*gfp*). A 340-bp putative promoter element, *kap1*, was isolated from a recombinant strain, KA2-5-1, that had been shown to have high fluorescence intensity. The promoter element of *kap1* was not repressed by 1 mM of sulfate and it had about twofold greater activity than the *rrn* promoter from *R. erythropolis*. *Kap1* stimulated cell growth with biodesulfurization activity without the repression of sulfate. In conclusion, *kap1* is a convenient tool for improving biodesulfurization in *Rhodococcus*.

Otherwise, screening for recombinant bacteria that cannot be inhibited by sulfate is another substitutable method. Tanaka et al. (2002) isolated two mutants of the

dibenzothiophene-desulfurizing *R. erythropolis* KA2-5-1 that express a high level of desulfurizing activity in the presence of sulfate using the transposome technique. The level of dibenzothiophene desulfurization by cell-free extracts prepared from mutants grown on sulfate was about fivefold higher than that by cell-free extracts from the wild-type. Gene analysis of the mutants revealed that the same gene was disrupted and that the transposon-inserted gene in these strains was the gene for cystathionine β-synthase, *cbs*. The *cbs* mutants also expressed high levels of Dsz enzymes when methionine was used as the sole source of sulfur.

5.3 Increasing the Expression of Key Enzymes

The reactions catalyzed by DszC and DszB have been widely recognized as rate-limiting steps in the microbial desulfurization pathway. Several approaches have been performed by genetic engineering to improve desulfurizing enzyme activities, including those of DszC and DszB.

Coco et al. (2001) used random chimeragenesis on a transient template (RACHITT) to improve the activity of DszC by 20 times, and it must have increased the rate of the whole pathway.

As described previously, the rate of desulfurization is limited by the last enzyme in the pathway, DszB. In the native *dsz* operon, the ratio of mRNA of *dsz*A, *dsz*B, and *dsz*C was 11:3.3:1, indicating that the translation levels of the desulfurization enzymes decreased according to their positions in the operon due to polar effects on *dsz* gene transcription; however, western blot analysis indicated that the expression level of *dsz*B was far lower than that of *dsz*C. These results suggest that the translation of *dsz*B mRNA was not as efficient as *dsz*A or *dsz*B mRNA. Gene analysis revealed that the termination codon of *dsz*A and the initiation codon of *dsz*B overlapped, whereas there was a 13-bp gap between *dsz*B and *dsz*C. Potential ribosome-binding sites were present upstream of each putative ATG initiation codon. In order to get a better, steady expression of DszB, Li et al. (2007b) removed the overlap structure by overlap polymerase chain reaction (PCR) and expressed the redesigned *dsz* operon in *R. erythropolis* without desulfurization activity, named *R. erythropolis* DR-2. Real-time PCR analysis confirmed that the transcription characteristics did not change in *R. erythropolis* DR-2 compared with *R. erythropolis* DR-1, which contains the original *dsz* operon. However, western blot analysis revealed that *R. erythropolis* DR-2 produced more DszB than *R. erythropolis* DR-1 did. The desulfurization activity of resting cells prepared from *R. erythropolis* DR-2 was about fivefold higher than that of *R. erythropolis* DR-1. That indicated that the enhanced expression level increased the metabolic rate of HBPS in the cells and contributed to the improved desulfurization rate of *R. erythropolis* DR-2.

To increase DszB production, Reichmuth et al. (2004) mutated the untranslated 5′ region of *dsz*B using degenerate oligonucleotides. Because neither DszB activity nor the amount of DszB protein produced could be directly measured, it was

difficult to determine the exact cause for the lack of HBP production. To clarify the results of our genetic manipulations, they chose to tag the production of the desulfurization transcripts and proteins by creating transcriptional and translational fusions with a fluorescent protein. This permitted a quick, straightforward, and direct determination of the amount of the desulfurization protein produced. This technique does not measure the activity of the proteins; however, activity screens could be used after protein production was optimized using fluorescent fusion tags. The protein used for those fusions was GFP. GFP has been widely used for the quantitative measurement of protein production and is known to be stable for a period of several days, allowing an integrative and quantitative measure of protein production (Albano et al. 1998; Cha et al. 2000). After screening only 96 mutants, several showed increased green fluorescence and two showed increased DszB activity. When cotransformed with the full *dsz*ABC operon, the mutant *dsz*B increased the rate of desulfurization ninefold relative to the native *dsz*B.

R. erythropolis KA2-5-1 can desulfurize DBT into 2-HBP through the 4 S pathway. Hirasawa et al. (2001) constructed an *E. coli–Rhodococcus* shuttle vector, and the desulfurization gene cluster, *dsz*ABC, and the related reductase gene, dszD, were cloned from KA2-5-1, reintroduced into KA2-5-1, and efficiently expressed. The DBT-desulfurization ability of the transformant carrying two *dsz*ABC and one *dsz*D on the vector was about fourfold higher than that of the parent strain, and the transformant also showed improved desulfurization activity for light gas oil. Matsui et al. (2001) also enhanced the desulfurization rate by 3.3 times, by increasing the copy number of *dsz* genes.

5.4 The Expression of Desulfurization Enzymes in Heterologous Hosts

In fossil fuels, there are many kinds of compounds inhibiting on desulfurization process and *Rhodococcus*. *Pseudomonas* was found to be an ideal candidate for biodesulfurization because they are organic solvent-tolerant and have a high growth rate. *Pseudomonas* sp. are among the best studied and most abundant microorganisms found in crude oil (Leahy and Colwell 1990), and a wide variety of genetic tools are now available for their molecular manipulation (Lorenzo and Timmis 1994). Furthermore, several biotechnological properties for the design of biocatalysts targeted to industrial biodesulfurization processes are present in *Pseudomonas* species. For example, while the solvent tolerance of *Rhodococcus* is the lowest reported (log *P* values from 6.0 to 7.0), that of the genus *Pseudomonas* (log *P* values from 3.1 to 3.4) is the highest known (Inoue and Horikoshi 1991), and several *Pseudomonas* strains that are highly resistant to heavy metals present in fossil fuels have been reported (Atlas 1994).

With the properties noted, *dsz*ABC genes from *R. erythropolis* XP were cloned into *P. putida* Idaho to construct a solvent-tolerant, desulfurizing *P. putida* A4. This strain, when contacted with sulfur refractory compounds dissolved in hydrocarbon

solvent, maintained the same substrate desulfurization traits as observed in *R. erythropolis* XP. Resting cells of *P. putida* A4 could desulfurize 86% of DBT at 10% (v/v) *p*-xylene in 6 h. In the first 2 h, the desulfurization occurred with a rate of 1.29 mM DBT (gdw cell)$^{-1}$ h^{-1}. No DBT reduction was noticed when the experiment was repeated with *R. erythropolis* or *P. putida* Idaho under identical conditions (Tao et al. 2006).

In the development of engineered strains with potential industrial or environmental applications, a high degree of predictability in their performance and behavior is desirable. To achieve this goal, stable chromosomal insertion of the genes conferring the new trait is required. Therefore, Gallardo et al. (1997) constructed *P. putida* EGSOX, which carried *dsz* genes stably inserted into the chromosome of the host cell.

To improve the biodesulfurization process, it would be interesting to design a recombinant biocatalyst that combines the Dsz phenotype with another trait of potential interest, such as the production of biosurfactants. To accomplish this goal, Gallardo et al. (1997) also transferred *dsz* genes into *P. aeruginosa* PG201 (Ochsner et al. 1995), which cannot use DBT as the sole carbon and/or sulfur source and produces rhamnolipid biosurfactants. These are of increasing industrial relevance because of their applications in emulsification, wetting, phase separation, and viscosity reduction. The final recombinant bacterium was named *P. aeruginosa* EGSOX, and carried *dsz* genes stably inserted into the chromosome of the host cell.

At 48 h of incubation, cultures of strain IGTS8 still contained DBT; however, this compound was exhausted by the two engineered *Pseudomonas* strains. *P. aeruginosa* EGSOX had the fastest metabolism of DBT, transforming 95% of the DBT at 24 h of incubation. Only 18% of the DBT was transformed by *R. erythropolis* IGTS8, and 40% was transformed by *P. putida* EGSOX. Remarkably, DBT depletion was concomitant with 2-HBP accumulation in all the three strains, indicating that 2-HBP is a dead-end metabolite that cannot be further catabolized or used as a carbon source. These data demonstrated that the IGTS8-derived *dsz* cassette was efficiently expressed, allowing the elimination of sulfur with no loss of DBT carbon atoms, both in *P. putida* EGSOX and *P. aeruginosa* EGSOX. Moreover, in comparison with wild-type *R. erythropolis* IGTS8, the two recombinant biocatalysts showed enhanced biodesulfurization ability.

However, many *Pseudomonas* strains were unable to desulfurize DBT in the oil phase, and this will restrict their application in industry. Darzins et al. (1999) found that *P. fluorescens* with *dsz*ABC genes cannot desulfurize DBT in the oil phase; but the whole cell lysate with the cell wall removed can. The results showed a lack of DBT uptake ability from the oil phase to the inside of the recombinant *Pseudomonas* strains. Noda et al. (2003) transferred the *dsz* desulfurization gene cluster from *R. erythropolis* IGTS8 into the chromosome of *P. aeruginosa* NCIMB9571 using a transposon vector. All of the recombinant strains completely desulfurized 1 mM DBT in *n*-tetradecane (*n*-TD) except one, named PARM1. PARM1 was unable to desulfurize DBT in *n*-TD, but was able to desulfurize it in water. The transposon tagging analysis indicated that the tranposon was inserted into *hcu*A of the open reading frames *hcu*ABC. The full-length *hcu*ABC genes, when transformed into

PARM1, achieved 87% recovery of the desulfurization activity of DBT in *n*-TD, but partial *hcu*ABC genes achieved only 0–12%. These results indicated that DBT desulfurization in the oil phase by recombinant *P. aeruginosa* NCIMB9571 required the full-length *hcu*ABC gene cluster. The *hcu*ABC gene cluster is related to DBT uptake from the oil phase into the cell.

5.5 *Rearranging the* dsz *Gene Cluster*

As described before, the levels of transcription and translation of *dsz*A, *dsz*B, and *dsz*C decreased according to the positions of the genes in the *dsz* operon. Furthermore, the translation of *dsz*B was repressed by an overlapping structure in the *dsz* operon. In order to get better and steady expression of the Dsz enzymes and optimize the metabolic flux of DBT, the overlapped structure was removed, and the expression level of *dsz*B was increased. The DBT-desulfurization rate was five times faster than that of the native *dsz* operon (Li et al. 2007b), but this is still low in comparison to the requirements of a commercial process.

The rate of an enzyme catalytic reaction is determined by the catalytic activity, the quantity of the enzyme, and the substrate concentration. Higher levels of mRNA are the precondition for higher levels of the encoded protein. Therefore, rearranging these genes according to the catalytic capabilities of the enzymes and their reaction orders could not only balance the catalytic capabilities but also increase the substrate concentrations for the enzymes. Li et al. (2008) introduced a genetic rearrangement strategy for optimizing the metabolic pathway of DBT. By using recombinant PCR, the *dsz* operon of *R. erythropolis* DS-3 was rearranged according to the catalytic capabilities of the Dsz enzymes and their reaction orders in the 4S pathway (Fig. 7).

The catalytic capabilities of the Dsz enzymes were approximately 25:1:5 (DszA: DszB:DszC). Hence, the *dsz* operon was rearranged according to the catalytic capabilities of the enzymes. The expression levels of *dsz*B and *dsz*C were improved by rearranging the order of the *dsz* genes to generate the operon *dsz*BCA, which contained *dsz*B, *dsz*C, and *dsz*A in tandem. After rearrangement, the ratio of *dszA*, *dszB*, and *dszC* mRNAs in the cells was changed from 11:3.3:1 to 1:16:5. The desulfurization rate of the recombinant strain containing the rearranged *dsz* operon was 12 times faster than that of the native *dsz* operon. The maximum desulfurization rate was only about 26 μmol DBT/g DCW/h for the strain containing the native *dsz* operon. After removing the overlapped structure before the initiation codon of *dsz*B, the rate was 120 μmol DBT/g DCW/h. The recombinant strain containing the rearranged *dsz* operon had the highest desulfurization rate, about 320 μmol DBT/g DCW/h. Therefore, the enhanced expression levels of DszC and DszB increased the desulfurization rate of the recombinant strain.

Feng et al. (2006) found that the function of the surfactant Tween 80 in the desulfurization was to decrease the product concentration associated with the cells, reducing product inhibition. The *dsz* genes of *R. erythropolis* DS-3 were also

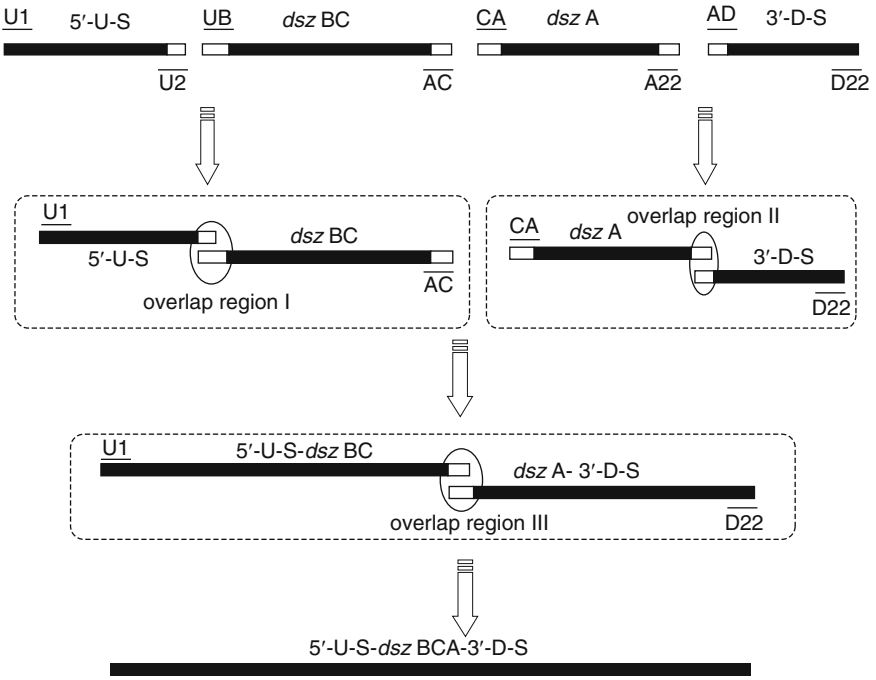

Fig. 7 Rearrangement of the *dsz* operon by overlap PCR. Fragments of the 400-bp 5′ upstream segment (5′-U-S) and the 400-bp 3′ downstream segment (3′-D-S) of *dsz*ABC and the *dsz*A and *dsz*BC segments, including the overlap regions, were amplified by PCR, then the ligated 5′ upstream-*dsz*BC segment (5′-U-S-*dsz*BC) and the ligated *dsz*A-3′ downstream segment (*dsz*A-3′-D-S) were produced by overlap PCR via their overlap regions, and finally, the 5′ upstream-*dsz*BC segment and the *dsz*A-3′ downstream segment were linked together by overlap PCR via their overlap region to yield the reconstructed *dsz* operon. *Black bars* represent genes, and *white bars* represent overlap regions

integrated into the chromosome of *Bacillus subtilis* ATCC 21332, which can secrete biosurfactant, yielding the recombinant strain *B. subtilis* M29, which has higher desulfurization efficiency than *R. erythropolis* DS-3 and showed no product inhibition (Ma et al. 2006b). It should be noted that the biosurfactant secreted from *B. subtilis* M29 significantly varied the interfacial tension of the supernatant. The biosurfactant therefore has an important function in the degradation of DBT.

6 Future Perspectives

Our understanding of how microorganisms metabolize sulfur heterocyclic compounds in petroleum has increased rapidly. All the studies outlined above are significant steps to explore the biotechnological potential for developing an efficient biodesulfurization process. However, these technologies have not yet been

available for large-scale applications. We still need a much better understanding of more aspects of this pathway to turn it into a commercial process earlier. Any progress that provides the possibility to remove sulfur in crude oil at higher temperature, with higher rate, or longer stability of desulfurization activity is considered to be a significant step toward industry level biodesulfurization. Microorganisms with a wider substrate range and higher substrate affinity in biphasic reaction containing toxic solvents or higher biodesulfurization activities could be engineered if the biocatalysts were to be used for petroleum treatment. In the next few years, the availability of the genome sequences of these biocatalysts will make it possible to regulate the metabolism engineering.

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Application of *Rhodococcus* in Bioremediation of Contaminated Environments

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Contents

Abstract Environmental pollution with anthropogenic organic compounds is the global problem of our planet. Bioremediation has a great potential to effectively restore polluted environments by using biodegradative activities of microorganisms. The genus *Rhodococcus* is a promising group of bacteria suitable for biodegradation of recalcitrant contaminants, such as petroleum hydrocarbons, chlorinated, nitrogenated, and other complex organics. *Rhodococcus* species are ubiquitous in pristine and contaminated environments, survive under harsh environmental conditions, compete successfully in complex bacterial populations, and therefore could be efficiently used in bioremediation applications. Some success in bioremediation of contaminated soils, waters, and air has been achieved using rhodococci either as bioaugmentation agents or members of indigenous microbial communities

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stimulated by nutrient and oxygen amendments. Laboratory and field-scale studies on *Rhodococcus* application in cleanup technologies are reviewed relating to in-situ subsurface and groundwater remediation, on site treatments of contaminated soils, sludges, wastewaters, and waste gases.

1 Introduction

Environmental pollution with anthropogenic organic compounds has become one of the most urgent problems worldwide. This negatively impacts economical and social developments, poses significant threats to human health and natural biodiversity. Potential sources of organic contaminants include industrial leaks and spills, improper application of pesticides and fire retardants, negligent disposal of industrial and domestic wastes, landfills, and garbage dumps. Oil spillage and oily waste deposits represent the major part of hydrocarbon contamination in our planet. Petroleum hydrocarbons are widespread soil and groundwater pollutants resulting from leaking of underground storage sites, spillage from the storage tanks and damaged pipelines. There are thousands of sites that have been seriously contaminated by petroleum products in oil-producing regions around the world (Etkin 2001). Moreover, marine oil spills from crashed tankers are responsible for the massive contamination of seawater and shorelines. Apart from oil industry, major sources of hydrocarbon contaminants, such as alkanes and polycyclic aromatic hydrocarbons (PAHs), are coal-mining sites and coking plants, gas processing plants, solid fuels for domestic heating, aircraft and car exhausts, and forest fires. Also, chlorinated hydrocarbons such as chlorobenzenes, chlorophenols, and polychlorinated biphenyls (PCBs) that are used commercially for a variety of purposes, including production of solvents, paint additives, pesticides, fire retardants, and insulating fluids, represent a large proportion in long-term persistent contamination of soils and groundwater. It should be noted that soil contamination by organic compounds is a complex process and difficult to treat due to many reasons, for example, a tendency to sorption of contaminants into the soil matrix, low water solubility, and limited rate of mass transfer. Remediation activities are often hampered by remoteness and low accessibility of contaminated sites, harsh environmental conditions, as well as high pollution levels and large amounts of contaminated material to be treated. Bioremediation is considered to be a nondestructive, cost- and treatment-effective, and sometimes logistically favorable cleanup technology capable of accelerating naturally occurring biodegradation of contaminants through optimization of limiting conditions (Alexander 1999). Currently, methods of biological remediation of contaminated sites gain ever increasing popularity owing to their sustainability, relatively low cost, and environmental safety (Fig. 1). Bioremediation of polluted environments is based on contaminant biodegradation, that is, metabolic abilities of microorganisms to transform or mineralize organic contaminants into less harmful, nonhazardous substances, which are further integrated into natural biogeochemical cycles (Alexander 1999;

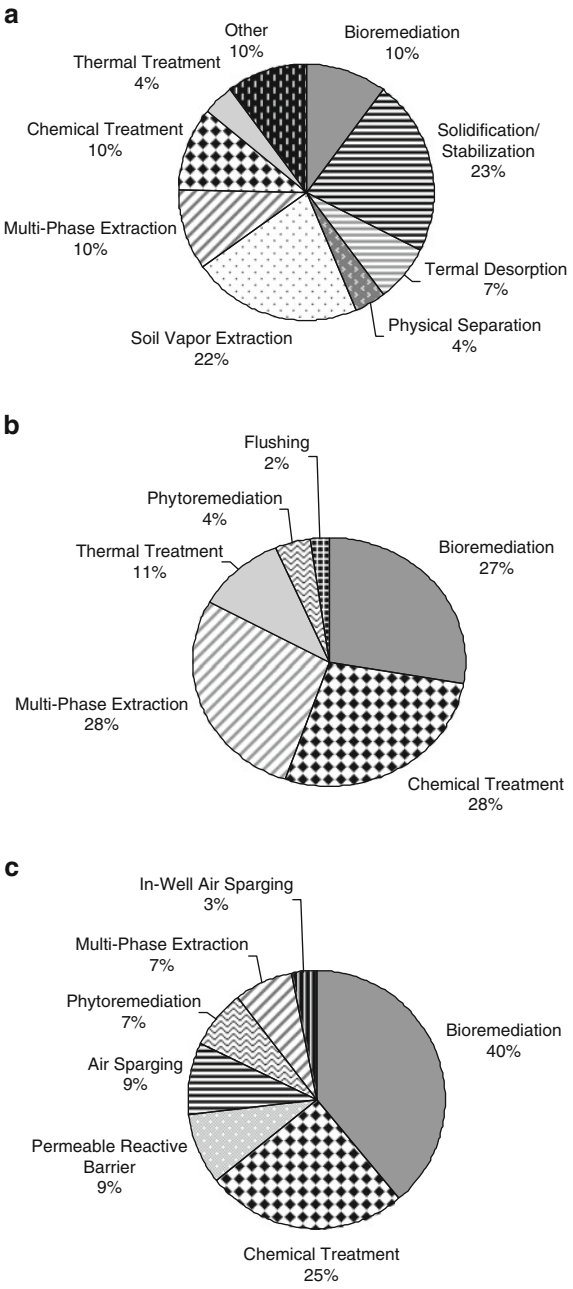

Fig. 1 Proportion of bioremediation among treatment technologies for site cleanup reported in the US EPA Annual Status Report (12th Edition) for 2002–2005. (**a**) Source control (soil, sludge, sediment) treatment. (**b**) Innovative source treatment technologies. (**c**) In situ groundwater treatment. Figures generated from (US EPA 2007)

Whyte et al. 2001). In most cases, treatment of contaminated environments has involved biostimulation, addition of nutrients, and other factors to stimulate spontaneous enrichment of the indigenous contaminant-degrading microbial population. However, there has been considerable debate over the efficacy of bioaugmentation (Aislabie et al. 2006), the addition of either indigenous or exogenous microorganism's cultures to enhance the remediation process. An application area for bioaugmentation could be contaminated environments deficient in microorganisms due to extreme climate conditions (e.g., polar and desert soils, low oxygen ground waters) or high levels of toxic contaminants (e.g., industrial waste dumps and wastewater streams). For example, low numbers of hydrocarbon-oxidizing bacteria in cold climate pristine soils coupled with short summer seasons may limit the spontaneous enrichment of oil-contaminated soils with autochthonous hydrocarbon-oxidizers when biostimulation alone is applied (Ruberto et al. 2005). Bioaugmentation, in the context of bioremediation, should be considered as inoculation of contaminated soil or water with specific strains or consortia of microorganisms to improve the biodegradation capacity of the system for specific organic pollutant(s) (Alexander 1999). There are two fundamental approaches to bioaugmentation of a contaminated site. The first involves increasing the genetic diversity of the residential microbial community by inoculation with allochthonous microorganisms, which may enhance the catabolic potential, and thereby the biodegradation rate (Dejonghe et al. 2001). The second approach applied in most bioremediation projects involves a serial enrichment of indigenous microbial populations isolated from the contaminated samples using laboratory cultivation with the contaminant(s) as the sole carbon source. This enriched inoculum containing mostly fast-growing organisms with exceptional degradative capacities is then returned back to the site to increase the rate of biodegradation. Various factors are known to influence the success or failure of bioaugmentation; the predictability, however, is beyond our scope yet. In many cases, contaminated environments are hostile to the introduction of allochthonous microorganisms, and large numbers will never survive unless they have a selective advantage. The controlling environmental factors are both biotic and abiotic (for reviews, see van Veen et al. 1997; Watanabe and Hamamura 2003). So, assuming bioaugmentation as a feasible bioremediation approach, it seems to be important to find the "proper" microorganisms for the bioaugmentation consortium. These microorganisms should possess high biodegrading abilities, be highly competitive in natural bacteriocenoses, as well as be nonpathogenic and environmentally friendly.

The genus *Rhodococcus* was regarded as one of the most promising groups of microorganisms suitable for the biodegradation of compounds not readily transformed by other organisms. The biochemical potential of rhodococci has been increasingly explored because of their broad catabolic versatility and unique enzymatic capabilities (Van der Geize and Dijkhuizen 2004; Martínková et al. 2009; chapter, "Genomes and Plasmids in *Rhodococcus*" by Larkin et al.). Xenobiotic compounds metabolized by rhodococci cover a wide range of structural groups, including aliphatic and aromatic hydrocarbons, oxygenated and halogenated compounds, nitroaromatics, heterocyclic compounds, nitriles, and various pesticides.

Many of these substrates are complex synthetic molecules with high chemical stabilities and toxicity. *Rhodococcus* species are ubiquitous in pristine and contaminated environments, possess remarkable metabolic activities, can persist under harsh environmental conditions, compete successfully in complex bacterial populations, and therefore could be considered as having great potential in bioremediation applications. The significance of rhodococci in environmental biotechnology was discussed in the reviews characterizing the genus *Rhodococcus* (Warhurst and Fewson 1994; Bell et al. 1998). This chapter describes the roles of rhodococci as members of natural hydrocarbon-oxidizing consortia and promising bioremediation agents, and also reviews laboratory and field-scale studies on *Rhodococcus* application in cleanup technologies for contaminated environments.

2 Why Are Rhodococci Considered as Most Suitable for Environment Bioremediation?

Rhodococcus species are naturally present in diverse temperate and extreme environments. They can persist and grow in highly contaminated soils and waters, and even under oxygen- and nutrient-limited conditions. *Rhodococcus* strains can be readily isolated from environmental samples and enriched in mixed or pure cultures by incubation with a particular contaminant of interest, which is important for preparing inocula for bioremediation. Their natural occurrence in contaminated environments, broad catabolic versatility, physiological and ecological adaptations to extreme environmental conditions imply that they may play a significant role in both natural degradation of persistent pollutants and bioremediation of contaminated ecosystems.

2.1 Pristine and Contaminated Environments Are Common Habitats for Rhodococcus *Species*

Rhodococci have been isolated from a large variety of natural sources, including clean and contaminated soils and rocks, surface and ground waters, marine sediments, from animals and plants. *Rhodococcus* strains are often isolated from environments where hydrocarbons are present. Since petroleum hydrocarbons are most widespread contaminants of terrestrial and marine ecosystems, a large variety of studies were undertaken attempting isolation, characterization, and cleanup application of petroleum-degrading bacteria (for a review, see Van Hamme et al. 2003). Some *Rhodococcus* species, particularly *R. rhodochrous* and *R. ruber*, are able to grow using gaseous hydrocarbons (such as propane, butane, and acetylene) as a sole carbon source (Ivshina et al. 1994). Ivshina et al. (1981) found large numbers of propane- and butane-degrading *R. rhodochrous* and *R. ruber* isolates in

soil, rock, and groundwater associated with oil-bearing sites, suggesting that these gas-oxidizing rhodococci are indicative of the presence of subterranean hydrocarbon deposits and thus could be used in oil prospecting. Crude oil contaminated soil and marine samples collected from different localities in Kuwait were screened for microorganisms capable of oil degradation (Sorkhoh et al. 1990). It was found that, among many bacterial isolates, *Rhodococcus* strains were the most abundant mesophilic hydrocarbon-oxidizing bacteria and most efficient oil degraders. Cold-tolerant *Rhodococcus* strains were isolated from oil-contaminated soils in Antarctica; they grew on a range of alkanes from hexane (C_6) through at least eicosane (C_{20}) and the isoprenoid compound pristane (2,6,10,14-tetramethylpentadecane) and retained metabolic activities at subzero temperatures of –2°C (Bej et al. 2000). Several cold-tolerant *Rhodococcus* strains isolated from a deep Greenland glacier ice core grew rapidly at 2°C (Miteva et al. 2004). A psychrotrophic *Rhodococcus* sp. from Arctic soil (Whyte et al. 1998) utilized a broad range of aliphatics (C_{10} to C_{21} alkanes, branched alkanes, and a substituted cyclohexane) present in diesel oil at 5°C. The strain mineralized short-chain alkanes (C_{10} and C_{16}) to a significantly greater extent (by a factor of about 2–3) than long-chain alkanes (C_{28} and C_{32}) at 0 and 5°C. The psychrotrophic halotolerant oil-degrading strain *Rhodococcus* sp. YHLT-2 isolated from gasoline-contaminated groundwater was able to grow at 7% NaCl and utilized short-chain alkenes (C_9 to C_{12}) as well as a broad range of long chain alkenes (C_{19} to C_{32}) present in crude oil at 4°C (Ryu et al. 2006). The study of Mergaert et al. (2001) showed that a large proportion (34%) of facultative oligotrophic and psychrotrophic strains isolated from Arctic and Antarctic seawaters were grouped according to their fatty acid profiles into the *Rhodococcus fascians* cluster. Phylogenetic analysis of 16S rRNA genes from alkane-degrading bacterial isolates indicated that *Rhodococcus* spp. from cold regions mostly group with *R. erythropolis* or *R. fascians* (Aislabie et al. 2006). We have isolated a large number of alkanotrophic *Rhodococcus* strains identified as *R. erythropolis*, *R. fascians*, *R. opacus*, *R. rhodochrous*, and *R. ruber* from soil, surface and spring water, snow, air, and core samples taken from ecologically and geographically diverse regions of the former Soviet Union (Ivshina et al. 1994, 1995).

However, most of the recent environmental microbiology studies using molecular genetic techniques focused on bacterial community structure and dynamics rather than on culture isolation. The occurrence of four alkane monooxygenase genotypes (*Pseudomonas putida* GPo1, Pp *alkB*; *Rhodococcus* sp. strain Q15, Rh *alkB1* and Rh *alkB2*; and *Acinetobacter* sp. strain ADP-1, Ac *alkM*) in hydrocarbon-contaminated and pristine soils from the Arctic and Antarctica was determined by both culture-independent (PCR hybridization analyses) and culture-dependent (colony hybridization analyses) molecular methods (Whyte et al. 2002a). PCR hybridization of the total soil community DNA revealed that Rh *alkB1* and Rh *alkB2* genes are common in both contaminated and clean soils, whereas Pp *alkB* is common in contaminated soil, while Ac *alkM* is rare. Furthermore, Rh *alkB1* was prevalent in culturable psychrotolerant bacteria, suggesting that *Rhodococcus* is the predominant alkane degrader in both pristine and contaminated polar soils.

Similar results for Rh *alkB1* and Rh *alkB2* prevalence in polar (Antarctica), Alpine (Austria), and tropical (Brazil) soils (Margesin et al. 2003; Luz et al. 2004) suggested that rhodococci are typical alkanotrophic soil bacteria through various ecological and climatic regions. Moreover, phylogenetic comparison of pristine and hydrocarbon-contaminated Alpine soils using DGGE fingerprinting of PCR-amplified 16S rRNA gene sequences indicated the abundance of the *Actinobacteria* phylum members, *Rhodococcus* and *Mycobacterium* (Labbé et al. 2007). Microbial communities of heavy fuel-impacted shoreline in north Spain analyzed by DGGE of PCR-amplified 16S rDNA also contained high proportions of *Rhodococcus* members associated with weathered and biotreated contaminations, suggesting that this genus may be important for biodegradation of high-molecular-weight hydrocarbons (Jiménez et al. 2007).

2.2 *Rhodococci Can Be Successfully Enriched in Laboratory Hydrocarbon-Oxidizing Consortia*

There are considerable numbers of studies appeared in recent years, showing that rhodococci play a leading role in aerobic biodegradation of mono- and polyaromatic compounds, highly toxic to many bacterial species (for a review, see Martínková et al. 2009). Taki et al. (2007) found that in pristine and trichloroethylene-contaminated soils incubated with *o*-xylene (the most recalcitrant isomer of xylenes) and mineral nutrients, the *R. opacus* was abundant, increasing by almost two orders of magnitude during an active *o*-xylene biodegradation as it was estimated by competitive PCR using a primer set specific for *R. opacus* and *R. koreensis*. These authors also isolated *o*-xylene-degrading *Rhodococcus* strains that may be effective in the bioaugmentation of soil polluted with BTEX (benzene, toluene, ethyl benzene, and xylene). A *Rhodococcus* sp. isolated from PAH-contaminated river sediment utilized anthracene, phenanthrene, pyrene, and fluoranthene as a sole source of carbon and energy (Dean-Ross et al. 2001). Recently, Fahy et al. (2008a) have investigated two groundwater samples from a BTEX-contaminated aquifer located below a petrochemical plant using 16S rDNA fingerprinting and found that aerobic benzene-degrading communities contain *Actinobacteria*, including *Rhodococcus* and *Arthrobacter*, which were enriched at high benzene concentrations. Alkali-tolerant benzene-degrading *R. erythropolis* strains were isolated; they have potential applications in bioremediation or natural attenuation of aromatic-contaminated alkaline waters (Fahy et al. 2008b). The prevalence of *Rhodococcus* in benzene- and toluene-degrading bacterial communities of compost-based biofilters treating air polluted with aromatic compounds was confirmed by cultivation-dependent (plate counts and isolated strain identification) and cultivation-independent [automated ribosomal intergenic spacer analysis (ARISA) and PCR-DGGE of 16s rRNA gene] methods (Juteau et al. 1999; Borin et al. 2006). It was hypothesized that *Rhodococcus* cells originally present in compost at very low concentrations were enriched during biofilter operation at

high benzene/toluene load and they have out-competed other aromatic-degrading bacteria, such as *Pseudomonas*.

Soils, sediments, and waters contaminated with chlorinated hydrocarbons and nitroaromatic compounds could also be a source for isolation of metabolically active *Rhodococcus* strains (Coleman et al. 1998; Wagner-Döbler et al. 1998; Poelarends et al. 2000; Seth-Smith et al. 2002; Petrić et al. 2007). For example, in the biphenyl-enriched microbial communities isolated from soils and sediments contaminated with PCBs, the majority of strains was identified as *R. opacus*, which out-competed other biphenyl-mineralizing bacteria in the microcosms during long-term enrichment, thereby demonstrating a great potential for use in bioremediation requiring long-term survival of inocula (e.g., for recalcitrant xenobiotic compounds, such as PCBs) (Wagner-Döbler et al. 1998). In a similar study of Petrić et al. (2007), the isolated *R. erythropolis* strain was characterized by the highest PCB-transformation potential comparable with that of the parental mixed culture obtained from PCB-contaminated soil. Several *Rhodococcus* strains able to aerobically degrade 2,4,6-trinitrophenol and hexahydro-1,3,5-trinitro-1,3,5-triazine were isolated from soils heavily contaminated with nitrophenols and explosives (Coleman et al. 1998; Seth-Smith et al. 2002; Shen et al. 2009b).

A large body of research showed that rhodococci are abundant and often predominant components of natural and industrial biofilms developing upon the contact with hydrocarbon contamination (Sorkhoh et al. 1995; Tresse et al. 2002; Di Lorenzo et al. 2005). For example, *Rhodococcus* members were most abundant among indigenous oil-degrading bacteria immobilized in cyanobacterial mats on crude oil-contaminated coasts of the Arabian Gulf (Sorkhoh et al. 1995). Microbial consortia of artificially developed oil-degrading biofilms on gravel particles and glass plates included hydrocarbon-oxidizing bacteria, namely nocardioforms (a group to which rhodococci belong) and *Acinetobacter calcoaceticus* partly attached to filaments of cyanobacteria (Al-Awadhi et al. 2003). The authors discussed a potential use of these biofilms for preparing trickling filters (gravel particles), and in bioreactors (glass plates) for biotreatment of oily wastes and oil-contaminated waters. Complex toluene-degrading biofilms developed on pumice granules in the laboratory bioreactor, following the inoculation with a microbial consortium obtained by enrichment of toluene contaminated water (Di Lorenzo et al. 2005). Interestingly, the identification of the species present in the biofilm based on 16S rDNA comparative analysis revealed that the majority (85%) of the attached cells was represented by *R. erythropolis*, whereas *Pseudomonas marginalis* represented only 10% of the entire consortium. On the contrary, in the inoculum used for biofilm development, *P. marginalis* was predominant (86%), and *R. erythropolis* was only 10% of the consortium. Apparently, adhesion to the pumice support promoted the growth of *R. erythropolis*, modifying the initial ratio between the two species.

From the results of numerous studies referenced above on the occurrence and frequent dominance of *Rhodococcus* members in diverse pristine and human-impacted ecosystems, it is clear that this genus is ubiquitous in the environment, associating with the presence of hydrocarbons and their substituted derivatives.

There are increasing numbers of *Rhodococcus* strains isolated from clean and contaminated environments able to degrade/transform hydrocarbon contaminants and xenobiotics; these strains are available from the culture collections worldwide (Home Pages of Culture Collections in the World 2009).

2.3 *Outstanding Physiological, Biochemical, and Ecological Properties of* Rhodococcus

Physiologo-biochemical and ecological properties of *Rhodococcus* suitable for environment bioremediation are listed in Table 1. First, rhodococci are characterized by high catabolic diversity and unique enzymatic capabilities (see chapters "Central metabolism of species of the genus Rhodococcus" by Alvarez, and "Catabolism of aromatic compounds and steroids by Rhodococcus" by Yam and Eltis). Chemical pollutants degraded by rhodococci range from aliphatic and aromatic hydrocarbons through chlorinated hydrocarbons and nitroaromatics to complex poly- and heterocyclic compounds. The reader is referred to the fundamental review of Warhurst and Fewson (1994) and more recent reviews (van der Geize and Dijkhuizen 2004; Larkin et al. 2005; Martínková et al. 2009). A number of studies on the degradation of most abundant environmental contaminants, crude oil, and its refinery products by rhodococci has been carried out (Whyte et al. 1998, 2001; Sharma and Pant 2000), and although many other bacteria can also degrade petroleum hydrocarbons, some novel catalytic pathways have been described in *Rhodococcus* (Whyte et al. 2002a, 2002b; Kim et al. 2004; van der Geize and Dijkhuizen 2004). Metabolic studies were focused on the rhodococcal pathways for alkane, cycloalkane, mono- and polyaromatic hydrocarbon biodegradation (Dean-Ross et al. 2001; Kim et al. 2004; Whyte et al. 2002a, 2002b; Larkin et al. 2005; Lee and Cho 2008), transformations of nitrogen and sulfur compounds (Xu et al. 2006; Shen et al. 2009a,b), and on the interactions between rhodococci and other oil-degraders (Van Hamme et al. 2003; Hamamura et al. 2006). Chlorophenols, chlorobenzenes, and polychlorinated biphenyls can be degraded by rhodococci (for review see Martínková et al. 2009), as well as other recalcitrant and toxic pollutants including nitriles (Baxter et al. 2006), sulphonated azo dyes (Joshi et al. 2008), herbicides (Nagy et al. 1995), metal cyanides (Baxter and Cummings 2006), benzothiazoles (Besse et al. 2001), hydrofurans (Daye et al. 2003) and many others.

2.3.1 Adaptation to Hydrocarbon Assimilation

Apart from remarkable biodegradative abilities, rhodococcal adaptation to hydrophobic contaminants is the important matter for bioremediation (Pieper and Reineke 2000). It is assumed that prolonged persistence of hydrophobic organic compounds in the environment is mainly determined by their solubilization-limited

Table 1 Physiological, biochemical, and ecological properties of *Rhodococcus* suitable for environmental bioremediation

Characteristics	Description	Advantage in bioremediation	Reference
High catabolic diversity	Degradation of wide range of chemicals, including aliphatic and aromatic hydrocarbons and their nitro- and halogenated derivates, oxygenates, and heterocyclic compounds	Biodegradation of complex contaminations, e.g., crude oil and industrial wastes. Biodegradation of recalcitrant xenobiotics	Warhurst and Fewson (1994), van der Geize and Dijkhuizen (2004), Martínková et al. (2009)
Aerobic and microaerophilic metabolism	Persistence in low-oxygen environments and activation upon oxygen supply	Bioremediation under well-aerated and oxygen-limited conditions (e.g., in high density soils, sediments, and hypoxic aquifers)	Travkin et al. (2002), Fahy et al. (2006, 2008a), Vogt et al. (2004), Joshi et al. (2008)
Oligotrophy and nitrogen-fixation	Growth under nutrient limitation conditions (C/N limitation) and resistance to long-term starvation	Bioremediation of resource-limited environments, e.g., low nutrient soils and ground waters	Ivshina et al. (1981), Elo et al. (2000), Mergaert et al. (2001), Priestley et al. (2006), Ohhata et al. (2007)
Lack of catabolic repression	Biodegradation of contaminants is not repressed by the presence of easily assimilable nutrients	Bioremediation of organic-rich environments, e.g., waste waters	Warhurst and Fewson (1994)
Adaptation to hydrophobic substrates	Cell-surface hydrophobicity, adhesion to hydrocarbons, and biosurfactant production	Enhanced biodegradation of hydrophobic pollutants	Lang and Philp (1998), Whyte et al. (1999)
Adhesion and biofilm formation	Adhesion to solid surfaces, cell aggregation, and biofilm formation	Application as immobilized or self-immobilized biocatalysts	Sorkhoh et al. (1995), Di Lorenzo et al. (2005), Borin et al. (2006)
Resistance to environmental stresses	Psychrotrophy, thermo-, xero- and galotolerance, pH-resistance, resistance to heavy metals and xenobiotics	Bioremediation in extreme environments and harsh industrial conditions	Whyte et al. (1999), Bej et al. (2000), Aislabie et al. (2006), Ryu et al. (2006), Fahy et al. (2008b)

(*continued*)

Table 1 (continued)

Characteristics	Description	Advantage in bioremediation	Reference
Ecological behavior	*K*-strategy and high competitiveness in communities under crowded, substrate-limited conditions	Survival in bioaugmentation inocula and indigenous communities	Juteau et al. (1999), Margesin et al. (2003), Borin et al. (2006)
Nonpathogenicity and lack of antagonistic properties	Low number of pathogenic species (only *R. equi* and *R. fascians*), lack of antimicrobial activity, plant biosafing	Biosafety and environmental safety	Bell et al. (1998), Nagy et al. (1995), Uroz et al. (2003), Aoshima et al. (2007)

bioavailability for microorganisms. Many bacteria can assimilate hydrophobic substances, for example, hydrocarbons, only in solubilized or emulsified forms. On the contrary, the hydrocarbon uptake by *Rhodococcus* occurs via the direct cell contact with large oil drops (Lang and Philp 1998). Thus, Whyte et al. (1999) observed physiological adaptations involved in alkane assimilation by *Rhodococcus* cells at a low temperature, these included production of cell-bound biosurfactants, increase in cell surface hydrophobicity, production of intracellular inclusions and extracellular polymers, and alteration of membrane fluidity. The authors suggested that high cell hydrophobicity and cell-associated biosurfactants promote the adhesion of rhodococcal cells not only to liquid alkanes, but also to hydrophobic solid surfaces, allowing direct uptake from sorbed/crystalline hydrocarbons, another important mechanism of microbial assimilation of hydrophobic pollutants in soils and sediments (Wattiau 2002). In our experiments, *Rhodococcus* biosurfactants desorbed crude oil from soil, thus facilitating its biodegradation by soil microorganisms (Kuyukina et al. 2005). It should be noted that many hydrocarbons, for example low molecular weight alkanes, monoaromatic, and chlorinated aliphatic compounds, are toxic to microorganisms primarily owing to the solvent effect on cell membranes. They destroy microbial cells and therefore abolish the desired biodegradative activity. Several *Rhodococcus* strains resistant to organic solvents have been isolated, and possible mechanisms of solvent tolerance, such as alterations in the composition of cell envelope, have been reported (for a review, see chapter, "Adaptation of *Rhodococcus* to organic solvents" by de Carvalho).

2.3.2 Ecological Plasticity

Another important *Rhodococcus* feature is the ability to persist and metabolize in microaerophilic and oligotrophic conditions. Rhodococci could be isolated from microaerophilic environments, for example, high density soils, deep-sea sediments, and hypoxic aquifers (Colquhoun et al. 1998; Hendrickx et al. 2005; Fahy et al. 2006).

The study of Travkin et al. (2002) reported the isolation of an enrichment culture and a *Rhodococcus* strain derived from it, transforming 3,4-halogenated anilines under nitrate-reducing conditions. Anaerobic bioconversion of these haloanilines by *Rhodococcus* sp. started with reductive deamination, resulting in production of dihalobenzene intermediates, which were further dehalogenated in the biodegradation pathway. A natural bacterial consortium consisting of *Aeromonas caviae*, *Proteus mirabilis*, and *R. globerulus* was reported to decolorize azo dyes under microaerophilic condition in the presence of the organic carbon source (Joshi et al. 2008). The consortium decolorized 16 azo dyes individually as well as in simulated mixed wastewater, suggesting its possible application in industrial wastewater treatment. Although the above-mentioned studies reveal the possibility of *Rhodococcus* application in microaerophilic/anaerobic biodegradation of contaminants, the vast majority of extensive researches resume that rhodococci can persist well in low-oxygen environments and rapidly increase in number upon the oxygen supply, thus contributing to aerobic (oxidative) environmental decontamination (Vogt et al. 2004; Fahy et al. 2008a). Ohhata et al. (2007) isolated a *R. erythropolis* strain N9T-4 from a crude oil sample, and found that this strain and some other collection *R. erythropolis* strains grew in extremely oligotrophic conditions, suggesting that the oligotrophy could be a common feature of *Rhodococcus* (Mergaert et al. 2001). Additionally, there are several reports on the abilities of rhodococci to grow and degrade organic contaminants under carbon- and nitrogen-limiting conditions (Priestley et al. 2006), to fix atmospheric nitrogen (Ivshina et al. 1981; Elo et al. 2000), and to oxidize complex pollutants even in the presence of more easily assimilable carbon sources (Warhurst and Fewson 1994). These features make rhodococci the promising candidates for bioremediation of both recourse-limited and organic-rich environments.

Many contaminated sites are characterized by harsh environmental conditions, for example, low or elevated temperatures, acidic or alkaline pH, high salt concentrations, or high pressure. Apparently, extremotolerant *Rhodococcus* members adapted to grow and thrive in these environments play an important role in bioremediation of polluted extreme habitats (Sorkhoh et al. 1990; Whyte et al. 1999; Aislabie et al. 2006; Ryu et al. 2006; Fahy et al. 2008b). A hydrocarbon-degrading potential of cold-adapted rhodococci was mentioned earlier in this chapter. It is assumed that low temperature greatly influences the process of hydrocarbon biodegradation in soil by affecting both the physical nature of spilled oil and microbial metabolism (Fig. 2). Particularly, at low temperatures, the oil viscosity is increased and the volatilization of toxic low-molecular weight compounds is reduced, thus decreasing the bioavailability and biodegradation of hydrocarbon pollutants. Additionally, soils of cold regions are often subject to drying because of low precipitation and long freezing periods. The low soil moisture results in nutrient diffusion limitations and, in addition to typically low available N and P contents of these soils, lead to development of oligotrophic conditions for soil microorganisms (Aislabie et al. 2006). On the one hand, it is important that complex physiological adaptations of rhodococci (see Fig. 2) allow them to survive in such extreme cold environments as well as to contribute to microbiological

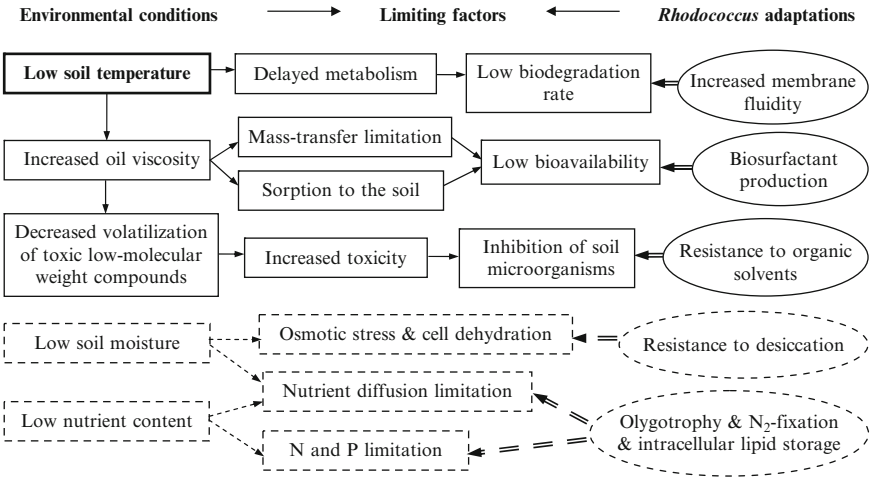

Fig. 2 Adaptations of *Rhodococcus* to cold soil conditions. Optional environmental conditions, factors and corresponding adaptations are shown by *dotted lines*

degradation of hydrophobic pollutants (Bej et al. 2000). On the other hand, indigenous mesophilic hydrocarbon-oxidizing rhodococci were suggested as promising bioremediation agents for hot climate regions, for example, for the Kuwaiti desert soil heavily contaminated with crude oil (Sorkhoh et al. 1990). Moreover, there are increasing numbers of reports on the isolation of heat-resistant enzymes, for example, specific dioxygenases, catalyzing initial steps of degradation of (poly) aromatic compounds by *Rhodococcus* cells (Gakhar et al. 2005; Yang et al. 2008). These enzymes, due to their high chemical and thermal stability, offer powerful tools for biological treatment of polluted environments and industrial wastes at elevated temperatures. One more important aspect of bioremediation is rhodococcal resistance to heavy metal ions and their bioaccumulation, including those that are radioactive (Bell et al. 1998; Ivshina et al. 2002). Since heavy metals are often present in hydrocarbon-contaminated soils associated with oil spills, petrochemical and other chemical waste discharges, and in industrial effluents, rhodococci can be used for bioremediation of such complex metallo-organic contaminations.

The ability of rhodococci to adhere to different surfaces and to form biofilms is widely used to develop immobilized biocatalysts suitable for various eco-biotechnological applications, namely bioreactor treatment of contaminated waters and sludges, and soil bioremediation (Prieto et al. 2002a; Di Lorenzo et al. 2005; Podorozhko et al. 2008; Kuyukina et al. 2009). It is assumed that microorganisms in biofilms are more resistant to environmental stressors than in a free-living state. Therefore, rhodococci with high adhesive activities, especially toward hydrophobic surfaces, tend to be more successful in colonizing of hydrocarbon-contaminated sites compared to less adhering microorganisms (Masák et al. 2004). Relative prevalence of *Rhodococcus* species in many natural and laboratory contaminant-degrading

microbial consortia may be also explained by their ecological behavior, particularly the *r-K* scheme, which suggests that evolution favors either adaptation to high rates of reproduction (*r* strategists) or optimal utilization of environmental resources (*K* strategists) (Margesin et al. 2003). Bacteria, such as pseudomonads, which rapidly grow in nutrient-rich media, are *r* strategists. Others, such as rhodococci, tend to be more successful in resource-limited, crowded environments are *K* strategists. Apparently, populations of *K* strategists would be more stable and permanent members of the communities of chronically contaminated biotopes or bioreactor microcosms, when easy-degradable substrates are depleted and the competition for nutrients is intensive.

2.3.3 Biosafety Aspects

One potential problem with bioaugmentation is that the inocula may contain microorganisms harmful for the human health or environment, for example, human, animal, and plant pathogens or strains producing toxins or antibiotics, which were present in indigenous populations or came from laboratory media contamination, and were enriched during the inoculum growth. To prevent the pathogen occurrence in bioaugmentation inocula, such consortia should contain only taxonomically defined microorganisms belonging to species known as nonpathogenic. Moreover, in some countries, national environmental and health authorities require an assessment of biosafety and environment safety of microbial inocula intended for bioremediation applications (Aoshima et al. 2007). The study of Aoshima et al. (2007) evaluated the safety of the hydrocarbon-oxidizing soil isolate *R. erythropolis* C2 for the application in open oil-contaminated ecosystems and found that this strain demonstrates the lack of pathogenicity, mutagenicity, or ecotoxicity. It therefore requires no special occupational health precautions during application processes, and has low environmental impact. These results are in agreement with other literature data indicating that only two *Rhodococcus* species, *R. equi* and *R. fascians*, are associated with pathogenicity for animals and plants, respectively (Bell et al. 1998; chapter "*Rhodococcus equi* and its pathogenic mechanisms" by Vázquez-Boland). Such relatively small proportion of pathogenic species is rare within the micolata group of actinobacteria harboring genera *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia*, and *Tsukamurella*, characterized by abundant presence of human pathogens. Additionally, literature and our research data suggest that rhodococci are unlikely to produce any toxins or antimicrobial compounds (Kitamoto et al. 2002; Kuyukina et al. 2007); this is another strong advantage of *Rhodococcus* applications in environment bioremediation technologies. Furthermore, the rhizospheric *R. erythropolis* strain W2 degrading *N*-acylhomoserine lactones was shown to be effective in quenching of quorum-sensing-regulated functions of plant pathogenic bacteria, thereby reducing their pathogenicity (Uroz et al. 2003). This bacterium and herbicide-degrading *Rhodococcus* strains applicable for plant biosafing (Nagy et al. 1995) could be used, for example, in phytoremediation projects for contaminated agricultural soils.

3 *Rhodococcus* Applications in Bioremediation Technologies

Biological remediation of terrestrial and aquatic habitats contaminated with hazardous compounds received increasing attention in early 1990s, with enhanced awareness of the potential harmful effects on human health and the environment (Alexander 1999). Various bioremediation techniques have been used at a large number of sites contaminated with organic compounds since the most well-known clean-up of oil spilt from the Exxon Valdez in Prince William Sound, Alaska, in 1989 (Van Hamme et al. 2003). A historical aspect of *Rhodococcus* application in environment bioremediation could be addressed to late 1990s, when first attempts were made to remediate oil-contaminated lands using naturally accruing rhodococci or laboratory *Rhodococcus* cultures (Sorkhoh et al. 1995; Koronelli et al. 1997; Christofi et al. 1998). Upon revealing new catabolic abilities of *Rhodococcus* species and isolation of environmental strains degrading a wide range of contaminants, these bacteria have been increasingly explored for bioremediation of soils, waters, and air polluted with different recalcitrant and toxic organic chemicals (Fig. 3).

3.1 In Situ Treatment

In-situ bioremediation comprises various techniques, which treat contaminated material in place (without excavation and transfer) and keep the material treated

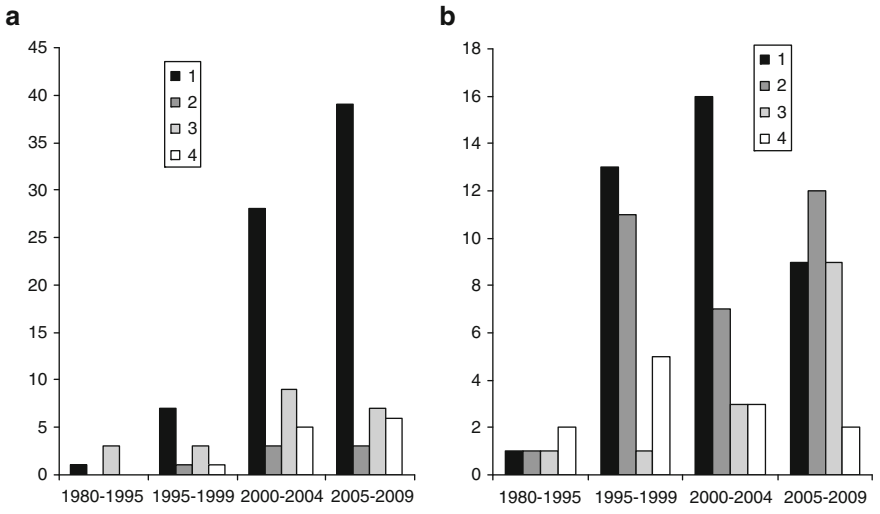

Fig. 3 Number of publications (**a**) and patents (**b**) concerned to *Rhodococcus* applications in environment bioremediation (according to http://www.scopus.com). Queries: Title/Abstract/Keywords: *Rhodococcus* and (1) soil bioremediation; (2) ground water bioremediation; (3) bioreactor degradation; (4) air biofilter. Nonrelevant papers/patents were removed from the query results

essentially undisturbed. These techniques are usually applied to the remote or difficult of access polluted environments, such as forest soils and wetlands, aquatic sediments and subsurface zones, as well as in cases when intrusive methods are inappropriate, for example, for remediation of unique landscapes and valued soils. Most in-situ processes involve stimulation of indigenous microbial populations through the addition of nutrients and other factors enhancing biodegradation (e.g., surfactants and oxygen source), and sometimes the augmentation with specifically adapted microbial cultures possessing high biodegradative abilities (Van Hamme et al. 2003). The limited number of laboratory and field studies referenced in Table 2 suggested that rhodococci, either as parts of bioaugmentation inocula or members of indigenous communities, can be successfully used for reducing in situ organic contaminant levels. Thus, laboratory soil microcosm study using an indigenous psychrotolerant alkane-degrading *Rhodococcus* strain ADH reported a positive effect of bioaugmentation on the biodegradation of diesel fuel (Ruberto et al. 2005). Although the natural microflora responded significantly to the pollutants, *Rhodococcus*-inoculated microcosms showed enhanced biodegradation compared to noninoculated soil and sterilized controls. The survival and activity of *Rhodococcus* sp. strain 1BN introduced into naphthalene-contaminated sandy-loam soil were studied by Cavalca et al. (2002). The naphthalene consumption and CO_2 production rates were the highest in the *Rhodococcus*-amended sterilized soil, although inoculation of nonsterile soil did not enhance significantly the biodegradation process, indicating a considerable bioremediation potential of the indigenous naphthalene-degrading bacteriocenosis. Nevertheless, the introduced *Rhodococcus* strain was well-established in the contaminated soil even in the presence of native naphthalene-degrading bacteria. Several treatability studies showed positive effects of *Rhodococcus* cocultures with other bacteria and fungi, as well as rhodococcal associations with higher plants and earthworms on biodegradation of petroleum hydrocarbons and polychlorinated biphenyls in soil and sediments (Luepromchai et al. 2002; Rodrigues et al. 2006; Hong et al. 2007; Kim and Lee 2007). Additionally, in situ bioremediation can be an economically advisable and environmentally harmless approach to cleanup shorelines contaminated as a result of marine oil-spills. During the field-scale trial carried out by Jiménez et al. (2007), some success in the removal of heavy fuel oil from the cobble beach on the Cantabrian coast (north Spain) polluted after the oil tanker *Prestige* crush in 2002 was achieved using the oleophilic fertilizer, and the biodegradative potential of the indigenous microbial community, including *Rhodococcus* representatives was established.

It is assumed that engineered in-situ bioremediation is a feasible and effective method for treating contaminants within the saturated zone of soil and contaminated groundwater. Engineering in-situ technologies involve drilling a series of wells for direct injection of appropriate solutions into the subsurface or the construction of reactive permeable barriers allowing the passage of groundwater while promoting the biodegradation of contaminants. Although polluted subsurface and groundwater systems are often very low in oxygen and nutrients and therefore characterized by slow biological oxidation rates, several laboratory and field-scale studies described successful applications of reactive biobarriers inoculated with

Table 2 Examples of *Rhodococcus* application for bioremediation of contaminated environments

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	Reference
In situ treatment – Treatability studies				
Antarctic soil contaminated with diesel fuel	Laboratory bioaugmented soil system exposed to natural climate conditions of Antarctica	Liquid culture of *Rhodococcus* sp.	+	Ruberto et al. (2005)
Naphthalene-contaminated soil from industrial area	Laboratory bioaugmented soil systems	Liquid culture of *Rhodococcus* sp.	+	Cavalca et al. (2002)
PAH-contaminated soil from petroleum refinery site	Laboratory bioaugmented soil systems	Liquid coculture of *Rhodococcus* sp. and *Aspergillus terreus*/*Penicillium* sp.	+	Kim and Lee (2007)
Diesel-contaminated soil	Soil inoculated with diesel-degrading *Rhizobacterium* and seeded with diesel-resistance *Zea mays*	Liquid culture of *Rhodococcus* sp.	+	Hong et al. (2007)
Aroclor 1242-contaminated soil	Laboratory soil column with mineral nutrient addition, bioaugmented with bacteria and earthworms	Liquid culture of *Ralstonia eutrophus* and *Rhodococcus* sp.	+	Luepromchai et al. (2002)
Aroclor 1242-contaminated sediment	Laboratory two-stage anaerobic/aerobic biotreatment (aerobic stage)	Liquid culture of recombinant *Burkholderia xenovorans* and *Rhodococcus* sp. strains	+	Rodrigues et al. (2006)
Benzene-contaminated groundwater	Laboratory groundwater microcosms	Indigenous bacterial community containing *Rhodococcus*	+	Fahy et al. (2006, 2008a)
In situ treatment – Field studies				
Shore-line contaminated with heavy fuel-oil	Plots on the cobblestone beach spread with oleophilic fertilizer S200 (IEP Europe)	Indigenous bacterial community containing *Rhodococcus*	+	Jiménez et al. (2007)
Crude oil-polluted river bay, lakes, wetland and marshy peat soil	Preliminary mechanical collection of spilled oil using skimmers followed by spraying with biopreparation and mineral nutrients	Liquid biopreparation *Rhoder* consisting of *R. erythropolis* and *R. ruber*	+	Murygina et al. (2005)
Groundwater polluted with methyl *t*-butyl ether and *t*-butyl alcohol	Biobarrier plots with O_2- or air-sparging	Mixed culture or pure *Rhodococcus* sp. culture isolated from polluted groundwater	+	Salanitro et al. (2001)
Chlorobenzene-polluted groundwater	Reactive barrier supplied with hydrogen peroxide and nitrate solution	Indigenous bacterial community containing *Rhodococcus*	+	Vogt et al. (2004)

(*continued*)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	Reference
On site treatment – Treatability studies				
Crude oil-contaminated Arctic soil spiked with *n*- hexadecane	Laboratory bioaugmented soil systems	Liquid culture of *Rhodococcus* sp.	+	Whyte et al. (1998)
Arctic soil contaminated with *n*-alkanes or diesel fuel	Laboratory soil systems with additions of inorganic/organic fertilizers and bulking agents	Indigenous bacterial community containing *Rhodococcus*	+	Whyte et al. (2001)
Arctic soil contaminated with weathered diesel fuel	Laboratory bioaugmented soil system	Liquid enriched indigenous culture containing *Rhodococcus*	+/−[a]	Thomassin-Lacroix et al. (2002)
Soil contaminated with crude oil	Laboratory soil systems with mineral nutrient addition	Indigenous bacterial community containing *Rhodococcus*	+	Peressutti et al. (2003)
Soils from geographically distinct areas contaminated with weathered crude oil	Laboratory soil systems with mineral nutrient addition	Indigenous bacterial community containing *Rhodococcus*	+	Hamamura et al. (2006)
Soil contaminated with fuel oil	Laboratory bioaugmented soil plots with bulking agent and mineral nutrient addition	Liquid biopreparation *Devoroil* containing *R. erythropolis* and *Rhodococcus* sp.	+	Sidorov et al. (1998)
Soil contaminated with BTEX	Laboratory bioaugmented soil systems with mineral nutrient addition	Liquid culture of *R. opacus*	+	Taki et al. (2004)
Soil contaminated with BTEX	Laboratory soil systems with mineral nutrient addition	Indigenous bacterial community with dominating *Rhodococcus* group	+	Taki et al. (2007)
Soil contaminated with disulfide oil	Laboratory bioaugmented soil systems with mineral nutrient and glucose addition	Vermiculite-immobilized culture of *Rhodococcus* sp.	+	Taheri et al. (2008)
On site treatment – Field studies				
Crude oil-contaminated desert soil	Bioaugmented landfarming cells with addition of mineral nutrients and lime	Liquid mixed culture containing *Rhodococcus*, removed from cyanobacterial mats floating in oil-polluted waters	+	Sorkhoh et al. (1995)
Tundra soil artificially contaminated with crude oil	Bioaugmented soil plots with mineral nutrient addition	Lyophilized culture of *R. erythropolis*	+	Koronelli et al. (1997)
Soil artificially contaminated with crude oil and oil-field brine	Bioaugmented landfarming cells with mineral nutrient addition	Liquid biopreparation *Devoroil* containing *R. erythropolis* and *Rhodococcus* sp.	+	Sidorov et al. (1997)

(*continued*)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	Reference
Crude oil-contaminated soil	Composted inoculated biopiles with addition of mineral nutrients and straw as bulking agent	Liquid culture of biosurfactant-producing *Rhodococcus ruber*	+	Christofi et al. (1998)
Arctic soil contaminated with weathered diesel fuel	Inoculated biopiles with addition of fertilizer (granular urea and diammonium phosphate), surfactant and cocoa-fiber bulking agent	Liquid enriched indigenous culture containing *Rhodococcus*	+/−[a]	Thomassin-Lacroix et al. (2002)
Crude oil-contaminated soil	Landfarming cells with addition of wood-chips and oleophilic biofertilizer	Oleophilic biofertilizer containing *R. erythropolis* and *R. ruber*	+	Kuyukina et al. (2003)
Polar marshy wetland polluted with crude oil	Landfarming + phytoremediation with addition of biopreparation, inorganic fertilizer and lime	Liquid biopreparation *Rhoder* consisting of *R. erythropolis* and *R. ruber*	+	Murygina et al. (2005)
Dehydrated oil sludge	Inoculated biopiles with addition of sand, woodchips, and inorganic fertilizer	Liquid biopreparation *Rhoder* consisting of *R. erythropolis* and *R. ruber*	+	De-qing et al. (2007)
Soil contaminated with fuel hydrocarbons	Inoculated biopile with addition of $(NH_4)_2HPO_4$	Liquid culture of *Pseudomonas putida*, *Acinetobacter johnsonii* and *R. erythropolis*	+	Genovese et al. (2008)
Bioreactor/biofilter treatment – Laboratory studies				
Soil from former coke works site contaminated with $K_2Ni(CN)_4$	Laboratory shaking-flask experiments (1 g soil/100 ml minimal salt medium) with glucose addition	Liquid culture of *Rhodococcus* sp.	+/−[a]	Baxter and Cummings (2006)
Soil contaminated with bis (2-ethylhexyl)phthalate	Slurry-phase batch and sequencing batch bioreactors with mineral nutrient addition	Mixed culture isolated from contaminated soil and containing *R. fascians*	+	Juneson et al. (2001)
Aged polychlorinated biphenyl-contaminated soils	Aerobic solid-phase and packed-bed loop reactors with addition of mineral nutrients, biphenyl and methyl-β-cyclodextrins	Indigenous bacterial community containing *Rhodococcus*	+	Fava et al. (2003)
Sediment contaminated with polycyclic aromatic hydrocarbons	Aerobic slurry-phase bioreactors with mineral nutrient addition	Indigenous community containing *Rhodococcus*	+	Ringelberg et al. (2001)

(*continued*)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	Reference
Phenol and formaldehyde contaminated synthetic and industrial wastewaters	Laboratory shaking-flask experiments with addition of phosphate, ammonium, and micronutrients	Liquid culture of *R. erythropolis*	+	Hidalgo et al. (2002a, b)
Phenol and formaldehyde contaminated synthetic and industrial wastewaters	Air-stirred and packed-bed column bioreactors with addition of nitrogen source and micronutrients	Biolite- and diatomaceous earth-immobilized cultures of *R. erythropolis*	+	Prieto et al. (2002a, b)
Aroclor 1242 and biphenyl–contaminated water	Granular biofilm reactor with limited aeration and addition of mineral nutrients, yeast extract, and microelements	*Rhodococcus* sp. culture immobilized on anaerobic sludge granules	+/−[b]	Tartakovsky et al. (2001)
Simulated azo dye-contaminated wastewater	Laboratory flask experiments under microaerophilic condition, with addition of yeast extract and glucose	Mixed culture isolated from dye-contaminated soil and sludge and containing *R. globerulus*	+	Joshi et al. (2008)
Model petroleum-contaminated water	Fluidized-bed column bioreactor	Sawdust-immobilized culture of *R. ruber* and *R. opacus*	+	Kuyukina et al. (2009)
2,4-Dinitrophenol-contaminated water	Column plug-flow bioreactor	Agar granule-immobilized culture of *R. erythropolis*	+	Kitova et al. (2004)
2,4,6-Trinitrophenol or picric acid-polluted wastewater or groundwater	Sequencing batch bioreactor	Liquid culture of *R. opacus*	+	Weidhaas et al. (2007)
Model wastewater containing 2,4,6-trinitrophenol	Pilot biological aerated filter with mineral nutrient addition	Liquid culture of *Rhodococcus* sp.	+	Shen et al. (2009a)
Methylhydrazine/hydrazine-contaminated wastewater	Fixed-film trickle-bed bioreactor	Coarse sand-immobilized culture of *Rhodococcus* sp.	+	Nwankwoala et al. (2001)
Artificial toluene-contaminated waste gas	Rotating biological contactor with mineral nutrient addition	Liquid culture of *Pseudomonas putida* and *R. erythropolis*	+	Vinage and von Rohr (2003)
Air polluted with isopropylbenzene	Two-phase partitioning bioreactor with addition of ethanol	Liquid culture of *R. erythropolis*	+	Aldric and Thonart (2008)
Air polluted with benzene	Compost-packed biofilter	Indigenous compost community containing *Rhodococcus*	+	Borin et al. (2006)
Bioreactor/biofilter treatment – Pilot/field studies				
Crude oil-contaminated soil	Field slurry-phase bioreactor with addition of oleophilic biofertilizer	Oleophilic biofertilizer containing *R. erythropolis* and *R. ruber*	+ +	Kuyukina et al. (2003)

(*continued*)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	Reference
Waste oil contaminated with polychlorinated biphenyls	Pilot plant consisting of UV-irradiation equipment and two successive bioreactors	Liquid culture of *Comamonas testosteroni* and *R. opacus*		Kimbara et al. (1998)
Industrial wastewater polluted with 1,3-dichloropropene and other chlorinated aliphatics	Pilot extractive membrane biofilm reactor	Mixed biofilm culture isolated from bioreactor and containing *R. erythropolis*	+	Katsivela et al. (1999)
Synthetic tetrahydrofuran-contaminated wastewater	Pilot membrane bioreactor	Mixed culture isolated from the industrial wastewater treatment plant and containing *R. ruber*	+	Daye et al. (2003)
Industrial wastewater contaminated with hydrocarbons, surfactants and heavy metals	Pilot biotreatment installation	Immobilized algal–bacterial coculture containing *Rhodococcus* sp.	+	Safonova et al. (2004)

[a]+/−, Effect of bioaugmentation did not exceed that of biostimulation; [b]+/−, Effect of *Rhodococcus* immobilized on anaerobic sewage did not exceed that of anaerobic sewage alone

Rhodococcus pure or mixed cultures (Salanitro et al. 2001), and supplied with oxygen and mineral nutrients (Vogt et al. 2004) for degradation of recalcitrant groundwater contaminants such as chlorinated benzene, methyl *t*-butyl ether, and *t*-butyl alcohol.

3.2 On Site Treatment

As evident from Table 2, the on-site bioremediation using *Rhodococcus* mostly involves the landfarming and biopile treatment of complex petroleum hydrocarbon mixtures, including crude oil, diesel fuel, and fuel oil. Historically, landfarming was one of the first forms of on-site contamination treatment and was widely used in oil industry for the disposal and neutralization of oily wastes. The waste material is applied to clean soil and landfarming area managed by tilling, fertilization, watering, and addition of bulking agents to maintain optimum soil conditions of nutrients, moisture, and pH. Microorganisms used in the biodegradation process are mostly indigenous soil populations. However, high concentrations of toxic contaminants present in oily wastes often hinder the development of resident oil-oxidizing microbial consortia. Therefore, the bioaugmentation with selected cultures of oil-degrading microorganisms adapted to high toxicant content and harsh environmental conditions may be used to enhance bioremediation process. It should be noted that while landfarming of oily sludges is no longer considered environmentally acceptable due to large volumes of clean soil contaminated during the process, and

the potential risk of contaminant leaching and emission from the treatment area, it is still being used in many countries (Van Hamme et al. 2003). More recently, ventilated and composting biopiles, which involve a greater degree of engineering and containment, have been developed for the oil-contaminated soil and sludge treatment. The contaminated material is removed to a specifically prepared area, which is usually lined with low permeability material such as high-density polyethylene or clay to minimize contaminant movement off-site. Soil biopile systems often include leachate-collecting and sometimes emission-control facilities. Construction of composting biopiles involving a succession of mesophilic and thermophilic microorganisms consists of piling the contaminated soil and mixing with an organic bulking agent such as composted agricultural waste, straw, or wood chips. The piles are aerated by either passive or forced ventilation or pile turning, and the temperature, pH, moisture, and nutrient contents are controlled.

Several laboratory studies have reported favorable effects of *Rhodococcus* augmentation on petroleum hydrocarbon biodegradation in soils at low (Whyte et al. 1998) and mesophilic (Sidorov et al. 1998; Taki et al. 2004; Taheri et al. 2008) temperatures. Thus, mineralization of ^{14}C-labeled hexadecane at 5°C was significantly greater in both crude oil-contaminated and pristine soil microcosms seeded with *Rhodococcus* sp. Q15 cells compared to noninoculated control soil microcosms (Whyte et al. 1998). Moreover, efficient removal of less degradable contaminants such as aromatic hydrocarbons (including most recalcitrant *o*-xylene) and fuel oil from soil was obtained upon soil inoculation with either pure culture of *R. opacus* (Taki et al. 2004) or mixed culture of hydrocarbon-oxidizing bacteria containing *R. erythropolis* and *Rhodococcus* sp. strains (Sidorov et al. 1998). Recently, Taheri et al. (2008) have performed a feasibility study for the soil polluted with disulfide oil, a waste product of liquefied petroleum gas desulphurization, and found that a vermiculite-immobilized *Rhodococcus* sp. strain previously isolated from disulfide oil-contaminated soil has a great potential for its bioremediation, although no comparison of immobilized and liquid forms of inoculum was made. There are also few reports on considerable bioremediation potential of indigenous bacterial communities inhabiting hydrocarbon-contaminated soils and containing large proportions of *Rhodococcus* representatives (Whyte et al. 2001; Hamamura et al. 2006), which could be enriched in laboratory microcosms during bioremediation process (Peressutti et al. 2003; Taki et al. 2007). However, the laboratory microcosm study of Thomassin-Lacroix et al. (2002) demonstrated low effect of bioaugmentation of fuel-contaminated Arctic tundra soil with enriched bacterial culture originated from the same soil and containing *Rhodococcus* members, indicating that biostimulation with a mineral nitrogen source was sufficient for the soil naturally rich in hydrocarbon-degrading microorganisms (including *Rhodococcus*).

It is now generally agreed that results of field bioremediation can differ significantly from the laboratory studies due to much stronger and more complex influence of environmental factors, both abiotic and biotic, on contaminant biodegradation process. Since bench-scale feasibility results often lack representativeness to field situations, field bioremediation trials must be conducted to corroborate findings of laboratory experiments. Small-scale field experiments conducted

by Koronelli et al. (1997) reported that introduction of a hydrocarbon-degrading strain of *R. erythropolis* into tundra soil artificially contaminated with crude oil resulted in increased counts of hydrocarbon-degrading bacteria and an increased rate of hydrocarbon degradation. Christofi et al. (1998) found that inoculation with biosurfactant-producing *R. ruber* increased counts of hydrocarbon-oxidizing bacteria persisted in composted crude oil-contaminated soil and enhanced oil biodegradation. Sorkhoh et al. (1995) have used naturally occurring bacterial consortia removed from cyanobacterial mats floating in oil-polluted waters in the Arabian Gulf to inoculate oil-contaminated sand. This increased removal of oil from the sand, and rhodococci appeared to predominate in microbial populations. Bacterial preparations consisting of two-component *R. erythropolis* and *R. ruber* cultures (Kuyukina et al. 2003; Murygina et al. 2005; De-qing et al. 2007), a three-component bacterial culture of *R. erythropolis*, *P. putida*, and *Acinetobacter johnsonii* (Genovese et al. 2008), and a complex bacterial–yeast consortium of *Dietzia* (former *Rhodococcus*) *maris*, *R. erythropolis*, *Rhodococcus* sp., *Pseudomonas stutzeri*, and *Candida* sp. (Sidorov et al. 1997) were successfully used in field trials on bioremediation of soils contaminated with crude oil, fuel, and oily wastes. It should be noted that in most field studies referenced above, contaminated soils were seeded with bacterial inocula and amended by addition of mineral fertilizers, bulking agents and other factors stimulating biodegradation process, thus suggesting that combination of two bioremediation approaches, bioaugmentation, and biostimulation is essential for the cleanup of hydrocarbon-contaminated soils.

3.3 Bioreactor Treatment

Bioreactors are widely used to degrade toxic compounds in industrial effluents to prevent environmental pollution. Furthermore, bioreactor treatment of contaminated soils and sludge is not yet mainstream, but growing technology that overcomes some rate-limiting and variability factors observed in landfarming and biopile processes. Particularly, bioreactor-based technologies allow more precise control and management of biodegradation parameters such as temperature, pH, oxygen, nutrient and water contents, homogenous distribution of contaminated material and biomass in the reactor volume, which leads to increased mass transfer and reaction rates (Van Hamme et al. 2003). Several laboratory studies reported the application of indigenous bacterial communities containing *Rhodococcus* representatives in bioreactors of different types for treatment of contaminated soils and sediments; these included solid-phase, slurry-phase, and packed-bed loop reactors and resulted in reducing the levels of phthalate esters, polychlorinated and polycyclic aromatic compounds (Juneson et al. 2001; Ringelberg et al. 2001; Fava et al. 2003). We have used an oleophilic biofertilizer in the form of concentrated emulsion of hydrocarbon-grown *R. erythropolis* and *R. ruber* cultures and mineral salt solution stabilized by a *Rhodococcus*-biosurfactant for successive treatment of crude oil-contaminated soil in a field slurry bioreactor and land farming plots, and found out that high

biodegradation rate for petroleum hydrocarbons can be achieved following stimulation of the degradation process in a slurry bioreactor (Kuyukina et al. 2003).

Rhodococci are also candidate organisms for use as inocula in contaminated water treatments, demonstrating promising results in laboratory simulations (see Table 2 for references). For example, inoculations with suspended and biolite/diatomaceous earth-immobilized *R. erythropolis* cells were shown to be efficient in the biotreatment of phenol- and formaldehyde-contaminated synthetic and industrial wastewaters (Hidalgo et al. 2002a, b; Prieto et al. 2002a, b). Sawdust-immobilized *R. ruber* IEGM 615 and *R. opacus* IEGM 249 cells degraded petroleum hydrocarbons (including aliphatic from C_{10} to C_{19} and polyaromatic) to a great extent when applied to a fluidized-bed bioreactor, and retained high catalytic activity during repeated bioreactor cycles (Kuyukina et al. 2009). A coarse sand-immobilized *Rhodococcus* sp. culture was successfully used in a fixed-film trickle-bed bioreactor treating wastewaters contaminated with methylhydrazine/hydrazine (Nwankwoala et al. 2001). It has been reported that a mixed culture isolated from azo dye-contaminated soil and containing *R. globerulus* cells was able to decolorize azo dyes under microaerophilic conditions (Joshi et al. 2008). However, another microaerophilic biodegradation study conducted by Tartakovsky et al. (2001) showed no significant difference in reduction of Aroclor 1242 levels in *Rhodococcus*-bioaugmented and nonbioaugmented reactors, although identification of indigenous bacterial populations of the nonbioaugmented reactor by 16S rDNA sequencing revealed *Rhodococcus* members among other biphenyl-degrading bacteria. Liquid and agar-immobilized *Rhodococcus* spp. cultures were efficiently applied to different type bioreactors treating di- and trinitrophenol-contaminated waters (Kitova et al. 2004; Weidhaas et al. 2007; Shen et al. 2009a). Several pilot-scale bioreactor studies referenced in Table 2 involved *Rhodococcus* applications to industrial waste and wastewater treatments (Kimbara et al. 1998; Katsivela et al. 1999; Daye et al. 2003; Safonova et al. 2004). Particularly, pilot membrane bioreactors inoculated with *Rhodococcus*-containing mixed cultures were used to treat tetrahydrofuran and dichloropropene wastestreams (Katsivela et al. 1999; Daye et al. 2003). Industrial wastewater inoculation with an algal–bacterial coculture containing a *Rhodococcus* sp. strain Ac-1267 and immobilized on capron fibers resulted in the formation of a stable microbial consortium and significant decrease of petroleum hydrocarbon, phenol, anionic surfactant, and heavy metal concentrations (Safonova et al. 2004). A pilot plant consisting of UV-irradiation equipment and two successive bioreactors was constructed to treat PCB-contaminated waste oil from high voltage transformers and condensers, and liquid cultures of *Comamonas testosteroni* TK102 and *R. opacus* TSP 203 were used as inocula for bioreactors providing complete biodegradation of PCBs partially dechlorinated by the UV-pretreatment (Kimbara et al. 1998).

Biological oxidation of volatile organic carbon vapors by microbial biofilms formed on a solid support in biofilters/bioreactors provides an effective and inexpensive alternative to physico-chemical methods (Vinage and von Rohr 2003). A modified rotating biological contactor inoculated with a suspension of *P. putida* F1 and *R. erythropolis* PWD1 was proposed by Vinage and von Rohr (2003) for the

biological treatment of artificial waste gas polluted with toluene vapors. The proposed system allowed proper control of the biofilm growth and long-term bioremediation performance for a year indicating its feasibility for industrial applications. Borin et al. (2006) investigated microbial succession in a compost-packed biofilter treating benzene-contaminated air and found out that the maximum benzene removal rate strongly correlated with the prevalence of *Rhodococcus* representatives in the bacterial community, thus suggesting their major role in benzene degradation. Aldric and Thonart (2008) evaluated the performance of a water/silicone oil two-phase partitioning bioreactor inoculated with *R. erythropolis* T902.1 cells for removing volatile organic compounds from gaseous effluents. They reported simultaneous degradation of isopropylbenzene and ethanol by rhodococci, suggesting that ethanol improves contaminant biodegradation process in the bioreactor.

4 Concluding Remarks

It could be resumed that some success in bioremediation of contaminated environments has been achieved using actinobacteria of the genus *Rhodococcus* either as bioaugmentation agents or members of indigenous microbial communities stimulated by nutrient amendments. Although possible bioremediation applications of genetically modified *Rhodococcus* were not discussed in this review, it should be noted that advanced methods and powerful tools for genetic engineering of rhodococci were developed (see chapter, "Genomes and Plasmids in *Rhodococcus*" by Larkin et al.), which could be used in constructing recombinant strains for improved bioremediation inocula. Indeed, the complete nucleotide sequences of several *Rhodococcus* genomes provided new insights that could facilitate biotechnological exploitation of this genus (see chapter, "Genomes and Plasmids in *Rhodococcus*" by Larkin et al.). However, up today, applications of genetically modified rhodococci in bioremediation fields are limited to laboratory biodegradation studies attempting to reveal their multitudinous catabolic pathways and regulatory mechanisms for different organic contaminants (Larkin et al. 2005). In the context of future applications of genetically engineered *Rhodococcus* strains in environmental biotechnology, it could be feasible to use biosynthesis products (e.g., enzymes and biosurfactants) rather than whole rhodococcal cells, which may help to overcome biosafety limitations associated with release of genetically modified microorganisms into open environments. Another possible perspective for molecular genetic approach to be applied to bioremediation is the use of oligonucleotide primers and DNA probes constructed for rhodococcal biodegradation genes to estimate their in situ functional activities (Whyte et al. 2002a). These techniques, as well as novel genomic and proteomic methods could be used to predict bacterial metabolism in contaminated environments and to enhance bioremediation. Moreover, correct prognosis of rhodococcal survival and biodegradation activity in contaminated environments would require further fundamental studies of interactions between

Rhodococcus cultures introduced and indigenous micro- and macroorganisms using physiological and molecular approaches (Watanabe and Hamamura 2003). Clearly, *Rhodococcus* application in bioremediation of contaminated sites is a promising and evolving field of environmental biotechnology, and its success depends on the increase in our fundamental knowledge of these remarkable bacteria.

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Physiology, Biochemistry, and Molecular Biology of Triacylglycerol Accumulation by *Rhodococcus*

Héctor M. Alvarez and Alexander Steinbüchel

Contents

Abstract Members of *Rhodococcus* genus are specialists in the accumulation of triacylglycerols (TAGs). Some of them can be considered oleaginous microorganisms since they are able to produce significant amounts of those lipids under certain

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conditions. In this context, *R. opacus* strain PD630 has become a model among prokaryotes in this research area. The basic knowledge generated for rhodococci could be also extrapolated to other related microorganisms with clinical importance, such as mycobacteria. The biosynthesis and accumulation of TAGs by *Rhodococcus* members and other actinomycetes seems to be a process linked to the stationary growth phase or as a response to stress. The chemical structure of rhodococcal TAGs can be controlled by the composition of the carbon source used. The biosynthesis and accumulation of novel TAGs containing unusual components, such as aromatic and isoprenoid fatty acids, by members of *Rhodococcus* and related genera have been reported. The low specificity of wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) enzymes, which catalyze TAG biosynthesis in prokaryotes, may contribute to the high variability of TAG composition. The occurrence of genes coding for WS/DGAT enzymes is highly redundant in rhodococcal genomes. The enrichment of genes and enzymes involved in TAG metabolism in rhodococci suggests the important role of these lipids in the physiology of these microorganisms. This article aims to summarize the most relevant achievements of basic research in this field, including the most recent knowledge that has emerged from studies on TAG accumulation by rhodococci and some unpublished results.

1 Introduction

Triacylglycerols (TAGs) are nonpolar, water-insoluble fatty acid triesters of glycerol, which are accumulated in most eukaryotic organisms, including animals, plants, yeast, and fungi. These compounds are the main reserve material in eukaryotes for energy and fatty acids required for membrane biosynthesis (Sorger and Daum 2002). Similarly, poly(3-hydroxybutyric acid) (PHB) or other polyhydroxyalkanoic acids (PHAs) mainly function as carbon and energy-reserve materials in most bacteria (Anderson and Dawes 1990; Steinbüchel 1991). PHAs are polyesters of alkanoic acids containing a hydroxyl group as a functional group in addition to the carboxyl group, which are accumulated by diverse bacteria as intracellular inclusions (Steinbüchel 1991). More than 150 different hydroxyalkanoic acids have been reported as constituents of bacterial PHAs (Steinbüchel 1991; Steinbüchel and Valentin 1995). Despite the wide occurrence of PHAs among prokaryotes, TAGs also occur as storage lipids in several groups of prokaryotes (Alvarez and Steinbüchel 2002; Alvarez 2006). Within the last decade, the reports on new TAG-accumulating bacteria have considerably increased. Gram-negative bacteria are able to accumulate neutral lipids composed of wax esters (WS) as main lipids, and TAGs only as minor components. WS and TAGs have been reported for Gram-negative members of the genera *Acinetobacter*, *Alcanivorax*, and *Marinobacter* (Makula et al. 1975; Alvarez et al. 1997a; Bredemeier et al. 2003; Rontani et al. 2003). Gram-positive bacteria belonging to the actinomycetes group seem to be the TAG-accumulating specialists among prokaryotes. TAG accumulation has been

reported for sporulating-actinomycete genera, such as *Streptomyces*, as well as for nonsporulating members, such as *Rhodococcus*, *Nocardia*, *Dietzia*, and *Mycobacterium* (Olukoshi and Packter 1994; Alvarez and Steinbüchel 2002; Alvarez 2006; Kaddor et al. 2009). Some members of these genera are able to accumulate significant amounts of TAGs as intracellular inclusions. Majority of the published research on the basic aspects of bacterial TAGs has been derived from studies on species of the genera *Rhodococcus* and *Acinetobacter*, with *R. opacus* PD630 and *A. baylii* ADP1 being the preferred bacterial models for these studies. Recently, new knowledge that has emerged from research mainly focused on the molecular characterization of genes and enzymes involved in TAG formation, for bacteria belonging to *Mycobacterium*, *Streptomyces*, *Alcanivorax*, and *Marinobacter* genera (Arabolaza et al. 2008; Daniel et al. 2004; Kalscheuer et al. 2007; Holtzapple and Schmidt-Dannert 2007). The knowledge of the physiology of such microorganisms may be useful for clinical and environmental biotechnology purposes. In this chapter, we summarize the current knowledge on the TAG metabolism, physiology, and molecular biology in members of the *Rhodococcus* genus.

2 Triacylglycerol Accumulation by *Rhodococcus*

The ability to accumulate TAGs is a characteristic feature among the species of the genus *Rhodococcus*. They are able to accumulate variable amounts of TAG during cultivation on diverse substrates (Table 1). Some strains can be considered as oleaginous bacteria since they accumulate more than 20% of their biomass as lipids (Table 1). *R. opacus* PD630, which is the best known TAG-accumulating member of the *Rhodococcus* genus, is able to accumulate very high levels of TAGs in the cells after cultivation on gluconate and other substrates (Alvarez et al. 1996). Figure 1 shows a cell of the strain PD630 containing several TAG granules in the cytoplasm. Voss and Steinbüchel (2001) used the *R. opacus* strain PD630 for high cell density cultivation to obtain high concentrations of TAGs in bioreactors, which contained sugar beet molasses and sucrose as sole carbon sources. This work demonstrated that inexpensive feedstock, such as organic wastes or residual materials from industry, can be also used for lipid production. In this context, Gouda et al. (2008) reported TAG accumulation by *R. opacus* and *Gordonia* sp. from agro-industrial wastes, such as carob and orange wastes and sugarcane molasses. Thus, cultivation of rhodococci on a cheap residual carbon source from agricultural products could be applied to the biotechnological production of interesting single-cell oils and probably other lipid-derived products as well (Voss and Steinbüchel 2001; Gouda et al. 2008).

In addition to TAGs, rhodococci are able to produce other storage compounds, such as wax esters, PHA, and glycogen, generally as minor compounds. The accumulation of small amounts of wax esters was reported for *R. opacus* PD630 and *R. jostii* RHA1 after cultivation on phenyldecane and a mixture of hexadecane and hexadecanol, respectively (Alvarez et al. 2002; Hernández et al. 2008).

Table 1 Biosynthesis and accumulation of TAG by members of the *Rhodococcus* genus

Bacterial strains	Carbon source	TAG content[a]	References
R. opacus PD630 (DSMZ 44193)	Gluconate	76.0	Alvarez et al. (1996)
	Fructose	40.0	
	Acetate	31.0	
	Propionate	18.0	
	Pentadecane	39.0	
	Hexadecane	38.0	
	Heptadecane	28.0	
	Octadecane	39.0	
	Olive oil	87.0	
	Sugar beet molasses	68.1	Voss and Steinbüchel (2001)
	Carob wastes	88.9*	Gouda et al. (2008)
	Sesame oil	11.3*	
R. opacus MR22 (DSMZ 3346)	Gluconate	48.0	Alvarez et al. (1997b)
	Hexadecane	43.0	
	Valerate	42.5	
R. jostii RHA1	Gluconate	56.9	Hernández et al. (2008)
	Glucosa	48.4	
	Acetate	21.2	
	3-Hydroxybutyric acid	32.5	
	Hexadecane	30.4	
R. ruber NCIMB 40126	Glucose	19.0	Alvarez et al. (1997b)
	Hexadecane	26.0	
	Valerate	12.2	
R. fascians D188-5	Glucose	3.8	Alvarez et al. (1997b)
	Hexadecane	18.1	
	Valerate	1.8	
R. fascians 123	Gluconate	3.8	Alvarez (2003)
	Pentadecane	4.8	
	Hexadecane	12.9	
R. erythropolis DSMZ 43060	Gluconate	21.0	Alvarez et al. (1997b)
	Hexadecane	17.6	
	Valerate	15.1	
R. erythropolis 17	Gluconate	7.7	Alvarez (2003)
	Pentadecane	56.8	
	Hexadecane	43.4	
R. aetherivorans IAR1	Toluene	24.0	Hori et al. (2009)
Rhodococcus sp 20	Gluconate	7.6	Alvarez (2003)
	Hexadecane	8.1	
Rhodococcus sp 602	Gluconate	71.2	Silva et al. (2010)
	Benzoate	64.9	
	Hexadecane	22.3	

[a]Expressed as percentage of total fatty acids by cellular dry weight, except values with *, which is expressed as mg/L

Rhodococci are able to accumulate PHAs containing short-chain-length monomer units, such as 3-hydroxybutyric acid (C_4) (3HB) or/and 3-hydroxyvaleric acid (C_5) (3HV) (Anderson et al. 1995; Pieper and Steinbüchel 1992; Alvarez et al. 1997b; Alvarez 2003). In general, PHAs represent minor components of the storage lipids

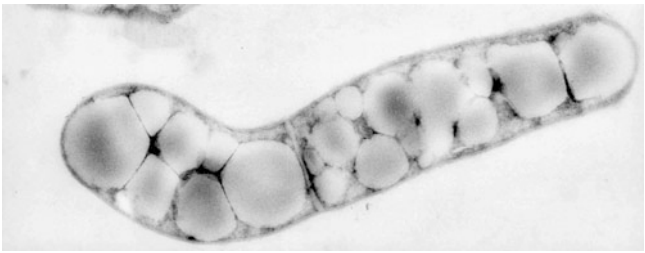

Fig. 1 Micrograph showing a cell of *Rhodococcus opacus* PD630 containing several TAG granules during growth on gluconate as sole carbon and energy source (Alvarez 2006). Picture: F. Mayer from the Georg-August University of Göttingen, Germany

accumulated by most rhodococci, with the exception of *R. ruber* and the related *Nocardia corallina*, which produce large amounts of both storage lipids, TAGs and the copolyester poly(3HB-*co*-3HV), during growth on glucose. Although the PHA content and composition vary among strains, most of rhodococci produce poly (3HB-*co*-3HV) with 3HV as major monomer unit of the copolyester. Some strains belonging to *R. erythropolis* and *R. fascians* accumulate a polyester containing only 3HB monomer units (Alvarez et al. 1997b).

Recently, Hernández et al. (2008) reported the occurrence of glycogen in *R. jostii* cells during growth on gluconate, in addition to TAGs and PHAs. The accumulation of glycogen seems to be a usual feature among rhodococci, since this material has also been identified in cells of *R. erythropolis*, *R. fascians*, *R. opacus*, and *R. equi* (Hernández and Alvarez, unpublished results).

3 Composition and Structure of Rhodococcal Triacylglycerols

Rhodococci are able to produce a variety of TAGs with a high variability of fatty acid composition depending of the carbon source used for cell cultivation. Chemical analyses of TAGs accumulated by diverse *Rhodococcus* species revealed the occurrence of saturated and unsaturated straight long-chain fatty acids, principally with a chain length between C_{14} and C_{18} (Alvarez and Steinbüchel 2002; Alvarez 2006). In general, palmitic acid ($C_{16:0}$) and octadecenoic acid ($C_{18:1}$) are the major fatty acids synthesized from nonrelated substrates such as glucose, gluconate, or acetate. Some strains belonging to *R. opacus*, *R. jostii*, and *R. erythropolis* produce significant amounts of odd-numbered fatty acids during growth on those substrates (from 25% to 40% of the total fatty acids). Substrates such as citrate and succinate, which are also intermediates of the tricarboxylic acid cycle (TCA), or acetate, which is fed to the TCA cycle, and odd-numbered organic acids such as propionate or valerate, promote an increase of the fraction of odd-numbered fatty acids in TAGs compared to lipids occurring in cells cultivated on glucose or gluconate (Alvarez et al. 1997b; Alvarez 2003). The mentioned strains posses an efficient mechanism for production

of the intermediate propionyl-CoA, which is presumably utilized as precursor for the biosynthesis of fatty acids containing an odd number of carbon atoms. Cells are able to produce substantial amounts of propionyl-CoA during growth on diverse substrates from succinyl-CoA via the methylmalonyl-CoA pathway (Anderson et al. 1995; Alvarez et al. 1997b). On the other hand, during cultivation of rhodococcal cells on *n*-alkanes, the main fatty acids produced are related to the chain length of the substrate, as well as to other fatty acids derived from the β-oxidation pathway. Thus, the degradation pathways of hydrocarbons are well coupled to the lipid metabolism in these hydrocarbon-degrading microorganisms.

Previous studies demonstrated that the biosynthetic pathway of TAGs is very flexible in rhodococci and related bacteria, being able to accept acyl residues with various chemical structures. During cultivation of *R. opacus* PD630 cells on phenyldecane as sole carbon source, a mixture of TAGs containing phenyldecanoic acid residues was detected (Alvarez et al. 2002). In addition, cells produced the wax ester phenyldecylphenyldecanoate by condensation of phenyldecanoic acid and phenyldecanol formed as intermediate during the catabolism of phenyldecane. Other related microorganisms were also able to incorporate unusual fatty acids into TAGs or wax esters. The *Nocardia globerula* strain 432 accumulated TAGs containing the branched fatty acid 4,8,12-trimethyl tridecanoic acid after cultivation of the cells on the recalcitrant branched alkane, pristane (Alvarez et al. 2001), whereas the *Mycobacterium ratisbonense* strain SD4 was able to produce a mixture of wax esters containing isoprenoid fatty acids and fatty alcohols, such as 2,6,10,14 tetramethylhexadecanoic acid and 2,6,10,14 tetramethylhexadecan-1-ol among others, after cultivation of cells on phytane (Silva et al. 2007). In another study, cells of *Rhodococcus* sp. 602, an indigenous strain isolated from a soil sample in Patagonia (Argentina), were cultivated under nitrogen-limiting conditions in the presence of naphthyl-1-dodecanoate as the sole carbon source. After 6 days of incubation, a mixture of novel TAGs containing only medium-chain-length fatty acids (C_8, C_{10}, and C_{12}) was identified in the cells (Silva et al. 2010). The results suggested the formation of 1-naphthol and dodecanoic acid residues by an esterase, and subsequent β-oxidation of the fatty acid during catabolism of naphthyl-1-dodecanoate. Thus, the TAG biosynthesis pathway of strain 602 was able to incorporate the catabolic intermediates into the storage lipids structure.

The composition and the properties of storage lipids can also be changed by alteration of genes/enzymes involved in lipid metabolism. One example of this is the mutant UFA4 of *R. opacus* PD630, which exhibited a defect in the fatty acid desaturation system. This mutant accumulated increased amounts of stearic acid (C18:0) and lacked odd-numbered fatty acids in TAGs during cultivation on gluconate, thus producing a cocoa-butter-like oil containing about 74% saturated fatty acids with a relatively high content of stearic acid (>18%) (Wältermann and Steinbüchel 2000). All these results demonstrate that the content and composition of rhodococcal TAGs can be influenced by the carbon source used for the growth of cells or manipulated by engineering procedures.

Wältermann et al. (2000) determined by stereospecific analysis the distribution of fatty acids in TAG for *R. opacus* PD630. The final acyl composition of TAGs and the

distribution of diverse acyl groups on the hydroxyl groups of the glycerol backbone depend on the differing specificities of the acyltransferases involved in the sequential acylation of the *sn*-1,2, and 3 positions of glycerol-3-phosphate during TAG biosynthesis. This study demonstrated that the enzymes involved in TAG biosynthesis in strain PD630 exhibit specificity for the acyl-CoAs different from the corresponding enzymes in eukaryotes. In eukaryotic TAGs (from mammals, plants and yeasts), unsaturated fatty acids are found in position *sn*-2 and saturated fatty acids are almost totally excluded from this central position. In contrast, *R. opacus* PD630 preferentially incorporated the shorter and saturated fatty acids in the *sn*-2 carbon atom and the unsaturated fatty acids were predominantly found at position 3. Brennan (1988) reported that fatty acids with more than 20 carbon atoms were predominantly located in the *sn*-3-position of the glycerol molecule, with C16 fatty acids occupying the 2-position and either octadecanoate, octadecenoate, or 10-methyloctadecanoate at the 1-position by TAG-accumulating mycobacteria.

4 Conditions for Triacylglycerol Accumulation and Mobilization

The biosynthesis and accumulation of TAGs by members of the genus *Rhodococcus* and by other actinomycetes seems to be a process linked to the stationary growth phase or as a response to stress (Olukoshi and Packter 1994; Alvarez et al. 2000). In general, the total content of TAGs accumulated by rhodococci depends on both the strain and the carbon source used for growth. However, the nutritional stress seems to be the main condition that influences TAG accumulation by rhodococci. Nitrogen-limiting conditions in the presence of an excess of a carbon source promote significantly TAG biosynthesis and accumulation by *Rhodococcus* members (Alvarez and Steinbüchel 2002). Almost a fourfold increase in the cellular TAG content occurred during cultivation of *R. opacus* PD630 on gluconate with only 0.05 g/L ammonium in the medium, as compared to cells cultivated in a medium containing 1 g/L ammonium (Alvarez et al. 2000). When the an N source lacking in the medium, the biosynthesis of N-containing compounds, such as proteins and nucleotides, is impaired; thus, the biosynthesis of compounds containing only C, O, and H, such as lipids or carbohydrates, is favored. In general, cells accumulate TAGs principally during the stationary growth phase. This is logical, considering that the fatty acids necessary for TAG biosynthesis are indispensable intermediates for biosynthesis of phospholipids and membranes, which are essential for cell growth and proliferation. Thus, TAG biosynthesis competes with cell growth. In contrast to many bacteria that block lipid metabolism under growth-restricting conditions (Huisman et al. 1993), rhodococci are able to maintain an active de novo fatty acid biosynthesis pathway under such conditions, generating acyl residues from the available carbon source, which are used for TAG formation.

Another nutritional stress that affects TAG metabolism in rhodococci is C-starvation. When cells of *R. opacus* PD630 and *R. ruber* were incubated in the presence of a nitrogen source and in the absence of any carbon source, they were able to mobilize the stored TAGs (Alvarez et al. 2000). This indicated that TAGs serve as endogenous carbon and energy sources during incubation of cells under starvation conditions.

Some studies suggest that conditions of limited aeration also promote TAG biosynthesis and accumulation by *Rhodococcus* members (Hernández, Alvarez, unpublished results). In this context, Daniel et al. (2004) reported that several genes involved in TAG biosynthesis in *Mycobacterium tuberculosis* are induced under oxygen-limiting conditions, when cells go into the nonreplicative drug-resistant state. Some of these genes show the highest induction and activity by hypoxia (Daniel et al. 2004). The authors concluded that TAG may be the form of energy storage for use during long-term dormancy in this microorganism. However, TAGs may act also as a sink for reducing equivalents under these conditions, since the fatty acid biosynthetic pathway includes pyridine-nucleotide-dependent reduction reactions. Thus, TAG biosynthesis may avoid accumulation of reduced pyridine nucleotides in the cells under oxygen-limiting conditions, which may inhibit some key enzymes of the central metabolism (Alvarez and Steinbüchel 2002).

Whether TAG accumulation by rhodococci is also promoted by other stress conditions remains to be investigated.

5 Triacylglycerol Biosynthesis by *Rhodococcus*

Despite the fact that the knowledge obtained on the biochemistry of TAG biosynthesis in rhodococci is still fragmentary, some generalizations are made in this section based on experimental and genomic data. In this section, we subdivide the biosynthesis of TAGs into three steps: (1) production of key metabolic precursors for fatty acids and TAG biosynthesis; (2) biosynthesis of fatty acids; and (3) sequential esterification of the glycerol moiety with fatty acyl-residues.

5.1 Production of Key Metabolic Precursors for Fatty Acid Biosynthesis

Biosynthesis of TAGs requires an efficient metabolic network capable of producing the necessary precursors and energy for the specific reactions. In general, the central metabolism of rhodococci possesses a great flexibility and diversity of metabolic reactions, which supports the energy-demanding TAG biosynthesis process under certain conditions from a diversity of carbon sources, as is shown in Table 1. The pathways of rhodococcal central metabolism are able to efficiently convert diverse

carbon sources to the key metabolic intermediates, such as pyruvate, acetyl-CoA, and glycerol-3-phosphate, to create reducing equivalents that are required by lipid biosynthesis pathways and to produce the necessary energy as adenosine triphosphate (ATP). For more detailed information on the central metabolism of rhodococci see chapter "Central metabolism of species of the genus *Rhodococcus*" by Alvarez. However, many bacteria that are not able to accumulate TAGs are also able to produce these metabolic intermediates, reducing equivalents, and ATP. Thus, an oleaginous microorganism must also be able to maintain a high carbon flux toward the lipid production pathways. Since TAG accumulation is a carbon- and energy-expensive process, rhodococcal cells are able to arrest cell growth and replication and shift their metabolism and carbon flux to lipid biosynthesis pathway. Such changes in cell metabolism depend on the stimuli from the environment, as mentioned above.

Diverse pathways may contribute to the production of the acetyl-CoA pool in rhodococci. The conversion of acetyl-CoA from glycolysis-derived pyruvate might be the major route of carbon flux to fatty acid biosynthesis. In general, sugars support significant TAG accumulation by oleaginous *Rhodococcus* members (Table 1). The intermediate acetyl-CoA might be produced alternatively by the reaction catalyzed by citrate lyase enzyme. Citrate lyase, which converts citric acid into acetyl-CoA and oxalacetate, is one of the key enzymes of the reductive TCA cycle. The presence of citrate lyase and 2-oxoglutarate synthase in genome databases of *R. jostii* RHA1 and *R. opacus* B4 suggests that these microorganisms are able to drive the TCA cycle in the reductive direction. This permits the metabolism to incorporate CO_2 for synthesis of intermediates, which may feed the lipid biosynthesis pathways under growth-restricting conditions. On the other hand, free acetate could be activated to acetyl-CoA by acetyl-CoA synthetase in an ATP-dependent reaction. This enzyme, together with acetate kinase and phosphotransacetylase enzymes, which were detected in genome databases of *R. jostii* RHA1 and *R. opacus* B4, may be involved in the maintenance of the intracellular pools of acetyl-CoA and acetyl-P in these microorganisms. The other metabolic intermediate required for fatty acid biosynthesis in cells of rhodococci is propionyl-CoA, which is generally used for the synthesis of odd-numbered fatty acids (Anderson et al. 1995; Alvarez et al. 1997b). Feisthauer et al. (2008) reported that *R. opacus* 1CP possesses an essential dependence on heterotrophic CO_2 fixation by anaplerotic reactions. Using $^{13}CO_2$ for cultivation experiments, the authors demonstrated that, during growth on glucose, the fixed CO_2 was directed principally to the biosynthesis of odd-numbered fatty acids probably via the methyl malonyl-CoA pathway using TCA cycle intermediates as precursors. Fatty acids containing an odd number of carbon atoms may account for up to 20–30% of the total fatty acids in many *Rhodococcus* strains (Alvarez et al. 1997b; Alvarez 2003).

The synthesis of fatty acids requires stoichiometric amounts of ATP and acetyl-CoA, NADPH and NADH for each C_2 addition to a growing acyl chain in the reactions catalyzed by acetyl-CoA carboxylase and fatty acid synthetase (Rawsthorne 2002). The necessary ATP might be generated by substrate-level phosphorylation in rhodococci through glycolisis, among other possible ATP-generating reactions.

The source of reducing equivalents for fatty acid biosynthesis in rhodococci is actually not known, although the pentose phosphate pathway might be one potential source of NADPH. Malic enzyme might be involved in the generation of NADPH on carbon sources, which likely have a low flux through the pentose phosphate pathway.

Little is known about the interaction of pathways that occur in cells of oleaginous rhodococci. Previous studies using inhibitors of lipid metabolism such as cerulenin and acrylic acid revealed that the biosynthesis pathways of PHAs and TAGs in cells of *R. ruber* and *N. corallina* compete for the common intermediates acetyl-CoA and propionyl-CoA during cultivation of cells under nitrogen-limiting conditions (Alvarez et al. 1997b). The inhibition of fatty acid synthesis by the addition of cerulenin in the medium caused an increase in the PHA content and altered the composition of the copolyester with an increase of the 3HB monomer units. In contrast, some mutants of *R. ruber* impaired in PHA accumulation produced increasing amounts of TAGs in comparison with the wild type (Alvarez et al. 1997b).

5.2 Biosynthesis of Fatty Acids

The first step for fatty acid biosynthesis in animals, plants, and prokaryotes is the synthesis of the intermediate malonyl-CoA by the acetyl-CoA carboxylase enzymatic complex (ACC). Malonyl-CoA is the central carbon donor for fatty acid biosynthesis (Wakil et al. 1983). The ACCs are highly conserved enzymes, which catalyze the carboxylation of acetyl-CoA to produce malonyl-CoA in eukaryotic and prokaryotic organisms (Wakil et al. 1983). The ACC is formed by three functional components, such as biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase (Cronan and Waldrop 2002). In general, the ACC found in chloroplasts and most prokaryotes is an enzyme formed by multiple subunits, whereas a unique enzyme with multiple domains is found in eukaryotic organisms. Moreover, a different type of ACC is found in actinomycetes bacteria, which contain ACC enzymes consisting in two subunits, one major subunit (α chain) with biotin carboxylase and biotin carboxyl carrier protein function, and a small subunit (β chain) with carboxyltransferase activity (Gago et al. 2006). The genome of *M. tuberculosis* contains three genes encoding the α subunit (*accA1–A3*) and six genes encoding the β subunits (*accD1–D6*) (Gago et al. 2006; Daniel et al. 2007). In addition, there is a gene encoding a third subunit (ε), which is present in diverse actinomycetes and is essential for the maximal activity of the complexes (Diacovich et al. 2002). A broad bioinformatics analysis of genomic databases revealed the occurrence of numerous homologous ACC genes in members of the genus *Rhodococcus* (Table 2). Rhodococcal genomes seem to posses more ACC genes than those of *M. tuberculosis*. As example, the genome of *R. jostii* RHA1 contains at least 7 α subunits and 11 β subunits (Table 2). As in *M. tuberculosis*, the homologous of β_6 in all rhodococcal genomes are located within the fatty acid

Table 2 Occurrence of genes homologous to ACC genes of *Mycobacterium tuberculosis* in the genome of *Rhodococcus* members

Mycobacterium tuberculosis	RHA1	Identity (%)	B4	Identity (%)	PR4	Identity (%)	SK121	Identity (%)
accA1 (Rv2501c)	ro06096	67	61530	66	18310	67	5924	67
accA2 (Rv0973c)	ro01930	68	16130	68	17550	67	6005	67
accA3 (Rv3285)	ro03742	81	35620	81	21210	77	4242	77
	ro06282	81	63450	80				
	ro08921	81						
accD1 (Rv2502c)	ro06095	84	61520	83	18300	82	5925	82
accD2 (Rv0974c)	ro01931	83	16140	82	17540	82	6006	82
accD3 (Rv0904c)	ro05011	65	50730	65	45670	66	2359	66
	ro02935	60	26590	60	58860	59	3376	59
accD4 (Rv3799c)	ro04066	68	39440	69	02220	63	5065	63
	ro06570	63						
accD5 (Rv3280)	ro06292	81	63560	81	4232	81		
	ro03744	82	35640	82				
	ro08919	82						
accD6 (Rv2247)	ro01202	75	09250	75	36750	74	4063	74

Identities were based on alignments of primary protein structure derived from full-length gene sequences

synthase II (FAS II) gene locus. In addition to the *acc* genes listed in Table 2, two genes (*ro10399* and *ro10400*), which code for one α and one β subunit, respectively, were detected in the plasmid called pRHL2 of strain RHA1. Curiously, these genes showed identities (between 51 and 57%) to the respective *acc* genes of Gram-negative bacteria, such as *Bordetella* or *Pseudomonas*. Daniel et al. (2007) demonstrated that the α_3, β_4, β_5, β_6, and ε genes were the main subunits regulated during cell growth in *M. tuberculosis*. Notably, three isoenzymes of α_3 and β_5 and two isoenzymes of β_5 occur in the RHA1 genome (Table 2). No genes orthologous to that encoding ε subunit in *M. tuberculosis* were found in rhodococcal genomes; therefore, the occurrence of genes coding for this subunit in rhodococci should be investigated in the future.

The biosynthesis of fatty acids is carried out by a multienzymatic complex known as fatty acid synthase (FAS). This complex catalyzes the successive reaction of condensation, reduction, dehydration, and reduction. Two alternative FAS complexes exist in organisms. FAS type II is present in most prokaryotes and some eukaryote organelles, such as mitochondria and chloroplasts and consists of independent proteins encoded by different genes (Bloch 1977). In contrast, the FAS type I consists of a unique, large protein with the different catalytic activities. FAS I enzymes are found in the cytoplasm of eukaryotic cells and in a subgroup of actinobacteria. FAS I is responsible for fatty acid biosynthesis in mycobacteria, which are used for phospholipids and TAG synthesis or for mycolic acid production after an elongation process mediated by FAS II (Bloch 1977; Zimhony et al. 2004). FAS II uses medium-chain-length fatty acids (C_{16} to C_{24}) as primers for synthesizing long-chain-length mycolic acids (Shweizer and Hofmann 2004). For more

details of FAS II in rhodococci, see chapter, "The rhodococcal cell envelope: composition, organisation and biosynthesis" by Sutcliffe et al. The FAS I multienzyme genes of mycobacteria and rhodococci seem to be structurally very similar. All rhodococcal enzymes are similar in size and amino acid sequences, comprising 3,128 amino acids in *R. jostii* RHA1 (*ro01426*), 3,107 in *R. opacus* B4 (*ROP_11350*), 3,100 in *R. erythropolis* PR4 (*RER_38730*), and 3,103 in *R. erythropolis* SK121 (*RHOER0001_5412*). The main products of rhodococcal FAS I may be C_{16}–C_{18} fatty acids, which may be utilized for phospholipid and TAG biosynthesis.

5.3 Biosynthesis of Triacylglycerols

The TAG biosynthesis in rhodococci has been proposed to occur via sequential acyl-CoA-dependent reactions referred to as the "Kennedy pathway," which has been described for yeast and plants (Fig. 2). This pathway has been well studied in

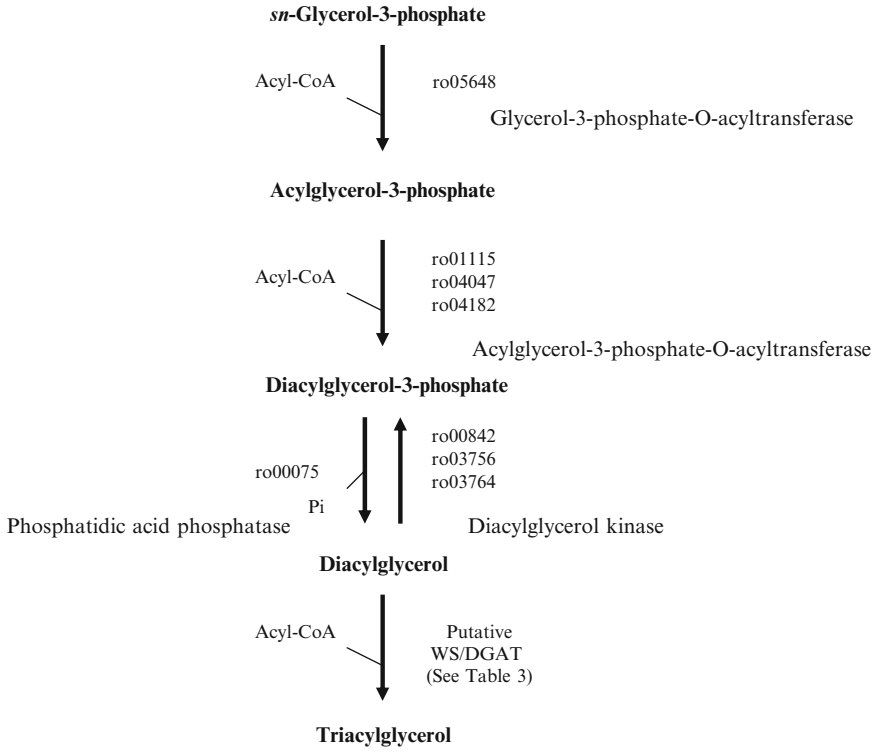

Fig. 2 Pathway for TAG biosynthesis in rhodococci. Genes encoding for putative enzymes for the Kennedy pathway occurring in *R. jostii* RHA1 genome are shown

eukaryotic cells, but not in prokaryotes. The pathway involves the sequential acylation of the *sn*-1, 2 positions of glycerol-3-phosphate, resulting in the formation of phosphatidic acid. The removal of the phosphate group catalyzed by the phosphatidic acid phosphatase enzyme occurs before the final acylation step. In the third acylation reaction, an acyl-residue is transferred to the vacant position of diacylglycerol, which is the final step of TAG biosynthesis (Fig. 2). The three acylation reactions are catalyzed by different acyltransferases. The differing specificities of the acyltransferase determine the distribution of diverse acyl groups on the hydroxyl groups of the glycerol backbone and, therefore, the final acyl composition of TAGs. Phosphatidic acid and diacylglycerol generated in the Kennedy pathway are also used for the synthesis of phospholipids occurring in the membranes. Thus, the third acylation step of the glycerol backbone is the unique enzymatic reaction to TAG biosynthesis. This reaction is catalyzed by a diacylglycerol acyltransferase (DGAT) enzyme. Kalscheuer and Steinbüchel (2003) identified the first prokaryotic DGAT in *Acinetobacter baylyi* ADP1, which exhibited simultaneously both DGAT and acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) activities. Strain ADP1 accumulates mainly wax esters and TAGs as minor compounds, amounting up to 6.9% and 1.4% of cellular dry weight, respectively. Interestingly, WS/DGAT from *A. baylyi* ADP1 represents a new class of TAG-synthesizing enzyme, which exhibits no extended sequence similarity to any known eukaryote acyltransferase (Kalscheuer and Steinbüchel 2003). A highly conserved motif HHxxxDG, which may be the catalytic site responsible for ester bond formation, is found in WS/DGAT from strain ADP1 and related proteins from other microorganisms (Kalscheuer and Steinbüchel 2003). Later, several WS/DGATs were described in various TAG- or WS-accumulating bacteria. Whereas only one or few WS/DGATs occur in Gram-negative bacteria capable of producing WS and TAG, a high redundancy of these enzymes occurs in most TAG-accumulating actinomycetes bacteria, such as the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus*. Daniel et al. (2004) identified 15 genes as putative WS/DGAT in *M. tuberculosis* strain H37Rv, which exhibited acyltransferase activity when expressed in *E. coli*. Eleven of these genes have the conserved active-site motif HHxxxDG, whereas three of them have modified versions of this motif, and one has no recognizable motif (Daniel et al. 2004). Alvarez et al. (2008) identified and cloned the first WS/DGAT gene (called *atf1*) in a *Rhodococcus* member, *R. opacus* PD630, when any rhodococcal genomic database was available. They obtained an 800-bp polymerase chain reaction (PCR) product from chromosomal DNA of strain PD630 by using degenerate primers designed from conserved stretches of WS/DGAT proteins of *A. baylyi* ADP1 and *M. smegmatis* mc2155. The *atf* gene fragment was used as a probe for a strain PD630 gene library, resulting in the identification of a 3,948-bp chromosomal DNA fragment containing the complete *atf1* gene (Alvarez et al. 2008). ATF1 exhibited high WS activity and only scant DGAT activity when expressed in *E. coli*. When *atf1* gene was disrupted in strain PD630, cells of the mutant showed a significant reduction of DGAT activity and accumulated up to 50% less fatty acids in comparison to the wild type during cultivation on gluconate under nitrogen-limiting conditions (Alvarez et al. 2008). Although the results of

this study demonstrated that ATF1 was mainly responsible for TAG biosynthesis in *R. opacus* PD630, it was clear that additional WS/DGAT contributed to the total DGAT activity and TAG content in this strain. Interestingly, TAGs accumulated by the *atf1*-disrupted mutant showed a significant reduction of oleic acid content in comparison to TAGs produced by the wild type, after cultivation on gluconate and oleic acid. These results suggested that WS/DGAT isoenzymes in actinomycetes are specialized for the selective incorporation of different fatty acyl residues into TAGs (Alvarez et al. 2008). When the genome database of *R. jostii* RHA1 was publicly available, nine additional *atf*-homologous genes were identified in the strain PD630 (*atf2* to *atf10*) using nondegenerate primers deduced from strain RHA1 sequence data. WS/DGATs of strain PD630 exhibited 88–99% sequence identity to the corresponding strain RHA1 enzymes (Alvarez et al. 2008). All deduced proteins showed the complete putative active-site motif HHxxxDG described for bacterial WS/DGAT enzymes. Those of Atf5 and Atf10 exhibited a modified active-site motif, in which the second histidine was replaced by serine or lysine, respectively (Alvarez et al. 2008). Interestingly, the atf2 gene exhibited a premature stop codon due to a point mutation in position 1107, thereby yielding a protein of only 374 instead of 453 amino acids in the RHA1 protein. All WS/DGAT of strain PD630 were heterologously expressed in *E. coli* for analyzing their acyltransferase activities. In general, all crude extracts of recombinant *E. coli* strains exhibited only low enzymatic activities compared to those obtained from the *R. opacus* (Alvarez et al. 2008). In addition to *atf1* as mentioned above, recombinant *E. coli* harboring plasmid pBluescriptSK::*atf2* exhibited WS as well as significant DGAT activities. However, crude protein extracts of *E. coli* strains expressing *atf3* to *atf10* exhibited no or only slightly increased WS/DGAT activities in comparison to the vector control cultivated under conditions used in that study (Alvarez et al. 2008).

R. jostii RHA1 is also able to accumulate significant amounts of TAGs, in addition to other storage compounds, such as PHAs, glycogen, and polyphosphate (Hernández et al. 2008). This strain possesses all necessary genes/enzymes for TAG biosynthesis via the Kennedy pathway as they occur in the genome of *R. opacus* B4 and *R. erythropolis* PR4 and SK121 (Fig. 2 and Tables 3, 4). A genome-wide bioinformatic analysis of key genes encoding metabolism of diverse storage compounds by *R. jostii* RHA1 (Mclead et al. 2006) identified 14 genes encoding putative WS/DGAT enzymes likely involved in TAG and wax ester biosynthesis; a total of 54 genes coding for putative lipase/esterase enzymes possibly involved in TAG and wax ester degradation; three sets of genes encoding PHA synthases and PHA depolymerases; six genes encoding key enzymes for glycogen metabolism; one gene coding for a putative polyphosphate kinase; and three putative exopolyphosphatase genes possibly involved in polyphosphate biosynthesis and degradation (Hernández et al. 2008). Eleven of these predicted WS/DGATs contain the putative active site motif of WS/DGATs (HHxxxDG), while in *atf4*, *atf10*, and *atf14*, the second histidine of the motif is replaced by lysine, serine, and proline; respectively. Eleven *atf* genes are located on the RHA1 chromosome, whereas *atf12*, *atf13*, and *atf14* are located on plasmid pRHL1. The WS/DGAT genes of strain RHA1 are not located in

Table 3 Putative genes from *R. jostii* RHA1 involved in the Kennedy pathway and their orthologous genes in the genome of other *Rhodococcus* members

Genes RHA1	Enzyme	*R. opacus* B4	*R. erythropolis* PR4	*R. erythropolis* SK121
ro05648	Glycerol-3-phosphate-*O*-acyltransferase	ROP_57110 (97%)[a]	RER_16280 (67%)[a]	RHOER0001_6138 (67%)[a]
ro01115	Acylglycerol-3-phosphate-*O*-acyltransferase	ROP_08430 (97%)	RER_35800 (81%)	RHOER0001_6439 (81%)
ro04047		ROP_39250 (97%)	RER_02010 (87%)	RHOER0001_5089 (87%)
ro04182		ROP_41110 (93%)	RER_13990 (71%)	RHOER0001_4788 (71%)
ro00075	Phosphatidic acid phosphatase	ROP_pROB01-01880 (47%)	RER_10170 (47%)	–
ro00842	Diacylglycerol kinase	ROP_05800 (89%)	RER_31720 (68%)	RHOER0001_1405 (68%)
ro03756		ROP_48990 (67%)	RER_57060 (65%)	RHOER0001_1820 (67%)
ro03764		ROP_48990 (66%)	RER_57060 (65%)	RHOER0001_1820 (65%)

[a]Identities based on alignments of primary protein structure derived from the respective full-length gene sequences of putative WS/DGAT genes of *R. jostii* RHA1

operons with other genes involved in TAG metabolism, and they are widely distributed throughout the genome, which seems to be common in TAG-accumulating actinomycetes (Daniel et al. 2004; Wältermann et al. 2007). However, some of the 14 RHA1 WS/DGAT genes are adjacent or proximal to other genes, likely involved in TAG or lipid metabolism (Hernández et al. 2008). Bioinformatic analysis of the available genomic databases showed the occurrence of a variable number of putative WS/DGAT genes in the genome of *R. opacus* B4, and *R. erythropolis* PR4 and SK121 (Table 4). Most of them were homologous genes to WS/DGAT genes of *R. jostii* RHA1, whereas few genes seemed to be specific putative WS/DGAT genes of *R. erythropolis* species (strains PR4 and SK121) (Table 4). The WS/DGAT gene number found in the rhodococcal genomes seems to be a strain-dependent feature.

The Kennedy pathway seems to be the main TAG biosynthesis pathway in rhodococci; however, alternative acyl-CoA-independent routes for TAG synthesis could occur in these microorganisms. Dahlqvist et al. (2000) reported a pathway that uses phospholipids as acyl donors and diacylglycerols as acceptor for TAG biosynthesis in plants and yeast. This reaction is catalyzed by a phospholipids: diacylglycerol acyltranferase (PDAT) enzyme. Interestingly, Arabolaza et al. (2008) demonstrated that phospholipids could act as acyl donors for TAG biosynthesis in *Streptomyces coelicolor* and that this reaction could be catalyzed by a PDAT enzyme. The absence of sequence similarities of eukaryotic PDATs to any of the genomic sequences makes it difficult to study such enzymes in TAG-accumulating actinomycetes and to establish their physiological role in cells. It has been proposed that the PDAT enzyme might function to modulate membrane lipid composition (Dahlqvist et al. 2000; Arabolaza et al. 2008). The occurrence of

Table 4 Putative WS/DGAT genes identified in *R. jostii* RHA1 and their orthologous genes in the genome of other *Rhodococcus* members

R. jostii RHA1	*R. opacus* PD630	*R. opacus* B4	*R. erythropolis* PR4	*R. erythropolis* SK121
ro00023 (436)[a]	atf4 (483)[a] (88%)[b]			
ro00024 (477)			RER_34070 (456)[a] (48%)[b]	
ro00039 (473)	atf1 (462) (89%)		RER_33950 (461) (55%)	
ro00087 (461)	atf10 (461) (98%)	ROP_02100 (461)[a] (95%)[b]		
ro00583 (430)	atf9 (422) (90%)	ROP_04650 (430) (84%)		
ro01601 (453)	atf2 (374) (95%)	ROP_13050 (453) (97%)	RER_11860 (458) (52%)	RHOER0001_1136 (458)[a] (52%)[b]
ro02966 (467)	atf6 (467) (99%)	ROP_26950 (468) (94%)		
ro05356 (463)	atf8 (463) (98%)	ROP_54550 (464) (95%)		
ro05649 (484)	atf3 (484) (98%)	ROP_57120 (484) (96%)	RER_16290 (461) (72%)	RHOER0001_6137 (488) (72%)
ro06332 (474)	atf5 (474) (99%)	ROP_63930 (474) (98%)	RER_21760 (469) (75%)	RHOER0001_4187 (468) (32%)
ro06855 (464)	atf7 (464) (98%)	ROP_68400 (463) (96%)	RER_28450 (459) (70%)	RHOER0001_6312 (468) (70%)
ro08369 (301)				
ro08645 (473)				
ro08660 (497)				
			RER_12460 (425)	RHOER0001_3973 (417)
			RER_15300 (481)	RHOER0001_1571 (486)
			RER_15290 (486)	RHOER0001_1570 (486)

[a]Length aa

[b]Identities based on alignments of primary protein structure derived from the respective full-length gene sequences of putative WS/DGAT genes of *R. jostii* RHA1

acyl-CoA-independent routes for TAG synthesis and PDAT-like enzymes in rhodococci remains to be investigated.

6 Biogenesis of TAG Inclusion Bodies

Lipid inclusions in bacteria lack small amphiphilic proteins, which, like phasins (Wieczorek et al. 1995) or oleosines (Murphy 2001), are bound to PHB granules in bacteria or often to lipid inclusions in plants, respectively. Despite the lack of such proteins, multiple discrete lipid inclusions occur in the cytoplasm. This indicates that nonproteinaceous compounds instead of phasins or oleosines stabilize these lipid suspensions in the cytoplasm. Phospholipids are the most likely candidates. Although phasins or oleosins are absent from the surface of lipid inclusions in *R. opacus* strain PD630, the major *Ralstonia eutropha* H16 phasin PhaP1 and also

PhaP1 fusions with enhanced green fluorescent protein (eGFP) or with the *E. coli* β-galactosidase (LacZ) were bound to the surface of lipid inclusions when expressed in *R. opacus* (Hänisch et al. 2006a). Other proteins could also be bound to lipid inclusions in vivo (Hänisch et al. 2006b). Interestingly, the *Zea maize* oleosin was not bound to the lipid inclusions, although it was expressed in the *R. opacus* strain PD630 (Hänisch et al. 2006b).

Many marine hydrocarbonoclastic bacteria, such as *Alcanivorax borkumensis*, do not only accumulate lipids in the cells but secrete lipids also into the medium (for example, Sabirova et al. 2006). Although some mutants which still synthesize the lipids and accumulate them in the cytoplasm but exhibite the phenotype lipid-export negative (Manilla-Pérez et al. 2010) were isolated, the mechanism of lipid secretion remains unknown and must be unraveled in the future. However, lipid secretion is unknown in members of the genus *Rhodococcus*.

Detailed studies on the formation of lipid inclusions in bacteria have been made in *A. baylyi* strain ADP1 and in *R. opacus* strain PD630. Lipid biosynthesis starts at the inner leaflet of the cytoplasmic membrane to which the acyltransferase is bound, as revealed by cytoimmunological studies using polyclonal antibodies raised against this enzyme and ultrathin sections of cells just starting lipid biosynthesis (Wältermann et al. 2005). Also, in transmission electron micrographs, a thin film of material emerged on the surface of the inner leaflet. From this film, small lipid droplets arose which conglomerated to lipid prebodies. When these prebodies reached a certain size, they were released from the cytoplasm membrane and became separate, discrete structures which further matured to the lipid inclusions in their final stage. These steps were, in principle, also indirectly observed when in vitro studies using an artificial membrane and the purified acyltransferase protein were, besides the other necessary compounds (substrates, etc.), used in combination with a quartz crystal microbalance with scanning force microscopy. The changes of the frequency of the quartz crystal and the changes at the surface could be interpreted as similar steps occurring in vitro (Wältermann et al. 2005).

These observations were in agreement with the formation of the lipid inclusions according to the membrane budding model. This mode of formation and its location at the cytoplasm membrane, together with other evidence, explain that lipid bodies are surrounded by a half-unit membrane of phospholipids. PHB granules are formed in the cytoplasm independently from the membrane according to the micelle model, and must be covered by amphiphilic proteins (Jurasek and Marchessault 2004; Pötter et al. 2004).

7 Physiological Functions of TAGs in *Rhodococcus*

Rhodococcus species, which are enriched in a particular class of lipids, such as TAGs, may be highly dependent on these compounds and their functions for successful survival in the environment. In this context, TAGs seem to play a key

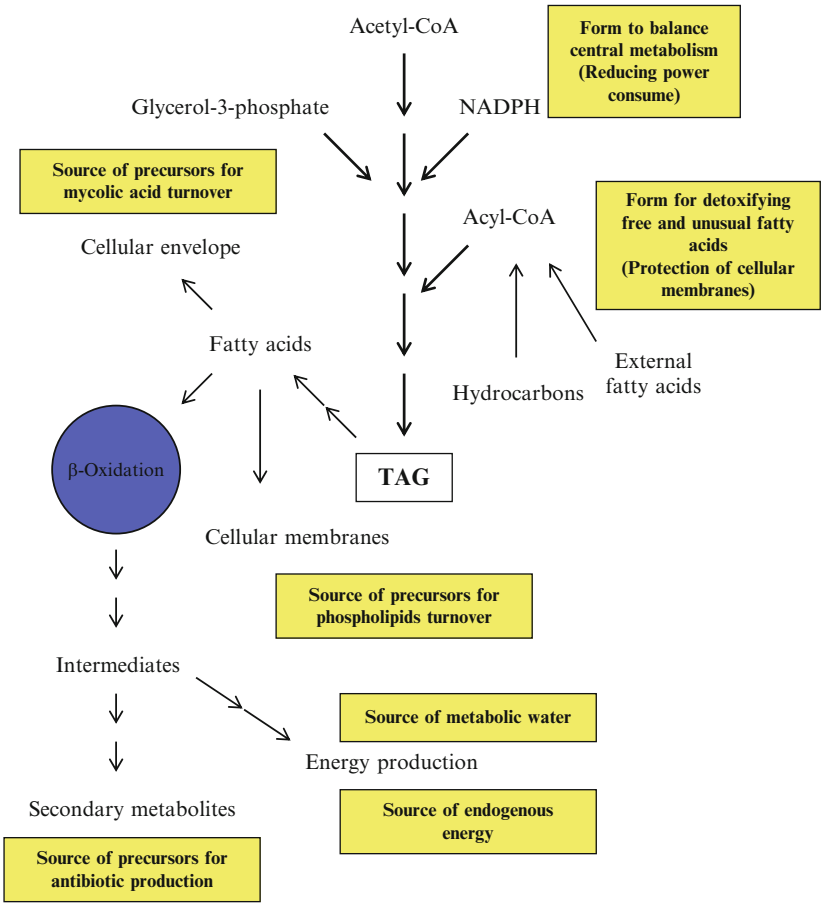

Fig. 3 Physiological functions of TAG proposed for TAG-accumulating rhodococci

role for the cells under growth-restricting conditions that frequently predominate in the environment (Fig. 3).

7.1 TAGs as Endogenous Carbon and Energy Sources

Rhodococci have been detected in different natural environments, such as tropical, arctic, and arid soils, as well in marine and very deep sea sediments (Whyte et al. 1998; Heald et al. 2001; Peressutti et al. 2003; Alvarez et al. 2004; Luz et al. 2004; Peng et al. 2008). Interestingly, these microorganisms and other related actinomycetes are frequently dominant components of microbial communities of arid environments (Skujins 1984).

Previous studies have revealed that species of the genera *Rhodococcus* and *Gordonia* belong to the autochthonous population in pristine and crude-oil-contaminated soils in semiarid Patagonia (Argentina), exhibiting high persistence in these environments (Pucci et al. 2000; Peressutti et al. 2003). In another study, Warton et al. (2001) identified 11 isolates as *Rhodococcus* spp. among a total of 18 Gram-positive bacteria, which were responsible for the biodegradation of the fumigant metham sodium in soil on a farm located in Western Australia. These strains were able to resist dry heat-treatments and to recover their degrading ability following dehydration (Warton et al. 2001). The frequent occurrence of rhodococci in arid sites around the world may reflect their adaptation to environments with poor nutritional conditions and tolerance to other extreme stresses. The accumulation of significant amounts of TAGs by rhodococci is a carbon-intensive and energy-demanding process, which competes with cell growth. Thus, the occurrence of such storage compounds in microorganisms that inhabit energy-poor environments must be an important feature of their physiology. It is known that TAGs are excellent reserve materials owing to their extremely hydrophobic properties, which allow their accumulation in large amounts in cells without changing the osmolarity of cytoplasm. In addition, oxidation of TAGs produces the maximum yields of energy in comparison with other storage compounds such as carbohydrates and PHAs, since the carbon atoms of the acyl moieties of TAGs are in their most reductive form (Alvarez and Steinbüchel 2002). Previous studies revealed that TAGs serve as carbon and energy sources during incubation of *R. opacus* PD630 cells under starvation and water-stress conditions (Alvarez et al. 2000, 2004). In addition, the metabolic activity of cells dropped after incubation under those conditions, whereas the cell counts remained constant. Profound metabolic suppression during unfavorable growth conditions allows a slow utilization of stored lipids, which are likely mobilized in a programmed manner. The energy obtained by the slow mobilization of stored TAGs may support the necessary biochemical and physiological adaptation mechanisms. This process may provide cells of energetic autonomy and a temporal independence from the environment and contribute for cell survival when they do not have access to energy resources in soil.

A similar function has been postulated for the virulent bacterium *M. tuberculosis,* which may use TAGs as a form of energy storage for its long-term survival under dormancy (Daniel et al. 2004). This microorganism survives for decades within the host in a state of nonreplicative, drug-resistant dormancy. This state results probably in a diminution in basal metabolic rate, which facilitates survival of cells at expenses of the accumulated TAGs.

In addition, TAGs may play other important role in TAG-accumulating bacteria such as rhodococci, which occur frequently in arid environments. These lipids may serve also as a reservoir of metabolic water under dry conditions, since fatty acid oxidation releases large amounts of water. Thus, the stored lipids in actinomycetes may be important not only for their energy potential but also for their metabolic water content.

7.2 TAGs as Source of Precursors for Membranes and Cell Envelope

TAGs may serve as precursors for mycolic acid biosynthesis during adaptation of mycolic-acid-producing actinomycetes to environmental stresses. Mycolic acids are long-chain-length fatty acids produced by elongation of normal fatty acids, which are key components for the integrity and function of the cellular envelope in these bacteria (See chapter, "The rhodococcal cell envelope: composition, organisation and biosynthesis" by Sutcliffe et al.). We investigated the physiological and morphological responses of *R. opacus* PD630 to water-stress conditions. During incubation of strain PD630 cells under desiccation conditions, no significant changes in the ultrastructure of the cellular envelope could be detected; thus, the adaptation of its fluidity and permeability may be the result of the variation of the lipid content in response to water stress by a controlled turnover of mycolic acids. Since mycolic acids are produced by elongation of fatty acids by the type II fatty acid synthase complex (FAS II), we studied the effect of isoniazid, which is an inhibitor of the FAS II system, on the survival of water-stressed cells. Cells pretreated with isoniazid (40 μg/mL) exhibited lower survival percentages, which were approximately 18% less than those of nontreated cells after 22 days under dehydration conditions. These results suggested that mycolic acid turnover using the preformed fatty acids contained in TAGs contributed to cell envelope adaptation under water-stress conditions in *R. opacus* PD630 (Alvarez et al. 2004).

TAGs may also play a role in regulating the fatty acid composition of membrane lipids, in order to adapt their fluidity to the environment. TAGs may serve as a donor of fatty acid for phospholipid biosynthesis under fluctuating nutritional conditions.

So far, there has been only indirect evidence on the role of TAGs as source of precursors, such as preformed fatty acids, for biosynthesis or turnover of membranes and cell envelope lipids. Specific studies on this topic are necessary to confirm this function in TAG-accumulating bacteria.

7.3 TAGs as a Form to Detoxify Free Fatty Acids

TAG formation may act to protect cells from sudden increases in fluxes of fatty acids in cells. In this context, Garton et al. (2002) proposed that the biosynthesis of TAGs may be a form of detoxification of free fatty acids, since they observed a rapid accumulation of lipid inclusion bodies by *Mycobacterium* species after transfer of the cells to oleic-acid-containing media. This may be relevant for pathogenic actinomycetes, since *M. tuberculosis* and *R. equi* normally sequester fatty acids from the host cells during the infection.

Another interesting aspect for consideration in hydrocarbon-degrading rhodococci is the role of TAG as acceptor of unusual fatty acids, which may be generated by the catabolism of cells, protecting the integrity and functionality of cellular membranes (Alvarez and Steinbüchel 2002; Alvarez 2006). We reported that

N. globerula 432 and *R. opacus* PD630 were able to degrade pristane and phenyldecane, respectively, and synthesize from them TAGs containing unusual fatty acids, under unbalanced growth conditions (Alvarez et al. 2001, 2002). In addition, cells of *Mycobacterium ratisbonense* SD4 were able to produce wax esters containing isoprenoid acyl- and alcohol residues during incubation of cells on phytane under nitrogen-starved conditions (Silva et al. 2007), whereas *Rhodococcus* sp. 602 accumulated a mixture of TAGs containing medium-chain-length fatty acids (C_8 to C_{12}) after cultivation on naphthyl-1-dodecanoate as sole carbon source (Silva et al. 2010). All these results suggest that TAGs serve as acceptor of unusual fatty acids, which would otherwise disturb membrane fluidity during degradation of hydrocarbons under conditions that normally occur in the environment (Alvarez and Steinbüchel 2002; Alvarez 2006). Thus, *Rhodococcus* spp. and related actinomycetes seem to possess metabolic mechanisms that permit cells to maintain the physiological conditions of cytoplasmic membranes during degradation of hydrocarbons under growth-restricting conditions.

7.4 TAGs as a Form to Balance Central Metabolism

The biosynthesis of TAGs by rhodococci may also be a means to balance the central metabolism dealing with an eventual excess of intermediates, such as acetyl-CoA, or reductive power, under fluctuating conditions as frequently found in natural environments. Previous studies revealed that oxygen-limiting conditions promote TAG accumulation by members of *Mycobacterium* and *Rhodococcus* genera (Daniel et al. 2004; Hernández and Alvarez, unpublished results). When the terminal electron acceptor is not sufficiently supplied during cultivation of cells under conditions of limited aeration, TAGs may serve as a sink for reducing equivalents in cells. Under oxygen-limiting conditions, the excess reducing power may inhibit some key enzymes of central metabolism in cells. The biosynthesis of fatty acids for TAG production, which consumes reduced pyridine nucleotides, may avoid their accumulation in cells. Thus, the biosynthesis of TAGs allows cells to balance their metabolism according to the changes of environmental conditions (Alvarez and Steinbüchel 2002; Alvarez 2006).

7.5 TAGs as Source of Intermediates for Secondary Metabolism

There is some evidence that TAGs may serve as a source of intermediates for the synthesis of compounds that are not essential for growth but for the survival of cells in the environment. Some authors demonstrated that TAGs act as carbon source for the biosynthesis of antibiotics from acetyl-CoA or malonyl-CoA precursors, as has been described by *Streptomyces* strains (Olukoshi and Packter 1994). Storage lipids accumulated by *S. coelicolor* provided carbon for the subsequent synthesis of the

acetate-derived antibiotic, actinorhodin, during nutrient deprivation (Banchio and Gramajo 2002). Whether this process also occurs in antibiotic-producing rhodococci must be investigated in the future.

On the other hand, TAGs may serve as a source of intermediates for the biosynthesis of the extracellular polymeric substance (EPS) produced as a response to diverse stress conditions, such as desiccation, in *Rhodococcus* members. Previous studies revealed that *R. opacus* PD630 was able to progressively accumulate an EPS at the surface of cells during incubation under desiccation conditions (Alvarez et al. 2004). Since the biosynthesis of polysaccharides is a carbon- and energy-intensive process and no external carbon source is available, cells must produce the protective EPS using an endogenous carbon and energy source, such as TAGs, among other possible mechanisms.

8 Biotechnological Significance of Rhodococcal TAGs

The world is currently facing a severe energy crisis. On the one hand, the known and accessible sources of crude oil and other fossil resources are being slowly but continuously depleted; on the other, the demand for fossil resources is rising owing to continuing global industrialization, in particular, also in countries with large populations such as China and India. Therefore, the possibilities to exploit alternative energies are currently intensively investigated. This includes regenerative energies and energy generation from renewable resources. One prominent example is ethanol, which is currently mainly produced from liquefied corn starch or sugarcane in particular in North and South America, respectively. TAGs are currently produced at large scale by agriculture for synthesis of fatty acid methylesters. In Europe, they are currently the preferred products from renewable resources and are referred to as "Biodiesel." Biodiesel is produced from synthetic methanol from the chemical industry and from TAGs by chemical transesterification yielding besides Fatty acid methyl esters (FAME) about 10% (wt/wt) glycerol as a byproduct (Röttig et al. 2010). Very small amounts of biodiesel are also enzymatically produced (Adamczak et al. 2009). Biodiesel and bioethanol currently constitute about 90% of the biofuel market (Antoni et al. 2007; Uthoff et al. 2009).

TAGs for biodiesel production are currently exclusively produced by agriculture; comparably very little amounts are obtained from the use of frying oil from fast food restaurants. The main crops for TAGs production are rapeseed in Europe, oil palm trees in South East Asia, and Soja in North America. TAGs could, however, also be produced from bacteria, in principle. Perhaps, one of the most suitable candidates is *R. opacus*, owing to its extraordinary high lipid content and the good growth of the cells. As already outlined earlier in this chapter, *R. opacus* strain PD630 has been investigated in much detail. Lipid contents of as high as 87% have been described for cells cultivated on a small scale (Alvarez et al. 1996). In first attempts, cells of *R. opacus* were also grown on a scale of 30 L and even of 500 L (Voss and Steinbüchel 2001). Using a medium of mineral salts supplemented

with beet molasses and sucrose, a cell density of 37.5 g cell dry matter per liter with a lipid content as high as 52% (wt/wt) was obtained at the 30-L scale. At the 500-L scale, which was only tried once, a cell density of 18.4 g cell dry matter per liter and a lipid content of 38.4% (wt/wt) were obtained (Voss and Steinbüchel 2001).

This oleaginous bacterium is therefore a promising candidate for the biotechnological production of TAGs from renewable resources. Production in bacteria gives a greater flexibility in comparison to that in plants because various renewable resources and in particular also residual carbon, which is not directly used for production of food and feed, may be used. If the residual carbon cannot be utilized by a strain, the metabolism of this strain may be engineered to utilize such carbon and energy sources for growth and lipid production. This is important to avoid a competition between the feed and food industry on one side and the chemical and energy industry on the other and to avoid a further increase in the emission of greenhouse gases and also for a sustainable production of lipids (Searchinger et al. 2008; Fargione et al. 2008).

The key enzyme of TAG or wax ester biosynthesis in bacteria is a novel type of acyltransferase which has so far been unknown in other groups of organisms. They occur frequently in multiple copies in bacteria, and also the *R. opacus* strain PD630 possesses several of these acyltransferases (Alvarez et al. 2008). One common feature of all of these acyltransferases is the low substrate specificity. The enzyme from *A. baylyi* seems to transfer acyl moieties of varying carbon chain lengths from the corresponding acyl coenzyme A thioesters to almost any hydroxyl group and even to some thiol groups (see above). Although no detailed biochemical studies on the substrate ranges of the acyltransferases from *R. opacus* have been made (Alvarez et al. 2008), data from preliminary enzymatic studies and physiological experiments clearly indicate a low substrate specificity of this bacterium also for the acyltransferases. This makes the enzymes from *R. opacus* also putative candidates for the synthesis of fine chemicals or oleochemicals comprising organic alcohols and thiols to which acyl moieties are covalently attached (Stöveken and Steinbüchel 2008).

9 Concluding Remarks

The accumulation of TAGs is a common feature among rhodococci. Some of them can be considered as oleaginous microorganisms because they produce significant amounts of TAGs as intracellular inclusion bodies. Although the knowledge acquired during the last decade on the production of TAGs in rhodococci has been considerable, many fundamental aspects remain to be clarified. The understanding of this topic in rhodococci is important because a member of this genus, *R. opacus* PD630, has been the preferred research model in this field, which can be extrapolated also to other actinobacteria with clinical importance, such as *M. tuberculosis*. The occurrence of storage lipids seems to be important for the survival of this microorganism in the host cells and, therefore, for the development

of the disease. Therefore, TAG biosynthesis may be a new target for developing drugs to prevent this important disease. Basic knowledge in this field is also relevant for predicting biotechnological applications of oleaginous bacteria in the industry, for example for the production of cosmetic products, biofuels, oleochemicals, lubricants, and other manufactured products. In addition, the advances in rhodococcal TAG research will permit better understanding of their physiology and relationship with the environment. TAGs may permit cells to survive under fluctuating and unfavourable conditions as occur normally in natural environments. In this context, the occurrence of TAGs could be one of the factors that determine the high water-stress resistance of rhodococci and their wide distribution in arid environments. The current availability of appropriate molecular tools and methods of analysis, as well as the availability of genome data bases of TAG-accumulating strains, will permit interesting advances in our understanding of the biology of the *Rhodococcus* genus.

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Rhodococcus Biosurfactants: Biosynthesis, Properties, and Potential Applications

Maria S. Kuyukina and Irena B. Ivshina

Contents

Abstract Members of the genus *Rhodococcus* produce biosurfactants in response to the presence of liquid hydrocarbons in the growth medium. These biosurfactants are predominantly cell-bound glycolipids containing trehalose as the carbohydrate. Physiological roles of these glycolipids are diverse and involve participation in the uptake of water-insoluble substrates, promotion of the cell adhesion to hydrophobic surfaces, and increased rhodococcal resistance to physicochemical influences. In terms of surfactant characteristics (e.g., surface and interfacial tension, critical micelle concentration, emulsifying activity), *Rhodococcus* biosurfactants compete favorably with other microbial and synthetic surfactants. Additionally, biological activities of trehalolipids from rhodococci were revealed, including immunomodulating and antitumor properties. Recently developed optimization procedures for their biosynthesis and recovery would broaden potential applications of *Rhodococcus* biosurfactants in new advanced technologies, such as environmental bioremediation,

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improved polymeric material construction, and biomedicine. The present chapter summarizes recent research on *Rhodococcus* biosurfactants, updating the comprehensive review of Lang and Philp (Antonie van Leeuwenhoek Int J Gen Mol Microbiol 74:59–70, 1998), and focuses on biosynthesis features, physicochemical and bioactive properties, and their application potential.

1 Introduction

Biosurfactants, surface-active compounds of biological origin, are of increasing interest for many industries because of their chemical diversity, multifunctional characteristics, and low toxicity in comparison to synthetic, petrochemical-derived surfactants (Maier 2003). All biosurfactants are amphiphilic in nature, containing at least one hydrophilic (usually sugar or amino acid) moiety and one hydrophobic (usually fatty acid) moiety in the molecule. Owing to their amphiphilic nature, surfactants tend to accumulate at surfaces and interfaces and, as a result, reduce surface (liquid–air) and interfacial (liquid–liquid and liquid–solid) tension. When surfactant monomers are added into a solution, the surface or interfacial tension will decrease until the surfactant concentration reaches what is known as the critical micelle concentration (CMC). At the CMC, surfactant monomers begin to spontaneously associate into structured aggregates, called micelles. The CMC of a biosurfactant could be regarded as an efficiency measure and is dependent on the surfactant structure as well as on pH, ionic strength, and temperature of the solution.

Microbial biosurfactants are a structurally diverse group of surface-active molecules synthesized by various microorganisms and they appear to play a role in many physiological processes occurring at interfaces, ranging from cell adhesion and surface colonization to microbial antagonism and pathogenesis (Neu 1996; Ron and Rosenberg 2001). According to Maier (2003), biosurfactants of phylogenetically distant microorganisms are functionally convergent, thus indicating their significant role in the vital functions of biosurfactant producers. At the same time, biosurfactants synthesized by the bacteria of different species belonging to the same genus are often different in terms of their structure and function. Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, the major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants (Banat et al. 2000).

Fields of application or potential use of biosurfactants are diverse and include oil, pharmaceutical, cosmetic, and food-processing industries. The most promising applications of biosurfactants are the environmental remediation technologies since product purity is of less concern (Lang and Philp 1998). In recent years, biosurfactants traditionally used as emulsifying and solubilizing agents for hydrophobic substances are attracting an increasing attention as possible biomedical agents with expressed biological activities (Ryll et al. 2001; Kitamoto et al. 2002;

Cameotra and Makkar 2004). It should be noted that, at present, the industrial use of biosurfactants is not generally competitive with synthetic surfactants because of their higher production cost. However, among biogenic surfactants, the products of microbial synthesis are the most promising candidates for biotechnological applications because of simple mineral media and available carbon sources used in fermentation processes as well as a shorter generation time compared to animal or plant growth (Lang 2002). Thereby, possible optimization of a biosurfactant synthesis process would allow increasing the product yield at relatively low material and energy expenditures (Banat et al. 2000). The possibility of in situ production of biosurfactants is essential for the bioremediation of water and soil contaminated with organic pollutants and heavy metals (Christofi and Ivshina 2002).

In terms of applied aspects, glycolipid biosurfactants structurally represented by mono- and disaccharide-based complexes having ester bonds with fatty acids are being thoroughly studied due to high productivity from renewable resources (e.g., carbohydrate biomass) and versatile biochemical properties compared to other types of biosurfactants (Kitamoto et al. 2002; Lang 2002). Most well-known glycolipid biosurfactants include rhamnolipids from *Pseudomonas aeruginosa*, sophorolipids from *Candida bombicola*, cellobiose lipids from *Ustilago maydis*; mannosylerythritol lipids from *Pseudozyma antarctica* and trehalolipids from *Rhodococcus erythropolis* (Kitamoto et al. 2002). Members of *Rhodococcus* species possessing unique biological properties and wide catabolic abilities are perspective candidates for biosurfactant producers (Ivshina 2001; Van der Geize and Dijkhuizen 2004).

2 Surfactant Production by *Rhodococcus* Species and Related Actinobacteria

The production of biosurfactants by rhodococci and related coryneform and nocardioform actinobacteria was studied intensively, but most of the research has been done on trehalolipid surfactants formed by *R. erythropolis* (Lang and Philp 1998), while other *Rhodococcus* species are also reported as active biosurfactant producers (Table 1). Comparing the data on surface-active properties of 35 strains covering four *Rhodococcus* species (namely, *R. erythropolis*, "*R. longus,*" *R. opacus*, and *R. ruber*), it was suggested that biosurfactant production is generalized throughout this genus (Ivshina et al. 1998; Philp et al. 2002). When grown on liquid alkanes, rhodococci produce surfactants capable of reducing the surface and interfacial tension of water to low values (Ivshina et al. 1998). Alkane growth of the most potent biosurfactant producers, *R. erythropolis* DSMZ 43215 and *R. ruber* IEGM 231 resulted in the biosurfactant yield of 32 and 10 g l^{-1}, respectively (Kim et al. 1990; Kuyukina et al. 2001). Bouchez-Naïtali et al. (1999) described several soil isolates belonging to *Rhodococcus equi* and *Corynebacterium* spp., which were

Table 1 Surfactant properties of *Rhodococcus* bacteria compared with other microorganisms

Microorganism	Surfactant product	Surface tension (mN m^{-1})	Interfacial tension (mN m^{-1})	CMC (mg l^{-1}) or CMD*	References
R. erythropolis (14 strains)	Whole culture broth	27.1 (0.55)[a]	6.3 (3.57)	60*	Ivshina et al. (1998), Philp et al. (2002)
R. erythropolis DSM 43215	Trehalose-dicorynomycolates	36	17	4	Kim et al. (1990)
R. erythropolis DSM 43215	Trehalose-monocorynomycolates	32	14	4	Kim et al. (1990)
R. erythropolis DSM 43215	Trehalose-2,2′,3,4-tetraester	26	<1	15	Kim et al. (1990)
R. erythropolis DSM 51T7	Trehalose tetraester	27.9	5	37	Marqués et al. (2009)
R. erythropolis MTCC 2794	Crude organic extract	33.8	n.d.	100	Pal et al. (2009)
R. erythropolis EK-1	Cell-free culture broth	30–39	n.d.	6*	Pirog et al. (2004)
"R. longus" (3 strains)	Whole culture broth	27.2 (0.40)	1.8 (0.1)	90*	Ivshina et al. (1998), Philp et al. (2002)
R. opacus (3 strains)	Whole culture broth	26.5 (0.61)	3.0 (1.75)	95*	Ivshina et al. (1998), Philp et al. (2002)
R. ruber (15 strains)	Whole culture broth	27.4 (0.57)	2.7 (1.61)	72*	Ivshina et al. (1998), Philp et al. (2002)
R. ruber IEGM 231	Crude organic extract	28.5–30.1	0.3–1.6	86–173	Kuyukina et al. (2001)
R. ruber IEGM 235	Glycolipid complex	26.8	0.9	54	Ivshina et al. (1998)
R. wratislaviensis BN38	Whole culture broth	28.6	5.3	n.d.	Tuleva et al. (2008)
R. wratislaviensis BN38	Trehalose tetraester	24.4	1.3	5	Tuleva et al. (2008)
Rhodococcus sp. 51T7	Cell free supernatant	30	n.d.	n.d.	Espuny et al. (1995)
Rhodococcus sp. H13-A	Octaacyl-trehalose	n.d.	0.02	1.5	Singer and Finnerty (1990)
Rhodococcus sp. SD-74	Succinoyl trehalolipid	19	n.d.	5.6×10^{-6} M	Tokumoto et al. (2009)
Pseudomonas aeruginosa	Rhamnolipids	29	0.25	50–200	Noordman et al. (2002)
Candida bombicola ATCC 22214	Sophorolipids	35	n.d.	40	Lang (2002)
Pseudozyma antarctica T-34	Mannosylerythritol lipids	28.2–28.4	2.1–2.4	$2.7–4.5 \times 10^{-6}$ M	Kitamoto et al. (2002)
Bacillus subtilis	Surfactin	27	<1	40×10^{-6} M	Lang (2002)

CMC Critical micelle concentration, *CMD* Critical micelle dilution, *n.d.* None determined

[a]Standard deviations

able to produce biosurfactants when grown on hexadecane. The *Rhodococcus rhodochrous* strain CF222 was reported to produce a surface-active complex of acidic polysaccharides and lipids, which increased bacterial tolerance to hexadecane (Iwabuchi et al. 2000). Recently, biosurfactant production of the alkanothrophic strain *Rhodococcus wratislaviensis* BN38 has been characterized (Tuleva et al. 2008). It should be noted that among 30 valid *Rhodococcus* species, only six species (and one invalid species – "*R. longus*") are reported to produce biosurfactants so far. However, members of these species (*R. equi*, *R. erythropolis*, *R. opacus*, *R. rhodochrous*, and *R. ruber*) are the most common environmental isolates and they represent the major portion of *Rhodococcus* holdings in culture collections. Biosurfactant production is also described in *Rhodococcus*-related genera, namely *Nocardia* (Kim et al. 2000), *Corynebacterium* (Cooper et al. 1979), *Gordonia* (Arenskötter et al. 2004), *Mycobacterium* (Abdelhay et al. 2009), *Tsukamurella* (Choi et al. 1999), and *Arthrobacter* (Passeri et al. 1991).

3 Structures and Physicochemical Properties

It is now assumed that the type of a biosurfactant produced is dictated by the producing microorganism. Thus, the best known glycolipid biosurfactants, rhamnolipids, are produced only by *P. aeruginosa* and few other pseudomonad species; sophorolipids and mannosylerythritol lipids are extracellular metabolites of yeasts belonging to *Candida* and *Pseudozyma*, respectively (Lang 2002). Trehalolipids are produced by members of closely related actinobacterial genera, including *Rhodococcus*, *Nocardia*, *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Tsukamurella*, and *Arthrobacter*. These compounds include α,ά-D-trehalose, a nonreducing disaccharide, which is linked by an ester bond to long-chain fatty acids. Among the latter α-branched β-hydroxy acids, so-called mycolic acids play a preferential role (Lang and Philp 1998). Trehalolipids from rhodococci are characterized by high structural diversity, and they often occur as a complex mixture, the composition of which varies depending on strain physiology and growth conditions. Trehalose monomycolates (Batrakov et al. 1981; Kretschmer and Wagner 1983), trehalose dimycolates (Rapp et al. 1979; Batrakov et al. 1981; Philp et al. 2002; Niescher et al. 2006), trehalose trimycolates (Tomiyasu et al. 1986), as well as mono-, di-, tetra-, hexa- and octa-acylated derivatives of trehalose (Kretschmer and Wagner 1983; Singer and Finnerty 1990; Philp et al. 2002) represent the nonionic trehalolipid type (Fig. 1a). Trehalose tetraesters (Ristau and Wagner 1983; Espuny et al. 1995; Rapp and Gabriel-Jürgens 2003; Tuleva et al. 2008; Marqués et al. 2009) and succinoyl trehalolipids (Tokumoto et al. 2009), which additionally harbor one or two succinoyl residues, represent the anionic trehalolipid type (Fig. 1b). Other biosurfactant types were described in rhodococci, such as glucolipids (Kurane et al. 1995; Peng et al. 2007) and polysaccharides (Iwabuchi et al. 2000), and the existence of additional chemical structures could be expected.

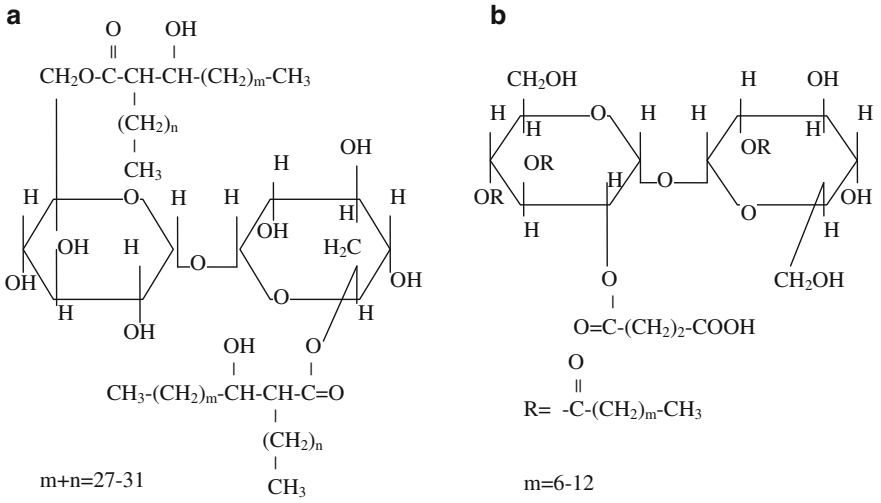

Fig. 1 Structures of nonionic (trehalose-dimycolates) and anionic (trehalose-tetraester) trehalolipids from *Rhodococcus erythropolis* (modified from Lang and Philp 1998)

Unlike synthetic surfactants having relatively simple chemical structure, biosurfactants are usually quite complex molecules. So, physicochemical properties of biosurfactants are more complex and "flexible" compared to synthetic surfactants. Evaluating the properties of *Rhodococcus* biosurfactants, it was observed that they lowered the surface tension of water from 72 mN m^{-1} to values between 19 and 43 mN m^{-1} and the interfacial tension of water/*n*-hexadecane system from 43 mN m^{-1} to values between 0.02 and 15 mN m^{-1} (Ivshina et al. 1998; Philp et al. 2002; Marqués et al. 2009; Tokumoto et al. 2009). Considering surface tension, interfacial tension, and CMC values, *Rhodococcus* biosurfactants compare well with other microbial and some synthetic surfactants (see Table 1). Another essential feature of biosurfactants from rhodococci is their emulsifying activity, i.e., the ability to emulsify various hydrocarbons and oils (Ivshina et al. 1998; Bicca et al. 1999). High emulsion indices with *n*-hexadecane were recorded for biosurfactants produced by *R. erythropolis*, "*R. longus,*" *R. opacus*, and *R. ruber* with a maximum E_{24} of 62.5% (Ivshina et al. 1998). Similar E_{24} values ranging from 20% to 60% were registered by Bicca et al. (1999) for the *R. ruber* biosurfactant with *n*-alkanes (pentane, hexane, heptane), aromatic hydrocarbons (benzene, toluene, xylene), and petroleum fractions (diesel, petrol, kerosene). Tuleva et al. (2008) showed high emulsification activities (E_{24} = 23–69%) of the purified trehalose tetraester from *R. wratislaviensis* BN38 toward various water-immiscible substrates including *n*-alkanes, toluene, benzene, xylene, kerosene, crude oil, mineral oils, sunflower oil, and almond oil. Hydrophilic–lipophilic balance (HLB) of biosurfactants, a relative hydrophobicity measure, is the important matter for their industrial applications. We calculated the HLB value of 8 from the chemical structure of purified trehalolipids produced by *R. ruber* IEGM 231, while the corresponding HLB of

6.4–7.5 was determined for the crude biosurfactant from this strain (Kuyukina et al. 2006). These HLB values, which are relatively low when compared to other bacterial and synthetic surfactants (Noordman et al. 2002), indicate prevailing hydrophobic properties of the *R. ruber* biosurfactant and confirm its potential as an oil-in-water emulsifier. Trehalolipids from *R. erythropolis* 51T7 with the HLB value of 11 were shown to produce stable emulsions with water and paraffin or isopropylmyristate (Marqués et al. 2009). Thermal, osmotic, and pH stabilities of *Rhodococcus* biosurfactants were also reported (Kuyukina et al. 2006; Marqués et al. 2009). However, there is still very little information available on physicochemical properties of *Rhodococcus* biosurfactants, thereby hindering their introduction to industrial fields.

4 Biosynthesis and Recovery

Metabolic pathways involved in trehalolipid synthesis in *Rhodococcus* are reviewed by Lang and Philp (1998). It seems that the trehalose moiety and the fatty (mycolic) acid moiety of trehalolipid molecules are synthesized independently and are subsequently etherified (Fig. 2). Trehalose monomycolate was postulated to be an intermediate of trehalose dimycolate biosynthesis (Kretschmer and Wagner 1983). The formation of the mycolate is considered to be a Claisen-type condensation of two fatty acids, a carboxylated acyl-coenzymeA and an activated acyl chain to yield a 3-oxo intermediate, which would then be reduced to form mycolic acid (Lang and Philp 1998). For the formation of the various mycolates, first the long-chain fatty acid constituting the major part of the molecule is synthesized before condensation with the second fatty acid. Fatty acids are synthesized in many metabolic pathways, for example via the successive oxidation of alkane substrates to alcohols, aldehydes, and carbonic acids followed by carbon chain elongation (for review, see chapter, "Central metabolism of species of the genus *Rhodococcus*" by Alvarez). Intermediates of the fatty acid β-oxidation could also be involved in the trehalolipid synthesis. The synthesis of the final resulting sugar residue, trehalose-6-phosphate, from UDP-glucose and glucose-6-phosphate proceeds apparently via the OtsA–OtsB pathway described for coryneform actinobacteria (Tzvetkov et al. 2003), although alternative trehalose biosynthesis pathways (TreY-TreZ pathway and TreS pathway) could also be involved (De Smet et al. 2000). *n*-Alkane substrates are first degraded to acetyl-CoA before glucose synthesis (see Fig. 2). Although the key pathways and enzymes of fatty (mycolic) acid and trehalose syntheses are described in *Rhodococcus* and related bacteria, only little is known about the final step of trehalolipid synthesis, trehalose acylation. This reaction is probably catalyzed by acyltransferases specific to particular fatty acid types. For example, Belisle et al. (1997) described mycolyltransferases responsible for the transfer of mycolic acids to α,ά-trehalose to form α,ά-trehalose monomycolate and α,ά-trehalose dimycolate in *Mycobacterium tuberculosis*. Similar enzymes and corresponding genes were later described in other mycobacteria and corynebacteria

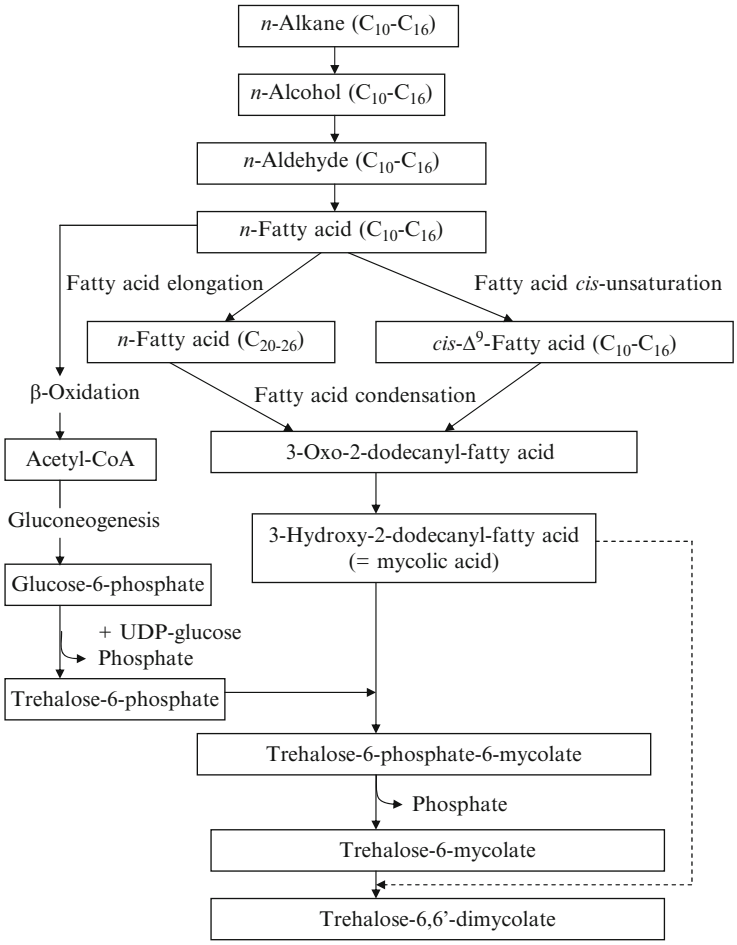

Fig. 2 Scheme of trehalose-mono- and -dimycolate synthesis from *n*-alkanes (modified from Lang and Philp 1998)

(Kacem et al. 2004), but not so far in rhodococci. Biochemical mechanisms of acylation leading to the formation of anionic trehalolipids such as trehalose tetraesters and succinoyl trehalolipids containing only straight-chain acids and succinate also remain unknown.

More research was performed on physiological aspects of biosurfactant production by *Rhodococcus* aimed to optimize growth conditions for surfactant overproduction. Rhodococci were reported to synthesize trehalolipids in growth associated, growth-limited, and resting cell conditions (Kim et al. 1990; Philp et al. 2002; Tuleva et al. 2008; Haddadin et al. 2009). It is assumed that biosurfactants cannot be produced in considerable amounts when *Rhodococcus* cells are grown with water-soluble substrates. We tested various hydrocarbons as the substrates for

Table 2 Growth, surface-active properties, and surfactant production of *R. ruber* IEGM 231 on different hydrocarbons

Growth substrate	Biomass ($g\ l^{-1}$)	Surface tension ($m\ Nm^{-1}$)	Surfactant concentration ($g\ l^{-1}$)
Hexane	–	63.1	–
Cyclohexane	–	65.9	–
Heptane	0.68	67.9	–
Octane	–	56.2	–
Nonane	0.34	56.7	–
Decane	1.24	56.5	–
Dec-1-ene	1.00	55.9	–
Undecane	2.15	26.9	0.18
Dodecane	4.53	29.7	0.59
Tridecane	4.93	32.1	2.23
Tetradecane	5.67	31.8	1.79
Pentadecane	4.53	31.2	4.01
Hexadecane	4.80	29.6	9.90

From Philp et al. (2002)

R. ruber biosurfactant production (Philp et al. 2002) and found that short chain *n*-alkanes ranging from hexane to nonane as sole carbon sources failed to support growth of *R. ruber* IEGM 231 (Table 2). Decane appeared to allow limited growth, but no biosurfactant was produced. The limited growth observed with dec-1-ene was visually identical to the growth on decane. Only from undecane upwards did the organism produce significant amounts of biomass and biosurfactant. The surfactant production of *R. ruber* increased with the increase in *n*-alkane chain length. Thus, during growth on undecane and dodecane, the surfactant concentrations were minimal, but increased from 1.8–2.2 $g\ l^{-1}$ on tridecane and tetradecane to 4.0 $g\ l^{-1}$ on pentadecane, reaching the maximal level (9.9 $g\ l^{-1}$) on hexadecane. These results are consistent with the data on biosurfactant production by *R. wratislaviensis* BN38, which also started from undecane and reached the maximal level of 3.1 $g\ l^{-1}$ on hexadecane (Tuleva et al. 2008). Among the four carbon sources tested (diesel, naphthalene, crude oil, benzene), the optimal biosurfactant production of *R. erythropolis* and *R. ruber* strains was obtained using naphthalene and diesel (Haddadin et al. 2009).

Kinetics of biosurfactant production by rhodococci grown with hydrocarbons was studied thoroughly in numerous batch and continuous culture experiments. Nonionic glycolipid production by *R. erythropolis* and *R. ruber* under nonlimiting conditions correlated with biomass formation and *n*-hexadecane consumption (Figs. 3 and 4), thus suggesting its growth-associated mode (Rapp et al. 1979; Philp et al. 2002; Haddadin et al. 2009). Nitrogen limitation and temperature shift (from 30 to 22°C) were shown to favor the formation of anionic trehalose tetraesters (up to 32 $g\ l^{-1}$) during growth of *R. erythropolis* DSM 43215 on hydrocarbons (Ristau and Wagner 1983; Kim et al. 1990), while *R. ruber* and *R. opacus* synthesized only nonionic trehalolipids under these conditions (Philp et al. 2002; Niescher et al. 2006). Since the overproduction began at a low nitrogen concentration, the complete separation of growth and production phase was attempted. Resting cell

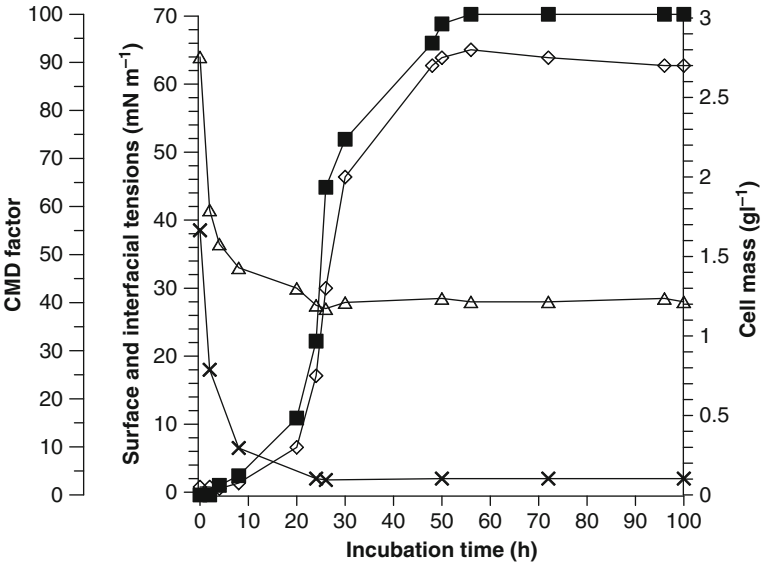

Fig. 3 Surfactant properties of *Rhodococcus ruber* IEGM 231 during batch culture growth on *n*-hexadecane. *filled square*, CMD factor; *open triangle*, surface tension; *times*, interfacial tension; *open diamond*, cell mass (from Philp et al. 2002)

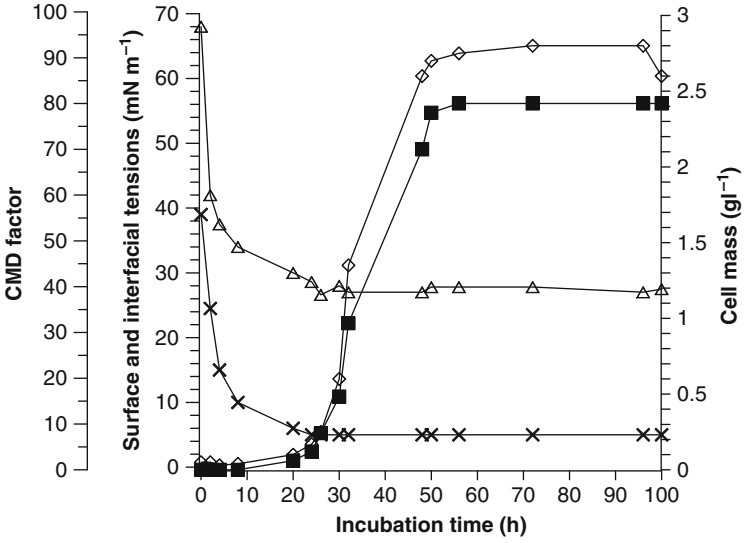

Fig. 4 Surfactant properties of *Rhodococcus erythropolis* IEGM 20 during batch culture growth on *n*-hexadecane. *filled square*, CMD factor; *open triangle*, surface tension; *times*, interfacial tension; *open diamond*, cell mass (from Philp et al. 2002)

experiments with *R. erythropolis* DSM 43215 resulted in the efficient conversion of the technical-grade C10 *n*-alkane to more than 20 g l^{-1} of trehalolipids (Kim et al. 1990). However, in the study of Peng et al. (2007), resting *R. erythropolis* 3C-9 cells incubated in a medium containing hexadecane, but lacking a nitrogen source, were not able to produce biosurfactants.

Mathematical modeling was applied to enhance the biosurfactant production of *R. erythropolis* MTCC 2794 (Pal et al. 2009). Two optimization techniques, such as the artificial neural network (ANN) coupled with genetic algorithm (GA) and the response surface methodology (RSM), were used for the optimization of medium components (sucrose, yeast extract, meat peptone, and toluene), resulting in the increased biosurfactant yield.

The recovery and concentration of biosurfactants from the fermentation broth largely determine their production cost. Often, low concentration and the amphiphilic nature of microbial surfactants limit their recovery (Desai and Banat 1997). Various techniques used for biosurfactant isolation include high-speed centrifugation, dia- and ultrafiltration, acid and salt precipitation, solvent extraction and adsorption chromatography (Bryant 1990; Desai and Banat 1997). However, surfactants produced by rhodococci under unrestricted growth conditions are predominantly cell-associated trehalolipids (Lang and Philp 1998), which can be effectively isolated only by the organic solvent extraction. A wide variety of organic solvents, for example, methanol, ethanol, diethyl ether, pentane, acetone, chloroform, and dichloromethane have been used, either singly or in combination, for biosurfactant extraction (Desai and Banat 1997). We proposed methyl tertiary-butyl ether (MTBE), a less toxic and inexpensive solvent with relatively low polarity for the extraction of biosurfactants from *Rhodococcus* cultures (Kuyukina et al. 2001). The combination of extraction procedure with an ultrasonic treatment of the extraction mixture resulted in a good trehalolipid recovery (Table 3).

Table 3 Yield and surface-active properties of biosurfactants from *R. ruber* IEGM 231 extracted using different solvent systems

Solvent system	Surfactant concentration (g l^{-1})	Surface tension (mN m^{-1})	Interfacial tension (mN m^{-1})	CMC (mg l^{-1})
MTBE	10.1	29.2	0.9	173
MTBE[a]	8.6	30.1	1.5	135
MTBE[b]	9.1	29.5	1.5	444
CH_2Cl_2	9.4	35.0	0.5	180
CH_2Cl_2[a]	8.1	29.9	1.4	86
CH_2Cl_2[b]	9.3	32.4	1.3	463
$CHCl_3$:CH_3OH (1:2)	9.8	28.9	1.0	171
$CHCl_3$:CH_3OH (1:2)[a]	9.6	30.0	1.6	90
$CHCl_3$:CH_3OH (1:1)[b]	12.2	30.9	2.2	119
$CHCl_3$:CH_3OH (2:1)	10.7	28.5	0.3	97
MTBE:$CHCl_3$ (1:1)	10.1	29.2	1.2	140
MTBE:$CHCl_3$ (1:1)[b]	10.7	30.3	7.6	320

MTBE tertiary-butyl ether; *CH_2Cl_2* methylene chloride; *$CHCl_3$* chloroform; *CH_3OH* methanol

[a]Extraction was performed by ultrasonic treatment (23 kHz, 10 min)

[b]Extraction was performed from the whole culture broth. From Kuyukina et al. (2001)

5 Physiological Roles and Biological Activity

Rhodococci are able to utilize aliphatic and aromatic hydrocarbons of extremely low water solubility as carbon and energy sources. The role of biosurfactants is related to the low water-solubility of *n*-alkanes as growth substrates and is determined by the ability to reduce interfacial tension between hydrocarbons and an aqueous phase (Ron and Rosenberg 2001). Although many bacteria can assimilate hydrophobic substrates in solubilized or emulsified forms, the hydrocarbon uptake by *Rhodococcus* occurs via the direct cell contact with large oil drops (Lang and Philp 1998). Production of cell-bound biosurfactants, together with an increase in cell surface hydrophobicity, formation of intracellular inclusions and extracellular polymers were considered as specific adaptive mechanisms of alkane assimilation by *Rhodococcus* cells, especially at a low temperature when the substrate bioavailability is extremely low (Whyte et al. 1999). Cell-associated biosurfactants promote the adhesion of rhodococcal cells not only to liquid hydrocarbons, but also to hydrophobic solid surfaces (Neu 1996), allowing effective cell colonization and direct uptake from sorbed/crystalline hydrocarbons (Whyte et al. 1999). A possible mechanism of biosurfactant-mediated cell detachment from used oil droplets proposed by Rosenberg and Ron (1999) for the *Acinetobacter calcoaceticus* strain RAG-1 could also act in rhodococcal cells.

Trehalose mycolates along with other cell-wall lipids are involved in cellular tolerance to antibiotics and organic solvents (Kuyukina et al. 2000; Sokolovska et al. 2003; Nguyen et al. 2005; chapter, "Adaptation of *Rhodococcus* to organic solvents" by de Carvalho), as well as to physical factors, for example, high temperature and desiccation (Sung et al. 2004; LeBlanc et al. 2008). We studied the protective effect of a trehalolipid biosurfactant on propane-oxidizing *R. ruber* cells during a long-term storage of freeze-dried cultures (Kamenskikh et al. 2004) and observed the two to threefold viability increase, when cell suspensions were added with 10% biosurfactant, 1.5% gelatin, and 0.1% agar prior to freeze-drying compared to the commonly used cryoprotectant (10% trehalose, 1.5% gelatin, and 0.1% agar). Interestingly, no viability increase of *R. ruber* cells treated with trehalose was registered, suggesting that although trehalose was reported among endogenous osmolytes protecting rhodococcal cells from the desiccation stress (Alvarez et al. 2004), the acylated trehalose would be a preferable form of the exogenous cell protectant. LeBlanc et al. (2008) performed transcriptomic analyses of air-dried *Rhodococcus jostii* RHA1 to identify genes involved in the regulatory response to desiccation. Among desiccation-specific up-regulated genes, 23 genes of lipid metabolism and cell envelope modification were revealed as well as the α,α-trehalose-phosphate synthase gene responsible for the synthesis of trehalose moiety of trehalolipids. In the study of Sung et al. (2004), genes of trehalose mycolyltransferases (Ag85B and alpha antigen) involved in the trehalolipid formation were strongly expressed in the heat stress-exposed cells and thus were associated with the heat resistance of *Mycobacterium paratuberculosis*.

There are considerable numbers of recent studies showing that biosurfactants play a significant role in fundamental biological processes, such as bacterial cell

signaling and quorum sensing, biofilm formation and cellular differentiation, bacterial pathogenesis and antagonism (for reviews, see Peypoux et al. 1999; Kitamoto et al. 2002; Lang 2002; Cameotra and Makkar 2004; Van Hamme et al. 2006). Although glycolipids from *Rhodococcus* and related bacteria showed no antagonistic activity against Gram-negative bacteria and yeasts (for review, see Kitamoto et al. 2002), trehalolipids from *R. erythropolis* DSMZ 43215 inhibited conidia germination of the fungus *Glomerella cingulata* (Kitamoto et al. 2002). Trehalose dimycolate from *M. tuberculosis* potentiated nonspecific resistance in mice to influenza virus infection (Hoq et al. 1997).

Many biological activities of trehalolipids from *Rhodococcus* and related bacteria are determined by their amphiphilic nature and resulted from the interaction with cellular membranes (Ortiz et al. 2009). Thus, Retzinger et al. (1981) hypothesized that biological responses evoked by trehalose dimycolate emulsions do not involve any recognition of specific chemical structures of the glycolipid, but are determined solely by the geometry and behavior of trehalose dimycolate at water–hydrophobe interfaces. The studies of Aranda et al. (2007) and Ortiz et al. (2008, 2009) showed that *Rhodococcus* trehalolipid interactions with phospholipid cellular membranes result in the altered structural and rheological membrane properties, which might contribute to the molecular mechanisms of trehalolipid biological activity.

It should be noted that cell-wall trehalose di- and monomycolates of pathogenic (*M. tuberculosis*, *Corynebacterium diphtheriae*) and opportunistic (*Mycobacterium avium – Mycobacterium intracellulare* group, *Nocardia asteroids*, *Corynebacterium matruchotii*, *Corynebacterium xerosis*) mycolata play a key role in the pathogenesis of infections caused by these actinobacteria, and they are characterized by high immunomodulating activity (Fig. 5). Particularly, they stimulate innate, early adaptive and both humoral and cellular adaptive immunity by inducing the production of cytokines (IL-12, IFN-γ, TNF-α, IL-4, -6, -10) and chemokines (MCP-1, MIP-1α, IL-8) (for review, see Ryll et al. 2001). Biological activity of these trehalolipids is significant; however, the apparent or potential pathogenicity of the producer strains and high toxicity of produced glycolipids limit their biomedical applications (Sakaguchi et al. 2000). Therefore, the search for trehalolipid producers among nonpathogenic actinobacteria is of importance. In our experiments, a trehalolipid biosurfactant from *R. ruber* stimulated interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) production of human monocytes (Kuyukina et al. 2007). Additionally, a trehalolipid from *R. ruber* displayed no cytotoxicity against human lymphocytes and therefore could be proposed as a potential immunomodulating and antitumor agent.

6 Industrial Potential

Current and potential application fields for microbial surfactants, including oil, pharmaceutical, food, and cosmetic industries as well as environmental technologies are reviewed by Banat et al. (2000), Christofi and Ivshina (2002),

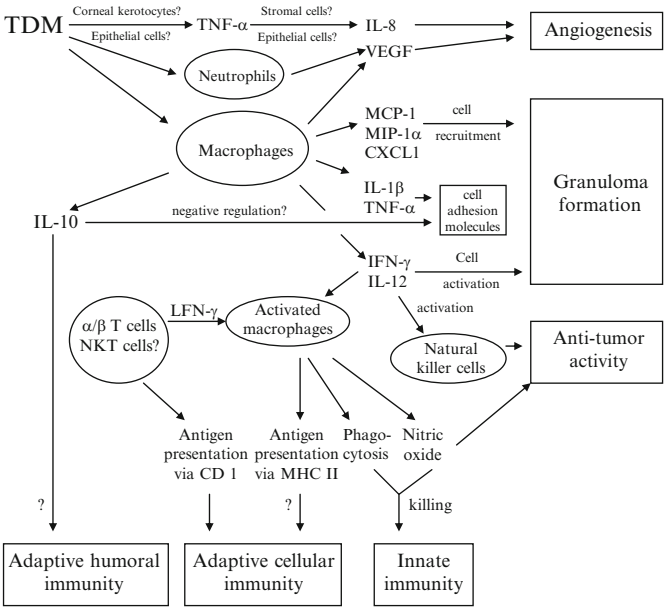

Fig. 5 Immunomodulating properties of trehalose dimycolate (TDM) from *Mycobacterium tuberculosis* (Modified from Ryll et al. 2001)

Kitamoto et al. (2002), Lang (2002), Mulligan (2005), Singh et al. (2007). Biosurfactants can be used as emulsifiers, deemulsifiers, wetting and foaming agents, functional food ingredients, detergents, and flocculating agents. However, despite of numerous advantages of biosurfactants, namely functional stability, low toxicity, and environmental safety, their proportion in the industrial surfactant market, currently occupied by synthetic, mainly petrochemical surfactants, does not exceed 2%; the major factor limiting the wide use of biosurfactants is their production cost. The largest application field for biosurfactants is the oil industry, including microbial enhanced oil recovery (MEOR), improvement of technological characteristics of refinery products, and clean-up of oil-contaminations (Singh et al. 2007). Enhanced oil recovery methods utilizing microorganisms and/or their metabolites for the residual oil extraction were developed in late 1970s, when Rapp et al. (1977) reported 30% increase in oil recovery from sandy rocks using trehalolipids from *R. erythropolis* (former *Nocardia rhodochrous*). However, since then, a constantly increasing global oil production has been associated with the growing environmental contamination by crude oil and refinery products.

6.1 Environmental Applications

In natural conditions, oil penetration through soil is an extremely complex process involving physical, chemical, and biological factors. Crude oil is a highly

hydrophobic material with most of its components having low water solubility. These components bind to soil particles and become nonbioavailable to microorganisms. To increase the bioavailability of hydrocarbon pollutants, surfactants may be used, allowing desorption and solubilization of petroleum hydrocarbons and thus facilitating their assimilation by microbial cells (for recent review, see Paria 2008). There are two mechanisms of surfactant-enhanced soil washing. One occurs below the CMC value, when surfactant monomers increase the contact angle between the soil and hydrophobic contaminant, thereby promoting the separation of contaminant from soil particles and finally displacing oil from the soil (roll-up mechanism). The other mechanism, solubilization, occurs above the CMC, when contaminants are partitioned from the soil into the hydrophobic core of surfactant micelles (Deshpande et al. 1999). Micellar phase bioavailability of hydrophobic organics means that contaminants partitioned into the micellar phase are biodegradable without being transferred to the dissolved phase first (Deshpande et al. 1999). Solubilization using surfactants at concentrations above their CMC values is currently explored in soil remediation (Mulligan 2005; Paria 2008). However, the inhibition of contaminant biodegradation in soil systems at surfactant concentrations above CMC has been reported (Billingsley et al. 2002). Moreover, many commonly used synthetic surfactants derived from petrochemicals are toxic and poorly biodegradable; their application may lead to the accumulation of ecologically harmful compounds in soil (Mulligan 2005). In recent decades, many studies have shown a great potential of biosurfactants for desorption and solubilization of hydrophobic organic contaminants in soil, and their subsequent biodegradation (for reviews, see Christofi and Ivshina 2002; Ron and Rosenberg 2002; Makkar and Rockne 2003; Mulligan 2005; Singh et al. 2007). In the use of biosurfactants, there is less need for product purity, and benefits are likely from in-situ production by indigenous or introduced microbial populations (Christofi and Ivshina 2002).

Kanga et al. (1997) compared the effect of (bio)surfactants on solubilization of naphthalene and methyl substituted naphthalenes and found that the synthetic surfactant (Tween 80) has a lower solubilization potential and higher toxicity than the glycolipid biosurfactant from *Rhodococcus* sp. H13-A. This result was later confirmed by Page et al. (1999) showing increased biosurfactant-mediated solubilization of three- and four-ring polyaromatic constituents of crude oil. Greater hydrocarbon solubilization was suggested to attribute to the larger micellar volume of the glycolipid biosurfactant (Kanga et al. 1997). Biosurfactant produced by various *Rhodococcus* species were found to partition high amount (up to 99%) of crude oil from the sand slurries into the aqueous phase under shaking conditions (Ivshina et al. 1998). Moreover, oil-removal activity of the *R. ruber* IEGM 231 biosurfactant tested in model soil-packed columns heavily contaminated with crude oil was 1.9–2.3 times greater than that of a synthetic surfactant Tween 60 (Kuyukina et al. 2005). The biosurfactant produced by *R. ruber* grown on dodecane was most effective for the oil removal from contaminated soil in colder conditions (at 15°C), but at higher temperatures (22°C and 28°C) the biosurfactant from hexadecane-grown cells was most effective (Fig. 6). Importantly, the glycolipid

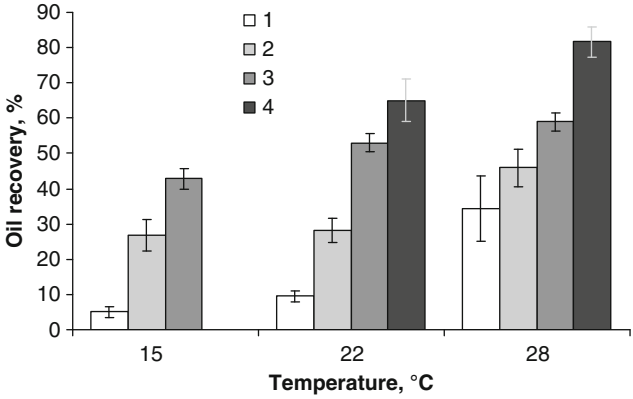

Fig. 6 (Bio)surfactant enhanced oil recovery from the model soil at different temperatures. Surfactants used: 1 – Water (control); 2 – Tween 60 (synthetic surfactant); 3 – *Rhodococcus* biosurfactant produced on *n*-dodecane; 4 – *Rhodococcus*-biosurfactant produced on *n*-hexadecane (from Kuyukina et al. 2005)

Table 4 The toxicity of biosurfactants compared to synthetic surfactants

Surfactant	IC_{50} of *Vibrio fischeri* (mg l^{-1})
Glycolipid complex from *R. ruber* IEGM 235	650
Trehalose dicorynomycolate from *R. erythropolis*	49
Trehalose tetraester from *R. erythropolis*	286
Rhamnolipids from *P. aeruginosa*	50
Nonylphenol-(ethylenoxide)$_9$-acetate (EQ 9)	78
Sucrose stearate (DK 50)	67
Finasol OSR-5	7
Corexit 9597	5
Inipol EAP 22	0.4

The toxicity results were calculated as inhibition concentration leading to a 50% light decrease measured in Microtox toxicity test. From Ivshina et al. (1998)

complex of *R. ruber* was less toxic than the purified biosurfactants of other bacterial strains, synthetic surfactants, and bioremediation formulations, such as Inipol EAP 22 (oleophilic fertilizer that is a microemulsion of urea and synthetic surfactants used to treat oil-contaminated soil) (Table 4). However, despite of the promising laboratory results, only limited studies have been performed so far on application of microbially produced biosurfactants in soil remediation processes (Christofi and Ivshina 2002).

Microbial surfactants were also used to remove heavy metals from soil (Mulligan 2005), although this potential for *Rhodococcus* biosurfactants is still to be revealed.

6.2 Other Potential Applications

The emulsifying activity of *Rhodococcus* biosurfactants was discussed earlier in this chapter. Considering a high physicochemical stability and a hydrophobic nature of the glycolipids produced by rhodococci, they can be effectively used as stabilizers of oleophilic emulsions and bases for creams, oily films, and pastes widely used in food and pharmaceutical industries, and cosmetics (Lang and Philp 1998). Additionally, several *Rhodococcus* (*R. globerulus*, *Rhodococcus* sp.) were shown to possess deemulsification properties (Singh et al. 2007), which can be effective in breaking both oil-in-water and water-in-oil emulsions, for example, formed during oil recovery and processing (Kosaric 1992). Kurane et al. (1995) isolated from the *R. erythropolis* S-1 culture and purified trehalo- and glucolipids with high flocculating activity; the authors suggested their possible application as bioflocculants widely used in many industrial processes, including wastewater treatment, downstream processing, food and fermentation technologies (Singh et al. 2007). However, rhodococci along with other mycolata group actinobacteria are reported as foaming-causative agents owing to their filamentous growth and biosurfactant production (Lang and Philp 1998). It should be noted that microbial foaming is among the most frequent and widespread problems associated with activated sludge wastewater treatment plants (Stainsby et al. 2005).

Utilization of biosurfactants in the production of new materials with improved physicochemical characteristics is another possible application area, at present covered by chemical surfactants. In our experiments, a glycolipid biosurfactant from *R. ruber* changed significantly thermal and rheological properties of the poly (vinyl alcohol) (PVA) cryogel, when added to the composite mixture prior to cryotropic gelation (Kuyukina et al. 2006). Particularly, biosurfactant addition distinctly increased the gel strength, elasticity, and plasticity (Table 5). Thus, instantaneous shear modulus (G_0) values of PVA cryogel increased from 3.1–4.7 to 5.6–6.0 kPa, and dynamic shear modulus (G_{30}) measured after 30-min exposure increased from 2.3–3.2 to 4.1–4.7 kPa, depending on biosurfactant concentration. In addition, gel fusion temperatures (T_f) did not change even in the presence of the highest (15%, v/v) biosurfactant concentration. Similar tests with synthetic surfactants, for example, sodium dodecylsulphate (SDS) and cetyl trimethylammonium

Table 5 Effect of the biosurfactant from *R. ruber* IEGM 231 on rheological properties of the PVA cryogel

PVA-cryogel sample	T_f (°C)	G_0 (kPa)	G_{30} (kPa)
Control PVA-cryogel (no additions)	69.5 ± 0.3	4.65 ± 0.28	3.21 ± 0.31
PVA cryogel + 5% (v/v) of water	68.6 ± 0.2	3.93 ± 0.72	3.19 ± 0.50
PVA cryogel + 5% (v/v) of biosurfactant	69.5 ± 0.1	6.04 ± 0.70	4.65 ± 0.27
PVA cryogel + 10% (v/v) of water	68.3 ± 0.2	3.72 ± 0.28	2.69 ± 0.10
PVA cryogel + 10% (v/v) of biosurfactant	69.6 ± 0.1	5.63 ± 0.60	4.14 ± 0.30
PVA cryogel + 15% (v/v) of water	67.0 ± 0.1	3.07 ± 0.21	2.30 ± 0.21
PVA cryogel + 15% (v/v) of biosurfactant	69.7 ± 0.1	5.63 ± 0.69	4.39 ± 0.33

From Kuyukina et al. (2006)

bromide (CTAB), showed weakening effects of surfactant additives on PVA cryogels (data not shown). It was also shown that incorporation of oleophilic polypropylene material Drizit into PVA cryogel weakened the mechanical strength of the resulting gel (Cunningham et al. 2004). Presumably, strengthening effect of biosurfactant additives could be used in polymeric gel formulations designed for various industrial and biotechnological applications.

Singh et al. (2007) reviewed biotechnological applications of surfactants, including bioprocessing and biocatalysis, which are determined by the surfactant effect on cellular membranes. Particularly, by interaction with cell membranes, surfactants can promote increased production of extracellular products during the fermentation and recovery of intracellular products due to cell lysis. In addition, they can facilitate the oxygen and mass transfer through the membrane and modulate the membrane-associated enzyme activity, specificity, and stability. Although all these applications have so far been confined to chemical surfactants, a growing volume of research on microbial surfactants, for example the above-mentioned studies of *Rhodococcus* trehalolipid interactions with phospholipid membranes (Aranda et al. 2007; Ortiz et al. 2008, 2009), would lead to a rapid biosurfactant introduction into this biotechnology market.

As mentioned earlier in this chapter, glycolipid surfactants produced by *Rhodococcus* and related bacteria exhibit biological activities toward various organisms ranging from prokaryotes to mammals, and thus have been investigated for their possible biomedical applications (Kitamoto et al. 2002). A challenge for the future in this field would be the identification of glycolipids with high biological (e.g., immunomodulating, antitumor) activity, but less toxic than the trehalose dimycolate from *M. tuberculosis* (Ryll et al. 2001). This could be achieved by introduction of nontoxic trehalolipids produced by nonpathogenic *Rhodococcus* species (Sakaguchi et al. 2000; Kuyukina et al. 2007). Although many fundamental issues of the trehalolipid influence on a human immune system and their interactions with immune cells and signal molecules still need more detailed investigations, it is reasonable to assume that these rhodococcal products will be used in clinical applications in the near future.

7 Conclusion

Actinobacteria of the genus *Rhodococcus* and closely related genera synthesize glycolipid (mostly trehalolipid) biosurfactants with diverse physicochemical properties and biological activities comparable with other microbial surfactants, which determine their potential applications in environmental and industrial biotechnologies. The only limitation for successful biosurfactant penetration in the biotechnology market is their high production cost, including bacterial fermentation and product recovery expenditures. Biosurfactant production from renewable resources is an attractive option currently unexploited for *Rhodococcus* surfactants. However, several recent studies showed that rhodococci are able to grow on vegetable oils

forming stable emulsions owing to intensive surfactant production (Haba et al. 2000; Sadouk et al. 2008). It should be noted that development of a cost-effective biotechnology for *Rhodococcus* biosurfactant production requires further fundamental research on the metabolic pathways involved in the glycolipid synthesis from different hydrophobic substrates and on the regulation mechanisms allowing a surfactant overproduction. This research would involve molecular biology tools currently available for *Rhodococcus* and developed on the base of complete genome sequences determined for several rhodococcal species (see chapter, "Genomes and Plasmids in *Rhodococcus*" by Larkin et al.). Another research area includes the modeling of cell growth conditions (e.g., mineral and organic medium components, temperature, pH and other physical factors, and chemical inducers) to obtain biosurfactants with target functional properties. Thus, in our experiments, a higher biosurfactant yield was recorded for *R. ruber* cells grown on hexadecane, although greater emulsification activity was registered for the biosurfactant produced using dodecane as a growth substrate (Kuyukina et al. 2005). In summary, development of cheaper fermentation processes for *Rhodococcus* biosurfactants using low-cost renewable raw materials and increased product yields through superactive, for example, genetically engineered strains would make them economically attractive and highly competitive with synthetic surfactants.

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Phytopathogenic Strategies of *Rhodococcus fascians*

Elisabeth Stes, Marcelle Holsters, and Danny Vereecke

Contents

Abstract *Rhodococcus fascians* is the only described phytopathogen in the genus and it induces a constellation of shooty symptoms on a very large range of plants. The main virulence factors, cytokinins, and functions that mediate more subtle aspects of the interaction with the host are encoded by a linear plasmid that shares a common origin with other rhodococcal linear replicons. The chromosome also plays an important role in the lifestyle of the bacterium. Chromosomal pathways are involved in producing auxin, delivering substrates for cytokinin production, and metabolizing nutrients retrieved from infected plants. The colonization of the plant tissues together with the secretion of various morphogens leads to a cascade of molecular responses that ultimately result in the establishment of disease symptoms, known as leafy galls.

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1 History of the *Rhodococcus fascians* Research

In 1927, in some greenhouses of New Jersey, Nellie Brown observed the formation of short, fleshy stems with misshapen and aborted leaves on sweet pea plants, a feature designated fasciation. The malformations were reminiscent of crown galls caused by *Agrobacterium tumefaciens*, but attempts to isolate the causative agent were unsuccessful (Brown 1927). Almost 10 years later, the fasciation-inducing organism, a bacterium, could finally be isolated and cultured (Tilford 1936; Lacey 1936a). The bacterium that was Gram-positive, rod-shaped, aerobic, without flagella, nonmotile, nonsporulating, and forming orange colonies was named *Phytomonas fascians* (Tilford 1936). For many years the taxonomic position of the bacterium was a matter of debate. After being renamed *Bacterium fascian*s (Lacey 1939), based on the characteristic post-division grouping and the occurrence of round intracellular bodies, the bacterium was classified as *Corynebacterium fascians* in the genus *Corynebacterium* of the order of the Actinomycetales (Dowson 1942). Discussions reinitiated when research showed the distinct nature and nonrelatedness of the bacterium within the corynebacteria (Starr et al. 1975; Goodfellow and Alderson 1977). Finally, supported by phenotypic, biochemical, and genetic data, the phytopathogen was repositioned within the Actinomycetales in the genus *Rhodococcus* and named *Rhodococcus fascians* (Goodfellow 1984).

After its discovery in fasciated sweet peas, increasingly more plant species were found to be susceptible to *R. fascians* infection, including monocotyledonous and dicotyledonous plants (Putnam and Miller 2007; Depuydt et al. 2008a), although disease occurrence proved to be limited by strain specificity and host susceptibility (Jacobs and Mohanty 1951). Histological studies of infected sweet pea plants revealed that the bacteria predominantly occurred in large colonies on the aerial plant surfaces and, to a lesser extent, penetrated the epidermal cells and invaded the intercellular spaces (Lacey 1936b). Once inside the plant tissues, the bacteria would lose their cell wall and convert into uncultivable L-forms (Lacey 1961).

Since the beginning, imbalances in plant hormone levels had been speculated to be involved in the *R. fascians*-induced shooty outgrowths. Cytokinins could partially reproduce symptoms on sweet pea (Roussaux 1966) and *R. fascians* was found to secrete bioactive cytokinins (Klämbt et al. 1966; Thimann and Sachs 1966; Rathbone and Hall 1972; Scarbrough et al. 1973; Armstrong et al. 1976; Murai et al. 1980). Moreover, some *R. fascians* strains were able to degrade auxins (Lacey 1948; Kemp 1978). Therefore, *R. fascians* has been postulated to redirect the hormone balance in the plant toward a high cytokinin/auxin ratio, with shoot formation as a consequence.

Here, we give an overview of the current knowledge of the different aspects of the *R. fascians* pathology that are schematically represented in Fig. 1.

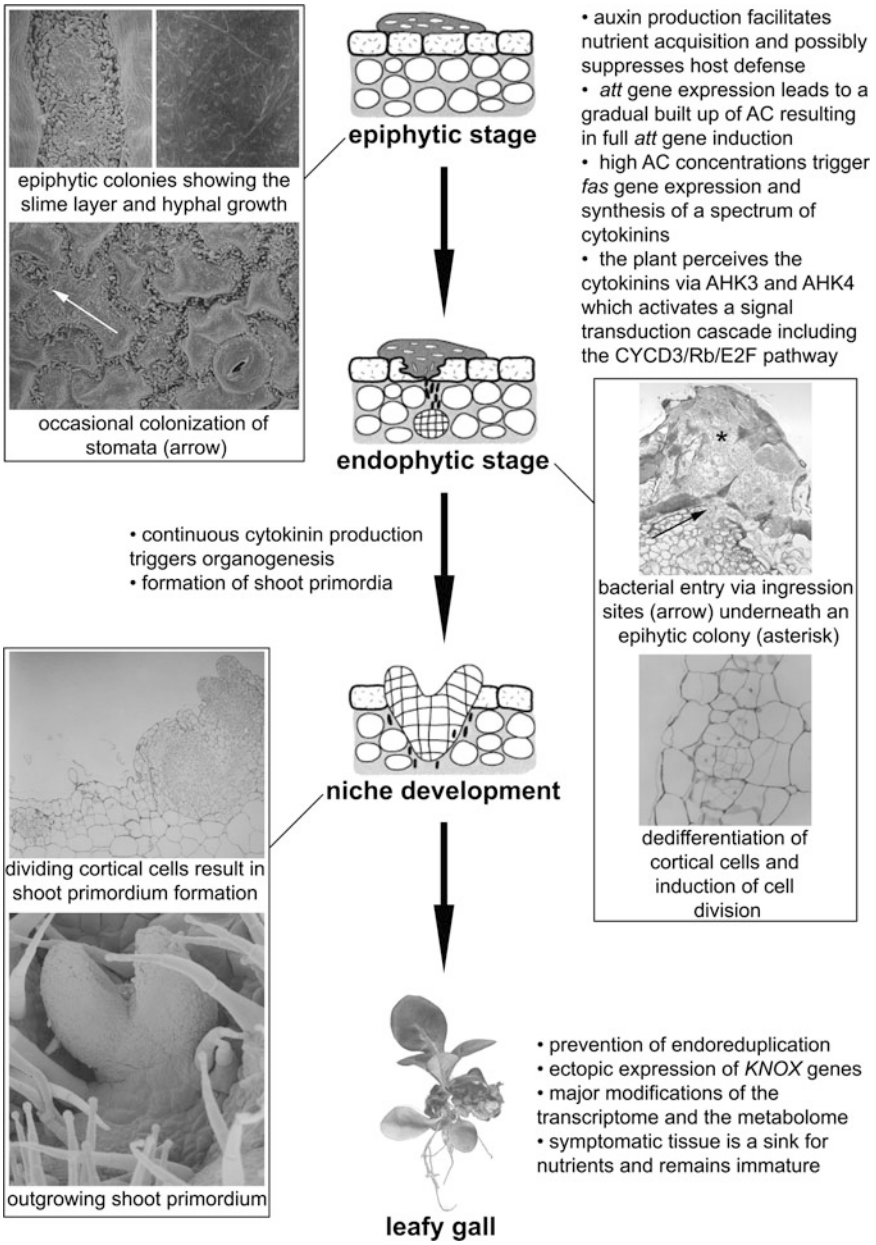

Fig. 1 Overview of the different aspects of the *R. fascians*–plant interaction. AC, autoregulatory

2 *Rhodococcus fascians* Infections Are a Threat for the Ornamentals Industry

Infection by *R. fascians* provokes an array of symptoms. Level and types of induced malformations depend on plant species or cultivar, plant age, bacterial strain, inoculation method, and growth conditions of both interacting partners (Vereecke et al. 2000). Misshapen leaves are small and are characterized by wrinkled, irregular lamina with swollen petioles and veins that result from secondary growth of the vasculature (Vereecke et al. 2000; de O Manes et al. 2004) (Fig. 2a). Witches' brooms consist of fleshy stems with deformed leaves growing from cotyledonary and axillary buds (Roussaux 1965). Leafy galls are differentiated galls that develop in the leaf axils, at the crown (Fig. 2b, c, f), or ectopically on the leaves from tissue flanking the vasculature. Typically, a leafy gall is a mass of meristematic tissue bearing many buds and small shoots that are inhibited in further outgrowth, and arise from the activation of existing meristems and the formation of new meristems from dedifferentiated cortical cells (de O Manes et al. 2001). Finally, shoot proliferations are loose collections of shoots of intermediary height (Putnam and Miller 2006). Although wounding is not required, more severe symptoms are established

Fig. 2 Symptoms on ornamental crops and tobacco grown in the field. (**a**) Control (*right*) and infected *Veronica* (*left*) plant showing fasciated stems and fleshy stems with small leaves; (**b**) Infected Hosta plant exhibiting multiple pale shoots at the crown; (**c**) Leafy gall at the base of a *Leucanthemum* plant; (**d**) and (**e**) Stunted growth of infected tobacco plants; (**f**) Stunted tobacco plant carrying galls with multiple pale shoots at the crown

when injured plants are inoculated (Lacey 1939). Deformations are always restricted to the inoculation site and both development and maintenance of the symptoms depend on the presence of metabolically active bacteria (Vereecke et al. 2000).

R. fascians infections seem to occur worldwide, but are observed especially in temperate regions (Depuydt et al. 2008b). Currently, 164 plant species belonging to 43 different plant families, comprising herbaceous and woody plants, have been registered as susceptible (Depuydt et al. 2008b). *R. fascians* is a biotrophic pathogen and infections do not shorten the lifespan of the host plants. Instead, infected plants have been reported to outlive uninfected controls (Vereecke et al. 2000). As such, *R. fascians* is seemingly not deleterious for the plant and, therefore, this interaction should be considered as mildly pathogenic. In contrast, in the ornamentals industry where profit is based on plant esthetics, *R. fascians* causes considerable financial losses. Infected plants develop leafy galls, exhibit stunted growth (Fig. 2a, d, e), produce fewer flowers, and sometimes display inhibited root growth. Incited malformations of herbaceous perennials can lead to the destruction of entire crops, and nurseries can suffer losses up to US$ 1 million per year. Crops can be contaminated either through infested soil, water and seeds, by vegetative propagation, or through transmission by insect vectors (Putnam and Miller 2007). An important reason for the large losses is that the leafy galls are often seen as shoot proliferations resulting from growth hormone treatments during vegetative propagation and consequently, are recognized too late as disease symptoms by the nursery grower. Moreover, the confusion on the identity of the agent provoking the shooty hyperplasia is still topical, because, in many cases, *R. fascians*-induced symptoms on ornamental crops in nurseries are mistakenly attributed to *A. tumefaciens*, viruses, eriophyid mites, or exposure to herbicides (Lacey 1939; Putnam and Miller 2006, 2007).

As no efficient eradication method exists growers are left with good sanitation practices to prevent the introduction of the disease in their nurseries (Kruyer and Boontjes 1982; Baker 1950; Depuydt et al. 2008b). Disease diagnosis is based on isolation and molecular or biochemical identification of *R. fascians* from symptomatic plants in combination with virulence assays of the isolates on the host of isolation and on indicator host species, such as *Nicotiana* spp. or *Arabidopsis thaliana* (Bradbury 1986; Gális et al. 2005; Goodfellow 1984; Hu et al. 1992; Stange et al. 1996).

3 A Linear Plasmid Carries Essential Virulence Functions

While searching for the genes that encoded the cytokinin production, a conjugative *f*asciation-*i*nducing linear plasmid pFiD188 was discovered in the *R. fascians* strain D188 that was essential for virulence (Crespi et al. 1992; Temmerman 2000). Similar to the linear plasmids of other actinomycetes, pFiD188 is of the invertron type with terminal inverted repeats and terminal proteins covalently linked to their

5′ ends (Chater and Kinashi 2007; Francis 2009). A linear plasmid-free derivative of strain D188 – strain D188-5 – is avirulent but not compromised in its growth; therefore, pFiD188 does not code for factors implicated in bacterial viability (Crespi et al. 1992). In planta, however, D188-5 can induce only small ingressions and the endophytic population is low. Hence, the linear plasmid is not only involved in pathogenicity sensu strictu, but also in penetration efficiency and endophytic colonization capacity (Cornelis et al. 2001).

Sequencing of the approximately 210-kb replicon revealed striking homologies with the linear plasmids pBD2 and pREL1 of *Rhodococcus erythropolis* strains BD2 and PR4, respectively, and pRHL2 of *Rhodococcus jostii* RHA1, indicative of a common origin. The *Rhodococcus* linear plasmids share four conserved regions, R1, R2, R3, and R6, that are mainly implicated in plasmid maintenance (Francis et al. 2007; Fig. 3). The conserved region R1 harbors genes involved in plasmid replication, natural transformation, and partitioning. In region R3, a locus with homology to the tight adherence locus (*tad*) of different organisms has been identified (Tomich et al. 2007). Tad proteins are necessary for the assemblage of adhesive pili, and their importance in the *R. fascians* pathology might lie in biofilm formation during colonization of the plant surface. Region R2 mostly consists of conserved hypothetical proteins, some of which presumably play a role in conjugation by forming a membrane-associated conjugation complex (Francis et al. 2007). Region R6 only encodes conserved hypothetical proteins, so its role in the linear plasmid biology is currently unknown (Francis et al. 2007).

Besides the four conserved regions, the linear plasmid pFiD188 harbors three unique regions U1, U2, and U3 (Fig. 3) that encode *R. fascians*-specific features. Functional analysis of some of the genes in the unique regions U2 and U3 have revealed that they are not involved in the pathology and probably play a role in the ecology of the interaction with the host (Francis 2009). Unique region U1, on the other hand, encodes the main virulence factors of *R. fascians*.

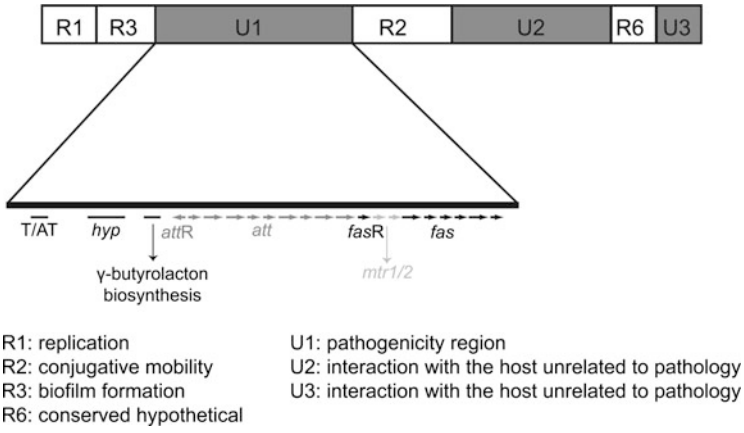

Fig. 3 Schematic representation and (putative) functions of the different regions of pFiD188

4 Unique Region U1 of pFiD188 Is the Pathogenicity Region

Region U1 is the largest of the three unique regions on pFiD188 and it contains at least three pathogenicity loci: *hyp*, *att*, and *fas* (Crespi et al. 1992). The observation that mutants in the *hyp*ervirulent (*hyp*) locus induce enhanced symptoms on plants (Crespi et al. 1992) suggests that the Hyp proteins negatively control the virulence signals or virulence gene expression. The locus encompasses three open reading frames (ORFs), of which two are hypothetical (ORF1 and ORF2) and one, ORF3, is homologous to RNA helicases and possibly controls virulence gene transcription, mRNA stability, or translation initiation (Goethals et al. 2001; Pertry 2009) (Fig. 3).

Mutants in the *att*enuated (*att*) locus cause smaller and loosely organized leafy galls on tobacco (Crespi et al. 1992). The locus carries eight genes (*att*A-*att*H) that are homologous to genes involved in the biosynthesis of arginine and β-lactam-containing carbapenem and clavulanic acid, a ninth gene (*att*X) that is probably implicated in the secretion of the produced compound, and a tenth regulatory gene (*att*R) that belongs to the LysR family of transcriptional regulators (Maes et al. 2001) (Fig. 3). Arginine is a precursor of clavulanic acid, suggesting that the Att proteins represent the biosynthetic pathway of a β-lactam-like compound. Despite the homologies, no antibiotic activity could be demonstrated for *R. fascians* cells or culture supernatants against specific test species. In contrast, the *att* locus produces an autoregulatory compound that is essential for activating virulence gene expression and that is secreted into the surroundings. Although the nature of the *att* compound remains to be identified, histidine as a functional analog implies some degree of structural resemblance (Maes et al. 2001).

Another locus, putatively involved in the biosynthesis of a γ-butyrolacton-like autoregulatory factor, is positioned between the *att* and the *hyp* loci (Fig. 3). Typically, these compounds are known as quorum sensing molecules of diverse Gram-positive bacteria (Nishida et al. 2007). Therefore, this locus might trigger virulence gene expression in a cell density-dependent manner.

Region U1 also contains a putative toxin–antitoxin system (Fig. 3). In the closely related *Mycobacterium tuberculosis*, such systems induce a state of metabolic dormancy during disadvantageous growth conditions in the host (Zhu et al. 2006). By analogy, this locus might provide a mechanism for survival of the bacteria inside the plant tissues (Francis 2009). Nonetheless, the most important pathogenicity locus in region U1 is the *fas*ciation (*fas*) locus.

5 The *fas* Locus of Region U1 Encodes the Production of Cytokinins, the Main Virulence Factors of *R. fascians*

Mutations in the *fas* locus provoke a complete loss of or a strongly reduced virulence (Crespi et al. 1992, 1994; Temmerman et al. 2000). The locus carries nine genes in three transcriptional units (Fig. 3): the first unit consists of the *fas*R

gene that codes for a transcriptional regulator of the AraC family; the second unit contains two genes that are homologous to *S*-adenosyl-methionine (SAM)-dependent methyltransferases (*mtr*1 and *mtr*2); the third unit comprises the six genes of the *fas* operon (*fas*A-*fas*F) that encodes the cytokinin biosynthetic machinery (Crespi et al. 1992; 1994; Temmerman et al. 2000; Pertry et al. 2010). Interestingly, the G+C content of all the genes of the *fas* locus is much lower than that of the genome (LeChevalier 1986; Pertry 2009), implying that the *fas* locus might have been acquired via horizontal gene transfer as a pathogenicity island. In support of this hypothesis, a *fas*-like operon, which has also a G+C content deviating from that of the genome, has been described in *Streptomyces turgidiscabies*, a recently evolved scab-causing Gram-positive pathogen that can induce leafy galls on different plant species (Joshi and Loria 2007).

Cytokinin profiling of supernatants of *R. fascians* strains D188 and D188-5 has revealed that basic levels of five cytokinin bases (isopentenyladenine [iP], 2-methylthio-isopentenyladenine [2MeSiP], *cis*-zeatin [cZ], 2-methylthio-*cis*-zeatin [2MeScZ], and *trans*-zeatin [tZ]) are produced by a chromosomal pathway (likely tRNA degradation). The Fas proteins are responsible for the enhanced production of all, except 2MeSiP, and the additional secretion of 2-methylthio-*trans*-zeatin (2MeStZ) (Pertry et al. 2010). Although these molecules individually affect the plant development, the combined secretion of this cytokinin mix has a strong synergistic effect (Pertry et al. 2009).

The enzyme central to the *fas*-encoded cytokinin biosynthesis pathway, an isopentenyltransferase (Ipt), is encoded by the *fas*D gene, and mutations in this gene lead to a complete loss of pathogenicity (Crespi et al. 1992). Biochemical characterization of the purified FasD protein showed that the preferred side chain acceptor was adenosine diphosphate (ADP), although adenosine monophosphate (AMP) could be used as well, and that both dimethylallyldiphosphate (DMAPP) and 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (HMBPP) could serve as side chain donors, indicating that FasD can produce both iP and tZ (Pertry et al. 2010). Equally important for pathogenicity is the *fas*A gene that is homologous to P450 cytochrome monooxygenase, implying that the hydroxylated cytokinins are essential for the induction of disease (Crespi et al. 1994; Pertry et al. 2010). The *fas*B gene product shows homology to two different proteins: the amino-terminus is similar to ferredoxins and the carboxy-terminus to the α-subunit of pyruvate dehydrogenase and FasC is homologous to the β-subunit of pyruvate dehydrogenase. Most probably, the FasB–FasC system generates the reducing power and electron transport system necessary for the functioning of the FasA protein (Crespi et al. 1994; Goethals et al. 2001). Mutations in *fas*E that encodes a cytokinin dehydrogenase/oxidase involved in cytokinin breakdown lead to reduced symptoms, suggesting that a correct balance in the produced cytokinins is determinative for induction and maintenance of the disease (Pertry et al. 2010). Strains defective in *fas*F, which codes for a lysine decarboxylase/phosphoribohydrolase, are not impaired in symptom initiation, but are unable to maintain the deformations. This observation implies that this protein is probably involved in the production of an additional level of cytokinins (Pertry et al. 2010). Altogether, these data show that

the Fas proteins play a differential role in virulence. The function of the SAM-dependent methyltransferases Mtr1 and Mtr2 remains to be determined, but their location in the *fas* locus would hint at their involvement in cytokinin biosynthesis (Pertry et al. 2009).

Determination of the cytokinin profiles of these *fas* mutants allowed us to propose the biosynthetic pathway encoded by the *fas* operon: FasD synthesizes iP (and possibly tZ), which serves as a precursor for the other cytokinins. FasA hydroxylates iP to tZ and cZ, as well as chromosomally produced 2MeSiP to 2MeScZ and probably 2MeStZ. FasF represents a complementary route for the production of tZ, cZ, and their 2MeS-derivatives. The exact role of *fas*E in the establishment of the cytokinin spectrum remains to be unraveled (Pertry et al. 2010).

6 Virulence Gene Expression Is Subjected to a Complex Regulation

The pathogenic interaction starts with the expression of the *att* genes encoding an autoregulatory compound required for induced virulence gene expression. The transcription of the *att* genes is activated by AttR, a LysR-type transcriptional regulator, while the coinducer needed for induced expression is provided by the Att proteins themselves or can be replaced by a combination of histidine and succinate/pyruvate (Maes et al. 2001). In planta, the *att* genes are mainly expressed in the epiphytic population at the early stages of the infection, while upon penetration of the plant tissues, *att* expression is shut down (Cornelis et al. 2002) (Fig. 1). Although the initial factors that control *att* expression in planta are unknown, recent data have shown that upon infection, the primary metabolism of the plant is modified in such a manner that the succinate and pyruvate levels increase, which would favor *att* gene expression (Depuydt et al. 2009b). Moreover, the initiation of the autoregulation system is probably stimulated by the aggregation of the bacteria in microcolonies that resemble biofilms at specific sites on the plant (Cornelis et al. 2002) (Fig. 1). Based on in vitro experimental data, a low basal expression of the *att* genes allows the gradual build-up of the autoregulatory compound until a threshold level is reached. The accumulating autoregulatory compound then activates the AttR protein, leading to a positive feed-forward loop that fully induces *att* gene transcription. The resulting very high levels of the autoregulatory compound would be necessary to trigger *fas* gene expression (Maes et al. 2001).

The ex planta transcription of the *fas* operon is independent of the growth conditions, but strongly upregulated at the translational level in response to specific triggers. Several transcriptional and translational regulators are implicated in *fas* gene expression, allowing the integration of environmental cues (pH, carbon and nitrogen sources, phosphate and oxygen content, and cell density) and the *att* autoregulatory compound (Temmerman et al. 2000; Maes et al. 2001). The AraC-type transcriptional regulator FasR is essential for *fas* gene expression and,

consequently, for virulence. FasR activates *fas* transcription, but predominantly controls *fas* induction at a posttranscriptional level, suggesting an indirect mechanism probably involving a translational regulator (Temmerman et al. 2000). Besides FasR, recent data point to the implication of another pFiD188-encoded transcriptional regulator in *fas* gene regulation, likely AttR (Temmerman et al. 2000; Pertry 2009). Previously the *hyp* locus was thought to encode the translational regulator (Temmerman 2000), but recently at least *fas*A induction has been shown to be independent of a functional *hyp* locus (Pertry 2009). Because FasR activates the expression of the locus encoding a γ-butyrolacton-like autoregulator (our unpublished data), it is tempting to speculate that this locus controls *fas* mRNA translation. Furthermore, translation of the *fas* mRNA strictly depends on the *att* autoregulatory component via an unknown mechanism (Maes et al. 2001). In planta, *fas* gene expression occurs both in the epiphytic and the endophytic populations and is maintained throughout the interaction (Cornelis et al. 2001), indicating that an *att*-independent control mechanism is active at the later time points of the interaction (Fig. 1).

7 The Chromosome Plays a Role in the Interaction

R. fascians is a well adapted epiphyte that forms large colonies on the leaf surface (Fig. 1). These epiphytic colonies are responsible for the onset of symptom development, while symptom maintenance is sustained by the endophytic population (Vereecke et al. 2002a). Although the linear plasmid-free strain D188-5 is unable to cause symptoms, it can very efficiently colonize the exterior of the plant (Cornelis et al. 2001). Therefore, chromosomally encoded proteins seem implicated in the epiphytic fitness of the bacteria and can thus influence pathogenicity. Such determinants are, for instance, the production of orange pigments that enhance UV tolerance (Sundin and Jacobs 1999) and the secretion of a colony-embedding slime layer (Fig. 1), which might protect the bacteria from desiccation and enhance attachment to the plant surface (Cornelis et al. 2001).

Upon penetration of the plant tissues, the bacteria encounter very different physiological conditions that require adjustment of their metabolism. A chromosomal gene, *vic*A, encoding a malate synthase involved in the glyoxylate shunt of the Krebs cycle, is imperative for this adaptation to the endophytic lifestyle because mutations in it lead to strongly reduced virulence and decreased endophytic colonization. Analysis with reporter gene fusions revealed that *vic*A expression is controlled by pFiD188 (Vereecke et al. 2002a). The glyoxylate cycle bypasses the steps in the citric acid cycle in which carbon is lost in the form of CO_2 and allows the use of two-carbon compounds as sole carbon source, suggesting that the bacteria are provided with such compounds in planta. Interestingly, the glyoxylate shunt seems to play a central role in persistent in-host survival of diverse pathogens and symbionts (Lorenz and Fink 2002; Garcia de los Santos et al. 2002; Vereecke et al. 2002b). The strategy to create a specific niche with customized nutritional

conditions gives a selective advantage over other plant-associated bacteria and is designated metabolic habitat modification (Vereecke et al. 2002a).

Besides being important for general fitness, the chromosome also plays a role in the production of morphogens. As discussed above, although the virulence-related levels of cytokinins depend on pFiD188, basic levels of five cytokinin bases originate from a chromosomal pathway (Pertry et al. 2009). Moreover, 2MeSiP, which is not produced by the Fas machinery, potentially serves as a substrate for FasA to produce 2MeSZ (Pertry et al. 2010). *R. fascians* not only produces cytokinins, but also synthesizes the auxin indole-3-acetic acid (IAA) via the indole-3-pyruvic acid pathway that is encoded by a still unidentified locus on the chromosome. However, monitoring of the kinetics of IAA production in strains D188 and D188-5 revealed that pFiD188-encoded determinants control the expression of the chromosomal pathway (Vandeputte et al. 2005). In symptomatic plant tissues, high levels of auxins are measured (Vereecke et al. 2000) and many aspects of the symptomatology are reminiscent of typical auxin effects (such as vascular overgrowth, increased lateral rooting, and inhibition of shoot outgrowth); nevertheless, until now, it is unclear whether this IAA is of bacterial or plant origin. Augmenting auxin levels or auxin signaling is a common strategy of plant-associated bacteria to enhance the exudation of nutrients, to increase surface colonization by stimulating tissue proliferation, and to suppress the plant's defense (Robert-Seilaniantz et al. 2007). Based on the biphasic kinetics of the IAA secretion by strain D188, a dual role for auxin in the interaction is proposed: the basal production increases the epiphytic fitness during the initial colonization and, later on, the stimulated IAA production is coresponsible for the induced morphological alterations (Vandeputte et al. 2005).

8 *Rhodococcus fascians* Infection Has a Strong Molecular Impact on the Host Plant

At the early stages of the interaction, *R. fascians* colonizes the plant epiphytically without causing disease but with a profound effect on the plant's primary metabolism. Of all amino acids, the accumulation of tryptophan is the strongest in the infected tissues, possibly feeding into the auxin biosynthetic pathway of the bacteria (Depuydt et al. 2009b). The resulting increased bacterial auxin production may aid the epiphytic colonization by stimulating nutrient release and preventing defense responses by the plant (Depuydt et al. 2009b). Moreover, pyruvate and succinate levels rise which might trigger *att* induction, resulting in the onset of virulence gene expression (Depuydt et al. 2009b).

As soon as the concentration of the autoregulatory compound is sufficiently high, the *fas*-encoded machinery is switched on and the array of cytokinins is produced. *Arabidopsis thaliana* perceives these morphogens via the cytokinin receptors AHK3 and AHK4. The latter is considered a root-specific receptor, but

infection with *R. fascians* leads to its ectopic expression in the aerial parts, thus increasing the sensitivity of the plant toward the bacterial signals (Pertry et al. 2009). Upon perception, a signaling cascade is activated that will ultimately result in the initiation of symptom development (Depuydt et al. 2009b). In an attempt to restore the cytokinin homeostasis, the cytokinin biosynthesis *IPT* genes of the plant are downregulated and the cytokinin-degrading *CKX* genes are upregulated (Depuydt et al. 2008a). However, within the bacterial cytokinin mix, cZ and 2MeScZ are resistant to degradation by the plant CKX proteins and, therefore, these molecules accumulate in the infected tissues (Pertry et al. 2009).

The locally high cytokinin concentrations stimulate the expression of the D-type cyclin genes in the plant which promotes B-type cyclin gene expression that together mediate a fast transition through G1-to-S phases and G2-to-M phases, stimulating proliferative cell divisions and preventing differentiation (Depuydt et al. 2009a). As a consequence, the symptomatic tissue never reaches maturity and remains a sink enriched in sugars – a physiological state that is favorable for the bacteria that can feed on the wealth of nutrients in these young tissues (Vereecke et al. 2002b; Depuydt et al. 2009a, b). The meristematic identity of the symptomatic tissue is maintained by the overexpression of *KNOX* genes (Depuydt et al. 2008a).

Although the cytokinins of *R. fascians* are recognized as the main effectors in the symptomatology, only low levels of cytokinins are measured in infected tissues (Depuydt et al. 2008a; Pertry et al. 2009). Possibly, to attain the very strong impact on plant development, the cytokinin response might be amplified by secondary signals from the plant. Indeed, polyamines and the disaccharide trehalose that are plant growth regulators have been found to accumulate upon infection (Depuydt et al. 2009b).

9 Concluding Remarks

Many aspects of the molecular basis of the pathology inflicted by the broad host range phytopathogen *R. fascians* have been elucidated. It is now clear that the interaction with the plant requires functions that are encoded by the linear plasmid as well as by the chromosome, and that the interplay between both replicons assists in a full expansion of the phytopathological potential. Moreover, the host seems also to contribute to the level of symptom development and provides the bacteria with a specific niche. Nevertheless, various issues remain to be resolved, such as the gene regulatory processes, the structures of autoregulatory compounds, the auxin biosynthetic pathway and its role in the interaction, some of which are the subject of ongoing research efforts.

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Rhodococcus equi and Its Pathogenic Mechanisms

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Abstract *Rhodococcus equi* is the only animal pathogen among the rhodococci. A soil inhabitant, *R. equi* is prevalent in the farm environment where it uses herbivore manure as growth substrate. In addition to its saprophytic lifestyle, *R. equi* has the ability to colonize animal host tissues, causing pyogranulomatous infections in a variety of mammals. Although clearly a multihost pathogen, *R. equi* is best known as the etiologic agent of a severe contagious bronchopneumonic disease in horses. It also causes deadly opportunistic infections in immunosuppressed individuals, particularly tuberculosis-like cavitary pneumonia in HIV-infected patients. *R. equi* is an intracellular parasite that replicates within a modified phagocytic vacuole in macrophages. Its pathogenicity depends on a horizontally-acquired genomic island carried on a virulence plasmid. This plasmid virulence locus encodes a family of surface-associated antigens, the Vap proteins. The products of the *vap* island confer the ability to survive within macrophages and recent evidence suggests they are also involved in infectious tropism towards specific animal host species (horses, pigs, and cattle). The complete DNA sequence of the *R. equi* genome has been recently determined and a detailed analysis will soon be published. This chapter reviews the current, "pregenomic" state of knowledge about the biology and virulence of this fascinating pathogenic actinomycete.

1 Introduction

The genus *Rhodococcus* is a taxon of the *Actinomycetales*, within the *Actinobacteria*, that comprises >40 species of organisms ubiquitously distributed in the environment, in particular in soil. Rhodococci belong to the mycolata group, which includes among others the genera *Mycobacterium*, *Nocardia,* and *Corynebacterium*, (Gurtler et al. 2004). Many *Rhodococcus* spp. are biotechnologically important owing to their outstanding metabolic versatility. They are useful in a number of applications such as biotransformation of hydrophobic compounds and xenobiotics, including chlorinated hydrocarbons and phenolics, fossil fuel desulfurization, and the industrial production of acrylates/acrylamides and bioactive steroids (Larkin et al. 2005; van der Geize and Dijkhuizen 2004). The environmental and biotechnological aspects of the rhodococci are extensively covered in this monograph and we refer the reader interested in these to the appropriate sections. The genus *Rhodococcus* also includes two species with parasitic lifestyles, the

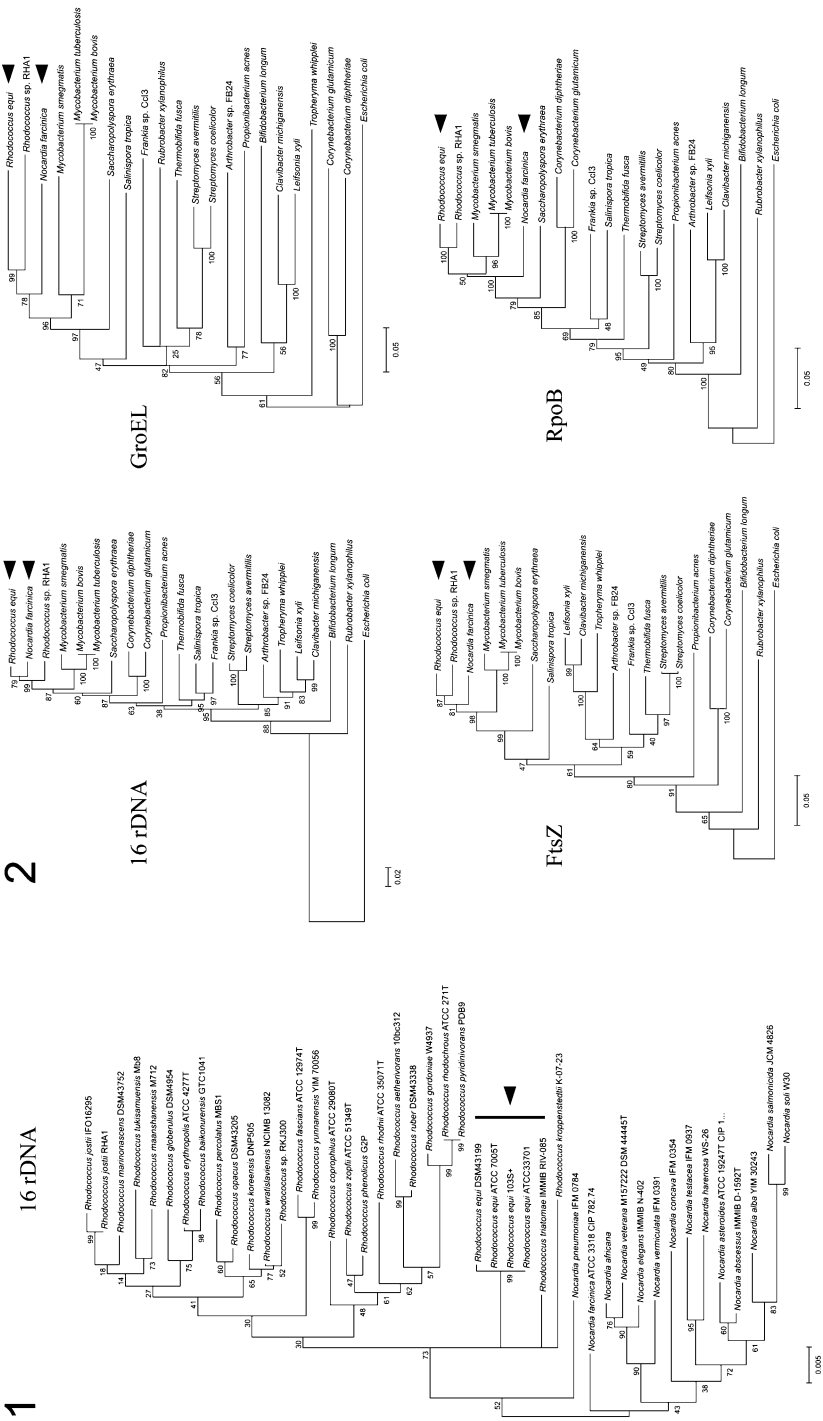
phytopathogen *Rhodococcus fascians* (Goethals et al. 2001), and the animal pathogen *Rhodococcus equi*, dealt with in this chapter.

2 *R. equi*: A Multihost Animal Pathogen

R. equi was first described by Magnusson in Sweden as the causal agent of a specific type of infectious pneumonia affecting foals (Magnusson 1923). Although best known in his facet as equine pathogen, *R. equi* can also cause purulent pulmonary and extrapulmonary infections in a variety of other mammalian species.

2.1 Taxonomic Position and Population Genetics

A strictly aerobic, nonmotile, Gram-positive coccobacillus, *R. equi* cells alternate between coccoid and rod-shaped phases. The latter adopt the typical "club" morphology of the coryneforms, on the basis of which the organism was initially classified as *Corynebacterium equi* (Magnusson 1938). Its current name came with the creation of the genus *Rhodococcus* (Goodfellow and Alderson 1977), in which *R. equi* occupies a peripheral position close to *Nocardia* according to 16S rDNA sequencing studies (Fig. 1). These studies have sometimes offered equivocal results, raising doubts as to whether *R. equi* was more closely related to the genus *Nocardia* or to members of the genus *Rhodococcus*, or even if it merited recognition as a separate genus (Goodfellow et al. 1998). 16S rDNA phylogenies, which rely on the assumption that the sequence divergence is representative of the genetic distance between two organisms, are not infallible and in some circumstances may not reflect the actual relationships – particularly when dealing with closely related species. This is possibly the case with *R. equi* and *Nocardia*. Whereas phylogenies based on 16S rDNA sequences are unstable, those derived from other molecular clocks consistently place *R. equi* among the rhodococci (Fig. 2). Whole genome comparative analyses with other rhodococci, *Nocardia farcinica*, and related actinomycetes fully support the inclusion of *R. equi* within the genus *Rhodococcus* (Letek et al. 2008a). It has also been claimed that *R. equi* is genetically diverse and heterogeneous (Cohen et al. 2003; Gurtler et al. 2004; Morton et al. 2001). However, data from our laboratory, based on the analysis by pulsed-field gel electrophoresis (PFGE) of a global collection of isolates from diverse geographical origins (25 countries) and sources (horse, human, pig, cattle, small ruminants, dog, cat, soil, ancillary) indicated that the *R. equi* taxon is in fact quite homogeneous. The existence of substantial genetic variability was confirmed, but traces of the same genomic backbone were very evident among *R. equi* isolates (Vazquez-Boland et al. 2008) (Fig. 2). Only a few, rare strains did not show the conserved signature pattern, but these were morphologically very different in culture and were clearly wrongly identified as *R. equi* by the source laboratories.


1
16 rDNA
Rhodococcus jostii IFO16295
Rhodococcus jostii RHA1
Rhodococcus marinonascens DSM43752
Rhodococcus tukisamuensis Mb8
Rhodococcus maanshanensis M712
Rhodococcus globerulus DSM4954
Rhodococcus erythropolis ATCC 4277T
Rhodococcus baikonurensis GTC1041
Rhodococcus percolatus MBS1
Rhodococcus opacus DSM43205
Rhodococcus koreensis DNP505
Rhodococcus wratislaviensis NCIMB 13082
Rhodococcus sp. RK300
Rhodococcus fascians ATCC 12974T
Rhodococcus yunnanensis YIM 70056
Rhodococcus coprophilus ATCC 29807
Rhodococcus equi ATCC 51134T
Rhodococcus phenolicus G2P
Rhodococcus rhodnii ATCC 35071T
Rhodococcus aetherivorans 10bc312
Rhodococcus ruber DSM43338
Rhodococcus gordoniae W4937
Rhodococcus rhodochrous ATCC 21T
Rhodococcus pyridinivorans PDB9
Rhodococcus equi DSM43199
Rhodococcus equi ATCC 7005T
Rhodococcus equi 103S+
Rhodococcus equi ATCC33701
Rhodococcus triatomae IMMIB RIV-085
Rhodococcus kroppenstedtii K-07-23
Nocardia pneumoniae IFM 10784
Nocardia farcinica ATCC 3318 CIP 782 74
Nocardia aficana
Nocardia veterana M157222 DSM 44445T
Nocardia elegans IMMIB N-402
Nocardia vermiculata IFM 0391
Nocardia concava IFM 0354
Nocardia testacea IFM 0937
Nocardia harenosa WS-26
Nocardia asteroides ATCC 19247T CIP 1...
Nocardia abscessius IMMIB D-15927
Nocardia alba YIM 30243
Nocardia salmonicida JCM 4825
Nocardia soli W30
0.005
2
16 rDNA
Rhodococcus equi
Rhodococcus sp. RHA1
Nocardia farcinica
Mycobacterium smegmatis
Mycobacterium bovis
Mycobacterium tuberculosis
Saccharopolyspora erythraea
Corynebacterium diphtheriae
Corynebacterium glutamicum
Propionibacterium acnes
Thermobifida fusca
Salinispora tropica
Frankia sp. Ccl3
Streptomyces coelicolor
Streptomyces avermitilis
Arthrobacter sp. FB24
Tropherma whipplei
Leifsonia xyli
Clavibacter michiganensis
Bifidobacterium longum
Rubrobacter xylanophilus
Escherichia coli
0.02
FtsZ
Rhodococcus equi
Rhodococcus sp. RHA1
Nocardia farcinica
Mycobacterium smegmatis
Mycobacterium bovis
Mycobacterium tuberculosis
Saccharopolyspora erythraea
Salinispora tropica
Leifsonia xyli
Clavibacter michiganensis
Tropherma whipplei
Arthrobacter sp. FB24
Frankia sp. Ccl3
Thermobifida fusca
Streptomyces avermitilis
Streptomyces coelicolor
Propionibacterium acnes
Corynebacterium diphtheriae
Corynebacterium glutamicum
Bifidobacterium longum
Rubrobacter xylanophilus
Escherichia coli
0.05
GroEL
Rhodococcus equi
Rhodococcus sp. RHA1
Nocardia farcinica
Mycobacterium smegmatis
Mycobacterium tuberculosis
Mycobacterium bovis
Saccharopolyspora erythraea
Salinispora tropica
Frankia sp. Ccl3
Rubrobacter xylanophilus
Thermobifida fusca
Streptomyces avermitilis
Streptomyces coelicolor
Arthrobacter sp. FB24
Propionibacterium acnes
Bifidobacterium longum
Clavibacter michiganensis
Leifsonia xyli
Tropherma whipplei
Corynebacterium glutamicum
Corynebacterium diphtheriae
Escherichia coli
0.05
RpoB
Rhodococcus equi
Rhodococcus sp. RHA1
Mycobacterium smegmatis
Mycobacterium tuberculosis
Mycobacterium bovis
Nocardia farcinica
Saccharopolyspora erythraea
Corynebacterium diphtheriae
Corynebacterium glutamicum
Frankia sp. Ccl3
Salinispora tropica
Thermobifida fusca
Streptomyces avermitilis
Streptomyces coelicolor
Propionibacterium acnes
Arthrobacter sp. FB24
Leifsonia xyli
Clavibacter michiganensis
Bifidobacterium longum
Rubrobacter xylanophilus
Escherichia coli
0.05

2.2 R. equi *Infection in Horses*

R. equi is one of the most important primary infectious agents of the horse and as such it deservedly ranks among the major bacterial veterinary pathogens. Primarily a soil dwelling saprotroph, *R. equi*, is able to colonize the lungs of young foals, causing life-threatening pyogranulomatous bronchopneumonia accompanied by gross lesions such as macroabscesses and cavitation (Fig. 3). Secondary enterocolotis

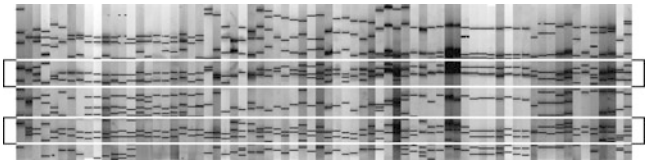


Fig. 2 PFGE genotyping (*Ase*I profiles) of *R. equi* isolates from a global strain collection from different sources and geographical origins. While there is substantial genetic diversity among *R. equi* isolates, traces of a same genomic backbone are clearly visible (examples of conserved band patterns are *boxed*). Generated with BioNumerics software v. 5.10 (Applied Maths, Sint-Martens-Latem, Belgium)

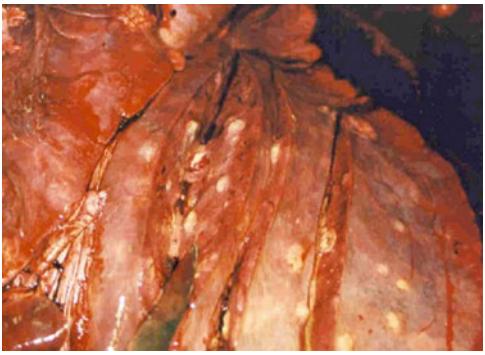


Fig. 3 Purulent bronchopneumonia due to *R. equi* in a foal (multifocal pulmonary absceses)

Fig. 1 Phylogeny and taxonomy of *R. equi*. Neighbor-joining unrooted phylogenetic trees constructed from ClustalW alignments, branchings are supported by the indicated bootstrap values (10,000 replicates). (1) Phylogeny of the *Rhodococcus* and *Nocardia* genera based on 16 rDNA sequence analysis. (2) Phylogenetic relationships of a selection of sequenced actinobacterial species based on 16 rDNA sequences and three other molecular clocks (protein sequences). Homologous *Escherichia coli* sequences were used as outgroups. Note that with the restricted population of 16 rDNA sequences used here, the position of *R. equi* flips and is clustered together with *N. farcinica*. *R. equi* is however grouped with *R. jostii* RHA1 in the phylogenies based on GroEL, FtsZ, and RpoB amino acid sequences

and mesenteric lymphadenitis are other common manifestations and are seen in adult horses as well. Less frequent forms of presentation include synovitis, uveitis, osteomyelitis, and septic arthritis. The disease typically affects foals between 1 and 6 months of age, follows an insidious, subacute or chronic, course with sudden onset of overt symptoms, and is generally fatal if antibiotic treatment is not rapidly administered (Prescott 1991).

The organism often becomes endemic on stud farms, where it represents a real challenge as there is no effective vaccine available. This is aggravated by the lack of sensitive diagnostic techniques for identifying the early stages of the infection in foals and the extent of *R. equi* subclinical carriage and farm endemicity (Muscatello et al. 2007). Efforts are being devoted however to develop new rapid and sensitive quantitative molecular methods (Harrington et al. 2005; Rodriguez-Lazaro et al. 2006). Another problem is the intrinsic lack of susceptibility of the organism to a number of antibiotics (e.g., penicillins, cephalosporins, sulfamides, quinolones, tetracyclines, clindamycin, and chloramphenicol) (Jacks et al. 2003; Makrai et al. 2000; McNeil and Brown 1992; Nordmann and Ronco 1992; Soriano et al. 1995). This, and the intracellular localization of the pathogen (see below), complicates the therapeutic management of *R. equi*, making it necessary to administer a lengthy antibiotic treatment of up to 3 months or more, with no guaranteed success.

Due to its high case-fatality rate, the lack of effective preventative measures, and the costs of the antibiotic treatments (which are often administered prophylactically in endemic studs), *R. equi* has a major economic impact and is recognized among the four most important infectious problems that afflict the equine industry (Ainsworth et al. 1998; Muscatello et al. 2007).

2.3 Other R. equi *Hosts*

R. equi is frequently isolated from submaxillary ganglia of pigs, often associated with pyogranulomatous adenitis (Komijn et al. 2007; Makrai et al. 2005; Pate et al. 2004; Takai et al. 1996a). It is not uncommonly found either in tuberculosis (TB)-like caseating abscesses in retropharyngeal, bronchial, or mediastinal lymph nodes of cattle (Soedarmanto et al. 1997). Abbatoir surveys in Ireland determined that up to 4% of suspected bovine TB cases were in fact *R. equi* infections (Flynn et al 2001). With the emergence of the AIDS pandemia, *R. equi* gained prominence as a human opportunistic pathogen. Human *R. equi*, infections are generally associated with immunosuppressive conditions and are most often seen in HIV-infected individuals or organ transplant patients, although apparently healthy persons can also be affected (Harvey and Sunstrum 1991; Linares et al 1997). Human rhodococcal infection is associated with high mortality (50–55%) (Kedlaya et al 2001), even in immunocompetent people (Gabriels et al 2006). In HIV-infected patients, it usually presents as a TB-like purulent cavitary pneumonia (Mosser and Hondalus 1996; Takai et al 2003b; Weinstock and Brown 2002). The intrinsic antibiotic resistance of *R. equi* restricts the therapeutic choice to certain combinations of antibiotics; these

are not always effective and surgical resection of the affected tissues is sometimes required (Stiles et al 2002). *R. equi* infections have been reported in a variety of other animal species, including dogs and cats (Davis et al 1999; Hong and Donahue 1995; Takai et al 2003a), but such cases remain anecdotal.

The number of reports of nonequine cases of *R. equi* infections is on the rise, probably due to increased awareness about this pathogen and the application of improved laboratory detection techniques. However, *R. equi* is still frequently misidentified as a "diphteroid" (Tuon et al 2007) or mycobacteria (Macias et al 1997). A careful differential diagnosis must always be carried out in any suspected cases of mycobacterial infection to exclude *R. equi* (Doig et al. 1991).

3 Pathogenesis and Epidemiology

Like many other actinomycetales, *R. equi* is a ubiquitous soil inhabitant. It is also an intracellular pathogen with the capacity to survive and multiply within macrophages, thus belonging to the group of organisms called facultative intracellular parasites.

3.1 Ecology and Transmission

R. equi is particularly prevalent in the farm environment, which is abundant in herbivore feces, the natural growth substrate of the pathogen. Multiplication in manure-rich soil occurs when the ambient temperature rises above 10°C. Foals contract the infection by inhalation of aerosolized soil particles carrying virulent *R. equi* bacteria. This explains the epidemiological pattern of foal rhodococcal pneumonia, with cases being more frequent during dry, warm weather, on densely populated farms with dusty paddocks. Mesenteric lymphadenitis and typhlocolitis may result from ingestion of *R. equi*, and multiplication in the fecal content of the intestine contributes to its dissemination in the environment. The infection can also be transmitted by inhalation of the breath of sick animals (Muscatello et al. 2007; Takai 1997).

3.2 Pathobiology of R. equi *Infection*

Upon entry into a mammalian host, *R. equi* is entrapped by local macrophages, particularly alveolar macrophages in the lung. Central to *R. equi* pathogenesis is the ability of these bacteria to survive and multiply within macrophages (Hondalus and Mosser 1994). This causes macrophage cytotoxicity, ultimately resulting in their destruction by necrosis rather than apoptosis (Luhrmann et al. 2004). The infectivity of *R. equi* seems to be restricted to cells of the monocyte–macrophage

lineage. Uptake by macrophages in vitro is enhanced by complement and involves Mac-1, a type 3 complement receptor (CR3) (Hondalus et al. 1993). The *R. equi* cell envelope component lipoarabinomannan (LAM) possesses as key structural features a linear α-1,6-mannan with side chains containing one 2-linked α-D-ManP residue and has been shown to bind mannose-binding protein (Garton et al. 2002). This may favor complement C3b fraction deposition on the bacterial surface and Mac-1-mediated uptake. Entry into macrophages may also proceed via the mannose receptor. These entry mechanisms bypass the opsonic receptor FcR-mediated pathway and thus may play a key role in promoting intracellular survival of the pathogen. Opsonic uptake mediated by specific antibodies normally results in macrophage activation, with release of proinflammatory mediators, increased phagosome–lysosome fusion, and enhanced *R. equi* intracellular killing by nitrogen and oxygen intermediates (Darrah et al. 2000).

A key intracellular survival mechanism of *R. equi* involves the active manipulation of the host cell endosomal pathway. As with *Mycobacterium tuberculosis* (*Mtb*), replication of the pathogen occurs inside a modified membrane-bound vacuole – dubbed the *R. equi*-containing vacuole (RCV). The RCV seems to transit normally through the early stages of phagosome maturation, with acquisition of typical early endocytic markers (early endosome antigen 1, transferring receptor, Rab5, coronin, and PI3P). At later stages however, although displaying some late endosome markers (LAMPs and Rab7), the mature RCVs lack the proton-pumping vacuolar ATPase, do not acidify (their pH remains at about 6.5), and the soluble lysosomal markers β-galactosidase and cathepsin D are absent. This suggests that *R. equi* establishes and maintains a safe intracellular replication compartment through prevention of vacuole acidification via exclusion of the proton-pumping v-ATPase and subsequent avoidance of RCV–lysosome fusion. The molecular mechanisms involved remain unknown, but formation of the RCV requires viable bacteria and the *R. equi* virulence plasmid (Fernandez-Mora et al. 2005). An unidentified soluble component(s) of the supernatant of virulent (plasmid-positive) *R. equi* apparently attenuates RCV acidification (Toyooka et al. 2005). Cytotoxicity for J774 murine macrophages was also shown to be strongly upregulated in plasmid-bearing *R. equi* bacteria (Luhrmann et al. 2004).

A major breakthrough in our current understanding of *R. equi* pathogenesis was the discovery that virulent *R. equi* bacteria from foals release 15–17 kDa surface antigens, called virulence-associated proteins (Vap), into the culture supernatant (Takai et al. 1991a). High levels of antibodies against the Vaps are detected in the sera of infected animals (Takai et al. 1996b), indicating that these proteins are strongly immunogenic and expressed in vivo during infection. Importantly, the occurrence of these antigens in the culture supernatant of *R. equi* was found to be associated with the presence of an 85–90 kb plasmid in the corresponding isolates (Takai et al. 1991b). Loss of the plasmid results in the inability to cause disease in foals and to replicate in macrophages in vitro and in mouse tissues in vivo, indicating it is essential for the virulence of *R. equi* (Giguere et al. 1999; Hondalus and Mosser 1994). The plasmid-encoded surface lipoprotein, VapA, appears to be a key mediator of these effects (see below).

3.3 Molecular Epidemiology

Clinical isolates from foals typically harbor 85–90 kb virulence plasmids associated with the *vapA* gene encoding VapA. At least 12 variants of this *vapA* plasmid have been identified based on restriction fragment length polymorphisms, and this is used in the molecular epidemiological analysis of *R. equi* (Ribeiro et al. 2005; Takai et al. 1999). Interestingly, whereas equine strains are almost invariably associated with *vapA* virulence plasmids, isolates from other animal species – particularly human and porcine isolates – are either reported to be plasmidless, or do have plasmids more variable in size (79–100 kb) associated with a variant Vap antigen/allele, designated VapB/*vapB* (Makrai et al. 2002, 2005; Takai et al. 1995, 2003b). The VapB antigen is immunologically related to VapA but has a slightly larger molecular mass (20 kDa). *R. equi* strains with *vapB* plasmids appear to be less virulent in experimentally-infected foals, but are capable of killing mice at doses comparable to *vapA* isolates (10^6–10^7 CFU, in contrast to $>10^8$ for plasmid-cured, avirulent strains) (Takai et al. 1991b, 1995, 2000a). Taking an "equinocentric" view, the *vapB* strains were considered to be "intermediately virulent" as opposed to the "virulent" (horse-pathogenic) isolates carrying *vapA* plasmids. Except soil isolates from horse-breeding farms, in which *vapA* plasmids are common, environmental isolates of *R. equi* do not usually possess plasmids, or carry smaller cryptic plasmids that do not express Vap proteins (Takai 1997).

While the distinction between *vapA* and *vapB* plasmid types is relatively easy using a simple PCR assay (Oldfield et al. 2004), the unambiguous discrimination between presence and absence of a plasmid in *R. equi* isolates poses more difficulties. Until recently this relied exclusively on plasmid DNA extraction and visualization, which is notoriously difficult in *R. equi* and involves methods that are cumbersome, time-consuming, and in some cases unreliable unless very carefully standardized and optimized. It was therefore unclear whether "plasmidless" isolates from nonequine pathological specimens were in all cases truly devoid of virulence plasmids. To address this question, our laboratory developed a universal virulence plasmid marker based on the *traA* gene, from the conserved conjugal transfer machinery, which facilitates the rapid and accurate discrimination between plasmid-positive and plasmid-negative *R. equi* by PCR (Ocampo-Sosa et al. 2007) (Fig. 4). This marker was incorporated into a novel multiplex PCR assay called TRAVAP, that also discriminates between *vapA* and *vapB* plasmid genotypes and classifies *R. equi* isolates into four major categories: $traA^+/vapA^+B^-$, $traA^+/vapA^-B^+$, $traA^+/vapAB^-$, and $traA^-/vapAB^-$ (plasmidless). The application of the TRAVAP typing scheme to a collection of field isolates of *R. equi* led to an interesting set of observations:

First, it demonstrated clear associations between specific plasmid types and specific animal hosts. Thus, $traA^+/vapA^+B^-$ plasmids were associated with equine isolates, confirming the strong connection between the *vapA* gene and horse infectivity (see above); the $traA^+/vapA^-B^+$ type was associated with swine isolates, consistent with previous observations on the frequent occurrence of *vapB* plasmids

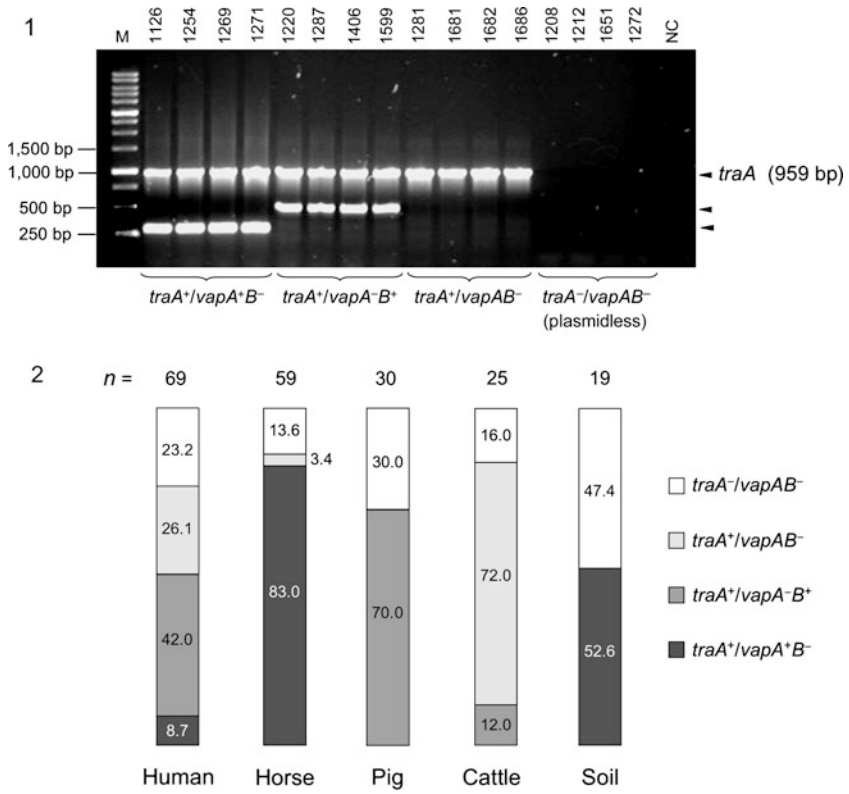


Fig. 4 *R. equi* virulence plasmid typing with TRAVAP, a multiplex PCR assay that targets the universal plasmid marker gene *traA* and the *vapA* and *vapB* allelic variants. (1) Ethidium bromide-stained egarose gel showing the band pattern of TRAVAP multiplex PCR. M: 100 bp DNA ladder (reference sizes indicated on the *left*); NC: negative control (PCR mix without DNA). (2) Classification of field isolates of *R. equi* in virulence plasmid pathotypes using the TRAVAP typing scheme. The number of isolates within each group is indicated *above the bars*; the percentages of the different plasmid categories are indicated *inside the bars*. Differences in plasmid category distribution between groups are statistically significant ($P < 0.001$). Note the high proportion (≈50%) of plasmidless *R. equi* among soil isolates. Soil isolates were from equine farm environments, as reflects the presence of a horse-pathogenic plasmid type (*traA*$^+$/*vapA*$^+$*B*$^-$) in the plasmid-positive isolates. Reproduced from Ocampo-Sosa et al. (2007) with permission

among *R. equi* strains from pigs (Makrai et al. 2005; Takai et al. 1996a); and *traA*$^+$/*vapAB*$^-$, defining a novel *R. equi* virulence plasmid type, was associated with bovine isolates (Fig. 4). Interestingly, bovine isolates were initially thought to be devoid of plasmids based on the lack of detection of *vapA*/*B* genes or cognate antigens (Flynn et al. 2001).

Whereas most isolates from pathological specimens contained a plasmid, nearly half of the soil isolates were plasmidless according to the *traA* marker (Fig. 4), consistent with the lack of importance of the virulence plasmid outside a host system. A greater percentage of porcine isolates lacked plasmids (30.0% vs. 13.6% in equine

isolates and 16.0% in bovine isolates) possibly reflecting their isolation from apparently healthy carriers (specimens collected randomly in abattoir surveys from pig tonsils showing no macroscopic lesions), in which the selective pressures for virulence plasmid maintenance are obviously weaker (Ocampo-Sosa et al. 2007).

Importantly, among plasmid-positive (*traA*$^+$) strains, no *vapB*$^+$ (porcine type) marker was found in equine isolates, no *vapA*$^+$ (equine type) marker was found in bovine isolates, and no *vapA*$^+$ (horse) or *vapAB*$^-$ (bovine type) plasmids were identified in porcine isolates. This suggests the interesting notion that the species-specific virulence plasmid types of *R. equi* are subject to strong selection determined by the host animal species. Given the strongly immunogenic character of the plasmid-encoded Vap proteins (Kohler et al. 2003), it is possible that this selection is driven by host species-specific aspects of the immune response.

The *vapA*$^+$ and *vapB*$^+$ markers were never associated in the same isolate, indicating that these genes (and plasmid types, by extension) do not occur simultaneously. This mutual exclusivity suggests that *vapA* and *vapB* are allelic variants of a same locus that divergently evolved in two different plasmid subpopulations.

Finally, all animal species-specific plasmid categories were commonly found in human isolates (frequencies: 8.7%, 42%, and 26% respectively, the rest being plasmidless) (Fig. 4). This possibly is a reflection of the predominantly opportunistic nature of *R. equi* infection in the human host. Importantly, it also points to the existence of a transmission flux between animals and people, revealing a new facet of *R. equi* as zoonotic agent (Ocampo-Sosa et al. 2007).

4 Molecular Determinants of Virulence

Given the conspicuous association between the plasmid-encoded VapA protein and horse pathogenicity, it is not surprising that most research efforts were focused on this major in vivo-expressed antigen. Bacterial virulence is however a multifactorial trait and multiple plasmid and chromosomal determinants, for the most part yet to be identified and/or characterized, are likely to contribute to *R. equi* pathogenesis.

4.1 Plasmid Virulence Genes: The vap *Pathogenicity Island*

Two virulence plasmids from different foal isolates, 103 and ATCC 3370, were sequenced and found to be identical (Takai et al. 2000b). This horse-associated extrachromosomal element was recently named pVAPA1037 using a new standardized nomenclature for the designation of *R. equi* virulence plasmids, and fully reannotated (Letek et al. 2008b). pVAPA1037 has a size of 80.6 kb and contains 73 coding sequences (CDS), including eight pseudogenes (Fig. 5). The *vapA* gene

encodes a 17.4-kDa secreted lipoprotein and lies in a horizontally acquired pathogenicity island (PAI) of 21.3-kb (not 27.5 kb as originally reported by Takai et al. (2000, b)) that contains additional *vap* gene homologs. Of the nine *vap* genes present in pVAPA1037, five (*vapA*, *-C*, *-D*, *-E*, *-G*, and *-H*) encode full-length Vap proteins while the rest are degenerate: *vapF* has a 5′ truncation and two frameshift mutations at the 3′ end, resulting in a Vap protein lacking the secretion signal and with a 39-residue nonconserved C terminus; *vapI* is truncated at both ends and encodes an 80-residue Vap polypeptide with no predicted signal sequence and lacking most of the conserved C-terminal domain; and *vapX* is a highly corrupted *vap* pseudogene that encodes a 28-residue peptide from the central region of the conserved Vap C-terminal domain (Fig. 6) (Letek et al. 2008b). Such

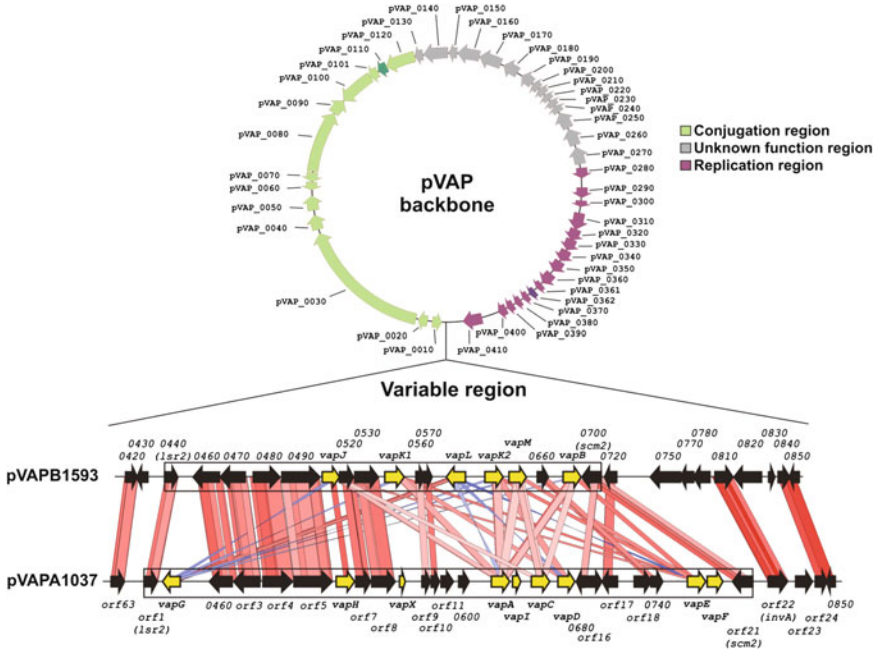


Fig. 5 Genetic structure of the circular virulence plasmids of *R. equi* and comparison of *vapA* and *vapB* type PAIs. The common backbone of the *R. equi* virulence plasmids pVAPA1037 and pVAPB1593 is represented as a circular diagram: conjugation region is in *green* (in *dark green* the *traA* gene targeted by the TRAVAP PCR; see Fig. 4), unknown function region in *gray* and replication region in *purple*. Comparison of the PAI carried out with Artemis Comparison Tool (ACT) (Carver et al. 2005); virulence-associated *vap* genes are in *yellow*, other genes in *black*. Regions with significant similarity (tBLASTx) are connected by *colored lines* (*red*, sequences in direct orientation; *blue*, sequences in reverse orientation; the color intensity indicates strength of sequence homology: *pink*/*light blue*, lowest; *red*/*deep blue*, highest). Gene designations for pVAPB1593 are according to standardized annotation nomenclature adopted for *R. equi* virulence plasmids (Letek et al. 2008b), those for pVAPA1037 are according to the nomenclature used by Takai et al. (2000, b) except for the newly identified genes, in which standardized nomenclature was used

accumulation of pseudogenes is unique in the virulence plasmid, suggesting that *vap* family genes are not under stringent selection and are relatively free to evolve.

Deletion mutants in the *vap* locus were shown to have reduced virulence in mice and to be unable to replicate in vivo and in murine bone marrow macrophages, to an extent similar to that observed for plasmid-cured bacteria. Complementation analysis indicated that the lack of *vapA* was responsible for the phenotype of the mutants, thus confirming the suspected major role of VapA in *R. equi* virulence (Jain et al. 2003). However, expression of *vapA* in a plasmid-cured strain of *R. equi* did not restore the capacity to proliferate in macrophages and to colonize the lungs of experimentally infected foals (Giguere et al. 1999), indicating that VapA alone is not sufficient for virulence and that other plasmid genes are required.

Besides the *vap* genes, the plasmid PAI contains other genes that may play a role in virulence. The recent sequencing of a *vapB* (porcine) type plasmid, pVAPB1593 from a human isolate, showed it had an identical housekeeping backbone to that of the *vapA* type plasmid but differed in *vap* PAI structure (Letek et al. 2008b). Computational analyses of base composition bias for prediction of horizontal

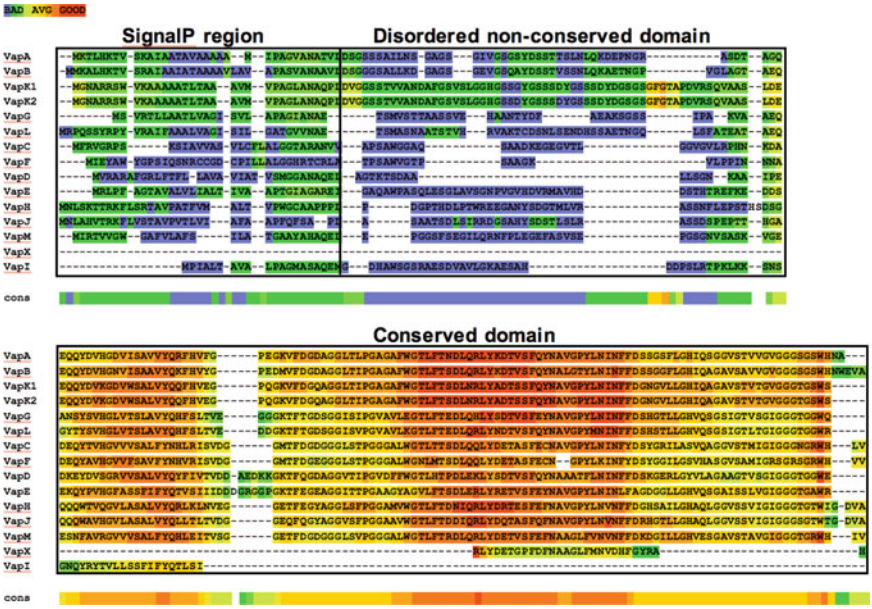


Fig. 6 Multiple alignment of the Vap protein family encoded in the *vapA* (horse-pathogenic)-type pVAPA1037 and the *vapB* (pig-associated)-type pVAPB1593 virulence plasmids (Letek et al. 2008b). Vap (lipo)proteins consist of an N-terminal signal sequence, a ≈50- to 60-residue nonconserved, structurally disordered region predicted to contain the lipidation site, and a conserved C-terminal region likely to form the functionally active site. Alignment constructed using T-coffee software v. 5.68 (Notredame et al. 2000). The *color scale* denotes the reliability of the alignment (*red*, highest likelihood; *blue*, less trustable). Reproduced from Letek et al. (2008b) with permission

gene transfer (HGT) DNA regions identified with precision the boundaries of the *vap* PAI in both plasmids. Despite the differences in genetic organization and structure, a number of non-*vap* genes are conserved between the *vapA* and v*apB* PAIs, suggesting they are important for virulence (Fig. 5). These include the 6.2-Kb five-cistron *vir* locus, which includes the only two regulatory genes found in the PAI, located at each end of the operon: *virR* (or *orf4*; normalized designation, pVAP_0480) encoding a LysR-type transcriptional regulator, and *orf4* (pVAP_0530) encoding an orphan two-component regulatory gene (see below). The *virR* operon also contains a *vap* gene (*vapH*/*J*) flanked by *orf5* (pVAP_0470), encoding a multi-facilitator-superfamily (MFS) membrane transporter of unknown substrate, and *orf7* (pVAP_0490) encoding a hypothetical protein (Fig. 5).

The conserved non-*vap* gene complement includes *orf3* (pVAP_470), encoding a SAM dependent methyltransferase that may also have a regulatory role, and at each end of the PAI, *lsr2* (*pVAP_0440,* or *orf1*) and *scm2* (pVAPA_0700/pVAPB_0800 or *orf21*) (Fig. 5). The former encodes a homolog of Lsr2 from *Mtb*, which has been involved in a number of functions, from protection against reactive oxygen intermediates to resistance to antibiotics and phage infections and modifications in the mycolic acid composition of the cell envelope (Arora et al. 2008; Chen et al. 2006; Colangeli et al. 2007, 2009). The mechanism of action of Lsr2 appears to be similar to that of the nucleoid-associated H-NS family proteins from Gram-negative bacteria, which modulate gene expression by binding to A+T-rich DNA regions (Chen et al. 2008). Lsr2 proteins may have evolved to fulfil a similar role to H-NS proteins in the high G+C content actinobacterial genomes. Interestingly, an H-NS-like protein has been shown to facilitate the conjugal transfer of plasmid pSf-R27 to new hosts with minimal costs to bacterial fitness (Doyle et al. 2007). It is tempting to speculate that Lsr2 from the pVAP plasmid plays an analogous role, facilitating the proper insertion of the plasmid (or PAI) genes in the regulatory network of *R. equi*, thus minimizing the impact of virulence plasmid acquisition and improving plasmid stability.

smc2 encodes an AroQ-type chorismate mutase (CM) that in contrast to the typical cytosolic CM enzymes possesses a secretion signal. CM (EC 5.4.99.5) mediates the interconversion of the shikimate pathway metabolite chorismate into prephenate, a key precursor in the synthesis of important molecules such as aromatic amino acids, folate, and siderophores. The presence of this secreted CM in the virulence plasmid *vap* PAI is intriguing. Although the existence of a periplasmic pathway for biosynthesis of phenylalanine from chorismate has been documented (Calhoun et al. 2001), mammals do not possess the shikimate pathway (Richards et al. 2006) and CM substrates (chorismate and prephenate) are unikely to be present in animal tissues, particularly in the macrophage vacuole. Interestingly, secreted CMs are present in other pathogens, e.g., *Mtb* and *Salmonella typhimurium* (Calhoun et al. 2001; Kim et al. 2006); in the latter the coding gene has been shown to be induced in vivo in infected mice, suggesting a role in virulence (Bumann 2002). These secreted enzymes may be important for scavenging shikimate metabolites that may leak from lysed *R. equi* bacteria (*R. equi* possesses a complete shikimate pathway) (Letek et al. 2008a) at the infection

point and which in the nutrient-deprived granulome/phagocytic vacuole environment could be rate-limiting for bacterial growth. Alternatively, the homology between the *smc2* product and the AroQ enzymes (ciclohexadienyl mutase) may simply reflect similarities in the catalytic mechanism of two unrelated enzymes with different substrates.

The rest of the non-*vap* genes present in the PAI encode products of unknown function (nine in pVAPA1037, five in pVAPB1593). Of these, *orf10* (pVAP_0570) is conserved in pathogenic mycobacteria and has been shown to be highly expressed in macrophages; however, its deletion did not affect the virulence of *R. equi* in mice (Ren and Prescott 2003, 2004), suggesting that it only has a secondary role, if any, in pathogenesis.

4.2 *Environmental Control of* vap *PAI Gene Expression*

The PAI genes are differentially expressed in cultured macrophages in comparison to in vitro growth conditions at 30°C in rich medium. Six *vap* genes, together with *orf9* and *orf10*, were found to be the most transcribed intracellularly in the pVAPA1037 PAI (Rahman et al. 2005; Ren and Prescott 2003). There is evidence that *R. equi* uses a variety of environmental cues to sense its entry into a mammalian host habitat – particularly that of the phagocytic vacuole – and transcriptionally activate the plasmid virulence genes. These signals include a change in temperature from 30 to 37°C, low pH (6.5), the presence of oxidizing agents, and variations in the concentration of divalent cations (iron, magnesium) (Benoit et al. 2001, 2002; Byrne et al. 2001; Jordan et al. 2003; Ren and Prescott 2003; Takai et al. 1992). The mechanisms underlying this regulation remain poorly understood.

The two regulatory genes in the PAI, *virR* and *orf8* (see above), appear to be involved since disruption of either gene in pVAPA1037 fully attenuated *R. equi* virulence (Ren and Prescott 2004). The synthesis of VapA from a complementation vector carrying the *vapA* gene in a plasmid-cured *R. equi* strain was also shown to require co-complementation with a second plasmid carrying the *virR* gene. In these experiments, complete restoration of wild-type VapA protein levels was only achieved in the presence of the full-length *vir* operon (Russell et al. 2004). Somewhat counterintuitively, however, a plasmid microarray study showed that the transcription of several *vap* genes, including *vapA*, was in fact enhanced in *virR* and *orf8* knock out mutants (Ren and Prescott 2004). The observed mutant attenuation despite the increased *vap* gene expression seems to indicate that *virR*- and *orf8*-mediated PAI gene regulation operates mainly through negative control. The lack of VapA protein synthesis in the absence of *virR* and *orf8* (Russell et al. 2004) when *vap* gene expression is upregulated (Ren and Prescott 2004) also suggests the involvement of posttranslational control mechanisms. Interestingly, the *virR* and *orf8* mutations affected similarly the expression of the same set of PAI genes (Ren and Prescott 2004), indicating either functional overlap of the two regulators or, more likely, that one controls the expression of the other. The *vir*

operon seems to be subject to a dual control, with the LysR-type VirR transcription factor negatively autoregulating its own expression, resulting in constitutively low levels of *virR* mRNA, while at the same time the downstream genes – *orf5*, *vapH*, *orf7,* and *orf8* – are induced from a second promoter in front of *orf5* (P*orf5*) in response to activating signals (high temperature and low pH) (Byrne et al. 2007). While Orf8 does not seem to control *virR* expression, it has been postulated that VirR regulates *orf8* transcription via P*orf5* (Byrne et al. 2007), but the evidence is weak and requires confirmation. It has also been suggested that the activity of the orphan two-component response regulator Orf8 is modulated by a hypothetical, chromosome-encoded sensor kinase, but no proof of this has been provided so far.

It remains therefore unclear how the upregulation of *orf8* and the other four *vir* genes is brought about, and also whether the activation of *orf8* is responsible for the observed upregulation of *vapA* and other PAI genes in response to virulence-activating conditions. Indeed, the currently available evidence is still too partial and fragmentary to build a coherent model of how *vap* PAI gene expression is controlled, and more systematic research is required to rigorously dissect the mechanisms involved in what appears to be a complex, multilayered regulation.

4.3 The vap *Multigene Family: A Role in Host Tropism?*

The available evidence indicates that the Vap proteins are important players in the immune response to *R. equi* (Kohler et al. 2003) and in key virulence traits associated with the plasmid, including the maintenance of the mildly acidic RCV (Fernandez-Mora et al. 2005). Their precise role in pathogenesis and mechanism of action remain however to be determined. Recent findings from our laboratory suggest a novel aspect of the contribution of Vap proteins to *R. equi* virulence, based on the observation that specific virulence plasmid types are associated with specific animal hosts (see Sect. 3.3). The detailed comparison of the horse type pVAPA1037 plasmid and the pig type *vapB* plasmid pVAPB1593 revealed that they only differ in PAI structure, particularly at the level of the *vap* genes (Fig. 5). The pVAPB1593 PAI harbored new members of the *vap* multigene family (besides *vapB*, *vapJ* to *–M*, with two copies of *vapK*), which encoded polypeptides that differed by 24–84% in amino acid sequence from the Vap proteins encoded in the *vapA* plasmid (Fig. 6). We have now sequenced a bovine (*vapAB*$^{-}$, or *vapN*)-type plasmid and it also carries a divergent copy of the PAI with novel *vap* multigene family members (Valero and Vazquez-Boland, unpublished data). These observations suggest the hypothesis that the different *vap* gene complements present in each of the host-associated plasmid types play an important role in the adaptation of *R. equi* to specific animal hosts. The Vap proteins have no homologs in other organisms and possibly account for much of the specificities of the parasitic lifestyle of *R. equi*.

4.4 Chromosomal Determinants

While most clinical isolates from foals harbor virulence plasmids, a significant fraction of human isolates apparently lack these (Makrai et al. 2002; Takai et al. 1994, 1995, 2003b). It can be argued that these investigations were carried out before the bovine virulence plasmid was identified and improved methods for pVAP plasmid detection via the *traA* maker were introduced, but a recent study incorporating these advances found that up to 23% of human isolates do not carry virulence plasmids (Ocampo-Sosa et al. 2007). In humans, the disease is clinically very similar to that seen in foals, with purulent cavitary pneumonia as prominent lesion, indicating that although plasmid determinants are necessary for the colonization of the equine host, chromosomal factors are also involved in *R. equi* pathogenesis.

Like all mycolate actinomycetes, *R. equi* possesses a lipid-rich cell wall. This feature has been described as an equivalent of the Gram-negative outer membrane and is thought to act as a permeability barrier (Hoffmann et al. 2008). The lipid cell envelope of *R. equi* contains mycolic acids composed of trehalose dimycolate and lipoarabinomannan, which are ester-linked to arabinogalactan polysaccharides, in turn bound to the peptidoglycan (Sutcliffe 1997). It appears that there is also an antigenically variable polysaccharide capsule, likely responsible for the >20 different capsular serotypes described in *R. equi* (Prescott 1991). In *Mtb* the mycolic-acid layer is believed to play a role in virulence by conferring protection against immune attack (Takayama et al. 2005). Mycolyl transferases are involved in the last steps of mycolic acid layer assembly and in *Mtb* also play a role as fibronectin-binding proteins. A *R. equi* mutant in a mycolyl transferase gene homologous to *Mtb* antigen 85 *fbpA* was constructed, but was found to be fully virulent in macrophages and mice (Sydor et al. 2008). A possible reason is that the genome of *R. equi* encodes other possible mycolyl transferases (Letek et al. unpublished data), and in *Mtb* only one of its three mycolyl transferases was found to be essential for intracellular survival (Takayama et al. 2005). The *R. equi* mycolyl transferase mutant showed a dry-colony phenotype, presumably due to loss of the capsule, suggesting that the extracellular polysaccharide material is directly or indirectly anchored to the mycolic acid layer (Sydor et al. 2008). An implication of these findings is that the *R. equi* exopolysaccharide capsule is not required for virulence.

The surface lipoglycan LAM, associated with both plasma and mycolic-acid membranes, may also play a role in *R. equi* virulence. *R. equi* LAM, ReqLAM, is smaller than mycobacterial LAM and lacks its extensive arabinan branching, but retains side-chains containing terminal mannose units. The mycobacterial mannose-capped LAM (ManLAM) is believed to be involved in the inhibition of phagosome maturation and apoptosis as well as in the modulation of the immune response (Briken et al. 2004; Welin et al. 2008). As discussed above, ReqLAM may

play a role in macrophage-nonactivating internalization of *R. equi* (see Sect. 3.2). Purified ReqLAM induced inflammatory and regulatory cytokine mRNA expression in equine macrophages in vitro in a manner similar to that induced by live virulent *R. equi*, suggesting it is involved in the macrophage cytokine response against the pathogen (Garton et al. 2002).

It has been long speculated that *R. equi* produces a difussible cytolytic factor involved in virulence. The activity of this "equi factor" is easily detectable on sheep blood agar in the form of a synergistic (CAMP-like) hemolysis reaction with sphingomyelinase-producing bacteria (e.g. *Staphylococcus aureus*, *B. cereus* or *Listeria ivanovii*) and is used as specific marker for the rapid presumptive identification of *R. equi* (Ladron et al. 2003; Navas et al. 2001; Prescott 1991). The cytolytic determinant involved, *choE* encoding a secreted cholesterol oxidase, was identified using a novel homologous recombination-based in-frame gene deletion strategy that has formed the basis for the targeted mutagenesis methods currently in use for *R. equi* (Navas et al. 2001). Despite ChoE being massively secreted by *R. equi* and highly immunogenic (Barbey et al. 2009), a *choE* mutation did not result in any apparent loss of virulence (Pei et al. 2006). However, the *R. equi* chromosome encodes three other cholesterol oxidases, two secreted (ChoA and ChoB) and one intracellular (ChoD) (Letek et al. unpublished data), and these may functionally compensate the loss of ChoE during infection. The *Mtb* ChoD homolog has been shown to be required for virulence (Brzostek et al. 2007), but it is unclear whether this reflects a role for the enzyme in membrane damage, interference with membrane-associated signaling, or cholesterol-derived lipid utilization as a carbon source.

Recently, an *mce* (*m*ammalian *c*ell *e*ntry) operon identified through partial genome sequencing of the strain ATCC 33701 (Rahman et al. 2003) has been shown to mediate cholesterol uptake (van der Geize et al. 2008). *mce* operons were discovered in *Mtb* and have been repeatedly shown to contribute to the virulence of the tubercle bacillus (Gioffre et al. 2005; Sassetti and Rubin 2003; Senaratne et al. 2008). Although recombinantly expressed Mce1A from *Mtb* promoted entry of latex beads into HeLa cells (Chitale et al. 2001), the mechanisms through which the *mce* loci are involved in virulence remained elusive. The findings with *R. equi mce2* confirmed previous observations with the homologous *mce4* locus from *Rhodococcus jostii* RHA1 (Mohn et al. 2008) and are consistent with the notion that *mce* clusters constitute a novel subfamily of ABC permeases specialized in lipid transport (Casali and Riley 2007). The *mce2* mutation did not affect *R. equi* survival in cultured murine macrophages (van der Geize et al. 2008), although this does not exclude a role for *mce*-mediated cholesterol utilization in vivo or in IFNγ-activated macrophages, as shown using an *Mtb* mutant in the homologous *mce* operon (Pandey and Sassetti 2008).

To date, the chromosomal determinant most clearly associated with *R. equi* virulence is *aceA* (*icl*) encoding the glyoxylate shunt enzyme, isocitrate lyase (Kelly et al. 2002). A *R. equi aceA* mutant was impaired in intracellular survival in mouse macrophages, was rapidly cleared from the organs of infected mice, and was unable to cause disease in experimentally infected foals (Wall et al. 2005).

The glyoxylate bypass plays a crucial role in diverting carbon skeletons from the citric acid cycle for biosynthetic purposes when bacteria are growing on lipids in the absence of carbohydrate or other carbon sources. Strong attenuation of virulence was previously observed with an *icl* mutant in *Mtb*, a pathogen that mainly feeds on host-derived lipids within granulomes (McKinney et al. 2000). Thus, as in the tubercle bacillus, lipids assimilated through β-oxidation and the glyoxylate bypass also appear to be a key in vivo source of carbon for *R. equi* during infection. The *R. equi aceA* gene is cotranscribed with *fadB*, which encodes a 3-hydroxyacyl-CoA dehydrogenase involved in β-oxidation of fatty acids. The *aceA* transcripts are stable and abundant, which may provide the high isocitrate lyase activity required for successful competition for isocitrate between the anaplerotic glyoxylate shunt enzyme and the degradative isocitrate dehydrogenase (Kelly et al. 2002).

Another important nutritional aspect relevant to virulence is the ability of pathogenic bacteria to acquire ferric iron from the host. Iron is an essential cofactor in key processes including central metabolism, energy generation, and detoxification of oxygen radicals. However, the Fe^{3+} ion is insoluble and is sequestered by iron-binding proteins and heme groups in animal hosts tissues, making it unavailable to bacterial parasites. The synthesis and release of iron scavenging systems with greater affinity for iron than the eukaryotic iron-binding proteins is a widespread strategy used by pathogenic bacteria to overcome iron limitation within the host (Miethke and Marahiel 2007; Neilands 1995). *R. equi* has been shown to acquire iron from different animal sources, including transferrin, lactoferrin, and hemoglobin, presumably via *iupABC* encoding a putative iron ABC transporter (Jordan et al. 2003; Miranda-Casoluengo et al. 2005). Two nonribosomal peptide synthetases (NRPS), IupS and IupU, are involved in the formation of catecholic siderophores, called "rhequibactins," under iron limitation. Knockout mutants of these NRPSs or the *iupABC* cluster were unable to grow in vitro in low iron conditions (Miranda-Casoluengo et al. 2005, 2008). However, virulence was not affected, indicating that other systems are involved in within-host iron uptake (the *R. equi* genome indeed encodes additional iron acquisition systems; Letek et al. unpublished data). Iron is also used by many pathogens as an important signal to control virulence gene expression. The actinobacterial iron-responsive transcriptional repressor IdeR (DtxR) has been shown to play a key role in the virulence of *Mtb* and *Corynebacterium diphtheriae* (Manabe et al. 1999, 2005). An IdeR homolog has been identified in *R. equi* (Boland and Meijer 2000), but its role in virulence remains to be determined.

Recently, four chromosomal determinants potentially involved in *R. equi* virulence were identified by disruption of candidate genes (from the partial genome sequences of strain ATCC 33701; (Rahman et al. 2003)) and mutant testing in mouse clearance infection assays. Two mutants, in *htrA* (temperature-induced serine protease, involved in the folding and maturation of secreted virulence proteins in Gram-negative bacteria) and *narG* (nitrate reductase subunit), showed complete attenuation, whereas in a third mutant, *pepD* (putative aminopeptidase), the effect was partial (Pei et al. 2007). A mutant in *phoPR* (putative divalent cation-responsive two-component regulatory system) showed increased survival in mice

compared to the wild type, and this correlated with upregulation of *vap* PAI genes (Ren and Prescott 2004). Although these are preliminary observations that require confirmation with clean in-frame mutants and additional mechanistic work to ascertain the role in virulence, this study highlights the potential of genome-based systematic approaches for rapid progress in understanding *R. equi* pathogenesis.

5 The *R. equi* Genome Project

A preliminary analysis of the *R. equi* genome based on 1,417 shotgun sequences from strain ATCC 33701 (0.25× coverage) was published in 2003. This study underscored the close genetic relationship between *R. equi* and *Mtb* (Rahman et al. 2003). In 2004, an international consortium of laboratories undertook to determine the complete genome sequence of *R. equi* in collaboration with the Wellcome Trust's Sanger Institute (UK). The strain chosen, 103, a prototypic clinical isolate from a case of foal pneumonia, belongs to one of the two major horse-associated PFGE genomic groups and, in contrast to ATCC 33701, is amenable to genetic manipulation (compared to strain 103, ATCC 33701 has a high frequency of illegitimate recombination and an unacceptably low transformation efficiency) (Navas et al. 2001). A frozen stock from the original isolate, designated clone 103S, was selected for sequencing to avoid mutations associated with prolonged in vitro passage.

R. equi 103S has a genome of just above five millions base pairs, with a circular chromosome of 5,043 Kb and a virulence plasmid of 80.6 Kb. The G+C content is 68.8%. Whole genome comparisons showed that it is most similar to that of the soil-restricted versatile biodegrader *R. jostii* RHA1 (9.7 Mb) (McLeod et al. 2006) and two recently sequenced environmental rhodococci, *Rhodococcus erythropolis* PR4 (6.89 Mb) and *Rhodococcus opacus* B4 (8.17 Mb) (published online by NITE, Japanese National Institute for Technology and Evaluation, http://www.nite.go.jp/index-e.html); it is however significantly smaller in size, presumably owing to genome expansion in the environmental rhodococci rather than genome reduction in *R. equi*. Much of the expanded genome of the environmental species is constituted by niche-adaptive extrachromosomal replicons (≈20% in *R. jostii*), including large linear plasmids of up to 1 Mb (see section below), which are lacking in *R. equi*. Interestingly, the rhodococcal chromosomes also differ in topology: circular for *R. equi* 103S and *R. erythropolis* PR4, and linear for *R. jostii* RHA1 and *R. opacus* B4 like in streptomycetes, which also have large genomes (>8.5 Mb). Next in overall genome similarity, but clearly more distantly related, is *N. farcinica* IFM 10152, followed by *Mtb*. *R. equi* 103S possesses a large set of genes involved in lipid metabolism, reflecting the importance of lipids as major carbon source for the organism. It lacks the extensive metabolic network and catabolic abilities characteristic of the environmental rhodococci, as well as the extensive secondary metabolism found in many other *Actinobacteria*. It has in contrast a larger than average secretome and many surface proteins (406 or 8.9% of genes) and regulators

(464, 10.26% of genes). HGT regions are underrepresented in the *R. equi* genome compared to the environmental *Rhodococcus* spp. The in silico analyses identified a number of chromosomal genes putatively involved in virulence, including members of the mycobacterial virulence-associated gene families *mce*, PE/PPE, and *esx*. Interestingly, some of the putative chromosomal virulence determinants are in HGT genomic islands. About 29% of the products encoded by *R. equi* 103S are of unknown function. The *R. equi* 103S genome sequence is publicly available since 2007 for Blast search (http://www.sanger.ac.uk/Projects/R_equi/). The manual annotation is now completed (Letek et al. 2008a) and a detailed analysis will soon be published.

6 Concluding Remarks and Perspectives

In this chapter, we have reviewed the current knowledge about the biology and virulence of *R. equi*, the only animal pathogen recognized within the ubiquitous actinobacterial genus *Rhodococcus*. Except that virulence and intracellular survival depends on plasmid-encoded products, very little is known – comparatively with other pathogens – about the infection mechanisms of *R. equi*. However fragmentary the available information, it already reveals *R. equi* as an interesting research model and, with its plasmid-driven host tropism, a true paradigm in microbial pathogenesis.

Progress into the molecular understanding of *R. equi* virulence has been marred for a long time by lack of genetic analysis tools. Such is no longer the case, and methods for random and site-directed mutagenesis are now available together with suitable complementation vectors and clean selection markers (Ashour and Hondalus 2003; Mangan and Meijer 2001; Mangan et al. 2005; Navas et al. 2001). Recent additions to the research armory include the development of a reliable counter-selection system to produce unmarked gene deletions (van der Geize et al. 2008) and a vector for site-specific integration in the chromosome (Hong and Hondalus 2008). The field is therefore ripe for rapid advances and this will be enormously facilitated by the recent completion of the genome sequence of strain 103S (Vazquez-Boland et al. 2009).

A better understanding of *R. equi* in genomic terms may lead to breakthroughs with potential impact on the management and prevention of rhodococcal foal pneumonia, a major unresolved problem that afflicts the equine industry. This problem is compounded by the limited choice of antibiotics available against *R. equi* due to its natural resistance to many antimicrobials and the recent emergence of acquired resistance to currently used drugs (Asoh et al. 2003; Hsueh et al. 1998; Niwa et al. 2006). The functional genomic analysis of *R. equi* by expression and proteomic profiling at the host–pathogen interface may facilitate the identification of novel vaccine targets. The genome may also provide critical insight into the mechanisms used by *R. equi* to survive and multiply in manure and soil to rationally design management procedures to control the prevalence and spread of virulent

R. equi strains in the farm environment. Besides these practical applications, with its dual lifestyle as soil saprophyte and intracellular parasite, *R. equi* offers a unique opportunity to study the evolutionary genomics of niche breadth in *Actinobacteria*. The comparative genomic analysis of *R. equi* and the environmental species of the genus will provide valuable insight into the mechanisms of niche-adaptive genome plasticity and evolution in rhodococci as well as valuable pieces of the puzzle to understand how virulence is shaped in the actinomycetes.

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