Microbiology Monographs

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Biology of Rhodococcus

Second Edition

Springer

Microbiology Monographs

Volume 16

Series Editor
Alexander Steinbüchel
Münster, Germany

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ISSN 1862-5576 ISSN 1862-5584 (electronic)
Microbiology Monographs
ISBN 978-3-030-11460-2 ISBN 978-3-030-11461-9 (eBook)
https://doi.org/10.1007/978-3-030-11461-9

Library of Congress Control Number: 2019934756

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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 about 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 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 and biotransformation of diverse compounds, biosynthesis of lipids and biosurfactants, adaptation and tolerance to solvents and the interaction with metals and biotechnological applications. Chapters dealing with its taxonomy, genomes and plasmids and oligotrophic and central metabolism are also included in this volume. Moreover, the book examines the basic aspects of the phytopathogenic member *R. fascians*.

I would like to express my thanks to all of the authors, who contributed with high-quality reviews of each topic, to the series editor, Alexander Steinbüchel, and to the staff at Springer Nature, especially Bibhuti Sharma, for supporting the second edition of 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, Argentina Héctor M. Alvarez

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Refined Systematics of the Genus *Rhodococcus* Based on Whole Genome Analyses

Vartul Sangal, Michael Goodfellow, Amanda L. Jones, Robert J. Seviour, and Iain C. Sutcliffe

Contents

Abstract The genus *Rhodococcus* has had a long and complicated taxonomic history. With the advent of 16S rRNA gene sequence analyses and, subsequently, whole genome-based phylogenomic studies, the heterogeneity within the genus has become progressively more apparent and better resolved. We here review recent advances in the systematics of the genus, which currently contains more than 50 described species. Important developments include the proposed reclassifications of *Rhodococcus equi* and *Rhodococcus kunmingensis* into novel genera as *Prescottella equi* and *Aldersonia kunmingensis*, respectively. Notably, phylogenomic studies consistently resolve the genus *Rhodococcus* into multiple species-groups of coherent composition which thus likely merit separate genus status alongside *Rhodococcus* sensu stricto. In particular, a well-sampled group of plant-associated species we here define as the "*Rhodococcus fascians* assemblage" likely represents a novel genus.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_1

The rhodococci are remarkable organisms: interest in their ecology and biological activities (and the pathogenicity of some strains) has sustained thousands of scientific studies, including what is now two editions of this monograph (Alvarez 2010, 2018). Consequently, it is all the more remarkable that we haven't yet answered definitively the question "are they all actually rhodococci"? This chapter will review progress in clarifying the systematics of the genus *Rhodococcus* in the light of new knowledge and will provide a convincing answer to that question.

1 Historical Perspective and Recent Developments in the Traditional Classification of Rhodococci

The long and intricate taxonomic history of the genus *Rhodococcus* has been the subject of several comprehensive reviews (Bousfield and Goodfellow 1976; Goodfellow and Wayne 1982; Goodfellow et al. 1998; Gürtler and Seviour 2010) and hence will only be considered briefly here. The genus was proposed by Zopf (1891) for two species of red-coloured bacteria that had been classified in the genus *Micrococcus* by Overbeck (1891). Early classifications based upon form and function are difficult to navigate as "rhodococci" were assigned to 11 genera, including *Bacillus, Micrococcus*, *Mycobacterium* and *Nocardia*. The reintroduction of the epithet "rhodochrous" for members of this extensive taxonomic diaspora by Gordon and Mihm (1959) represented an important milestone in rhodococcal systematics as the strains belonging to this taxon were considered to represent a definitive species, albeit one that was tentatively classified in the genus *Mycobacterium*. However, it soon became apparent from a succession of studies based on chemotaxonomic and numerical phenetic methods that this taxon merited generic status (Bousfield and Goodfellow 1976). Following an extensive numerical taxonomic study by Goodfellow and Alderson (1977), the genus *Rhodococcus* was reintroduced for "rhodochrous" strains with *Rhodococcus rhodochrous* (Zopf 1891) Tsukamura 1974 as the type species, along with nine additional species. Several of the latter have since been assigned to the genus *Gordonia* (http://www.bacterio.net/rhodococcus.html).

The taxonomic status of most of the 32 validly named *Rhodococcus* species considered by Gürtler and Seviour (2010) and in the current edition of *Bergey's Manual of Systematic Bacteriology* (Jones and Goodfellow 2012) is underscored by a combination of genotypic and phenotypic data, although it is clear from associated 16S rRNA gene sequence studies that the genus is heterogeneous. Indeed, Jones and Goodfellow (2012) assigned the type strains of 30 rhodococcal species to three lineages, the *Rhodococcus equi*, *Rhodococcus erythropolis* and *R. rhodochrous* subclades. Similar intra-generic structure was also evident in other studies, although the position of *R. equi* strains tended to be unstable (Rainey et al. 1995; Goodfellow et al. 1998; McMinn et al. 2000; Gürtler et al. 2004; Gürtler and Seviour 2010). At the time of publication of Gürtler and Seviour (2010), the genus contained 32 validly

named species. Since then, an additional 22 *Rhodococcus* species with either validly or effectively published names have been described (Table 1). In addition to the description of these new species, it has also been demonstrated that the biotechnologically important strain *Rhodococcus* sp. RHA1 (Yam et al. 2011; Ceniceros et al. 2017) belongs to the species *Rhodococcus jostii* (Jones et al. 2013a), whereas *Rhodococcus jialingiae* is a later synonym of *Rhodococcus qingshengii* (Táncsics et al. 2014). Furthermore, recent genome sequence data have been used to emend the descriptions of 23 rhodococcal species with more precise measures of genomic DNA G + C content and estimated genome sizes (Nouioui et al. 2018).

It is evident from 16S rRNA gene sequence analyses (as exemplified in Fig. 1) that the type strains of most of these species can be assigned to several multimembered clades that correspond to a sister clade that encompasses representative *Nocardia* type strains. Genotypic data acquired from the analyses of nucleic acids and associated phenotypic data from chemotaxonomic and other expressed features (polyphasic taxonomy) has led to considerable improvements in the classification of rhodococci and other mycolic acid containing Actinobacteria classified in the order *Corynebacteriales* (Goodfellow and Jones 2012). Nevertheless, problems remain, notably the difficulty of distinguishing between genera within this taxon and between closely related species within individual genera (Goodfellow and Jones 2012; Baek et al. 2018). 16S rRNA gene sequence analysis in particular has played an important role in improving the classification of the genus although it is equally clear that this marker lacks sufficient resolution for distinguishing between closely related taxa. Classifications based on whole genome sequences and associated bioinformatic methods are proving to be effective ways of addressing such challenges, as they are based upon massively expanded amounts of sequence data, thereby providing a step change not only in information but also in reliability (Sangal et al. 2014b; Whitman 2014; Chun et al. 2018; Baek et al. 2018). However, it is important that whole genome sequence-based classifications (phylogenomics; see below) adhere to both minimal standards for sequencing quality (Chun et al. 2018) and sound taxonomic practices, such as the need to follow the nomenclatural type concept and the requirement to deposit type strains in a minimum of two public collections in different countries (Parker et al. 2019).

In addition to the application of whole genome sequence analysis to systematics, future improvements to the classification and identification of rhodococci will likely also derive from the refinement and implementation of matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry methods for rapidly phenotyping bacteria (Hsueh et al. 2014; Schumann and Maier 2014; Pasciak et al. 2015; de Alegria Puig et al. 2017). The integration of phylogenomic and high-throughput MALDI-TOF-based approaches has much potential for microbial systematics generally (Ramasamy et al. 2014) and in application to the rhodococci and relatives specifically.

Table 1 *Rhodococcus* species described since 2010

Name	Type strain	16S rRNA accession number	Source	Reference
Rhodococcus artemisiae	YIM 65754^T	GU367155	Endophyte of *Artemisia annua* L.	Zhao et al. (2012)
Rhodococcus nanhaiensis	SCSIO 10187^T	JN582175	South China Sea sediment	Li et al. (2012)
Rhodococcus canchipurensis	MBRL 353^T	JN164649	Limestone quarry, India	Nimaichand et al. (2013)
Rhodococcus cerastii	C5^T	FR714842	Phyllosphere of *Cerastium holosteoides*	Kämpfer et al. (2013)
Rhodococcus trifolii	T8^T	FR714843	Phyllosphere of *Trifolium repens*	
Rhodococcus defluvii	Ca11^T	KC788572	Bioreactor wastewater, Germany	Kämpfer et al. (2014)
Rhodococcus enclensis	NIO-1009^T	HQ858009	Marine sediment sample, Goa	Dastager et al. (2014)
Rhodococcus kronopolitis[a]	NEAU-ML12^T	KF887492	*Kronopolites svenhedind* Verhoeff	Liu et al. (2014)
Rhodococcus biphenylivorans	TG9^T	KJ546454	Polychlorinated biphenyl-contaminated sediment, China	Su et al. (2015)
Rhodococcus soli	DSD51W^T	KJ939314	Park soil, Japan	Li et al. (2015)
Rhodococcus aerolatus	PAMC 27367^T	KM044053	Rainwater from above the Bering Sea	Hwang et al. (2015)
Rhodococcus agglutinans	CFH S0262^T	KP232908	Soil sample, Vietnam	Guo et al. (2015)
Rhodococcus lactis	DW151B^T	KP342300	Dairy waste sludge, India	Singh et al. (2015)
Rhodococcus antrifimi	D7-21^T	LN867321	Dried bat dung, South Korea	Ko et al. (2015)
Rhodococcus degradans	CCM 4446^T	JQ776649	Polluted soil, Switzerland	Švec et al. (2015)
Rhodococcus pedocola	UC12^T	KT301938	Forest topsoil, South Korea	Nguyen and Kim (2016)
Rhodococcus humicola	UC33^T	KT301939	Forest topsoil, South Korea	
Rhodococcus sovatensis	H004^T	KU189221	Hypersaline and heliothermal lake water, Romania	Táncsics et al. (2017)
Rhodococcus gannanensis	M1^T	NR_152643	Root endophyte of *Helianthus annuus* L.	Ma et al. (2017)
Rhodococcus olei	Ktm-20^T	MF405107	Oil-contaminated soil, Nepal	Chaudhary and Kim (2018)

(continued)

Table 1 (continued)

Name	Type strain	16S rRNA accession number	Source	Reference
Rhodococcus psychrotolerans[a]	CMAA 1533^T	KY317932	Rhizosphere of *Deschampsia antarctica*	Silva et al. (2018)
Rhodococcus electrodiphilus	JC435^T	LT630357	Marine coral reef	Ramaprasad et al. (2018)
Rhodococcus daqingensis[a]	Z1^T	MH205096	Petroleum-contaminated soil	Wang et al. (2018)

[a]Effectively published name at the time of writing

2 Reclassification of *R. equi* as *Prescottella equi*

Based on 16S rRNA gene trees, it is now clear that *R. equi* represents a distinct phylogenetic lineage, although as noted above its position is unstable with regard to other members of the genus *Rhodococcus*. Indeed, Gürtler and Seviour (2010) noted that "whether *R. equi* should continue to be placed in the genus *Rhodococcus* or moved to a separate or alternative existing genus still awaits resolution", while Jones and Goodfellow (2012) raised the question as to "whether it should be recognized as a genus in its own right". This matter was addressed with the proposed reclassification of *R. equi* as the type species of the genus *Prescottia* (Jones et al. 2013b), although the rules of nomenclate required that it was necessary subsequently to propose the replacement of the illegitimate genus name *Prescottia* with *Prescottella* (Jones et al. 2013c), with the type species *Prescottella equi*. The justification for the reclassification was based upon extensive phylogenetic, molecular fingerprinting and numerical taxonomic analyses (Jones et al. 2013b), which have since been supported by phylogenomic data (Sangal et al. 2016; see below). However, the question of the correct nomenclature has been complicated by the formal reclassification of *R. equi* into the species *Rhodococcus hoagii* (Kämpfer et al. 2014). This move has been challenged in a "Request for an Opinion" from the Judicial Commission of the International Committee on Systematics of Prokaryotes, which requested the retention of the species epithet *equi* (Garrity 2014). Until such an Opinion is issued, the correct name of the taxon remains *R. hoagii*, and the species name *P. equi* cannot be validated. As reviewed in Goodfellow et al. (2015), a pragmatic solution is to refer to the organism as "*Rhodococcus equi* (*Rhodococcus hoagii*/*Prescottella equi*)" at first usage in scientific communications, while the matter is under consideration by the Judicial Commission. We note that the community can use any of these names although currently *R. hoagii* is the "correct" one (Tindall 2014a).

While 16S rRNA gene sequence analysis recovered 26 *R. equi* strains in a single cluster, the molecular fingerprinting and numerical taxonomic analyses of Jones et al. (2013b) suggested that the species could be resolved into two subclusters. However, subsequent genomic analysis has indicated a high degree of relatedness

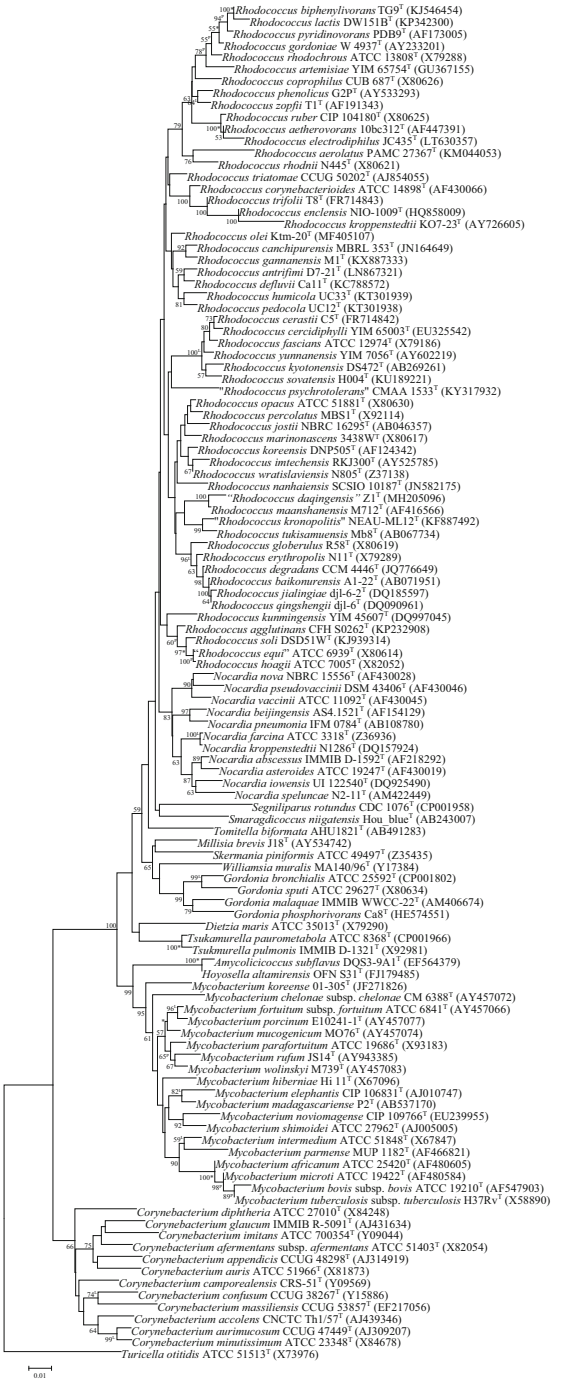

Fig. 1 16S rRNA gene sequence analysis of members of the genus *Rhodococcus* and representatives of related genera in the order *Corynebacteriales*. The tree was constructed using the neighbour-joining algorithm in MEGA. Nodes supported by greater than 50% of 1000 bootstrap replications are indicated. Nodes labelled L or P were also supported in the maximum likelihood or maximum parsimony trees, respectively. Nodes labelled * were supported by all three tree-making algorithms

between strains of *R. equi* (Sangal et al. 2014a, 2016; Anastasi et al. 2016), indicative of a clonal population, although six sub-clades were evident (Anastasi et al. 2016). Similarly, a multilocus sequence typing scheme for *R. equi* based on seven housekeeping loci (https://pubmlst.org/rhodococcus/) supported a clonal population structure and identified 37 sequence types within the species, 16 of which could be grouped into 6 clonal complexes (Duquesne et al. 2017).

3 Is the Name *Rhodococcus* Zopf 1891 "Legitimate"?

Apart from the above complications regarding the nomenclature of *R. equi*, Tindall (2014b) has reported that the genus name *Rhodococcus* Zopf 1891 is illegitimate under the rules of nomenclatural priority in the International Code of Nomenclature of Prokaryotes (Parker et al. 2019), as it post-dates the establishment of the algal genus *Rhodococcus* Hansgirg 1884. However, the algal genus *Rhodococcus* Hansgirg 1884 is currently regarded as synonymous with the earlier cyanobacterial genus *Chroococcus* Nägeli 1849 (http://www.algaebase.org/search/genus/detail/?genus_id=42893). Thus, the legitimacy (or otherwise) of the bacterial name *Rhodococcus* Zopf 1891 will ultimately depend on both confirming the assignment of *Rhodococcus* Hansgirg 1884 to *Chroococcus* Nägeli 1849 and also on complex revisions to both the International Code of Nomenclature of Algae, Fungi and Plants (ICNafp) and International Code of Nomenclature of Prokaryotes regarding which rules should apply to cyanobacteria (Tindall 2014b; Oren and Ventura 2017). Such revisions will undoubtedly be time-consuming, and so, in the meantime, it seems prudent that researchers studying members of the current genus *Rhodococcus* maintain the use of this name until these matters are resolved (e.g. by the International Committee on Systematics of Prokaryotes and its sister committees governing nomenclature). Ironically, the reclassification of various rhodococcal species-groups into new genera (see below) will help to provide these new taxa with legitimate names, so long as the rules of the International Code of Nomenclature of Prokaryotes are correctly followed.

4 Insights from Phylogenomic Analyses

Gürtler et al. (2004) posed the question "Can whole genome analysis refine the taxonomy of the genus *Rhodococcus*?" It is now clear that this question can be answered in the affirmative. Four phylogenomic studies have defined several robust

species-groups within the genus. In a study focussed on plant pathogenic rhodococci and the intraspecific structure of *Rhodococcus fascians*, Creason et al. (2014) generated a phylogenetic tree based on average nucleotide identity (ANI) divergence for 59 genome-sequenced rhodococci, which was resolved into 7 clades with intra-group ANI values >70–75% (a threshold they considered relevant to the delineation of bacterial genera). The 25 plant-associated isolates included were recovered in two related clades. Eighteen of these isolates (including the type strain of *R. fascians*) were recovered in a clade with four recognisable subclades (1i–1iv). The remaining 34 rhodococci analysed were recovered in five clades, including Clade 5 containing *R. equi* strains. However, the clustering method and absence of outgroup taxa prevented relationships between these clades and other mycolic acid-containing bacteria from being inferred.

Subsequently, three further phylogenomic studies have confirmed the heterogeneity within *Rhodococcus*. In a study focussed on understanding relationships between 29 *R. equi* strains, Anastasi et al. (2016) used PhyloPhlAn to analyse the genomes of these strains, along with those of 46 rhodococci (discounting a sequence misattributed to "*Rhodococcus rhodnii*" NRRL B-16535^T; see below) and 57 other genomes representing strains belonging to 11 genera of the order *Corynebacteriales*. The genome of *Streptomyces albus* NBRC 1304^T was used as the out-group. This analysis recovered all the *Rhodococcus* strains in a single lineage but also identified five well-supported species-groups within this phyletic line, four of which were each resolved into two subclusters. Contemporaneously, we used PhyloPhlAn to analyse 100 rhodococcal genomes and 15 genomes representing seven genera of the order *Corynebacteriales* (Sangal et al. 2016). In this study, seven rhodococcal species-groups were identified, several of which were separated by lineages containing representatives of other genera, i.e. indicating *Rhodococcus* to be paraphyletic. Similar to Creason et al. (2014), we also found the ANIb threshold for separating potential genera to be ~74.8%. To explore this further, we have extended our PhyloPhlAn analysis to include 116 rhodococcal genomes and 15 genomes representing 7 genera of the order *Corynebacteriales* (Fig. 2; Table 2). Notably, this tree now includes genomes for type strains of 29 of the 52 currently validly named rhodococcal species. This analysis generated a tree with the same overall topology as that in Sangal et al. (2016), with the *Rhodococcus* strains recovered in the same seven species-groups (Table 3). In addition, *R. rhodnii* strain LMG 5362, which was considered to be a possibly misclassified singleton by Sangal et al. (2016), is now shown to belong to a newly defined species-group (H), along with *R. rhodnii* NBRC 100604^T and the type strain of *Rhodococcus triatomae* (Fig. 2). As noted by Anastasi et al. (2016), a genome deposited as "*R. rhodnii* NRRL B-16535^T" (Genbank GCA_000720375.1) is clearly misclassified, as it forms a lineage distant from the other two *R. rhodnii* strains (Fig. 2). As in the Sangal et al. (2016) analysis, *Rhodococcus kunmingensis* DSM 45001^T was recovered as a singleton lineage in Fig. 2, whereas it is typically loosely affiliated with *R. equi* (*Prescottella*) in 16S rRNA gene trees (e.g. Fig. 1).

Finally, a recent large-scale phylogenomic analysis of the phylum *Actinobacteria*, based on 1142 actinobacterial type strain genomes, has also

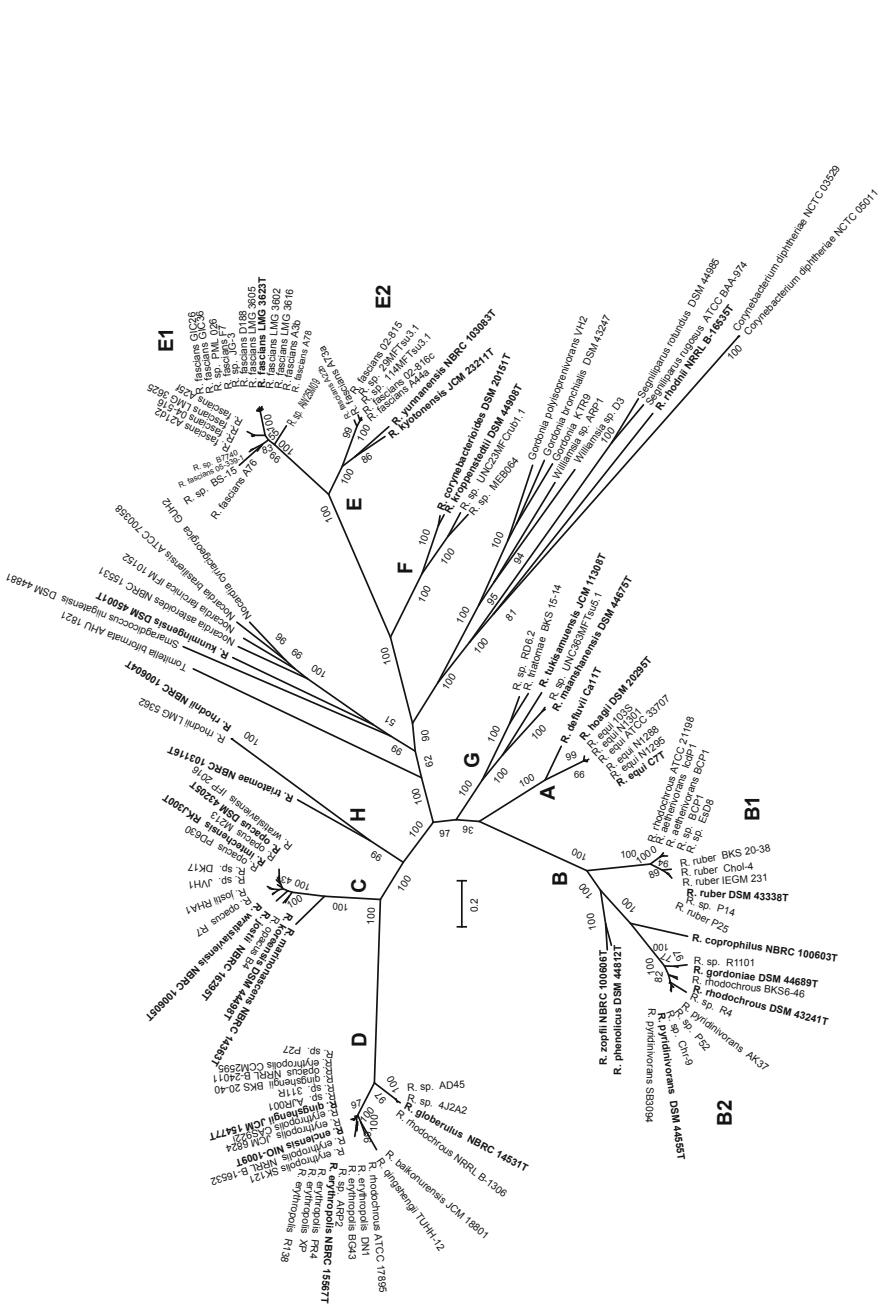

Fig. 2 Phylogenomic analysis of members of the genus *Rhodococcus* and representatives of related genera in the order *Corynebacteriales*. The genome sequence data were obtained from the GenBank and re-annotated using RAST server (Aziz et al. 2008). The phylogenetic tree was constructed from the protein sequences using PhyloPhlAn (Segata et al. 2013). PhyloPhlAn extracts a subset of amino acids from 400 universal proteins and constructs a maximum likelihood tree from the concatenated amino acid sequence alignment. The scale bar shows a normalised fraction of the total branch lengths as described by Segata et al. (2013)

Table 2 Data on rhodococcal and outgroup genomes analysed in this study

Taxon no.	Organism	Strain	Accession no.	Genome size (Mb)	GC%	Group
1	*Corynebacterium diphtheriae*	NCTC 03529	AJGI00000000	2.48	53.6	–
2	*Corynebacterium diphtheriae*	NCTC 05011	AJVH00000000	2.38	53.6	–
3	*Gordonia* sp.	KTR9	NC_018581	5.44	67.8	–
4	*Gordonia bronchialis*	DSM 43247	NC_013441	5.21	67.1	–
5	*Gordonia polyisoprenivorans*	VH2	NC_016906	5.67	67.0	–
6	*Nocardia asteroides*	NBRC 15531	BAFO02000000	6.96	69.9	–
7	*Nocardia brasiliensis*	ATCC 700358	NC_018681	9.44	68.1	–
8	*Nocardia cyriacigeorgica*	GUH2	NC_016887	6.19	68.4	–
9	*Nocardia farcinica*	IFM 10152	NC_006361	6.02	70.8	–
10	*Rhodococcus aetherivorans*	IcdP1	NZ_CP011341.1	5.92	70.6	B (B1)
11	***Rhodococcus aetherivorans***	**BCP1**	**AVAE00000000**	**6.23**	**70.4**	**B (B1)**
12	***Rhodococcus baikonurensis***	**JCM 18801**	**BBBO00000000**	**6.82**	**62.4**	**D**
13	***Rhodococcus coprophilus***	**NBRC 100603^T**	**BDAM00000000**	**4.55**	**66.9**	**B (B2)**
14	*Rhodococcus corynebacterioides*	DSM 20151^T	LPZL00000000	3.9	70.3	F
15	*Rhodococcus defluvii*	Ca11^T	JPOC00000000	5.14	68.7	A
16	***Rhodococcus enclensis***	**NIO-1009^T**	**FMBB00000000**	**7.48**	**62.3**	**D**
17	*Rhodococcus equi*	103S	NC_014659	5.04	68.8	A
18	*Rhodococcus equi*	N1288	LRQY00000000	5.17	68.8	A
19	*Rhodococcus equi*	N1295	LRQZ00000000	5.31	68.7	A
20	*Rhodococcus equi*	N1301	LRRA00000000	5.65	68.5	A
21	*Rhodococcus equi*	ATCC 33707	NZ_CM001149	5.26	68.8	A
22	*Rhodococcus equi*	C7^T	APJC00000000	5.2	68.8	A
23	*Rhodococcus erythropolis*	SK121	ACNO00000000	6.79	62.5	D
24	*Rhodococcus erythropolis*	CAS922i	LDPN01000000	7.21	62.3	D
25	*Rhodococcus erythropolis*	PR4	NC_012490	6.52	62.3	D
26	*Rhodococcus erythropolis*	CCM2595	NC_022115	6.28	62.5	D

27	*Rhodococcus erythropolis*	BG43	NZ_CP011295.1	6.33	62.4	D
28	***Rhodococcus erythropolis***	**NBRC 15567^{T}**	**BCRM00000000**	**6.59**	**62.4**	**D**
29	*Rhodococcus erythropolis*	JCM 6824	BBLL01000000	7.02	62.3	D
30	*Rhodococcus erythropolis*	NRRL B-16532	JOIL01000000	6.94	62.4	D
31	*Rhodococcus erythropolis*	XP	AGCF00000000	7.23	62.3	D
32	*Rhodococcus erythropolis*	DN1	AUZK00000000	6.55	62.4	D
33	*Rhodococcus erythropolis*	R138	ASKF01000000	6.77	62.3	D
34	*Rhodococcus fascians*	A22b	JOKB01000000	5.91	64.3	E (E1)
35	*Rhodococcus fascians*	A44a	JMEX01000000	5.95	64.5	E (E1)
36	*Rhodococcus fascians*	A73a	JMEW01000000	5.93	64.4	E (E1)
37	*Rhodococcus fascians*	02-815	JMFF01000000	6.24	64.3	E (E1)
38	*Rhodococcus fascians*	02-816c	JMFE01000000	6.08	64.5	E (E1)
39	*Rhodococcus fascians*	A76	JMEV01000000	6.03	64.6	E (E2)
40	*Rhodococcus fascians*	05-339-1	JMFC01000000	5.73	64.7	E (E2)
41	*Rhodococcus fascians*	04-516	JMFD01000000	5.82	64.2	E (E2)
42	*Rhodococcus fascians*	A21d2	JMFA01000000	5.98	64.1	E (E2)
43	*Rhodococcus fascians*	A25f	JMEZ01000000	5.87	64.1	E (E2)
44	*Rhodococcus fascians*	LMG 3625	JMEM01000000	5.94	64.1	E (E2)
45	*Rhodococcus fascians*	F7	LFDS01000000	5.25	64.7	E (E2)
46	*Rhodococcus fascians*	A3b	JMEY01000000	6.03	64.2	E (E2)
47	*Rhodococcus fascians*	A78	JMEU01000000	6	64.3	E (E2)
48	*Rhodococcus fascians*	GIC26	JMES01000000	5.34	64.5	E (E2)
49	*Rhodococcus fascians*	GIC36	JMER01000000	5.56	64.5	E (E2)
50	*Rhodococcus fascians*	D188	JMET01000000	5.44	64.6	E (E2)
51	*Rhodococcus fascians*	LMG 3602	JMEQ01000000	5.36	64.5	E (E2)
52	*Rhodococcus fascians*	LMG 3605	JMEP01000000	5.44	64.5	E (E2)
53	*Rhodococcus fascians*	LMG 3616	JMEO01000000	5.76	64.3	E (E2)

(continued)

Table 2 (continued)

Taxon no.	Organism	Strain	Accession no.	Genome size (Mb)	GC%	Group
54	*Rhodococcus fascians*	LMG 3623^T	JMEN01000000	5.78	64.4	E (E2)
55	***Rhodococcus globerulus***	**NBRC 14531^T**	**BCWX00000000**	**6.74**	**61.7**	**D**
56	*Rhodococcus gordoniae*	DSM 44689^T	LPZN00000000	4.82	67.9	B (B2)
57	*Rhodococcus hoagii*	DSM 20295^T	LRRF00000000	4.97	68.8	A
58	*Rhodococcus imtechensis*	RKJ300^T	AJJH00000000	8.23	67.2	C
59	*Rhodococcus jostii*	RHA1	NC_008268	7.8	67.5	C
60	***Rhodococcus jostii***	**NBRC 16295^T**	**BCWY00000000**	**9.73**	**66.9**	**C**
61	***Rhodococcus koreensis***	**DSM 44498^T**	**FNSV00000000**	**10.31**	**67.4**	**C**
62	*Rhodococcus kroppenstedtii*	DSM 44908^T	LPZO00000000	3.91	70.1	F
63	*Rhodococcus kunmingensis*	DSM 45001^T	LRRB00000000	5.62	66.2	*Aldersonia*
64	***Rhodococcus kyotonensis***	**JCM 23211^T**	**FZOW00000000**	**6.31**	**64.2**	**E2**
65	***Rhodococcus maanshanensis***	**DSM 44675^T**	**FOAW00000000**	**5.67**	**69.2**	**G**
66	***Rhodococcus marinonascens***	**NBRC 14363^T**	**BCXB00000000**	**4.92**	**64.4**	**C**
67	*Rhodococcus opacus*	DSM 43205^T	LRRG00000000	8.53	67.3	C
68	*Rhodococcus opacus*	B4	NC_012522	7.91	67.9	C
69	*Rhodococcus opacus*	M213	AJYC00000000	9.2	67.0	C
70	*Rhodococcus opacus*	R7	CP008947.1	8.47	67.2	C
71	*Rhodococcus opacus*	PD630	AGVD00000000	9.16	67.0	C
72	*Rhodococcus opacus*	NRRL B-24011	JOIM01000000	6.38	62.4	D
73	*Rhodococcus phenolicus*	DSM 44812^T	LRRH00000000	6.28	68.4	B
74	*Rhodococcus pyridinivorans*	SB3094	NC_023150	5.23	68.0	B (B2)
75	*Rhodococcus pyridinivorans*	AK37	AHBW00000000	5.25	67.9	B (B2)
76	*Rhodococcus pyridinivorans*	DSM 44555^T	LRRI00000000	5.18	67.9	B (B2)
77	*Rhodococcus qingshengii*	BKS 20-40	AODN00000000	6.6	62.4	D
78	*Rhodococcus qingshengii*	TUHH-12	JNCU01000000	7.43	61.7	D

79	*Rhodococcus qingshengii*	JCM 15477^T	LRRJ00000000	7.26	62.4	D
80	*Rhodococcus rhodnii*	LMG 5362	APMY00000000	4.39	69.7	H
81	*Rhodococcus rhodnii*	NRRL B-16535^T	JOAA01000000	12.42	66.3	See main text
82	***Rhodococcus rhodnii***	**NBRC 100604**	**BCXD00000000**	**4.46**	**69.7**	**H**
83	*Rhodococcus rhodochrous*	ATCC 21198	AZHI00000000	6.48	70.2	B (B1)
84	*Rhodococcus rhodochrous*	DSM 43241^T	LRRK00000000	5.18	68.2	B (B2)
85	*Rhodococcus rhodochrous*	BKS6-46	AGVW00000000	6.22	67.4	B (B2)
86	*Rhodococcus rhodochrous*	ATCC 17895	ASJJ00000000	6.87	62.3	D
87	*Rhodococcus rhodochrous*	NRRL B-1306	JNWS01000000	6.78	61.7	D
88	*Rhodococcus ruber*	DSM 43338^T	LRRL00000000	5.3	70.7	B (B1)
89	*Rhodococcus ruber*	IEGM 231	CCSD01000000	5.99	70.2	B (B1)
90	*Rhodococcus ruber*	P25	LDUF01000000	5.73	70.5	B (B1)
91	*Rhodococcus ruber*	Chol-4	ANGC00000000	5.4	70.6	B (B1)
92	*Rhodococcus ruber*	BKS 20-38	AOEX00000000	6.13	69.7	B (B1)
93	*Rhodococcus* sp.	BCP1	AVAE00000000	6.21	70.4	B (B1)
94	*Rhodococcus* sp.	EsD8	CAVJ000000000	6.63	70.1	B (B1)
95	*Rhodococcus* sp.	P14	AJFC00000000	5.68	70.4	B (B1)
96	*Rhodococcus* sp.	R1101	AJVB00000000	4.66	67.9	B (B2)
97	*Rhodococcus* sp.	Chr-9	JTIZ01000000	5.35	67.7	B (B2)
98	*Rhodococcus* sp.	R4	AFAQ00000000	8.86	69.5	B (B2)
99	*Rhodococcus* sp.	P52	JPJJ01000000	5.29	67.9	B (B2)
100	*Rhodococcus* sp.	JVH1	AKKP00000000	9.18	67.0	C
101	*Rhodococcus* sp.	DK17	AJLQ00000000	9.11	67.1	C
102	*Rhodococcus* sp.	311R	CFHW01000000	6.35	62.6	D
103	*Rhodococcus* sp.	AJR001	LPZM00000000	7.26	62.4	D
104	*Rhodococcus* sp.	ARP2	LEKE01000000	6.3	62.4	D
105	*Rhodococcus* sp.	P27	AVCO00000000	6.26	62.4	D

(continued)

Table 2 (continued)

Taxon no.	Organism	Strain	Accession no.	Genome size (Mb)	GC%	Group
106	*Rhodococcus* sp.	AD45	JYOP01000000	6.79	61.7	D
107	*Rhodococcus* sp.	4J2A2	CEDU01000000	6.45	61.8	D
108	*Rhodococcus* sp.	114MFTsu3.1	ARTN00000000	5.55	64.7	E (E1)
109	*Rhodococcus* sp.	29MFTsu3.1	ARND00000000	5.58	64.6	E (E1)
110	*Rhodococcus* sp.	BS-15	BAOX01000000	5.51	64.4	E (E2)
111	*Rhodococcus* sp.	B7740	NZ_CP010797.1	5.34	64.9	E (E2)
112	*Rhodococcus* sp.	PML 026	JZIS01000000	5.17	64.7	E (E2)
113	*Rhodococcus* sp.	JG-3	AXVF00000000	5.27	64.6	E (E2)
114	*Rhodococcus* sp.	AW25M09	CAPS00000000	5.64	64.1	E (E2)
115	*Rhodococcus* sp.	UNC23MFCrub1.1	JMLQ01000000	4.7	68.5	F
116	*Rhodococcus* sp.	MEB064	JXQS01000000	4.67	67.0	F
117	*Rhodococcus* sp.	UNC363MFTsu5.1	JMLO01000000	5.67	69.3	G
118	*Rhodococcus* sp.	RD6.2	CVQP01000000	5.57	68.4	G
119	*Rhodococcus triatomae*	BKS 15-14	AODO00000000	5.83	69.0	G
120	***Rhodococcus triatomae***	**NBRC 103116^{T}**	**BCXF00000000**	**4.74**	**68.7**	**H**
121	***Rhodococcus tukisamuensis***	**JCM 11308^{T}**	**FNAB00000000**	**5.49**	**69.8**	**G**
122	*Rhodococcus wratislaviensis*	IFP 2016	ANIU00000000	9.7	66.9	C
123	*Rhodococcus wratislaviensis*	NBRC 100605^{T}	BAWF01000000	10.41	66.8	C
124	***Rhodococcus yunnanensis***	**NBRC 103083^{T}**	**BCXH00000000**	**6.37**	**63.9**	**E2**
125	***Rhodococcus zopfii***	**NBRC 100606^{T}**	**BCXI00000000**	**6.3**	**68.2**	**B**
126	*Segniliparus rotundus*	DSM 44985	NC_014168	3.16	66.8	–
127	*Segniliparus rugosus*	ATCC BAA-974	ACZI00000000	3.57	68.1	–
128	*Smaragdicoccus niigatensis*	DSM 44881	AQXZ00000000	5.32	64.3	–
129	*Tomitella biformata*	AHU 1821	BAVQ00000000	4.71	68.1	–
130	*Williamsia* sp.	ARP1	JXYP00000000	4.75	68.6	–
131	*Williamsia* sp.	D3	AYTE00000000	5.62	64.6	–

Type strains are labelled ^{T}. Genomes in bold were not included in the analysis presented in Sangal et al. (2016)

Table 3 Species-groups evident within the current genus *Rhodococcus* as determined by phylogenomic analyses

Representative species[a]	Species-group[b] (Fig. 2)	Anastasi et al. (2016) clade	Creason et al. (2014) clade[b]	Affiliated species from 16S rRNA analysis[c]
"R. equi" *R. defluvii*	A	3a	5	*R. agglutinans* *R. soli*
R. ruber	B1	1b	6	*R. aetherivorans*
R. coprophilus *R. gordoniae* ***R. rhodochrous*** *R. phenolicus* *R. pyridinivorans* *R. zopfii*	B2	1a	7	*R. artemisiae* *R. biphenylivorans* *R. lactis*
R. imtechensis *R. jostii* *R. koreensis* *R. opacus* ***R. marinonascens*** *R. wratislaviensis*	C	2b	4	*R. percolatus*
R. enclensis ***R. erythropolis*** *R. qingshengii* *R. globerulus*	D	2a	3	*R. baikonurensis* *R. degradans*
R. fascians *R. kyotoensis* *R. yunnanensis*	E	5a	1 and 2	*R. cerastii* *R. cercidiphylli* *R. sovatensis*
R. corynebacterioides *R. kroppenstedtii*	F	5b	Not included	*R. trifolii*
R. maanshanensis *R. tukisamuensis*	G	3b	Strain BKS-51-14 associated with clade 5	*R. kronopolitis* *R. daqingensis*
R. rhodnii *R. triatome*	H	4 (*R. rhodnii* LMG 5362)	*R. rhodnii* LMG 5362 associated with clade 5	
R. kunmingensis[d]	Singleton		Not included	

[a]The earliest described species is shown in bold

[b]All these species-groups were also recovered as well-separated clusters in a seven protein multilocus sequence analysis (maximum likelihood tree) of 199 members of *Rhodococcus* (Savory et al. 2017)

[c]Species likely to belong to the same clusters/clades as judged from a consistent affiliation with related species in 16S rRNA gene trees

[d]Now reclassified as *Aldersonia kunmingensis* (Nouioui et al. 2018)

confirmed the species-groups indicated in Table 3 (Nouioui et al. 2018). Whole proteome analysis using the Genome BLAST Distance Phylogeny (GBDP) was used to determine both intra- and supra-generic relationships within the phylum. With the exception of *R. kunmingensis*, the 29 rhodococcal type strains included were recovered as a lineage within the family *Nocardiaceae*. Importantly, within this "core"

Rhodococcus lineage, the type strains were resolved into eight lineages in exact agreement with groups A–H in Table 3. However, similar to Fig. 2, *R. kunmingensis* DSM 45001^T was recovered as a singleton well separated from any of these species-groups, showing a poorly supported relationship with *Skermania piniformis*. Consequently, Nouioui et al. (2018) have proposed the reclassification of *R. kunmingensis* DSM 45001^T as the type species representing a novel genus, *Aldersonia kunmingensis* gen. nov., comb. nov.

It is notable that all of the above phylogenomic analyses recover well-supported species-groups of very similar composition (Table 3). *R. equi* strains, along with the type strain of *R. defluvii*, are recovered in a well-separated group corresponding to the proposed genus *Prescottella* (Jones et al. 2013b, c; Sangal et al. 2015, 2016). Group B (Fig. 2) contains the type strain of *R. rhodochrous*, the type species of the genus, and can therefore be defined as *Rhodococcus* sensu stricto. The formal proposal of novel genera in addition to *Aldersonia* and *Prescottella* may require further analyses (notably of genomes of as yet unsequenced rhodococcal type strains). However, it is already evident that Group E (Fig. 2) containing soil and plant-associated strains related to the *R. fascians* (Clades 1 and 2 sensu Creason et al. 2014) should be reclassified into at least one novel genus containing *R. fascians*, *Rhodococcus kyotonensis*, *Rhodococcus yunnanensis* and multiple novel species (Creason et al. 2014; Sangal et al. 2016). Indeed, multilocus sequence analysis combined with ANI analyses of a larger collection of plant-associated strains that map onto Group E indicated that Clades 1 and 2 may contain at least 13 novel species in addition to *R. fascians* (Savory et al. 2017). For now, we refer to these strains as the *R. fascians* assemblage. It is notable that Savory et al. (2017) also concluded that this grouping contains *R. kyotonensis* and is well separated from other rhodococci (*R. yunnanensis* was not included in their analysis). Their multilocus sequence analysis of 199 *Rhodococcus* strains (based on translated sequences for *ftsY*, *infB*, *rpoB*, *rsmA*, *secY*, *tsaD* and *ychF*) yielded a maximum likelihood tree with major groupings strikingly concordant with Groups A–H in Table 3 and also highlighted the distant relationship of *R. kunmingensis* DSM 45001^T to the rhodococci.

It is evident from the above, and Table 3, that several independent research groups using differing phylogenomic methodologies have all delineated at least eight groups of species currently classified in the genus *Rhodococcus*, aside from the reclassification of *R. kunmingensis* into the genus *Aldersonia* (Nouioui et al. 2018). As expanding our analysis from 100 to 116 strains, mostly with genome data for type strains, has not added to the number of clusters identified within the genus, it is likely that as further strains are sequenced and analysed they will fit mainly into the eight species-groups defined in Table 3. Indeed, the consistent association in 16S rRNA gene sequence trees (e.g. Fig. 1) of some species that have yet to have representative genomes sequenced with taxa already assigned to species-groups A–H strongly predicts that these species will eventually be assigned to the corresponding groups (Table 3). For example, 16S rRNA gene analyses (e.g. Guo et al. 2015; Li et al. 2015; Chaudhary and Kim 2018; Fig. 1) consistently indicate that *Rhodococcus agglutinans* and *Rhodococcus soli* are probably members of the genus *Prescottella*.

Similarly, 16S rRNA gene analyses (e.g. Silva et al. 2018; Táncsics et al. 2017) consistently associate *Rhodococcus cerastii*, *Rhodococcus cercidiphylli* and *Rhodococcus sovatensis* with *R. fascians*, *R. kyotonensis* and *R. yunnanensis*, as in Fig. 1.

5 Concluding Comments

Clearly, remarkable progress has been made recently in resolving the complex systematics of the genus *Rhodococcus*, in particular through the application of phylogenomic approaches. The heterogeneity within the genus is confirmed and the separation of two pathogenic species into the genus *Prescottella* (associated with animal hosts) and the *R. fascians* assemblage (plant-associated) is especially notable. A wealth of genomic data are now available for rhodococcal strains, including more than 50% of the currently recognised type strains. Completion of genome sequencing data for the remaining rhodococcal type strains will no doubt bring further insights into the groupings described herein (Table 3), along with important insights into their biology and defining characteristics. The division of *Rhodococcus* into multiple genera may be considered controversial as one persons' phylogenomically defined genus is another's intra-genus "species-group". Nevertheless, the taxonomic status of *Rhodococcus* seems to be currently poised like a game of Jenga: once blocks such as *Aldersonia*, *Prescottella* and the *R. fascians* assemblage have been moved, the tower will tumble into multiple genera, as was the case with the aerobic endospore-forming bacilli and, more recently, *Mycobacterium* (Gupta et al. 2018). Thus, it seems likely that a future edition of this monograph will need to be called "Biology of *Rhodococcus*, *Prescottella* and closely related genera".

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Genomics of *Rhodococcus*

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Contents

Abstract Members of the genus *Rhodococcus* have metabolic versatility and unique adaptation capacities to fluctuating environmental conditions, enabling the colonization of a wide variety of environments; they also play an important role in nutrient cycling and have potential applications in bioremediation, biotransformations and biocatalysis. *Rhodococcus* spp. are mainly distributed in soil, water and marine sediments, although some of them are also pathogens for humans, animals and plants. Consistent with the wide catabolic diversity, *Rhodococcus* spp. possess large and complex genomes (up to 10.1 Mbp), which contain a multiplicity of catabolic genes, high genetic redundancy of biosynthetic pathways and large catabolic plasmids, the latter encoding peculiar metabolic and physiological traits. Recently, the progress in sequencing technology led to a dramatic increase in the

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_2

number of sequenced *Rhodococcus* genomes, which have been investigated through diverse bioinformatic approaches. In particular, whole-genome comparative and genome-based functional studies were associated to *omic* technologies for the study of the global *Rhodococcus* cell response with the aim of providing insight into the genetic basis of specific catabolic capacities and phenotypic traits. Lastly, genome-based advances in *Rhodococcus* engineering led to the first design of molecular toolkits for tunable and targeted genome editing. Besides this, genome-based metabolic models were developed to make metabolic predictions of the *Rhodococcus* cell response to specific growth conditions. Both the synthetic and system approaches offered new opportunities for genome-scale rational design of *Rhodococcus* cell for environmental and industrial applications.

1 Introduction

The *Rhodococcus* genus comprises of Gram-positive, nonmotile, nonsporulating, aerobic bacteria, with a high G+C content and a mycolic acid-containing cell wall. Members of *Rhodoccocus* genus are genetically and physiologically diverse bacteria, widely distributed in soil, water and marine sediments; some *Rhodococcus* spp. are also pathogens for plants (*R. fascians*), animals and humans (*R. equi*). Members of *Rhodococcus* have also been recovered from extreme environments such as the deep sea, oil-contaminated soils and freeze-thaw tundra on glacial margins (Sheng et al. 2011; Shevtsov et al. 2013; Konishi et al. 2014).

Rhodococcus genus is featured by a broad metabolic versatility and environmental persistence supporting its clinical, industrial and environmental significance. In particular, *Rhodococcus* strains have peculiar degradative capacities towards a variety of organic compounds, including toxic and recalcitrant molecules like chlorinated hydrocarbons (Gröning et al. 2014; Cappelletti et al. 2017), herbicides (Fang et al. 2016), 4,4′-dithiodibutyric acid (DTDB) (Khairy et al. 2016) and dibenzothiophene (DBT) (Tao et al. 2011) as well as the ability to resist to various stress conditions (desiccation, radiation, heavy metals) (LeBlanc et al. 2008; Taketani et al. 2013; Cappelletti et al. 2016). They are also able to mediate a broad range of biotransformation, including enantioselective syntheses, and to produce biosurfactants, which facilitate the cell contact with hydrophobic substrates (Martìnkovà et al. 2009). Due to their metabolic flexibility and their tolerance to various stresses, they play an important role in nutrient cycling and have potential applications in bioremediation, biotransformations and biocatalysis.

Because of their biotechnological applications, the massive application of high-throughput genomic technologies has dramatically increased the number of sequenced *Rhodococcus* genomes, and a great effort has been directed towards the computational analysis of genomic data. Since the first complete genome (of *R. jostii* RHA1) published in 2006, an exponentially increasing number of genomes of *Rhodococcus* strains have been sequenced due to the progress of the sequencing

technologies and to the reduction of the DNA sequencing costs. In July 2018, 218 complete genomes of *Rhodococcus* are available in NCBI (www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=1827), most of them being available as draft genomes. Collectively, the analysis of genome sequences has provided insights into the genetic basis for diversity, plasticity and adaptation characteristic of *Rhodococcus* spp.

2 The *Rhodococcus* Genome: General Features and Structure

Rhodococcus genomes show high GC amount (61–71%) and range in size from around 4 Mb to over 10 Mb. In particular, the genome size seems to be at least partially dependent on the *Rhodococcus* strain lifestyle and niche complexity; indeed, the pathogenic *R. equi* has a substantially smaller genome than the soil-associated versatile biodegrader *R. jostii* RHA1 (9.7 Mb) and the other two environmental rhodococci, *Rhodococcus erythropolis* PR4 (6.9 Mb) and *Rhodococcus opacus* B4 (8.2 Mb) (Letek et al. 2010). Indeed, obligate pathogens live inside a host, reducing the need to adapt to sudden environmental changes and presence of competitors, which may contribute to genome minimization (Moran 2002).

Among *Rhodococcus* strains of the same species, the amount of GC in the genome was quite constant, showing the highest values (>70% GC-content) in *R. aetherivorans* and *R. ruber* species (Table 1). On the other hand, a remarkable variability was observed in the rhodococcal genome structure and plasmid presence.

Chromosomal and plasmid DNA of *Rhodococcus* strains is either linear or circular. Both *R. jostii* RHA1 and *R. opacus* B4 have linear chromosomes, while *R. erythropolis* PR4 and *R. equi* chromosomes are circular (Table 1). Interestingly, chromosome topology does not seem to correlate with taxonomy or phylogenetic relationship, as *R. equi* and *R. erythropolis* belong to different subclades, and the latter is considered the prototype of the "*erythropolis* subclade", which also includes *R. opacus* (Letek et al. 2010). Further, not all actinomycetales genera present linear chromosomes. *Streptomycetes* typically have linear chromosomes of very large size (>8.5 Mb), so linearization appears to have occurred independently in different actinobacterial lineages during evolution, apparently in association with increasing genome size (Bentley et al. 2002).

The co-existence of large linear and circular plasmids of different sizes within the same cell is also a characteristic of several actinomycetes genera; however, linear plasmids are mainly described in *Streptomyces* and *Rhodococcus* strains. The linearity of DNA molecules in *Rhodococcus* strains has been mainly associated to the presence telomere-like structures comprising of terminal inverted repeats (TIRs) and proteins bound to their 5′ ends (Kikuchi et al. 1985; Dib et al. 2015). This structure, named as invertron, has been detected in both chromosomes and plasmids of *Rhodococcus* and has been extensively studied in *Streptomyces* strains (Ventura

Table 1 Genomic features of *Rhodococcus* strains selected on the basis of the completeness of the genomes sequence and/or on the availability of functional studies[a]

Strain	WGS accession no. (NCBI)	Genome size (Mb)	Presence of plasmid DNA (count)	G+C content (%)	Total no. of genes (CDS)[c]	Main features
R. jostii RHA1	CP000431	9.7	Yes (3)	67.5	9256	PCB degrader, lipid accumulation
R. opacus B4	AP011115	8.83	Yes (5)	68	8227	Lipid accumulation
R. opacus PD630	CP003949	9.17	Yes (9)	67	8402	Hydrocarbon degrader, lipid accumulation
R. opacus R7	CP008947	10.12	Yes (5)	67	8621	Hydrocarbon degrader
R. opacus 1CP	CP009111	8.64	Yes (2)	67	7973	Enantioselective oxygenation of styrene
R. opacus M213	AJYC00000000	9.19	Yes (2)	67	8680	PAH degrader
R. erythropolis PR4	AP008957	6.89	Yes (3)	62	6491	Hydrocarbon degrader
R. erythropolis CCM2595	CP003761	6.37	Yes (1)	62.5	5895	Phenol degrader
R. erythropolis R138	CP007255–7	6.8	Yes (2)	62	6301	Quorum-sensing signal degrader and plant protection capacities
R. erythropolis DN1	AUZK00000000	6.55	–[b]	62	6331	Crude oil degrader
R. erythropolis BG43	CP011295	6.86	Yes (3)	62	6396	Quorum quencher
R. erythropolis XP	AGCF00000000	7.23	–	62	6826	Oil desulfurizer
R. erythropolis JCM 6824	BBLL00000000	7.02	–	62	6635	Antibiotic producer
R. erythropolis VSD3	MLKO00000000	6.55	–	62	6026	Hydrocarbon degrader and plant growth-promoting bacterium
R. aetherivorans BCP1	AVAE00000000	6.23	Yes (2)	70.3	5713	Hydrocarbon and chlorinated alkane degrader, metal-nanoparticle producer
R. aetherivorans IcdP1	CP011341	5.92	No	70.6	5388	High-molecular-weight and chlorinated hydrocarbon degrader
R. ruber P14	CP024315	5.52	No	70.5	5053	High-molecular-weight PAH degrader
R. ruber YYL	CP024890	5.59	Yes (2)	70.5	5200	Tetrahydrofuran degrader

R. ruber SD3	CP029146	5.37	No	70.6	4905	Phenol and chlorobenzene degrader
R. ruber Chol-4	ANGC00000000	5.46	–	70.4	4970	Steroid degrader
R. qingshengii djl-6-2	CP025959	6.70	Yes (2)	62	6332	Carbendazim degrader
R. qingshengii TUHH-12	JNCU00000000	7.43	–	62	6903	Crude oil degrader
R. wratislaviensis IFP 2016	ANIU00000000	9.69	–	67	9589	Diesel and gasoline oil additive degrader
R. wratislaviensis NBRC 100605	BAWF00000000	10.4	–	67	9528	–
R. pyridinivorans SB3094	CP006996	5.59	Yes (2)	68	5165	Methyl ethyl ketone degrader
R. pyridinivorans GF3	CP022915	5.3	No	68	4966	Anthraquinone dye degrader
R. rhodochrous ATCC 17895	ASJJ00000000	6.9	No	62	6609	Biocatalysis
R. rhodochrous NCTC 10210	LT906450	5.27	No	68	4668	–
R. rhodochrous J3	FXAV00000000	6.11	–	68	5639	–
R. rhodochrous DSM 43241	LRRK00000000	5.18	–	68	4790	Carotenoid producer
R. imtechensis RKJ300	AJJH01	8.23		67	7962	Nitrophenol degrader
R. equi 103S	FN563149	5.04	Ye (1)	69	4649	Pathogen for animals
R. equi ATCC 33707	CM001149	5.26	No	69	4899	Pathogen for humans
R. equi DSSKP-R-001	CP027793	5.25	Yes (2)	69	5118	Pathogen for animals
R. fascians D188	CP015235	5.5	Yes (2)	65	5149	Phytopathogen
R. fascians NBRC 12155 = LMG 3623	JMEN00000000	5.77	–	64	5433	Phytopathogen
R. fascians A44A	JMEX00000000	5.95	–	64.5	5566	Phytopathogen

(continued)

Table 1 (continued)

Strain	WGS accession no. (NCBI)	Genome size (Mb)	Presence of plasmid DNA (count)	G+C content (%)	Total no. of genes (CDS)[c]	Main features
Rhodococcus sp. AD45	CM003191	6.79	Yes (1)	62	6252	Isoprene degrader
Rhodococcus sp. DK17	AJLQ00000000	9.11	Yes (3)	67	8615	Aromatic hydrocarbon degrader

[a]Genome data are collected from NCBI genome database
[b]"–" stands for not determined
[c]CDS = coding sequence

et al. 2007). Most commonly, both ends of linear plasmids are the same or featured by only few mismatches, giving recognizable terminal inverted repeats (TIRs). For instance, plasmid pHG207 of *R. opacus* MR22 carries an imperfect terminal inverted repeat (TIR) of 583/560 bp (Kalkus et al. 1993). The RHA1 replicons have been described as typical actinomycete invertrons, containing two sets of inverted repeats flanking the GCTXCGC central motif with covalently associated proteins (Warren et al. 2004). The chromosomal inverted repeats (of around 10 Kp in RHA1) are much longer than those of the plasmids (e.g. the pRHL1 telomeres are 500 bp). The RHA1 telomeric inverted repeats are similar to those of *Streptomycetes*, particularly over the first 300 bp. The replication of linear *Streptomyces* chromosomes and plasmids is initiated from a fairly centrally located replication origin rich in DnaA box sequences and proceeds bidirectionally towards the telomeres. The telomeres themselves are replicated by a mechanism that includes priming from the terminal proteins covalently bound to the 5′ ends. It has been proposed that linear plasmids evolved from bacteriophages and that linear chromosomes originated from single-crossover recombination between an initially circular chromosome and a linear plasmid (McLeod et al. 2006; Ventura et al. 2007).

2.1 *Genomic and Metabolic Traits of Reference* Rhodococcus *Strains*

The analysis of single bacterial genomes and its annotation confer a wide range of knowledge in relation to the general genome features and chromosomal structure but also in relation to identification of specific genes in a genome and the detection of regions containing functionally connected genes. Although most of them are still in a draft version, the sequencing of individual *Rhodococcus* genomes has often provided important inputs for wet-lab experiments aimed at investigating the relevant metabolic capacities shown by the isolates.

Table 1 reports the genome features of representative *Rhodococcus* strains belonging to different species featured by specific metabolic and physiological traits.

Members of *Rhodococcus jostii*, *R. opacus* and *R. wratislaviensis* species possess genomes of very large size, ranging from 8.5 to over 10 Mb (Table 1). *R. jostii* RHA1 is considered the model of the genus as its genome was the first completely sequenced from a *Rhodococcus* strain. *R. jostii* RHA1 was isolated from lindane-contaminated soil (McLeod et al. 2006) and was widely investigated for the strong ability to degrade a wide variety of aromatic compounds and polychlorinated biphenyls (PCBs), for lipid accumulation and for stress condition resistance (Gonçalves et al. 2006; LeBlanc et al. 2008; Patrauchan et al. 2012; Costa et al. 2015).

R. opacus R7 and *R. wratislaviensis* NBRC 100605 have the largest *Rhodococcus* genomes described up to date (10.1 and 10.4 Mbp, respectively) and two of the largest bacterial genome reported in the literature. While NBRC 100605 metabolic

features have been scarcely investigated (Guo and Wu 2017), *R. opacus* R7 has been thoroughly studied considering both the broad degradative abilities towards aromatic and aliphatic hydrocarbons and the associated genome features (Di Gennaro et al. 2001, 2010; Zampolli et al. 2014; Orro et al. 2015; Di Canito et al. 2018). Based on the terminal sequence signatures defining the possible linearity of replicons, R7 possesses one linear chromosome and additional five linear replicons, ranging in size from 20 Kb to 430 Kb (Tables 1 and 2) (Di Gennaro et al. 2014). Other *Rhodococcus opacus* strains described from a genomic point of view have broad organic compound degradative abilities and capacity to produce and accumulate lipids for possible biodiesel application. In particular, *R. opacus* PD630 is the most studied bacterium for its ability to produce and accumulate lipids (mostly triacylglycerols, TAGs) using different carbon sources (Alvarez et al. 1996; Castro et al. 2016). Indeed, it was shown to accumulate significant amounts of lipids, namely, 76 and 87%, of its cellular dry weight, when grown on gluconate and olive oil, respectively (Alvarez et al. 1996; Voss and Steinbuchel 2001). *R. opacus* B4 was the first *R. opacus* with a completely sequenced genome; it was described to be able to transform and degrade several types of hydrocarbons, to stabilize water-oil phases and to accumulate TAG (Na et al. 2005; Honda et al. 2008; Sameshima et al. 2008). The analysis of *R. opacus* B4 genome revealed an 8.8-Mb-long genome composed by one linear chromosome, two linear plasmids of pROB series of size range of 2–4 Kb and three circular plasmids of pKNR series of size range of 110–550 Kb (Table 2).

Members of *R. erythropolis* are largely distributed in the environment and exhibit a remarkable metabolic versatility related to their capacity to degrade complex compounds, such as quorum-sensing signals N-acylhomoserine lactones, phenols, sterols and fuel derivatives (Table 1). Moreover, many *R. erythropolis* strains have been isolated from petroleum-contaminated sites and have been described for their ability to degrade various hydrocarbons using multiple catabolic pathways (de Carvalho and da Fonseca 2005). All the *R. erythropolis* strains whose complete genome has been sequenced showed the presence of plasmids ranging in length from 3 Kb to 261 Kb (Table 2).

The genomes of *R. aetherivorans* and *R. ruber* species showed the highest amount of GC, being around 70% in all the strains described up to date. Members of these two species have been described for different biosynthetic and biodegradative capacities. *R. aetherivorans* BCP1 (formerly *Rhodococcus* sp. BCP1) is able to degrade a wide range of alkanes, naphthenic acids and chlorinated alkanes (Frascari et al. 2006; Cappelletti et al. 2011, 2015; Presentato et al. 2018a; Ciavarelli et al. 2012) and to produce metal-based nanostructures (Presentato et al. 2016, 2018b, c). BCP1 has a genome of 6.2 Mbp composed by one chromosome and two plasmids (of 100–120 Kb size) (Tables 1 and 2). *R. aetherivorans* IcdP1 was isolated from an abandoned coking plant; it was proven to be able to degrade numerous high-molecular-weight polycyclic aromatic hydrocarbons and organochlorine pesticides (Qu et al. 2015). Although their genome data are not available, *R. aetherivorans* IAR1 was described to accumulate polyhydroxybutyrate PHB, while *R. aetherivorans* IG24 was employed by

Table 2 Details on the plasmid content of reference *Rhodococcus* genomes from Table 1[a]

Strain	WGS accession no. (NCBI)	Replicon	Size (Mbp)	Topology	G+C content (%)	Total no. of genes (CDS)[c]
R. jostii RHA1	CP000431	Chromosome	7.8	L	67.5	7262
	CP000432	pRHL1	1.12	L	65	1186
	CP000433	pRHL2	0.44	L	64	474
	CP000434	pRHL3	0.33	L	65	334
R. opacus B4	AP011115	Chromosome	7.91	L	68	7287
	AP011118	pKNR	0.004	C	65	7
	AP011119	pKNR01	0.002	C	64	2
	AP011120	pKNR02	0.55	C	64	598
	AP011116	pROB01	0.24	L	66	232
	AP011117	pROB02	0.11	L	64	101
R. opacus PD630	CP003949	Chromosome	9.17	C	67	7715
	CP003950	Plasmid 1	0.17	C	65	166
	CP003951	Plasmid 2	0.09	C	64	95
	CP003952	Plasmid 3	0.08	L	65	75
	CP003953	Plasmid 4	0.06	L	65	63
	CP003954	Plasmid 5	0.09	L	66	91
	CP003955	Plasmid 6	0.04	L	66	35
	CP003956	Plasmid 7	0.04	L	66	36
	CP003957	Plasmid 8	0.16	L	64	142
	CP003958	Plasmid 9	0.04	L	65	30
R. opacus R7	CP008947	Chromosome	10.1	L	67	7353
	CP008948	pPDG1	0.65	L	65	486
	CP008949	pPDG2	0.43	L	64	310
	CP008950	pPDG3	0.35	L	66	307
	CP008951	pPDG4	0.19	L	65	148
	CP008952	pPDG5	0.02	L	63	17
R. opacus 1CP	CP009111	Chromosome	8.63	C	67	7101
	CP009112	pR1CP1	0.88	L	64	815
	CP009113	pR1CP2	0.06	L	64	57
R. erythropolis PR4	AP008957	Chromosome	6.89	C	62	6092
	AP008932	pREC1	0.10	C	63	104
	AP008933	pREC2	0.003	C	62	5
	AP008931	pREL1	0.27	L	62	290
R. erythropolis CCM2595	CP003761	Chromosome	6.37	C	62.5	5793
	CP003762	pRECF1	0.09	C	62	102
R. erythropolis R138	CP007255	Chromosome	6.8	C	62	5759
	AP008932	pCRE138	0.09	C	61	7
	AP008933	pLRE138	0.48	L	60	33
R. erythropolis BG43	CP011295	Chromosome	6.33	C	62	5875

(continued)

Table 2 (continued)

Strain	WGS accession no. (NCBI)	Replicon	Size (Mbp)	Topology	G+C content (%)	Total no. of genes (CDS)[c]
	CP011296 CP011297 CP011298	pRLCBG43 pRLLBG43 pRSLBG43	0.24 0.26 0.03	C C C	60 61 59	225 273 23
R. aetherivorans BCP1	CM002177	Chromosome	6.2	L	70.3	5506
	CM002178 CM002179	pBMC1 pBMC2	0.12 0.10	L L	69 65	108 99
R. ruber YYL	CP024890	Chromosome	5.59	C	70	5200
	CP024892 CP024891	pYYL1.1 pYYL1.2	0.24 0.09	C L	67 66	206 91
R. qingshengii djl-6-2	CP025959	Chromosome	6.52	C	62	6154
	CP025960 CP025961 CP025962	pDJL1 pDJL2 pDJL3	0.08 0.08 0.02	C C C	62 62 61	87 73 18
R. pyridinivorans SB3094	CP006996	Chromosome	5.23	C	68	4818
	CP006997 CP006998	Unnamed Unnamed2	0.36 0	C C	65 66	344 3
R. equi 103S	FN563149	Chromosome	5.04	C	69	4649
	FN563149	pVAPA1037	0.08	C	65	73
R. equi DSSKP-R-001	CP027793	Chromosome	5.25	C	69	4872
	CP027794 CP027795	Plasmid 1 Plasmid 2	0.09 0.1	C C	69 66	151 95
R. fascians D188	CP015235	Chromosome	5.14	C	65	4808
	CP015236 CP015237	pFiD188 Unnamed	0.2 0.16	L C	62 64	176 165
R. sp. AD45	JYOP	Chromosome	6.45	C	62	5949
	JYOP	Unnamed	0.34	C	61	303
R. sp. DK17	AJLQ01	Chromosome	9.1	–[b]	67	–
	AJLQ01	pDK1 pDK2 pDK3	0.38 0.33 0.75	– –	– –	– –

[a]Genome data are collected from NCBI genome database
[b]"–" stands for not determined
[c]CDS = coding sequence

Merck Corporation for the production of the anti-HIV drug Crixivan™ (Treadway et al. 1999; Hori et al. 2009).

Rhodococcus pathogenic strains belong to *R. equi* and *R. fascians* species, which cause disease to animals and plants, respectively. In line with their lifestyle inside a

host, their genomes are generally smaller (<6 Mb) compared to the environmental *Rhodococcus* strains (Table 1). Recently, numerous genomes of both these two species have been sequenced to conduct phylogenomic analyses and to investigate on the evolutionary mechanisms of virulence traits (Letek et al. 2010; Creason et al. 2014a, b). In particular, virulence genes have been associated to plasmids. Several *R. equi* strains have been described to possess circular plasmids, whereas *R. fascians* members are featured by linear plasmids. The presence of linear plasmids in *R. fascians* has been correlated with phytopathogenicity, although it was described not to be strictly necessary (Creason et al. 2014a).

Compared to the other species, only few genomic studies are available for *R. qingshengii*, *R. pyridinivorans* and *R. rhodochrous.* Nevertheless, members of these species have been reported to possess peculiar metabolic activities such as degradative capacity towards rubber, fungicide and other hydrocarbon-related complex molecules. In relation to this, additional genome sequencing efforts promise to expand the knowledge on the genetic and metabolic diversity of *Rhodococcus* bacterial strains (Table 1) (Xu et al. 2007; Dueholm et al. 2014; Watcharakul et al. 2016).

3 Comparative Genomics of *Rhodococcus*

Comparative genomics allow providing genomic basis for the differences observed among microorganisms in terms of phenotypes, metabolic capacities and cellular response to specific environmental conditions. Recent studies have described genome-wide comparative analyses of *Rhodococcus* strains with the aim of defining the phylogeny and evolutionary relationship, determining the *Rhodococcus* genus core genome, analysing their catabolic potentials and stress response (Orro et al. 2015; Cappelletti et al. 2016). Genome comparative analysis is performed through pairwise or multiple (>3 genomes) whole-genome alignments. Among the applicable programs, Artemis Comparison Tool (ACT), available within the Integrated Microbial Genomes (IMG) system, was used for genome comparison of strain *R. opacus* M213 with different *Rhodococcus* strains (Pathak et al. 2016). Mauve (http://darlinglab.org/mauve/mauve.html) performs multiple sequence alignment and has been used for comparative genomic analyses of different *Rhodococcus* strains, e.g. *R. opacus* R7 and *R. aetherivorans* BCP1 (Orro et al. 2015), *R. opacus* M213 (Pathak et al. 2016) and *Rhodococcus fascians* strains (Creason et al. 2014b) (Fig. 1).

Comparative genome analysis allows to detect genomic regions that are conserved or syntenic (Pathak et al. 2016), representing conserved genome sections that have not undergone internal rearrangements and inversions. This conservation typically reflects phylogenetic correlation and represents the indication of functional evolutionary constraints. In comparative studies concerning *Rhodococcus*, the conservation of collinear blocks in different *R. fascians* strains was shown to be in accordance with their phylogenetic relationship (Creason et al. 2014b; Pathak et al.

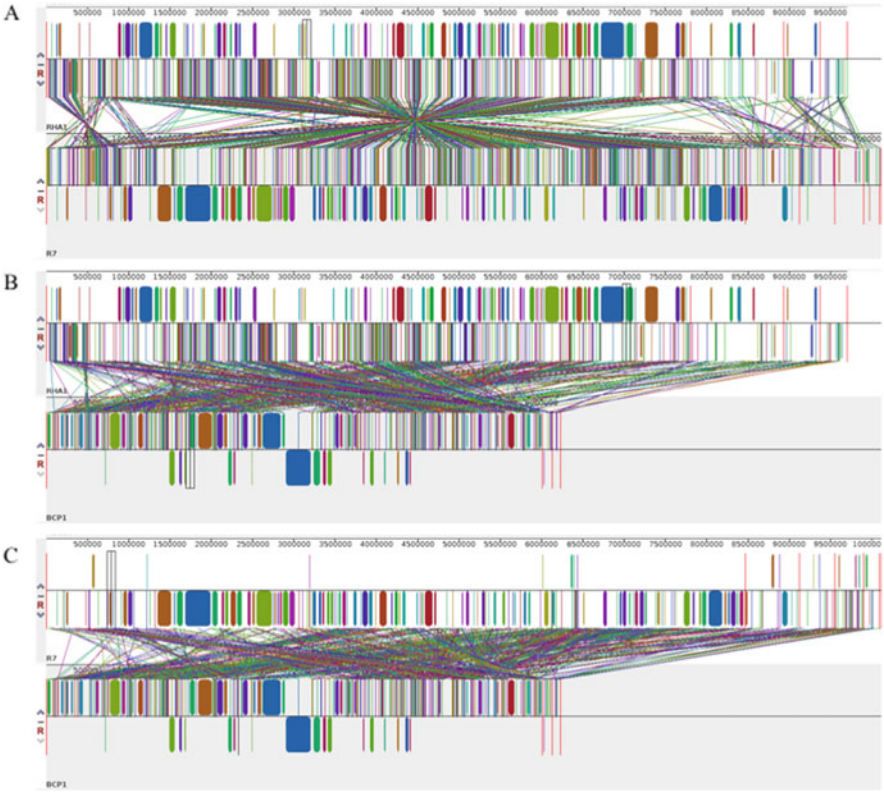

Fig. 1 Whole-genome sequence alignment with program Mauve diagram. The alignment of three pairs of genomes is shown: *R. jostii* RHA1 and *R. opacus* R7 (panel A), *R. jostii* RHA1 and *R. aetherivorans* BCP1 (panel B), *R. opacus* R7 and *R. aetherivorans* BCP1 (panel C). The coloured region represents a block of sequences that is collinear to a corresponding block of sequences in the other genome sequence. [Modified from Orro et al. (2015)]

2016). The 16S rRNA-based affiliation of *Rhodococcus* sp. M213 to *opacus* species was challenged by the higher M213 syntenic affiliation with *R. jostii* RHA1 compared to *R. opacus* strains B4 and PD630 (Pathak et al. 2016).

Another useful way to compare whole genomes is by computing a value that summarizes their similarity or distance. The average nucleotide identity (ANI) value calculated from whole-genome alignments was described to provide a tool for taxonomy and evolutionary studies and species definition (Creason et al. 2014b; Anastasi et al. 2016). Whole-genome alignment analysis of three *Rhodococcus equi* strains allowed to detect single nucleotide polymorphisms (SNPs) in specific coding regions that were suggested to possibly reflect differences in the lifestyle of these three isolates (Sangal et al. 2014).

In addition to gene/protein sequence-based alignment, gene content comparison can be done in terms of function using, for instance, the concept of functional

categories (available in RAST subsystem) or COG (cluster of orthologous groups of proteins). Typically, statistical tools analyse gene functions (or annotations) as categorized by COGs and determine whether certain functions or functional categories are statistically enriched in certain genomes compared to others. In this respect, the *R. opacus* M213 genome analysis at functional (COG) level further reinforced the closer relationship of M213 with *R. jostii* RHA1 and other *Rhodococcus* strains (e.g. *R. wratislaviensis* IFP 2016, *R. imtechensis* RKJ300, *Rhodococcus* sp. strain DK17) compared to *R. opacus* strains (Pathak et al. 2016). On the other hand, by comparing the RAST-based functional categories, *R. opacus* R7 genome resulted to be enriched in genes belonging to all the functional categories, compared to *R. aetherivorans* BCP1, except for those involved in some central metabolic process (e.g. DNA, iron, potassium and phosphorous metabolism, dormancy and sporulation, motility and chemotaxis) (Orro et al. 2015).

3.1 Phylogenomics

The use of the only 16S rRNA sequence as taxonomic marker was proved to limit the resolution of *Rhodococcus* phylogenetic studies (Gürtler et al. 2004; Pathak et al. 2016; Creason et al. 2014b) and more in general to lead to artefacts in the bacterial phylogenetic tree construction (Ventura et al. 2007). More recent phylogenetic investigations have been carried out using alternate gene/protein sequences (e.g. functional genes or molecular chaperone-encoding genes) as phylogenetic markers, which often evolve faster than the rRNA operon. This provides a larger phylogenetic resolution that can help to distinguish closely related bacterial species. For this purpose, the alkane monooxygenase (*alkB*) gene was applicable as phylogenetic marker of *Rhodococcus* strains (Táncsics et al. 2015).

The availability of genome sequences has provided new opportunities to get information on applicable phylogenetic markers, and new approaches arose to examine rhodococcal phylogeny based upon multiple gene or protein sequences. Among these approaches, the construction of phylogenetic trees based on concatenated sequences for large numbers of proteins (also called multilocus sequence analysis—MLSA) has proven particularly useful for evolutionary relationship studies (Gao and Gupta 2012). Phylogenetic tree based upon multiple conserved (or slow-evolving) genes/proteins was demonstrated to properly assign environmental isolates to existing *Rhodococcus* species (Orro et al. 2015) and to resolve evolutionary relationships of *Rhodococcus* strains belonging to the same species (Creason et al. 2014b; Letek et al. 2010). MLSA mainly employed sequence concatenamers of housekeeping genes including 16S rRNA, *recA*, *gyrB*, *rpoB*, *rpoC* and *secY* (Orro et al. 2015; Kwasiborski et al. 2015). Orro et al. (2015) assigned the species to two *Rhodococcus* strains on the basis of MLSA with four of these marker genes (Fig. 2). MLSA provided a framework for defining *Rhodococcus* genus within the *Actinobacteria* phylum using seven marker genes (*ftsY*, *infB*, *rpoB*, *rsmA*, *secY*, *tsaD* and *ychF*) (Creason et al. 2014b). In line with the whole-genome based studies

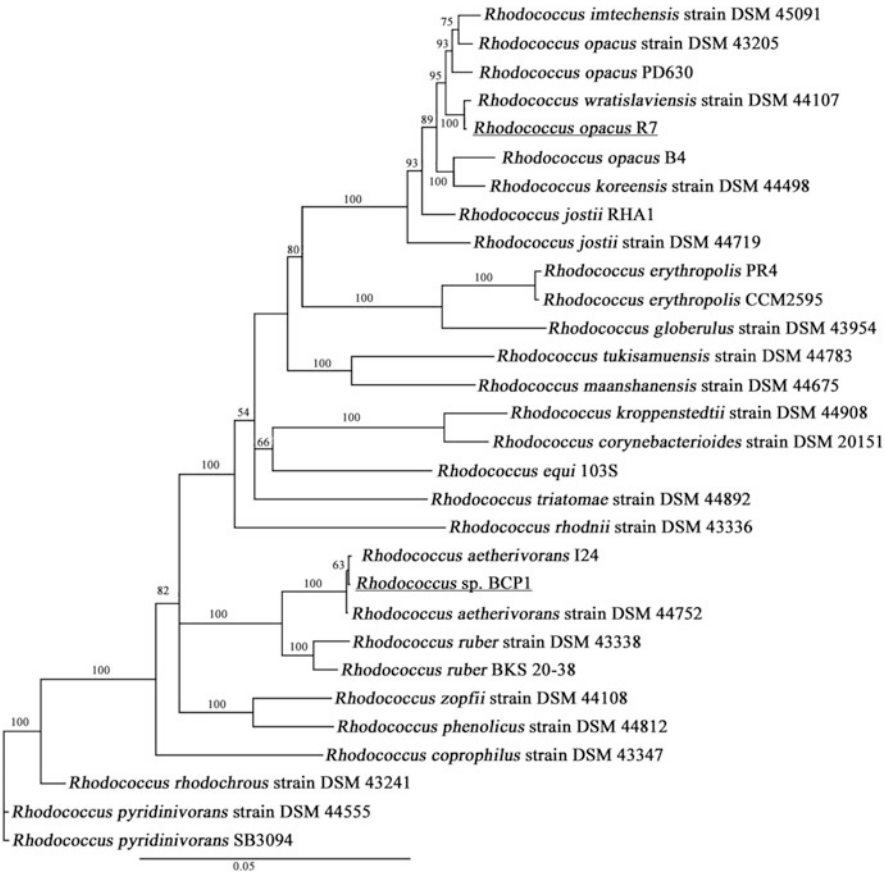

Fig. 2 Multilocus sequence analysis-based tree of 28 *Rhodococcus* strains. Phylogenetic tree of 28 *Rhodococcus* strains based on MLSA using the sequence of the four marker genes, 16S rRNA, *secY*, *rpoC* and *rpsA*. [Modified from Orro et al. (2015)]

by Letek et al. (2010), this study placed the *R. equi* species within the *Rhodococcus* genus, which was in contrast with other inconclusive phylogenetic studies based on 16S rDNA sequences (Goodfellow et al. 1998). More genome sequences and informative marker sequences might help in improving MLSA-mediated taxonomy and discern the finer details within the suborder. In this respect, available genome sequences were used to discover novel molecular characteristics that could be used for evolutionary and systematic studies of *Rhodococcus* genus. In this regard, novel specific conserved signature indels (CSIs) and conserved signature proteins (CSPs) have been proposed as possible molecular markers on the basis of their conservation among different *Rhodococcus* species (Gao and Gupta 2012).

In addition to phylogenomic analysis based on the concatenation of different taxonomic markers, *Rhodococcus* genome-based trees have been constructed on the basis of average sequence similarity. The genome similarity is at the basis of the

wet-lab DNA-DNA hybridization (DDH) technique, which represents the gold standard for bacterial taxonomy classification (Richter and Rosselló-Móra 2009). In the era of genomics, different attempts have been made to replace the traditional wet-lab DDH with in silico genome-to-genome comparison (Goris et al. 2007; Richter and Rosselló-Móra 2009). Among these, the average nucleotide identity (ANI) estimates the average nucleotide identity between two genomic datasets, and an ANI value in the range of 94–96% has been proposed to be the criterion for bacterial species delineation (corresponding to a wet-lab DDH value of 70%), while ANI values of 70–75% identified members of the same genus (Konstantinidis and Tiedje 2005; Goris et al. 2007; Richter and Rosselló-Móra 2009). Creason et al. (2014b) conducted phylogeny study of *Rhodococcus* genus by using the average nucleotide identity (ANI) approach and found that the genus *Rhodococcus* can be represented by as many as 20 distinct species (Fig. 3).

Using 27 de novo sequenced *R. equi* genomes, the ANI value was 99.13%, well above the consensus 95–96% threshold for prokaryotic species definition (Anastasi et al. 2016). This result highlighted the high genetic homogeneity of *R. equi* group and was found to correspond to a 16S rRNA sequence identity of 100%.

3.2 Core and Accessory Genome

Whole-genome comparative analysis can be used to obtain information on the pan genome, core genome and accessory genome of a group of bacterial strains belonging to a single species or different species belonging a single genus. The pan genome comprises all the genes present in a given bacterial group (the collection of all genetic material). The core genome is defined as the set of all genes shared by the genomes as orthologs and therefore considered as conserved by all the bacterial strains under analysis (Ventura et al. 2007). The genes included within the core genome are supposed to have strong functional significance for cell physiology and replication. Moreover, as the core genome can be considered to define the members of the bacterial group under analysis, the core genes are suitable as molecular targets to infer phylogeny (Ventura et al. 2007). On the other hand, the accessory or variable genome is represented by the genes, which are confined to a single member of the bacterial group and are therefore specific for a single strain (Ali 2013). The accessory genome therefore represents those gene families that can be associated with phenotypic traits that differentiate each member within a given bacterial population under analysis.

Comparative genomic studies on *Rhodococcus* strains have assessed the core genome shared by either strains belonging to the same species or strains belonging to different species. The resulting core-genome size and identity reported in each work was dependent on the *Rhodococcus* strains under analysis. Within the *Rhodococcus* genome set composed by *R. opacus* R7, *R. aetherivorans* BCP1, *R. jostii* RHA1, *R. opacus* PD630, *R. opacus* B4 and *R. pyridinivorans* SB3094, the core genome corresponded to around 50% of all the identified CDSs in each strain (Orro et al.

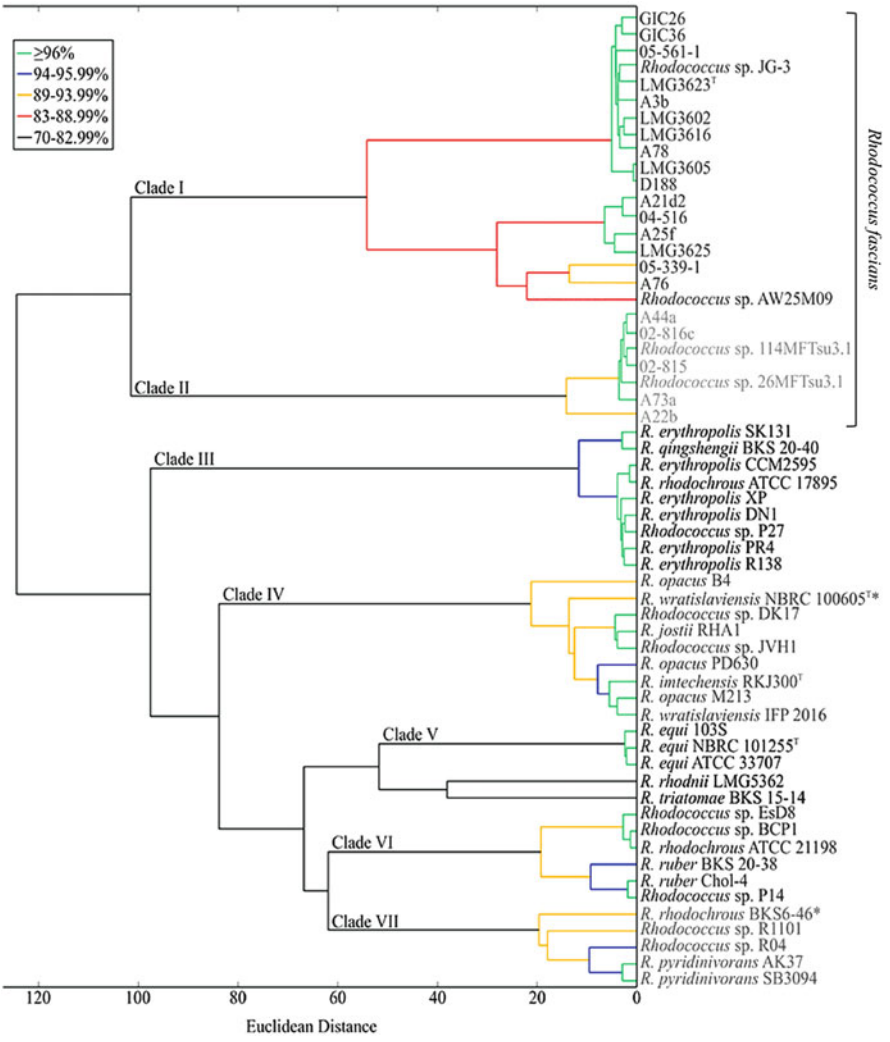

Fig. 3 Average nucleotide identity (ANI) dendrogram for 59 isolates of *Rhodococcus*. Complete genome sequences for 59 isolates of *Rhodococcus* were used to generate an ANI matrix. The matrix was used to calculate an ANI divergence dendrogram. Branches are coloured based on the ANI threshold values. [Modified from Creason et al. (2014b)]

2015) (Fig. 4a). This indicated a large genetic variability among these *Rhodococcus* strains, some of them belonging to different species. The portion of core genes increased by considering *Rhodococcus* strains belonging to the same species, such as *R. opacus* R7, B4 and PD630 (Fig. 4a) (Orro et al. 2015), *R. erythropolis* (Kwasiborski et al. 2015) and *R. equi* (Anastasi et al. 2016). In particular, the genome of *R. erythropolis* R138 was compared to the genomes of other ten

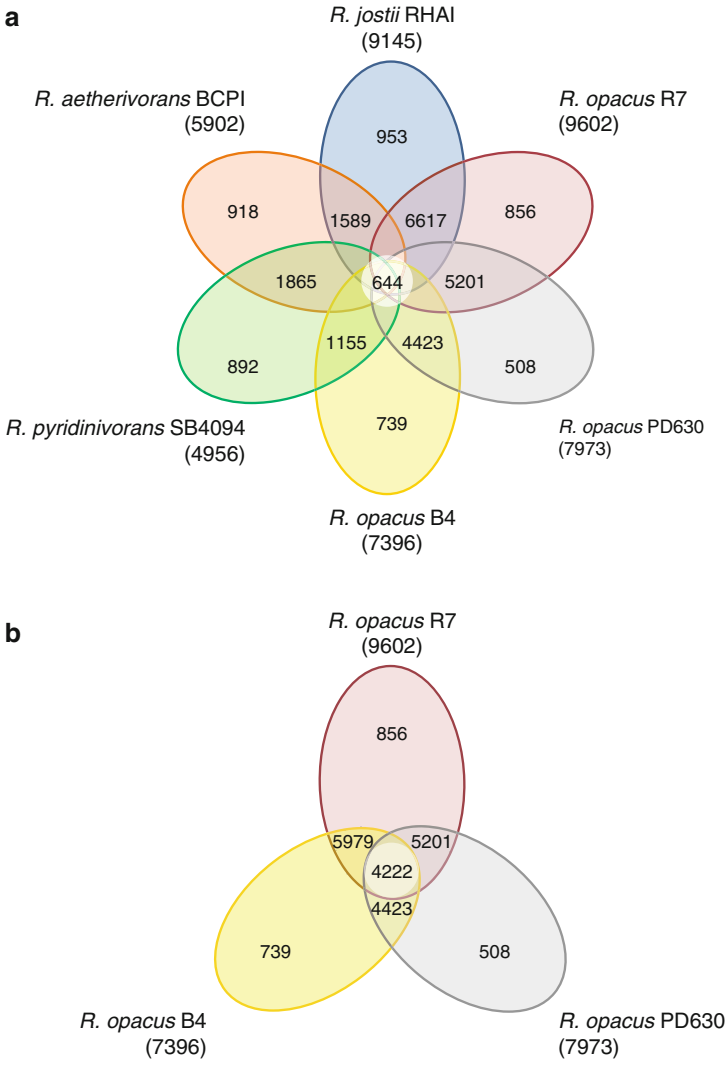

Fig. 4 Venn diagram displaying the core-genome and the unique genes (accessory genome) specific to each strain. The number of genes shared by all strains included within each genome set (i.e. the core genome) is in the centre. Numbers in nonoverlapping portions of each oval show the number of genes unique to each strain. The total number of protein coding genes found in each genome is listed below the strain name. Panel (A), core-genome and unique genes resulting from the comparison of the genomes from *R. opacus* R7, *R. aetherivorans* BCP1, *R. jostii* RHA1, *R. opacus* PD630, *R. opacus* B4, *R. pyridinivorans* SB4094; panel (B), core-genome and unique genes resulting from the comparison of the genomes from *R. opacus* R7, *R. opacus* PD630 and *R. opacus* B4

R. erythropolis strains, either available in the database or newly sequenced. The core-genome genes represented up to 87% of all the CDSs identified in *R. erythropolis* R138. A large core genome was also identified in a comparative study with 29 *R. equi* strains, which showed to have ~80% of shared genes in their genome (Anastasi et al. 2016). Some of these comparative studies also found that the core genes were mainly localized on the chromosome, whereas the unique genes (accessory genome) were harboured by the endogenous plasmids in each strain (Orro et al. 2015; Kwasiborski et al. 2015). For instance, considering *R. erythropolis* strains, up to 97% of the shared sequences were located on the circular chromosome of R138 strain (Kwasiborski et al. 2015). These results are general indications of a core set of functions localized on the *Rhodococcus* chromosome, whereas plasmids have variable genetic contents. The variable accessory genome underlies the peculiar capacities of each *Rhodococcus* strain and contributes functions for niche adaptation (Kwasiborski et al. 2015). In line with this, considering *R. equi* strains, the core genome included traits necessary for bacterial physiology, virulence and niche adaptation, including tolerance to desiccation and oxidative stress (Anastasi et al. 2016). Similarly, *R. erythropolis* core genome was highly represented by gene categorized within the primary metabolism of amino acids, carbohydrates, cofactors and proteins (Kwasiborski et al. 2015). Interestingly, genes encoding enzymes involved in the degradation of complex molecules (i.e. N-acylhomoserine lactones, phenol, catechol and sterol derivatives) were also shared by the 11 *R. erythropolis* under analysis by Kwasiborski et al. (2015). On the other hand, functions encoded by unique genes (accessory genome) were associated to degradative enzymes in *R. opacus* M213 (Pathak et al. 2016), *R. aetherivorans* BCP1 and *R. opacus* R7 (Orro et al. 2015). In particular, *R. aetherivorans* BCP1 possessed unique genes involved in short-chain *n*-alkanes degradation which were not present in the other *Rhodococcus* strains under analysis (Orro et al. 2015) Conversely, *R. opacus* R7 genome had a high number of unique genes involved in aromatic degradation metabolism. These genetic findings generally correlated with the different types of contaminants persisting in each *Rhodococcus* strain isolation source. (Di Gennaro et al. 2001; Frascari et al. 2006; Orro et al. 2015).

3.3 Functional Genomics

Genome sequence-based analysis relies on homology-based gene annotation and protein domain identification to annotate open reading frames (ORFs) and to perform functional predictions. Bioinformatic tools have been applied for the functional analysis of *Rhodococcus* genomes to determine the genetic basis possibly involved in relevant metabolic activities and phenotypic traits, e.g. degradation abilities of specific xenobiotics, plant growth-promoting effect and stress tolerance (Francis et al. 2016; Cappelletti et al. 2016; Pathak et al. 2016). For instance, genome-based analyses assessed the occurrence and distribution of key genes and pathways involved in the synthesis and accumulation of triacylglycerols (TAG), wax esters,

polyhydroxyalkanoates, glycogen and polyphosphate (Hernández et al. 2008; Villalba et al. 2013). In line with the extensive experimental work demonstrating the ability of *Rhodococcus* strains to synthesize and accumulate neutral lipids, numerous genes/enzymes predicted to be involved in TAG biosynthesis and degradation and fatty acid β-oxidation were identified in the genomes of *R. jostii* RHA1, *R. opacus* PD630, *R. opacus* B4, *R. erythropolis* PR4, *R. equi* 103S and *R. fascians* F7 (Villalba et al. 2013). The comparative genome analysis of psychrophilic *Rhodococcus* sp. JG3 and other mesophilic rhodococci allowed to detect the cold adaptive traits which confer JG3 with the ability to survive the extremely arid, cold and oligotrophic conditions of permafrost (Goordial et al. 2016). A bioinformatic analysis of the genomes of 20 *Rhodococcus* strains allowed identifying numerous biosynthetic gene clusters encoding pathways involved in the possible production of novel secondary metabolites (Ceniceros et al. 2017).

Several works have assessed a large range of metabolic abilities of strains using Phenotype Microarray (PM) technologies. PM results were interpreted using genomic analysis to predict the genes involved in central metabolic pathways, xenobiotic degradation metabolisms and stress response (Letek et al. 2010; Holder et al. 2011; Orro et al. 2015; Cappelletti et al. 2016).

The large-scale functional analysis of *Rhodococcus* genomes has been further integrated with the results obtained through *omic* technologies. In particular, high-throughput experiments were conducted to obtain global datasets on the expression of genes (transcriptomic analyses through RNA-seq or microarray) and on the protein profiles induced under specific growth conditions or after specific cell treatments. Transcriptomic studies were performed to define the genes involved in the degradation of aromatic compounds and steroids (chlorate and cholesterol) (Gonçalves et al. 2006; van der Geize et al. 2007; Swain et al. 2012), of PCBs and diesel oil (Puglisi et al. 2010, Laczi et al. 2015) and of synthetic polymers (Gravouil et al. 2017) but also in lipid metabolism and accumulation (Chen et al. 2014; Amara et al. 2016) and isoprene metabolism (Crombie et al. 2015). Proteome studies were combined with genome analysis for the study of the degradation of xenobiotic 4,4-dithiodibutyric acid (DTDB) by *R. erythropolis* MI2 (Khairy et al. 2016) and the metabolism of short-chain alkanes in *R. aetherivorans* BCP1 (Cappelletti et al. 2015) and to study the global response to stress conditions such as desiccation and carbon starvation in *R. jostii* RHA1 (LeBlanc et al. 2008; Patrauchan et al. 2012). In many of these studies, the involvement of multiple homologous genes was identified in specific metabolic pathways, providing clues to the redundancy of the different catabolic pathways in *Rhodococcus* (Gonçalves et al. 2006; Swain et al. 2012). Further, some omic studies gave indications on the involvement of possible regulators in the specific catabolic pathways under analysis (Crombie et al. 2015). In *R. ruber* C208, transcriptomic results indicated the involvement of alkane degradation pathway and β-oxidation of fatty acids as the main catabolic route for polyethylene degradation; further, it also indicated metabolic limiting steps which could represent molecular target to optimize the biodegradation process (Gravouil et al. 2017). In addition to catabolic genes/proteins, many genes/proteins involved in stress response (e.g. chaperone-like proteins, superoxide dismutase, catalase/

peroxidase) were reported to be induced during the degradation of organic compounds (Tomás-Gallardo et al. 2006; Puglisi et al. 2010; Khairy et al. 2016). The microarray analysis of *R. aetherivorans* I24 cells exposed to PCBs reported the transcriptional response to be primarily directed towards reducing oxidative stress rather than catabolism (Puglisi et al. 2010).

4 The Genomic Basis of Metabolic Versatility in *Rhodococcus*

The extraordinary metabolic versatility of *Rhodococcus* strains is reflected in their genomic attributes, like (1) large genome sizes (up to 10 Mbp) encoding numerous catabolic pathways for a variety of chemical compounds; (2) a significant degree of gene redundancy that ensures functional robustness and free-to-evolve genetic reservoirs; and (3) the occurrence of circular and linear (mega)plasmids that generally evolve more rapidly than the chromosome and consist of an additional pool of DNA that can evolve and can be easily transferred (Redenbach and Altenbuchner 2002; Van der Geize and Dijkhuize 2004). In this context, the broad *Rhodococcus* adaptability is related to their genome "flexibility" which refers to the genomic rearrangements mainly occurring on the large linear plasmids and due to still mostly unknown molecular mechanisms promoting the frequent non-homologous illegitimate recombination. This last aspect has been discussed in several review articles by Larkin and collaborators (Larkin et al. 1998, 2010; Kulakov and Larkin 2002). Additionally, recent works have underlined how the equipment of genes for the transport of many different substrates (de Carvalho et al. 2014) and the numerous genes encoding oxygenases, typically catalysing the hydroxylation and cleavage of organic compounds, are at the basis of the wide metabolic capacities of *Rhodococcus* strains. Only a few portion of the oxygenase genes have been acquired through horizontal gene transfer events, while most of them were chromosomally located, suggesting their fundamental role in *Rhodococcus* physiology (Mcleod et al. 2006).

4.1 Catabolic Gene Redundancy

Since the first complete genome sequence obtained from a *Rhodococcus* strain, the presence of multiple catabolic genes encoding homologous enzymes, called isozymes, attracted research interest. Recently, the analysis of whole-genome sequences of several rhodococci confirmed the redundancy in catabolic pathways initially only hypothesized for this genus (McLeod et al. 2006). Catabolic gene redundancy is often considered at the basis of the *Rhodococcus* catabolic versatility, functional robustness, adaptation to polluted and extreme environments and high-performing environmental competition (Mcleod et al. 2006; Pérez-Pérez et al. 2009).

Several examples of genetic redundancy originated either from gene duplication or horizontal gene transfer events have been described in *Rhodococcus* strains, the latter being supported by the proximity of transposase and invertase sequences to duplicated gene clusters (Taguchi et al. 2007). In general, the duplicate genes have been described to evolve and to encode isoenzymes possessing similar sequences but different substrate specificities and induction patterns. On the other hand, some duplicates have been described to be, at some extent, functionally redundant (Zhang 2012), catalysing the same metabolic reaction and presenting overlapping substrate range and inducing profiles. This type of duplication has probably occurred in relative recent period of time or has not been subject to strong selective pressure. For instance, in *R. jostii* RHA1, it has been hypothesized that the multiple homologous genes involved in the central aromatic pathways have received low selective pressure for functional diversification or gene removal (McLeod et al. 2006).

In this framework, the presence of multiple homologous genes strongly contributes to the wide *Rhodococcus* versatility in terms of catabolic activities including aliphatic and aromatic hydrocarbon catabolism, chlorinated hydrocarbons transformation and steroid degradation. Further, the presence of multiple functional homologs was found in central metabolic pathways (e.g. tricarboxylic acid cycle) (Van der Geize and Dijkhuize 2004).

While the presence of multiple copies of specific genes was not a universal feature of *Rhodococcus*, genomic redundancy generally characterizes the members of this genus in relation to metabolic and physiological traits useful for the adaptation to the environmental niche from which each *Rhodococcus* strain was isolated. For instance, the genome analysis of the psychrophilic *Rhodococcus* sp. JG3 isolated from a permafrost soil with a possible alkane source showed genomic redundancy in relation to genes involved in the adaptation to cold temperatures such as those associated to osmotic stress and to genes encoding alkane 1-monooxygenases (Goordial et al. 2016).

Functional redundancy has referred to the multiple alkane hydroxylase genes found in *Rhodococcus* strains featured by versatile alkane degradation capacity and typically isolated from petroleum-contaminated (sediment or marine) sites (van Beilen et al. 2002) (Fig. 5a). In particular, *alkB* gene encodes the membrane alkane hydroxylase AlkB, which catalyses the initial oxidation of alkanes. Four *alkB* homologous genes have been detected in *R. erythropolis* NRRL B-16531 and *R. qingshengii* Q15 (Whyte et al. 2002); three to five *alkB* genes were found in different *R. erythropolis* strains from various contaminated soils and in *Rhodococcus* sp. strain TMP2 (van Beilen et al. 2002; Takei et al. 2008). The multiple *alkB* genes found in *Rhodococcus* strains were mainly localized on the chromosome, and some of them showed high sequence divergence and different flanking regions (Whyte et al. 2002). The different AlkBs often catalysed the oxidation of different ranges or classes of alkanes and were differently expressed depending on the substrate and growth condition (Takei et al. 2008; Laczi et al. 2015). Because of this, the presence of multiple AlkB hydroxylases has been associated to broad metabolic capacities of *Rhodococcus* strains towards medium- and long-chain alkane and also to branched alkanes. In *R. aetherivorans* BCP1, two homologous gene clusters encoding alkane

Fig. 5 Examples of genetic redundancy in *Rhodococcus*. Genetic organization of (**a**) alkB genes in *Rhodococcus* strain NRRLB-16531, (**b**) catabolic island including *tpa*, *pat* and *pad* gene that is duplicated in the two linear plasmids in *R. jostii* RHA1, (**c**) the three homologous gene clusters (*bphA*, *etb1A* and *etb2A* genes) encoding dioxygenase systems involved in the initial hydroxylation of substituted benzenes in *R. jostii* RHA1. The plasmidic or chromosomal localization is indicated in correspondence to each gene cluster. [Modified from Whyte et al. (2002), Hara et al. (2007), Gonçalves et al. (2006), respectively]

monooxygenases of SDIMO (soluble di-iron monooxygenases) family were involved in the oxidation of short-chain alkanes (Cappelletti et al. 2013, 2015). This functional redundancy was correlated with the strong capacity of BCP1 strain to utilize gaseous alkanes and to co-metabolize chlorinated alkanes (Frascari et al. 2006; Cappelletti et al. 2012). In some cases, alkane hydroxylation functional redundancy was also associated to the co-existence in a single *Rhodococcus* strains of genes encoding different alkane hydroxylases, AlkB, SDIMOs and cytochrome P450 belonging to the CYP153 family. For instance, the versatile and efficient

degradation of alkanes by *R. erythropolis* PR4 was associated to the presence of four *alkB* genes, two CYP153 genes and other genes coding P450 on its genome, which are differently expressed on alkanes and hydrocarbon mixtures (Laczi et al. 2015).

Catabolic gene redundancy in some *Rhodococcus* strains has been extensively reported for aromatic compound degradation pathways. In particular, *R. jostii* RHA1, isolated from γ-hexachlorocyclohexane (lindane)-contaminated site, was described to be featured by high redundancy of catabolic pathways involved in aromatic hydrocarbon catabolism, which are also responsible for the co-metabolic transformation of polychlorinated biphenyls (PCBs). Several homologous genes were predicted to encode enzymes involved in the upper and lower degradation pathways of substituted benzenes, like ethylbenzene (ETB) and biphenyl (BPH). In RHA1, three homologous gene clusters (*bphA*, *etb1A* and *etb2A* genes) were found to encode dioxygenase systems, which are all induced by BPH and ETB by a possible common regulatory system (Fig. 5b). Nevertheless, these dioxygenase systems have shown distinct substrate specificity in terms of both aromatic hydrocarbons and PCB congeners (Iwasaki et al. 2006, 2007; Patrauchan et al. 2008). The gene cluster *bphA* is localized on the large linear plasmid pRHL1, while *etb1A* and *etb2A* are carried on the other linear plasmid pRHL2 and share high sequence similarity. Multiple copies of *bph* genes (*bphC-G*) were also predicted to be involved in the benzene ring cleavage downstream of the initial hydroxylation. Among these multiple homologs, only a few numbers of genes were transcriptionally induced during the growth on BPH and ETB, while the most part was expressed at constitutive levels (Gonçalves et al. 2006). In particular, only three out of the eight *bphEFG* clusters were up-regulated during RHA1 growth on BPH and ETB, independently on their genome localization (Gonçalves et al. 2006; Patrauchan et al. 2008). This indicated that out of the eight *bphEFG*, five homologous clusters produced paralog enzymes involved in distinct physiological processes (Irvine et al. 2000; Taguchi et al. 2004). Other *Rhodococcus* strains, isolated from the termite ecosystem typically exposed to plant-derived lignin and aromatics, showed multiple copies of *bph* gene clusters involved in PCB/biphenyl degradation; many of these were present on linear plasmids and in the proximity of transposase and invertase sequences (Taguchi et al. 2007). In addition to the catabolic enzymes, functional redundancy was found in the regulatory system responsible for the growth of RHA1 on aromatic compounds. In particular, two copies of the gene cluster *bphS* and *bphT* encode the two-component systems, BphS1T1 and BphS2T2, which showed high similarity in the amino acid sequence (>92%) and the same substrate spectrum, except for biphenyl (Takeda et al. 2010).

Additional genomic redundancy found in the genome of aromatic *Rhodococcus* degraders was related to genes involved in the catabolism of phthalate (*pad*), terephthalate (*tpa*), catechol (*cat*), protocatechuate (*pca*) and benzoate (*ben*) (Fig. 5c). In particular, two identical copies of a catabolic island including the *pad* and *tpa* clusters were found on the linear plasmids of *R. jostii* RHA1 and *Rhodococcus* sp. DK17, flanked by transposase-encoding genes (Patrauchan et al. 2005; Choi et al. 2005). The duplicated phthalate-degrading operons resulted to be simultaneously expressed and equally functional during the DK17 growth on

phthalate, allowing this strain to achieve the maximal degradation of phthalate (Choi et al. 2007). Genes involved in naphthalene and phthalate were found duplicated on two separate genomic islands in *R. opacus* M213, and, in *Rhodococcus* sp. TFB, it was demonstrated that naphthalene degradation probably results from the activities of different isozymes (Tomás-Gallardo et al. 2006). Genetic redundancy in aromatic degradation pathways was observed in *R. opacus* R7 possibly in relation to both the size of the genome and the degradation abilities towards different aromatic classes (Di Gennaro et al. 2014; Orro et al. 2015) and in *R. opacus* M213.

The catabolic redundancy was also associated to different substrate specificities and to possible mechanisms of metabolic intermediate detoxification in the steroid metabolism (involving *kshAB* and *kshD* genes) by some *Rhodococcus* strains. Four homologous *ksh* gene clusters are present in *R. jostii* RHA1 genome; the cluster 1 was shown to encode enzymes involved in cholesterol catabolism (van der Geize et al. 2007), while the homologous cluster 3 supported the catabolism of cholate (Swain et al. 2012). In *R. erythropolis* SQ1, *ksh* cluster 1 was involved in steroid metabolism, while *ksh* cluster 2 was supposed to have a role in limiting the intracellular accumulation of toxic metabolic intermediates (van der Geize et al. 2008). The genome analysis of *R. rhodochrous* DSM43269A revealed five *kshA* homologous genes (*kshA1* to *kshA5*) which were phylogenetically distinct, and each one showed a unique steroid induction pattern and substrate range, ensuring a fine-tuned steroid catabolism (Petrusma et al. 2011).

The redundancy of genes encoding (chloro)phenol hydroxylases supported the ability of *R. opacus* 1CP to metabolize a large spectrum of phenolic and chlorophenolic compounds (Gröning et al. 2014). All the three homologous phenol hydroxylases were able to convert the tested phenolic substrates at significant rates. This was probably due to the broad substrate specificity of these phenol hydroxylases and, therefore, low specialization (Gröning et al. 2014). Multiple genes (from 3 to 5) encoding phenol hydroxylases (*pheA1*/*pheA2*) were identified in other *Rhodococcus* strains (Gröning et al. 2014) In particular, in *R. jostii* RHA1, differences in the transcriptional regulation of two *pheA1*/*pheA2* clusters suggested their activation under different growth conditions (Szőköl et al. 2014).

Interestingly, the functional redundancy found in *Rhodococcus* was strongly associated to evolutionary mechanisms related to niche adaptation. In this respect, the genome and microarray analysis of *R. aetherivorans* I24 study of this strain to PCB/biphenyl exposure indicated the involvement of the only core enzymes in the substituted benzene metabolism, while most of the isozymes found in RHA1 were missing (Puglisi et al. 2010). This was associated to the fact that, unlike RHA1, I24 strain was not isolated from a PCB-contaminated site. Therefore, as previously mentioned, despite the gene redundancy is recognized as a trait of *Rhodococcus* genus, the presence of functional isozymes involved in specific catabolic pathways reflects the selective pressure imposed by the environmental habitat and is also in part associated to the size of the genomes, as in the case of *R. jostii* and *R. opacus* strains featured by larger genomes compared to *R. aetherivorans* and *R. ruber* (Table 1).

4.2 Plasmids

Plasmids are circular or linear extrachromosomal DNA elements, which are capable of semi-autonomous or self-replication; they do not typically encode essential genes for the host but instead carry genes that may help the organism to adapt to novel environments or nutrient sources (Aminov 2011; Carroll and Wong 2018). In this respect, most of the *Rhodococcus* genes belonging to the variable accessory genome have plasmidic localization (see Par. 3.2) (Creason et al. 2014a; Orro et al. 2015; Pathak et al. 2016). Plasmids are considered a major driving force in prokaryotic evolution as they can be transferred between cells, as mobile genetic elements, mediating horizontal gene transfer events (Hülter et al. 2017). Accordingly, in bacteria, plasmids are thought to play critical roles in the evolution, propagation and assembly of catabolic pathways, antibiotic and metal resistance mechanisms, antimicrobial biosynthetic pathways and pathogenicity (Meinhardt et al. 1997; Shimizu et al. 2001).

Many *Rhodococcus* strains harbour plasmids, which can be linear or circular, of small (of few Kbp) or large size (of hundreds of Kbp), with cryptic or catabolic functions. Some *Rhodococcus* strains have been described to simultaneously possess several plasmids of different types and sizes, e.g. *R. erythropolis* PR4 contains one linear plasmid, pREL1 (~270 Kb), and two circular plasmids, pREC1 (~100 Kb) and pREC2 (~3.5 Kb) (Sekine et al. 2006); *R. opacus* B4 possesses two linear plasmids of pROB series (pROB01 of ~560 Kb, pROB01 of ~240 Kb) and three circular plasmids of pKNR series (pKNR of 111 Kb, pKNR01 of 4.4 Kb, pKNR02 of 2.8 Kb) (Na et al. 2005). Many *Rhodococcus* plasmids, both linear and circular, encode catabolic functions associated to xenobiotic degradation (Shimizu et al. 2001), chemolithoautotrophical growth on gaseous hydrogen and carbon dioxide, hydrogen production (Grzeszik et al. 1997), metal toxicity resistance (Cappelletti et al. 2016) and pathogenicity (Letek et al. 2010). In this respect, deletions in several *Rhodococcus* plasmids have resulted in the loss of degradative genes and specific growth deficiencies; e.g. deletions on pRHL1 and pRHL2 in *R. jostii* RHA1 and on pTA422 in *R. erythropolis* TA421 affected biphenyl degradation (Fukuda et al. 1998; Kosono et al. 1997). In some cases, catabolic plasmids were also found to harbour homologous genes, and their mutation was found to exert moderate or null phenotypic effects (Patrauchan et al. 2005). In this framework, catabolic plasmids are thought to play a key role in *Rhodococcus* catabolic versatility and efficiency by harbouring unique set of genes involved in specific catabolic pathways but also contributing to *Rhodococcus* multiple pathways and functional redundancy (Kim et al. 2018). Additionally, *Rhodococcus* plasmids have been described to have a much higher density of DNA mobilization genes (e.g. insertion sequences, transposase genes), pseudogenes, unique species-specific genes and niche-specific determinants (e.g. genes involved in pathogenesis in *R. equi* and in peripheral aromatic clusters in *R. jostii* RHA1 and *R. opacus* R7) than the corresponding chromosomes. Rhodococcal plasmids are therefore under less stringent selection

and are key players in rhodococcal genome plasticity and niche adaptability (Sekine et al. 2006).

Linear plasmids, which are often very large, are widespread and diverse among *Rhodococcus* strains (Ventura et al. 2007). As they can reach lengths of several hundreds of kilobases, they are frequently referred to as megaplasmids. For instance, pRHL2 from *R. jostii* RHA1 is 443 kb (Shimizu et al. 2001), pPDG1 from *R. opacus* R7 is 656 kb (Di Gennaro et al. 2014), and pRHL1 from *R. jostii* RHA1 is 1.12 Mb (McLeod et al. 2006). The reason of extensive research attention on *Rhodococcus* large linear plasmids is connected with the fact that they are associated with catabolic genes and that they confer advantageous abilities on their hosts (Meinhardt et al. 1997). For instance, linear plasmids have been described to encode enzymes involved in the catabolism of naphthalene (Kulakov et al. 2005; Orro et al. 2015; Pathak et al. 2016), biphenyl (Taguchi et al. 2004), toluene (Priefert et al. 2004), alkylbenzene (Kim et al. 2002), isopropylbenzene and trichloroethylene (Meinhardt et al. 1997), phthalate (Patrauchan et al. 2005), gaseous *n*-alkanes (Cappelletti et al. 2015), chloroaromatic compounds (Konig et al. 2004), isoprene (Crombie et al. 2015) and triazine compounds (Dodge et al. 2011) and also in the desulphurization of organosulphur compounds (Denis-Larose et al. 1997), resistance to toxic metals (Meinhardt et al. 1997; Cappelletti et al. 2016), pathogenicity towards bovines (Valero-Rello et al. 2015) and phytopathogenicity (Francis et al. 2012). In many cases, catabolic genes present in linear plasmids contribute to degradation pathways together with genes located on the chromosome (Gonçalves et al. 2006).

As a distinctive feature, linear megaplasmids from *Rhodococcus* strains are capable of conjugal transfer, being responsible for genetic information sharing through HGT (Meinhardt et al. 1997; Dib et al. 2015). The importance of linear elements is also associated to their higher "flexibility" compared to circular plasmids. In particular, the telomeres are considered to be frequently subject to recombinational events (Volff and Altenbuchner 2000; Chen et al. 2002). In the case of HGT, intermolecular recombination events can take place between plasmids but also between host chromosomes of compatible species. Both the frequent observation of linear plasmids and illegitimate recombination in *Rhodococcus* have led to the hypothesis of the hyper-recombinational gene storage strategy. This is related to the function of the plasmids as storage of large number of catabolic genes that can represent recombination sources to respond and adapt to novel compounds in the native soil environments.

As for most bacteria, circular plasmids are also very common in *Rhodococcus* species. Circular plasmids in *Rhodococcus* have been generally shown to possess smaller size compared to the linear ones (Table 2). However, they often contribute to the catabolic capacities of the *Rhodococcus* host strain. Several circular plasmids harbour genes encoding part or complete xenobiotic degradation pathways in *Rhodococcus* strains such as pRTL1 (100 Kb) encoding haloalkane degradation enzymes in *R. rhodochrous* NCIMB13064 (Kulakova et al. 1995); pREC1 contains a complete set of genes for the β-oxidation of fatty acids (Sekine et al. 2006) and the large circular plasmids pKNR (111 kb) in *R. opacus* B4 (Honda et al. 2012).

Additionally, many *Rhodococcus* plasmids are self-transmissible but phenotypically cryptic. The cryptic plasmids described in *Rhodococcus* strains have principally small size and circular structure, e.g. pREC2 (3.6 Kb) in *R. erythropolis* PR4, pB264 (4.9 Kb) in *Rhodococcus* sp. B264-1. In some cases, these plasmids have been isolated to develop vector systems for DNA manipulation and protein expression in *Rhodococcus* strains. For instance, pKA22 (4.9 Kb) from *R. rhodochrous* NCIMB13064 (Kulakov et al. 1997), pFAJ2600 (5.9 Kb) from *R. erythropolis* NI86/21 (De Mot et al. 1997), pAN12 (6.3 Kb) from *R. erythropolis* AN12 and pKNR01 (4.4 Kb) from *R. opacus* B4 are some of the small circular cryptic plasmids, described in the literature, that have been used to construct *E. coli-Rhodococcus* shuttle plasmids (Kostichka et al. 2003). The first two plasmids require the activity of two replication proteins, RepA and RepB, to replicate. As these two replication proteins resemble replication proteins of the theta-type replicating *Mycobacterium* plasmid pAL5000 (Ventura et al. 2007), pKA22 and pFAJ2600 are categorized as replicons of pAL5000 family. The plasmid pAN12 is of the pIJ101/pJV1 family, which usually replicates thanks to a single replication. Detection of ssDNA intermediates in several of the pIJ101/pJV1 family of plasmids suggested that they replicate by rolling circle mechanism (Kostichka et al. 2003).

5 Towards Genome-Scale Modification of *Rhodococcus* Through System and Synthetic Biology

Genetic manipulation (or genetic engineering) methods have been applied to *Rhodococcus* strains in order to obtain new strains that express additional genetic properties to acquire new catabolic capacities (Xiong et al. 2012, 2016; Venkataraman et al. 2015; Rodrigues et al. 2001; Hirasawa et al. 2001), to perform promoter activity studies (van der Geize et al. 2008; Cappelletti et al. 2011) and to introduce genetic mutations or deletions to determine the phenotypic alterations and the function of a specific gene or gene cluster (van der Geize et al. 2008; Amara et al. 2016).

Genetic manipulation methods are based on the development of efficient *Rhodococcus* transformation procedure, as biochemical and genetic characteristics of this genus have for long time limited the genetic manipulation of this strain (Sallam et al. 2006; Cappelletti 2010). Although protoplast-mediated transformation methods have also been used for some *Rhodococcus* strains (Singer and Finnerty 1988; Duran 1998), the use of electroporation procedure showed the highest efficiency, and it is presently the method that is most widely used for *Rhodococcus* transformation (Shao et al. 1995; Sekizaki et al. 1998; Kalscheuer et al. 1999). Several genetic manipulation strategies during the last three decades led to the development of (1) *E. coli-Rhodococcus* shuttle vectors from cryptic plasmids of *Rhodococcus* strains (Kostichka et al. 2003; Na et al. 2005; Matsui et al. 2006), (2) expression vectors for heterologous gene expression and protein production

(Kalscheuer et al. 1999; Nakashima and Tamura 2004a, b), (3) random mutagenesis methods using transposons or spontaneous illegitimate recombination (Desomer et al. 1991; Sallam et al. 2006; Crespi et al. 1994) and (4) targeted gene disruption methods based on unmarked mutagenesis deletion systems with SacB as counter-selection (van der Geize et al. 2001).

Recently, advances in genomics and genome editing offered new opportunities to engineer *Rhodococcus* in a directed and combinatorial manner through a genomic-scale rational design of the cell through system and synthetic biology approaches.

With the aim of developing synthetic biology platforms for *Rhodococcus* engineering, a first BioBrick™-compatible plasmid system was designed for *R. opacus* PD630 by Ellinger and Schmidt-Dannert (2017). Generally, the BioBrick™ toolbox allows convenient and reproducible assembly of multigene pathways into series of vectors and the possibility to quickly mobilize any cloned gene into vectors with different features for gene expression and protein purification (Vick et al. 2011). Currently, the majority of the available BioBricks were designed for the Gram-negative model organism *Escherichia coli*. However, recently, novel BioBrick™ tools have been designed for the engineering of other bacteria, e.g. *Bacillus* (Radeck et al. 2013). The first BioBrick™-compatible vector designed for a *Rhodococcus* strain pSRKBB derived from the backbone of the *E. coli-Rhodococcus* shuttle vector pSRK21, which was modified by removing all BioBrick™-incompatible restriction, while it retained the capacity to replicate in *R. opacus* PD630 (Ellinger and Schmidt-Dannert 2017) (Fig. 6). This BioBrick-compatible plasmid was demonstrated to enable robust heterologous protein expression in *R. opacus* PD630 from the lac promoter, giving a first demonstration of the use of *Rhodococcus* as platform for synthetic biology.

Moreover, in order to expand the molecular toolkit for *Rhodococcus* genetic engineering, a recent work analysed the genetic tools to obtain gene expression control in *R. opacus* PD630 by characterizing a constitutive promoter library, optimizing antibiotic resistance markers and defining the copy numbers of different gene expression plasmids (DeLorenzo et al. 2018). The same work also reported methods for genome editing by using recombinase-based technique for site-specific genomic modifications and by describing the methodology to identify neutral integration sites for stable heterologous expression. Lastly, a tunable and targeted gene repression system was developed by utilizing for the first time in *Rhodococcus* the CRISPR interference method (DeLorenzo et al. 2018). Taken together, the heterologous gene expression tools along with the CRISPRi method open new prospective for the optimization of the bioconversion abilities of *R. opacus* and for the possible development of molecular tools for other *Rhodococcus* species with relevant environmental and/or industrial characteristics.

At the same time, genomic information have been used to develop genome-scale metabolic models, i.e. mathematical representations of the stoichiometry of the biochemical networks occurring in *Rhodococcus* cell. In this regard, an in silico genome-scale metabolic model (iMT1174) was developed to make metabolic predictions on the behaviour of *R. jostii* RHA1 in relation to the accumulation of three types of carbon storage (i.e. glycogen, polyhydroxyalkanoate and triacylglycerols),

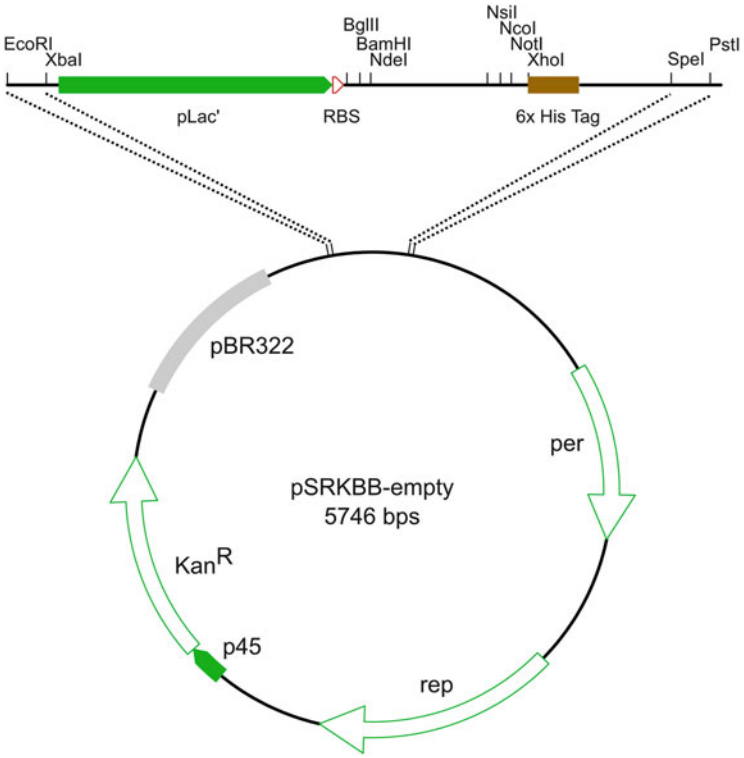

Fig. 6 Diagram of BioBrick™-compatible vector pSRKBB for *Rhodococcus.* The enlarged region includes the lac promoter and the ribosome binding site (RBS) upstream of the multi-cloning site where coding sequences can be cloned for constitutive expression. Further details are provided by Ellinger and Schmidt-Dannert (2017)

using different carbon sources (glucose or acetate) and under growth conditions typically occurring in activated sludge bioreactor systems for wastewater recovery (Tajparast et al. 2015, 2018). The simulations were compared with the experimentally measured metabolic fluxes through ^{13}C-labelling assays, and the predictive capacity of the model was established. These works represent the first steps towards systems biology approaches enabling to simulate and predict *Rhodococcus* metabolic fluxes leading to the production of industrially interesting metabolites. More efforts are needed to extend system and synthetic biology tools for genome-scale engineering of *Rhodococcus* species different from the model ones, i.e. *R. opacus* PD630 and *R. jostii* RHA1, in order to get deep into the extraordinary physiological and metabolic diversity featuring different *Rhodococcus* strains and to provide new opportunities for their utilization in targeted bioconversion and biodegradation processes.

6 Conclusions

Due to the biotechnological significance of this genus, a dramatic increase of sequenced *Rhodococcus* genomes has been published and become available in the database. The comparative and functional analyses of these genome data provided the opportunity to get insight into the genetic basis of the extraordinary metabolic diversity and versatility of *Rhodococcus* genus members. The high versatility and adaptability of members of *Rhodococcus* was found to be the reflection of the size and complexity of their genomes. The co-existence of linear and circular replicons (chromosome and plasmids) and the presence of large catabolic plasmids have strong implications in rhodococcal genome plasticity, fluidity and metabolic diversity. Further, the genetic and functional redundancy is a general characteristic of *Rhodococcus* genus, which supports metabolic robustness and versatility towards the degradation of different classes of substrates and organic compounds. The specific type of genomic redundancy was found to reflect the peculiar niche adaptation need of each *Rhodococcus* strain, and, at least in part, it was related to the size of the genome.

Lastly, advances in *Rhodococcus* genomics and genome editing provided new opportunities for the application of directed and combinatorial approaches to *Rhodococcus* engineering. Recent breakthroughs in genetic engineering of *Rhodococus* have included the use of synthetic biology platforms and new approaches for genome editing (CRISPR/Cas9 tool) of *R. opacus* PD630. Systems biology approaches have also been applied to *R. jostii* RHA1 to develop genome-scale metabolic models to predict cell metabolic fluxes for the production of relevant metabolites and storage compounds. Despite more efforts are needed to expand system and synthetic biology tools for genome-scale engineering of *Rhodococcus* species different from the model ones, i.e. *R. opacus* PD630 and *R. jostii* RHA1, the novel molecular toolkits along with the extended knowledge on genomic and functional genomics have largely contributed in making *Rhodococcus* strains relevant for future novel industrial applications.

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Central Metabolism of Species of the Genus *Rhodococcus*

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

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_3

1 Introduction

Members of the genus *Rhodococcus* are aerobic non-sporulating bacteria widely distributed in diverse natural environments. They have been detected in tropical, artic, and arid soils, as well as 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, members of *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 shown a significant increase 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 recent reviews (Larkin et al. 2005; Alvarez and Steinbüchel 2002; Alvarez 2006; Martínková et al. 2009; Yam et al. 2011). Despite the importance of the central metabolism for understanding the biology of rhodococci, this area has received little attention in comparison with the other metabolic processes mentioned above. The pathways of central metabolism are highly conserved among 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 in which cells do not grow but are still metabolically active. Under stress conditions, the physiology of rhodococci seems to depend on the metabolism of diverse storage compounds. Members of *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, 2017).

During the last years, numerous complete genome sequences of several *Rhodococcus* species such as *R. opacus* (strains B4 and PD630), *R. rhodochrous*, *R. pyridinivorans*, *R. ruber*, *R. hoagie* (*equi*), *R. erythropolis*, *R. qingshengii*, and *R. fascians*, among others, have been uploaded in public database. This fact reflects the importance of this genus as resources for biotechnological purposes or in the case of *R. hoagie* and *R. fascians* for their association with some pathologies of animals, humans, and plants. Among them, the genome sequence of *Rhodococcus jostii* strain RHA1 was 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 became a good model for understanding the genetics, physiology, and metabolism of the *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. Based on peer-reviewed literature and a thorough genome examination of *R. jostii* RHA1 as model, this chapter summarizes some aspects of the central metabolism of species of the *Rhodococcus* genus, including glycolytic pathways and NADPH-generating systems, gluconeogenesis, and phosphoenolpyruvate-pyruvate-oxaloacetate node, the tricarboxylic acid cycle (TCA), and the glyoxylate shunt. In addition, some new information about the energy pathways is given.

2 Glycolytic Pathways and NADPH-Generating Systems

Glycolysis (**E**mbden-**M**eyerhof-**P**arnas pathway, EMP) is a common pathway for glucose oxidation used by eukaryotic cells and some aerobic and facultative anaerobic bacteria, but not by Archaea. In this pathway, glucose is split into two molecules of pyruvic acid with the formation of two ATP molecules. Many aerobic bacteria and archaea use an alternative glycolytic pathway called **E**ntner-**D**oudoroff pathway (ED pathway), which produces glyceraldehyde 3-P, pyruvate, and only one ATP molecule from each molecule of glucose. This pathway is found in prokaryotes such as *Pseudomonas*, *Azotobacter*, *Rhizobium*, and other Gram negatives. However, the occurrence of the ED pathway has been also described in actinobacteria (Gunnarsson et al. 2004; Borodina et al. 2005; Juarez et al. 2017).

The analysis of the *R. jostii* RHA1 genome revealed the occurrence of key genes involved in glycolytic pathways. RHA1 strain is able to use both EMP and ED glycolytic pathways, to catabolize carbohydrates. Figure 1 and Table 1 show an overview on the pathways and enzymes/genes implicated in the glucose oxidation by this strain.

The RHA1 genome also contains all necessary genes/enzymes for the **P**entose **P**hosphate **P**athway (PPP) (Fig. 1 and Table 1). This pathway produces the precursor ribose 5-phosphate used in nucleic acid biosynthesis as well as erythrose 4-phosphate for the synthesis of aromatic amino acids. In addition, the PPP has been linked with the production of NADPH used in anabolic processes (Spaans et al. 2015).

Whereas the glucose metabolism by EMP generates two reducing equivalents in the form of NADH, the ED and PPP pathways generate one and two reducing equivalents in the form of NADPH, respectively. The initial step of ED and PPP pathways involves the conversion of glucose 6-phosphate in 6-phosphogluconolactone catalyzed by the enzyme glucose-6-phosphate dehydrogenase (Zwf), in which NADPH is generated.

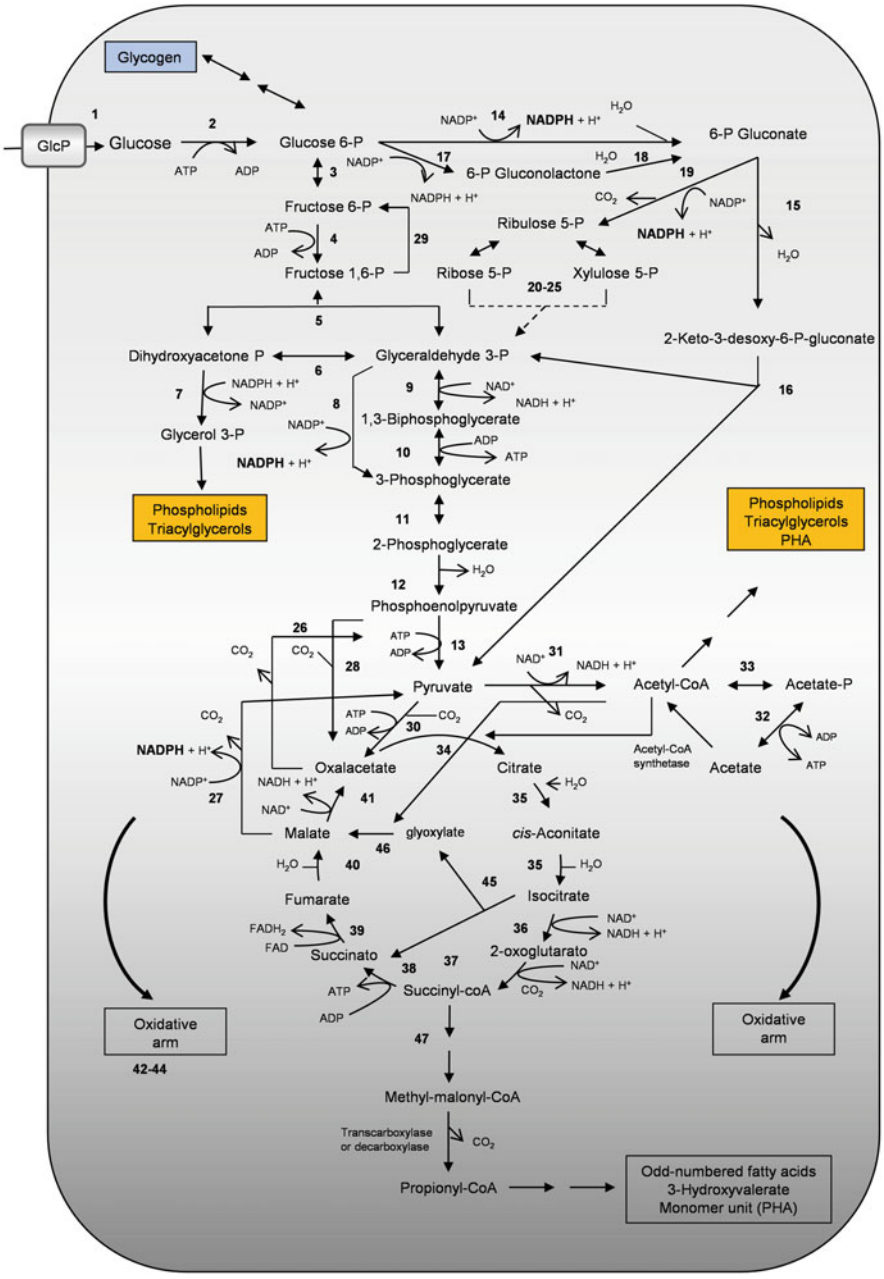

Fig. 1 Main pathways involved in central metabolism of *Rhodococcus jostii* RHA1 deduced from the genome database

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

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
1	*RHA1_RS11555*	*glcP*	Glucose transporter major facilitator superfamily (MFS)	Glucose uptake
2	*RHA1_RS05385*		Glucokinase	Glycolysis (EMP-ED), glycogen degradation
2	*RHA1_RS06585*		Glucokinase	Glycolysis (EMP-ED), glycogen degradation
2	*RHA1_RS20805*		Glucokinase	Glycolysis (EMP-ED), glycogen degradation
3	*RHA1_RS43575*	*pgi*	Glucose-6-phosphate isomerase	Glycolysis (EMP), gluconeogenesis
3	*RHA1_RS43120*	*pgi*	Glucose-6-phosphate isomerase	Glycolysis (EMP), gluconeogenesis
3	*RHA1_RS44310*	*pgi*	Glucose-6-phosphate isomerase	Glycolysis (EMP), gluconeogenesis
3	*RHA1_RS27160*	*pgi*	Glucose-6-phosphate isomerase	Glycolysis (EMP), gluconeogenesis
4	*RHA1_RS00295*		Phosphofructokinase	Glycolysis (EMP)
4	*RHA1_RS31690*	*pfkA*	6-Phosphofructokinase	Glycolysis (EMP)
4	*RHA1_RS33150*		1-Phosphofructokinase	Glycolysis (EMP)
5	*RHA1_RS27000*	*fba*	Class II fructose-bisphosphate aldolase	Glycolysis (EMP), gluconeogenesis
5	*RHA1_RS26975*	*fba*	Fructose-bisphosphate aldolase	Glycolysis (EMP), gluconeogenesis
6	*RHA1_RS35040*	*tpiA*	Triose-phosphate isomerase	Glycolysis (EMP)
7	*RHA1_RS31815*	*gpsA*	Glycerol-3-phosphate dehydrogenase [NAD(P)+] 2	Glycerol 3-phosphate synthesis
7	*RHA1_RS11710*	*gpsA*	Glycerol-3-phosphate dehydrogenase [NAD(P)+] 1	Glycerol 3-phosphate synthesis
8	*RHA1_RS16630*	*tadD*	NADP+-dependent Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis (EMP/ED)
9	*RHA1_RS35030*	*gap2*	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis (EMP), gluconeogenesis
10	*RHA1_RS35035*	*pgk*	Phosphoglycerate kinase	Glycolysis (EMP), gluconeogenesis
11	*RHA1_RS04205*		Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis
11	*RHA1_RS06270*	*gpmA*	Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
11	*RHA1_RS31045*		Phosphoglycerate mutase	Glycolysis (EMP), gluconeogenesis
12	*RHA1_RS28200*	*eno*	Enolase	Glycolysis (EMP), gluconeogenesis
13	*RHA1_RS04835*	*pyk*	Pyruvate kinase	Glycolysis (EMP-ED)
13	*RHA1_RS12545*	*pyk*	Pyruvate kinase	Glycolysis (EMP-ED)
13	*RHA1_RS15635*	*pyk*	Pyruvate kinase	Glycolysis (EMP-ED)
13	*RHA1_RS23435*	*pyk*	Pyruvate kinase	Glycolysis (EMP-ED)
14	*RHA1_RS12070*		PQQ-dependent sugar dehydrogenase	Glucose degradation (PPP-ED pathway)
14	*RHA1_RS31700*		PQQ-dependent sugar dehydrogenase	Glucose degradation (PPP-ED pathway)
15	*RHA1_RS11570*	*edd*	Phosphogluconate dehydratase	Glucose degradation (ED)
16	*RHA1_RS11565*	*eda*	KHG/KDPG aldolase	Glucose degradation (ED)
17	*RHA1_RS02710*	*zwf1*	Glucose-6-phosphate 1-dehydrogenase	PPP/ED pathway
17	*RHA1_RS11575*	*zwf2*	Glucose-6-phosphate 1-dehydrogenase	PPP/ED pathway
17	*RHA1_RS27530*	*zwf3*	Glucose-6-phosphate 1-dehydrogenase	PPP/ED pathway
17	*RHA1_RS49040*	*zwf4*	Glucose-6-phosphate 1-dehydrogenase (NADP+)	PPP/ED pathway
17	*RHA1_RS44335*	*zwf5*	Glucose-6-phosphate 1-dehydrogenase	PPP/ED pathway
17	*RHA1_RS43115*	*fgd*	Glucose-6-phosphate dehydrogenase coenzyme-F420	PPP/ED pathway
17	*RHA1_RS43570*	*fgd*	Glucose-6-phosphate dehydrogenase coenzyme-F420	PPP/ED pathway
18	*RHA1_RS35055*	*pgl*	6-Phosphogluconolactonase	PP pathway
19	*RHA1_RS02715*	*gnd*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
19	*RHA1_RS17785*	*gnd*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
19	*RHA1_RS35365*	*gnd*	Phosphogluconate dehydrogenase ((NADP(+)-dependent, decarboxylating))	PP pathway

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
19	*RHA1_RS43110*	*gnd*	Phosphogluconate dehydrogenase (decarboxylating)	PP pathway
20	*RHA1_RS34980*	*rpe*	Ribulose-phosphate 3-epimerase	PP pathway
21	*RHA1_RS14120*		Ribose-5-phosphate isomerase	PP pathway
21	*RHA1_RS06675*		Ribose 5-phosphate isomerase	PP pathway
22	*RHA1_RS49045*	*tal*	Transaldolase	PP pathway
22	*RHA1_RS36885*	*tal*	Transaldolase	PP pathway
23	*RHA1_RS03880*		Transketolase, C-terminal subunit	PP pathway
23	*RHA1_RS03885*		Transketolase, N-terminal subunit	PP pathway
23	*RHA1_RS18640*		Transketolase, N-terminal subunit	PP pathway
23	*RHA1_RS18645*		Transketolase, C-terminal subunit	PP pathway
23	*RHA1_RS35075*		Transketolase	PP pathway
24	*RHA1_RS16375*		Sugar phosphate isomerase/ epimerase	PP pathway
24	*RHA1_RS25680*		Sugar phosphate isomerase/ epimerase	PP pathway
24	*RHA1_RS22195*		Sugar phosphate isomerase/ epimerase	PP pathway
24	*RHA1_RS14555*		Sugar phosphate isomerase/ epimerase	PP pathway
24	*RHA1_RS10065*		Sugar phosphate isomerase/ epimerase	PP pathway
25	*RHA1_RS22585*		Class II aldolase	PP pathway
25	*RHA1_RS34445*		Class II aldolase	PP pathway
26	*RHA1_RS25350*		Phosphoenolpyruvate carboxykinase	Gluconeogenesis/ anaplerotic pathway
27	*RHA1_RS02430*		NAD-dependent malic enzyme	Gluconeogenesis/ anaplerotic pathway
27	*RHA1_RS12570*		NAD-dependent malic enzyme	Gluconeogenesis/ anaplerotic pathway
27	*RHA1_RS29320*		NADP-dependent malic enzyme	Gluconeogenesis/ anaplerotic pathway
27	*RHA1_RS44255*		NADP-dependent malic enzyme	Gluconeogenesis/ anaplerotic pathway
27	*RHA1_RS39800*		NADP-dependent malic enzyme	Gluconeogenesis/ anaplerotic pathway
28	*RHA1_RS35050*	*ppc*	Phosphoenolpyruvate carboxylase	Anaplerotic pathway

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
29	*RHA1_RS28640*	*glpX*	Fructosa 1,6-bisphosphatase class II	Gluconeogenesis
30	*RHA1_RS31870*	*pycA*	Pyruvate carboxylase	Anaplerotic pathway
31	*RHA1_RS02650*		Pyruvate dehydrogenase E1 component	Acetyl-CoA synthesis
31	*RHA1_RS02655*		Dihydrolipoyllysine-residue succinyltransferase (PDH)	Acetyl-CoA synthesis
31	*RHA1_RS05135*		Pyruvate oxidase	Acetyl-CoA synthesis
31	*RHA1_RS05795*	*aceE1*	Pyruvate dehydrogenase (acetyl-transferring), homodimeric type	Acetyl-CoA synthesis
31	*RHA1_RS07665*	*pdhA*	Pyruvate dehydrogenase E1 component alpha subunit	Acetyl-CoA synthesis
31	*RHA1_RS07670*		Alpha-ketoacid dehydrogenase subunit beta	Acetyl-CoA synthesis
31	*RHA1_RS07675*		2-Oxo acid dehydrogenase subunit E2	Acetyl-CoA synthesis
31	*RHA1_RS10445*	*lpdA*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS12550*	*aceE*	Pseudo	Acetyl-CoA synthesis
31	*RHA1_RS14255*	*lpdA*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS16000*		MFS transporter	Acetyl-CoA synthesis
31	*RHA1_RS16020*		Pyruvate dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS16085*	*lpdA*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS16090*		Alpha-ketoacid dehydrogenase subunit beta	Acetyl-CoA synthesis
31	*RHA1_RS16095*	*pdhB1*	Pyruvate dehydrogenase E1 component beta subunit	Acetyl-CoA synthesis
31	*RHA1_RS16100*	*pdhA*	Pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	Acetyl-CoA synthesis
31	*RHA1_RS16380*	*lpdA*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS16385*		2-Oxo acid dehydrogenase subunit E2	Acetyl-CoA synthesis
31	*RHA1_RS16390*		Alpha-ketoacid dehydrogenase subunit beta	Acetyl-CoA synthesis

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
31	*RHA1_RS16395*	*pdhA*	Pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	Acetyl-CoA synthesis
31	*RHA1_RS27135*		NAD(P)/FAD-dependent oxidoreductase	Acetyl-CoA synthesis
31	*RHA1_RS30615*		NAD(P)H-quinone dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS37395*		Hypothetical protein	Acetyl-CoA synthesis
31	*RHA1_RS42945*	*lpdA*	Dihydrolipoyl dehydrogenase	Acetyl-CoA synthesis
31	*RHA1_RS42970*		2-oxo acid dehydrogenase subunit E2	Acetyl-CoA synthesis
31	*RHA1_RS42975*		Alpha-ketoacid dehydrogenase subunit beta	Acetyl-CoA synthesis
31	*RHA1_RS42980*		Thiamine pyrophosphate-dependent dehydrogenase E1 component subunit alpha	Acetyl-CoA synthesis
32	*RHA1_RS10720*		Acetate kinase	AK-PTA pathway
33	*RHA1_RS10725*		Phosphate acetyltransferase	AK-PTA pathway
34	*RHA1_RS01765*	*citA1*	Citrate (Si)-synthase	TCA cycle (oxidative)
34	*RHA1_RS07835*		Possible citrate synthase, C-terminal	TCA cycle (oxidative)
34	*RHA1_RS24420*	*citA2*	Citrate (Si)-synthase	TCA cycle (oxidative)
34	*RHA1_RS24440*		Citrate (Si)-synthase	TCA cycle (oxidative)
34	*RHA1_RS34010*		Citrate synthase	TCA cycle (oxidative)
35	*RHA1_RS11725*	*acnA1*	Aconitate hydratase	TCA cycle (oxidative/reductive)
35	*RHA1_RS35180*	*acnA2*	Aconitate hydratase	TCA cycle (oxidative/reductive)
35	*RHA1_RS36835*	*acnA3*	Aconitate hydratase	TCA cycle (oxidative/reductive)
36	*RHA1_RS02995*		Isocitrate dehydrogenase (NADP+)	TCA cycle (oxidative/reductive)
36	*RHA1_RS30490*		Isocitrate dehydrogenase (NADP+)	TCA cycle (oxidative/reductive)
37	*RHA1_RS29380*	*odhA*	2-Oxoglutarate dehydrogenase, E1 and E2 components	TCA cycle (oxidative)
38	*RHA1_RS27190*	*sucC*	Succinate CoA ligase β-subunit	TCA cycle (oxidative)

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
38	*RHA1_RS27195*	*sucD*	Succinate CoA ligase α-subunit	TCA cycle (oxidative)
38	*RHA1_RS17525*		CoA binding protein (previously annotated as probable succinate CoA ligase)	TCA cycle (oxidative)
39	*RHA1_RS05045*	*sdhB1*	Succinate dehydrogenase iron-sulfur protein	TCA cycle (oxidative)
39	*RHA1_RS05050*	*sdhA1*	Succinate dehydrogenase flavo-protein subunit	TCA cycle (oxidative)
39	*RHA1_RS05055*		Possible succinate dehydrogenase	TCA cycle (oxidative)
39	*RHA1_RS23305*		Hypothetical protein	TCA cycle (oxidative)
39	*RHA1_RS27810*		FAD-binding protein	TCA cycle (oxidative)
39	*RHA1_RS30525*	*sdhB2*	FAD-binding protein	TCA cycle (oxidative)
39	*RHA1_RS30530*	*sdhA2*	Succinate dehydrogenase	TCA cycle (oxidative)
39	*RHA1_RS30535*		Probable succinate dehydrogenase hydrophobic membrane anchor protein	TCA cycle (oxidative)
39	*RHA1_RS30540*		Probable succinate dehydrogenase (cytochrome)	TCA cycle (oxidative)
39	*RHA1_RS36845*		Ferredoxin	TCA cycle (oxidative)
39	*RHA1_RS39385*		Probable succinate dehydrogenase hydrophobic membrane anchor protein	TCA cycle (oxidative)
39	*RHA1_RS47620*	*sdhC*	Succinate dehydrogenase cyto-chrome β-subunit	TCA cycle (oxidative)
39	*RHA1_RS39395*		Ferredoxin	TCA cycle (oxidative)
39	*RHA1_RS39400*	*sdhB3*	Succinate dehydrogenase Fe-S protein subunit	TCA cycle (oxidative)
40	*RHA1_RS28635*	*fumC*	Fumarate hydratase class II	TCA cycle (oxi-dative/reductive)
40	*RHA1_RS28815*		Fumarate hydratase class I	TCA cycle (oxi-dative/reductive)
40	*RHA1_RS39380*		Fumarate hydratase, class I	TCA cycle (oxi-dative/reductive)
41	*RHA1_RS30520*	*mdh*	Malate dehydrogenase	TCA cycle (oxi-dative/reductive)
41	*RHA1_RS32320*		Malate dehydrogenase (acceptor)	TCA cycle (oxi-dative/reductive)

(continued)

Table 1 (continued)

Reaction number[a]	Gene ID	Gene name	Predicted function based on gene annotation	Metabolic pathway
42	*RHA1_RS00955*		Citrate lyase	TCA cycle (reductive)
42	*RHA1_RS01540*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS03190*		Aldolase	TCA cycle (reductive)
42	*RHA1_RS03695*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS03710*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS11960*		Possible citrate lyase beta subunit	TCA cycle (reductive)
42	*RHA1_RS13995*		CoA ester lyase (previously annotated as citrate (pro-3S)-lyase)	TCA cycle (reductive)
42	*RHA1_RS14330*		Citrate lyase beta subunit	TCA cycle (reductive)
42	*RHA1_RS29250*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS29815*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS32175*		CoA ester lyase	TCA cycle (reductive)
42	*RHA1_RS38600*		Possible citrate lyase beta subunit (C-terminal)	TCA cycle (reductive)
42	*RHA1_RS39235*		Aldolase	TCA cycle (reductive)
43	*RHA1_RS11675*		2-Oxo acid ferredoxin oxidoreductase subunit beta	TCA cycle (reductive)
44	*RHA1_RS11825*		FAD-binding dehydrogenase	TCA cycle (reductive)
44	*RHA1_RS40180*		FAD-binding dehydrogenase	TCA cycle (reductive)
44	*RHA1_RS40490*		FAD-binding dehydrogenase	TCA cycle (reductive)
45	*RHA1_RS10355*		Isocitrate lyase	Glyoxylate pathway
46	*RHA1_RS04300*	*glcB*	Malate synthase G	Glyoxylate pathway
46	*RHA1_RS12540*		Malate synthase G	Glyoxylate pathway
47	*RHA1_RS35295*	*mutA*	Methylmalonyl-CoA mutase small subunit	Methylmalonyl-CoA pathway
47	*RHA1_RS35300*	*mutB*	Methylmalonyl-CoA mutase	Methylmalonyl-CoA pathway

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

Nguyen et al. (2017) have characterized an alternative enzyme that catalyzes this step in *R. jostii* RHA1 called F420-dependent glucose-6-phosphate dehydrogenase (FGD). This enzyme uses low redox potential cofactor that could play an important role in the central metabolism and xenobiotics degradation.

The NADPH is an essential reducing equivalent in most biosynthetic pathways such as the fatty acid biosynthesis (Spaans et al. 2015). Recent proteomic and transcriptomic analyses (Dávila Costa et al. 2015; Juarez et al. 2017) of *R. jostii* RHA1 under lipid accumulation conditions (low nitrogen levels) showed a significant induction of genes/proteins that participate in ED and PPP pathways. Likewise, studies conducted in *R. opacus* PD630 using ^{13}C also show the activation of these pathways during lipid accumulation (Hollinshead et al. 2015). In addition, MacEachran and Sinskey (2013) identified and characterized an enzyme that contributes to the formation of NADPH, a non-phosphorylative glyceraldehyde dehydrogenase enzyme, which catalyzes the direct conversion of the glyceraldehyde 3-P to 3-phosphoglycerate (Fig. 1). This enzyme was also induced specifically during lipid accumulation. On the other hand, the occurrence of $NADP^+$-dependent malic enzymes (reaction 27 in Fig. 1 and Table 1) and their effect on lipid accumulation have been described recently in the RHA1 strain (Hernández and Alvarez 2018). Then, the NADPH necessary for lipid biosynthesis seems to come from several pathways in these bacteria.

Glycolytic pathway also contributes with key precursors for lipid biosynthesis such as triacylglycerols (TAG) in oleaginous *Rhodococcus* species. For example, the glycolytic intermediate dihydroxyacetone phosphate is converted to glycerol 3-phosphate by the NADP-dependent glycerol 3-phosphate dehydrogenase. This enzyme is upregulated in *R. jostii* RHA1 under TAG-accumulating condition (Dávila Costa et al. 2015).

All genes involved in the carbohydrate metabolism are widely distributed throughout the RHA1 genome. However, some genes such as the *tpiA*, *gap2*, and *pgk* involved in the glycolytic pathways are clustered in the genome. Similarly, the genes *RHA1_RS11570* coding for a phosphogluconate dehydratase (Edd) and *RHA1_RS11565* coding for a KHG/KDPG aldolase (Edda), both involved specifically in the ED pathway, are clustered in RHA1 genome with the gene coding for glucose-6-phosphate dehydrogenase (Zwf). In this same cluster, it is located in the gene *glcp* (*RHA1_RS11555*), involved in glucose uptake components in *R. jostii* RHA1 (Araki et al. 2011).

3 Glycogen Synthesis and the Link with the Central Metabolism

The ability to synthesize and accumulate glycogen has been studied based on bioinformatics and/or experimental approaches in different members of the *Rhodococcus* genus such as *R. jostii* (Hernández et al. 2008; Tajparast and Frigon

2015), *R. opacus* (Hernández and Alvarez 2010; Hernández et al. 2013), *R. erythropolis*, *R. ruber*, *R. equi*, and *R. fascians* (Hernández and Alvarez 2010; Yano et al. 2016). In general, the total content of glycogen in those microorganisms reached up to 2–6% of cellular dry weight. The studied strains accumulated glycogen during exponential growth phase, and the content tends to decrease during stationary growth phase. The occurrence of glycogen accumulation during exponential growth phase has been reported a long time ago in other actinomycetes such as *Mycobacterium smegmatis* (Belanger and Hatfull 1999) and *Corynebacterium glutamicum* (Seibold et al. 2007).

Similarly to other microorganisms, genes of glycogen synthesis and degradation in *Rhodococcus* species are usually arranged in clusters and seem to be non-redundant (*glgC*, *glgA*, *glgB*, *glgX*) (Hernández et al. 2008; Hernández and Alvarez 2010). In addition, as occur in other actinobacteria such as *Mycobacterium* and *Streptomyces* (Kalscheuer et al. 2010; Elbein et al. 2010), *Rhodococcus* species also contain the alternative GlgE α-glucan pathway mediated by the *glgE* gene (Hernández et al. 2008). 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 part of a sensing/signaling mechanism. Persson et al. (2007) proposed that the expression of some genes in *E. coli*, such as the universal stress protein *uspA* involved in the carbon starvation conditions or stationary phase, 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.

Transcriptome and proteome analyses in RHA1 and PD630 strains have shown that gene expression and/or protein abundance associated with glycogen synthesis remains constant between culture conditions with high or low nitrogen levels, which suggest that glycogen synthesis is not essentially regulated at the transcriptional level in those conditions (Dávila Costa et al. 2015; Chen et al. 2014). On the contrary, proteins associated with glycogen synthesis seem to be regulated at a posttranslational level. In this context, Cereijo et al. (2016) reported an allosteric regulation of ADP-glucose pyrophosphorylase of RHA1 strain, a key regulatory step in bacterial glycogen metabolism, being the glucose-6P, mannose-6P, fructose-6P, ribose-5P, and phosphoenolpyruvate, the major activators, whereas the NADPH and 6P-gluconate behaved as main inhibitors of this enzyme. The authors also suggested a potential role of glycogen as a temporal reserve that provides a pool of carbon able to be re-routed to produce long-term storage of lipids under certain conditions in oleaginous species of *Rhodococcus*. In contrast, Dávila Costa et al. (2015) reported a higher abundance of enzymes involved in glycogen degradation, such as glycogen phosphorylase (GlgP, EC 2.4.1.1), glycogen debranching enzyme (GlgX,

EC3.2.1.33), and phosphoglucomutase (PGM,EC5.4.2.2) during cell cultivation under conditions leading to lipid accumulation (low-nitrogen conditions), which suggest that glycogen degradation can be regulated at a transcriptional level. In conclusion, glycogen metabolism seems to respond to a complex regulation at both transcriptional and posttranslational levels and probably in orchestration with the synthesis of other compounds such as exopolysaccharides and lipids. In this context, recent studies have described the potential role of a capsular α-glucan-like compound exported from internal glycogen in the pathogenic strain of *M. tuberculosis* in virulence by evading the host immune response (Koliwer-Brandl et al. 2016). Although a capsular α-glucan-like compound have not been specifically described in *Rhodococcus*, it is known that several species of this genus are able to produce exopolysaccharides of varied nature (Perry et al. 2007; Hernández et al. 2017).

4 Gluconeogenesis and the Phosphoenolpyruvate-Pyruvate-Oxaloacetate Node

The metabolic link between glycolysis/gluconeogenesis and the TCA cycle is represented by the PEP-pyruvate-oxaloacetate 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 enters to the central metabolism via the acetyl-CoA or as TCA cycle intermediates, the intermediates malate and oxaloacetate must be converted to pyruvate and phosphoenolpyruvate (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 oxaloacetate 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 oxaloacetate decarboxylase and/or malic enzyme in combination with PEP synthetase. As in *C. glutamicum* (Sauer and Eikmanns 2005), PEP carboxykinase (RHA1_RS25350) is probably the only enzyme responsible for PEP synthesis from TCA cycle intermediates in *R. jostii* RHA1, since the gene coding a PEP synthetase is lacking in its genome. Nevertheless, some PEP syntheses are present in other *Rhodococcus* species such us *R. equi* (REQ_RS07445), *R. pyridinivorans* (Y013_RS04990), and *R. aetherivorans* (AAT18_RS13085). In *M. tuberculosis*, the role of Pck enzyme in gluconeogenesis has been demonstrated in presence of lipid substrates, being essential for intracellular growth and survival in macrophages during infection (Basu et al. 2018). On the other hand, pyruvate can be provided

from malate by the action of some malic enzymes (ME). The ME enzymes have been associated also with the production of reducing equivalents for anabolic processes in several organisms (Spaans et al. 2015). In RHA1 strain as well as other *Rhodococcus* species, there are several genes coding for these kinds of enzymes, and some of them seem to be associated also with the generation of NADPH for lipid biosynthesis in oleaginous species (Hernández and Alvarez, unpublished results) (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 oxaloacetate (Sauer and Eikmanns 2005). In *M. tuberculosis* H37Rv, it has been demonstrated that these anaplerotic functions involve not only pyruvate carboxylase (PCA) but also Pck and ME enzymes. The anaplerotic node was analyzed through different variant mutants of these genes, showing they are also essential for intracellular growth of *M. tuberculosis* strain. Particularly it was demonstrated that PCA is required for growth on pyruvate and that the phenotype could be complemented by addition of aspartate (as proxy for oxaloacetate) or by growing cultures in presence of 5% CO_2. These results showed the anaplerotic role of PCA replenishing oxaloacetate into TCA cycle and that this function could be complemented by CO_2 addition probably by action of either ME or Pck (Basu et al. 2018).

R. jostii RHA1 seems to possess PEP carboxylase and pyruvate carboxylase in its genome as anaplerotic enzymes (Fig. 1 and Table 1). However, as occurs in *M. tuberculosis H37Rv*, Pck enzyme of *R. jostii* RHA1 would be operating in the carboxylating direction (anaplerotic) or in gluconeogenic direction (Basu et al. 2018). It is known that pyruvate carboxylase plays a major anaplerotic role in mammals, yeast, and in a few prokaryotes such as *Rhodopseudomonas spheroids*, *Arthrobacter globiformis*, and *Mycobacterium smegmatis*. Many other bacteria such as *Pseudomonas citronellolis*, *Azotobacter vinelandii*, or *C. glutamicum* are able to use both PEP carboxylase and pyruvate carboxylase as anaplerotic enzymes (Sauer and Eikmanns 2005).

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

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 amino acids (principally the aspartate family) contained in *R. opacus* 1CP were much higher enriched in ^{13}C than those in strain B13 (Feisthauer et al. 2008). The authors concluded that *R. opacus* 1CP 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-negative bacteria (Feisthauer et al. 2008). Altogether, these results emphasize the role of the PEP carboxylase and pyruvate carboxylase enzymes in *R. jostii* RHA1 metabolism, a very close species to *R. opacus*. The occurrence of additional anaplerotic enzymes as Pck enzyme (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-oxaloacetate 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 be not 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 reactivate back 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 with 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 downregulated 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. 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. Otherwise, other reactions could contribute with the intracellular acetyl-CoA pool, such as the

reaction catalyzed by citrate lyase enzyme, which converts citric acid into acetyl-CoA and oxaloacetate and the eventual activation of acetate to acetyl-CoA by an acetyl-CoA synthetase. Transcriptomic analysis of *R. jostii* RHA1 under conditions that lead to TAG accumulation revealed a partial repression of pyruvate dehydrogenase enzyme to generate acetyl-CoA; however, it was determined the induction of several enzymes acetyl-CoA synthetases (Juarez et al. 2017).

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.

5 The Tricarboxylic Acid Cycle (TCA)

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 *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 oxaloacetate, whereas the 2-oxoglutarate synthase produces 2-oxoglutarate from succinyl-CoA. This allows the metabolism to incorporate CO_2 and to serve as an engine for synthesis instead of energy production (Srinivasan and Morowitz 2006). CO_2 fixation also occurs in *R. erythropolis* N9T-4 which possess an alternative TCA oxidative cycle. In Yano et al. (2015), the authors propose a bypass of oxoglutarate to succinate through succinic semialdehyde instead of the classic succinyl-CoA in this strain. They also demonstrate a shortcut to prevent CO_2 losses that occur within the TCA cycle. The possible autotrophic metabolism in *Rhodococcus* will be exposed deeper in a specific chapter of this book by Yoshida et al. 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 been also reported for other actinomycetes, such as *Mycobacterium tuberculosis* and *Streptomyces coelicolor* (Srinivasan

and Morowitz 2006). In addition, the genes coding for key enzymes of the reductive TCA cycle seem also 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 environmental conditions, for example, (a) starvation as demonstrated for RHA1 that showed a decrease in seven enzymes involved in the oxidative and reductive TCA cycle due to the shutdown of energy production or carbon-containing compounds (Patrauchan et al. 2012) or (b) pollutant stress as shown in a rhodococcal strain isolated from lithium-mining areas that overexpress TCA enzymes involved in the synthesis of intermediates of the TCA cycle (Belfiore et al. 2017). The authors postulate that these metabolites act as scavengers of ROS. A similar behavior was observed during degradation of tetrahydrofuran by *Rhodococcus* sp.YYL (He et al. 2014).

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 to replenish oxaloacetate 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. Members of the genus *Rhodococcus* are able to accumulate variable amounts of triacylglycerols, which are degraded during carbon starvation (Alvarez et al. 2000; Alvarez 2006). The upregulation of isocitrate lyase 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 (Patrauchan et al. 2012). The synthesis of triacylglycerols seems to be an important metabolic pathway in some species of rhodococci for the maintenance of energy homeostasis. Triacylglycerols are synthesized in 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 glyoxylate cycle plays a pivotal role in the persistence of *Mycobacterium tuberculosis* in mice by sustaining intracellular infection in inflammatory macrophages (Eisenreich et al. 2010). 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). Furthermore, it was recently described the importance of malate synthase for the *M. tuberculosis* growth and survival on even fatty acids by detoxification of glyoxylate, arising from acetyl-CoA metabolism, by assimilation of a second molecule of acetyl-CoA (Puckett et al.

2017). This function is also important in *R. fascians*, the only phytopathogen described so far, to avoid the toxicity of the glyoxylate produced when bacteria catabolize compounds from plants as nutrient factors during issues of infection (Vereecke et al. 2002). On the other hand, Yano et al. (2015) described the relevance of gluconeogenesis and glyoxylate cycle to oligotrophic growth and CO_2 fixation in strain *R. erythropolis* N9T-4; this pathway avoids the loss of carbon as CO_2 molecules and preserves CoA units in the TCA cycle.

7 Litoautotrophic Processes in *Rhodococcus*

Rhodococcus bacteria are usually considered chemoheterotrophic microorganisms, which use organic compounds as sources of carbon and energy. However, there is bioinformatic and experimental evidence that members of this genus are rather facultative chemolithoautotrophs. 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. On the other hand, *R. equi* was able to consume H_2 in the late exponential and stationary phase (Meredith et al. 2014). The examination of genomic data revealed that *R. jostii* RHA1 possesses a gene cluster (*RHA1_RS22440* to *RHA1_RS22555*) encoding a putative hydrogenase system. Hydrogenase genes also occur in the genome of *R. opacus* B4 and *R. erythropolis* PR4. Altogether, these results indicated that *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 (*RHA1_RS21555-RHA1_RS21565*) 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 contain three clusters including genes coding for putative CO dehydrogenases (CODH). Patrauchan et al. (2012) reported a 250-fold increase in CO dehydrogenase concurrent with a 130-fold increase in CODH activity during

cultivation of RHA1 strain under carbon starvation conditions, suggesting a switch from heterotrophic to autotrophic metabolism. The authors suggested that CODH enables assimilation of CO_2 or CO into cellular building blocks under these environmental conditions. Autotrophic growth has also been demonstrated in *R. erythropolis* N9T-4, a bacterial strain isolated from stored crude oil (Ohhata et al. 2007). This strain was able to grow on a basal medium without any additional carbon, nitrogen, sulfur, and energy sources but required CO_2 for oligotrophic growth. The authors suggested that N9T-4 possesses a novel CO_2 fixation pathway linked to the formaldehyde metabolism (Yoshida et al. 2011). Strain N9T-4 can utilize carbon, nitrogen, and sulfur compounds, directly from the atmosphere. It has been suggested that N9T-4 can utilize traces amounts of atmospheric ammonia as a nitrogen source through the strong expression of an ammonium transporter gene (*amtB*) (Yoshida et al. 2014).

Altogether, these studies confirmed the existence of diverse litoautotrophic pathways in rhodococci that allow them to survive and thrive in oligotrophic environments. CODH, hydrogenase, thiocyanate hydrolase, and other still unknown metabolic 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 cells. Rhodococci seem to possess the ability to conserve useful energy during catabolism, distributing the carbon flux of the substrate between the energy production and 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 others. 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 another essential feature of rhodococci. These microorganisms seem to have the potential to use alternatively different glycolytic pathways, such as EMP or ED pathways, the

PPP 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 with 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-oxaloacetate node may play a key role in the C flux distribution within the overall cell metabolism.

Despite the numerous *Rhodococcus* genomes now available, the biochemistry and the molecular biology of the central metabolism of the *Rhodococcus* genus are still poorly known. 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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Oligotrophic Growth of *Rhodococcus*

Nobuyuki Yoshida

Contents

Abstract We can relatively easily isolate "super" oligotrophs from various environments, which can grow on minimal medium without additional carbon and energy sources. The super oligotrophs isolated to date have all belonged to the genera *Rhodococcus* and *Streptomyces*. This chapter primarily describes the genetics and biochemistry of one of the super oligotrophs, *R. erythropolis* N9T-4, which was isolated from crude oil. It is suggested that C2 metabolism starting from acetaldehyde is the key to oligotrophic carbon metabolism of N9T-4. Intriguingly, this bacterium is also oligotrophic for nitrogen and sulfur sources. The most unequivocal evidence for the oligotrophy of N9T-4 relates to the utilization of nitrogen, in which ammonium transporter is involved in the incorporation of atmospheric ammonia under oligotrophic conditions. A unique intracellular structure found in the oligotrophically grown super oligotroph is also introduced.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_4

1 What Are Oligotrophs?

An oligotroph is a microorganism that can grow at very low concentrations of nutrients. Kuznetsov defined an oligotroph as one that develops at the first cultivation from nature on a medium with minimal organic matter, containing about 1–15 mg of carbon per liter (Kuznetsov et al. 1979). However, this definition is still nebulous, and there may be no sense in arguing whether concentration of nutrients is high or low for microbial growth in various environments, since low nutrient concentrations would be enough for the growth of oligotrophs. Recent metagenomic analyses have revealed that numerous microorganisms exist in various oligotrophic environments (Bonilla-Rosso et al. 2012; Siering et al. 2013). However, there are few research groups combining the biochemical, genetic, and molecular biological approaches required to investigate microbial oligotrophy. In general, autotrophs, which use CO_2 as their sole carbon source in conjunction with some energy or reducing power, such as sunlight, reducing metal, and molecular hydrogen, are not categorized as oligotrophs. However, it is possible that some oligotrophs fix atmospheric CO_2 to use low concentrations of in situ organic carbon sources effectively.

We have suggested that oligotrophs are useful as hosts for production of useful compounds, since the cost of their cultivation can be restrained. Oligotrophs would also be suitable for in situ bioremediation, because such microorganisms can grow on the contaminated site without additional nutrients. Thus, oligotrophs have a biotechnological potential, motivating us to attempt their isolation from various environments.

First, we used 1000 times-diluted nutrient broth to isolate numerous oligotrophs from various natural samples. However, during the screening, we surprisingly found that oligotrophs could be relatively easily isolated using an inorganic basal medium without carbon sources (BM), which was composed of 1% $NaNO_3$, 1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2{\cdot}2H_2O$, and a vitamin mixture (Ohhata et al. 2007). Most of the oligotrophs isolated using BM did not grow under CO_2-limiting conditions, in which a CO_2 absorbent was placed with a petri dish containing BM inoculated by an isolated oligotroph in a plastic bag. However, even under the CO_2-limiting conditions, growth was observed on BM containing $NaHCO_3$ or restored by injection of CO_2 gas into the plastic bag; this indicated that oligotrophic growth depends on CO_2 in the atmosphere. It should be noted that we have not used the term "autotrophic" but rather "super oligotrophic" for such CO_2-requiring oligotrophs as described above, since the CO_2 fixation mechanism is still unknown and any additional energy sources to fix CO_2 are not required for the oligotrophic growth. Indeed, the reason why $NaNO_3$ has been used as the nitrogen source is to avoid isolation of ammonia-oxidizing autotrophs, which could be isolated when NH_4^+ was used as the nitrogen source.

Most organisms, from bacteria to man, have CO_2-dependent carboxylase reactions in their metabolic pathways: acetyl-CoA carboxylase and anaplerotic enzymes such as phosphoenolpyruvate carboxylase, pyruvate carboxylase, and malic enzyme.

In that sense, all organisms are CO_2-dependent, though the CO_2 does not necessarily come from the atmosphere. Meanwhile, microorganisms that require a higher concentration of CO_2 (ca. 5%) are known as capnophiles (Al-Haideri et al. 2016; Bury-Moné et al. 2008; Sahuquillo-Arce et al. 2017). Such capnophilic bacteria lack a carbonic anhydrase activity, which catalyzes the reversible hydration of CO_2 to bicarbonate (HCO_3^-). Capnophilic bacteria should be dependent on nonenzymatically produced HCO_3^- from CO_2 due to the shift of the chemical equilibration under the high concentrations of CO_2. Unlike the capnophiles, super oligotrophs can grow on BM under the ambient air. Is CO_2 used as the main carbon source or in the anaplerotic pathways for super oligotrophs? What is the energy source for the CO_2 fixation associated with oligotrophic growth? The process of solving this puzzle has begun and continues to date.

2 Super Oligotrophs in *Rhodococcus*

The first super oligotroph to be discovered was *R. erythropolis* N9T-4, isolated from crude oil stored in a Japanese oil stockpile (Ohhata et al. 2007). N9T-4 grew well on BM solidified with agar or silica gel without any additional carbon and energy sources. This bacterium did not show oligotrophic growth under the CO_2-limiting conditions described above but grew on a BM plate containing $NaHCO_3$ (Fig. 1). Intriguingly, the only organic compounds that could sustain the growth under the CO_2-limiting conditions were *n*-alkanes, such as *n*-tetradecane and *n*-hexadecane, whereas sugars (glucose, fructose, and galactose) and alcohols (ethanol and methanol) did not restore the growth. The reason why only *n*-alkanes reversed the growth defect under CO_2-limiting conditions is unclear but is suggested to be due to C2 metabolism in N9T-4 as mentioned in the next section. The draft genome of N9T-4 has been sequenced, and it reveals that there are no genes encoding the key enzymes involved in the six known microbial CO_2 fixation pathways and cycles (Berg 2011). Recently, a seventh CO_2 fixation pathway has been found in a phosphite-oxidizing chemolithoautotroph (Figueroa et al. 2018). The gene encoding a formate-tetrahydrofolate ligase, which is the key enzyme in the seventh pathway, is also not found in the genome of N9T-4.

It is interesting to consider whether such unique oligotrophy of N9T-4 is due to specific environmental conditions, such as crude oil, where N9T-4 was isolated, or is widely distributed across environments. After the discovery of N9T-4, further isolation of super oligotrophs was attempted extensively from various environments (Yoshida et al. 2007). As a result, three super oligotrophic *Streptomyces* spp. were isolated from soil samples, which were close to *S. viridobrunneus*, *S. bikiniensis*, and *S. exfoliatus*. Super oligotrophs were also isolated from some culture collections, and besides *R. erythropolis*, *R. rhodochrous*, *R. opacus*, *R. ruber*, *and R. wratislaviensis* showed similar oligotrophic growth to that of N9T-4 (Fig. 2). As for *Streptomyces* sp., *S. aureus* NBRC 100912, *S. griseus* NBRC 15421, *S. bikiniensis* NBRC 14598, and *S. venezuelae* NBRC 13096 could also grow on BM (Yoshida et al. 2007). Thus,

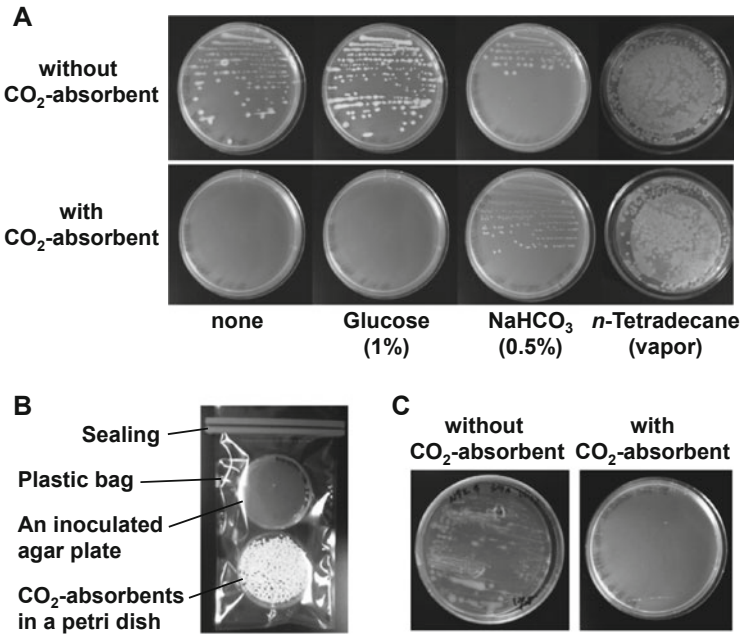

Fig. 1 Oligtorphic growth of *R. erythropolis* N9T-4. (**a**) N9T-4 formed its colonies on BM medium solidified by agar after 5-day cultivation at 30 °C. The oligotrophic growth was repressed when CO_2 was removed from the culture environment by a CO_2 absorbent, while $NaHCO_3$ and *n*-tetradecane, which was soaked in two filter papers on the lid of a glass petri dish and provided as the vapor, sustained the growth under the CO_2-limiting conditions. (**b**) CO_2-limiting conditions were constructed by using a CO_2 absorbent, which was sealed with a BM plate inoculated by N9T-4 in a plastic bag. (**c**) Inorganic growth of N9T-4 was verified using a BM solidified by silica gel

to date, super oligotrophs have been confined to the genera of *Rhodococcus* and *Streptomyces*. These results suggest that certain properties of actinomycetes are responsible for the super oligotrophy.

3 Genetics and Biochemistry of a Super Oligotroph

To elucidate the oligotrophic metabolism of N9T-4, differential two-dimensional electrophoresis (2-DE) was first performed using the cells grown on BM without carbon source and that with *n*-tetradecane as the sole carbon source (Ohhata et al. 2007). The protein profiles on the 2-DE showed that six proteins were highly induced in the oligotrophically grown cells. From analysis of their amino acid sequences, four of them were found to be similar to chaperones such as ClpB, DnaK, and DnaJ. The other two proteins with the apparent molecular masses of 45 kDa and 55 kDa were very close to methanol: *N,N′*-demethyl-4-nitrosoaniline (NDMA) oxidoreductase (MNO) from *Rhodococcus* sp. strain NI86/21 (Nagy et al.

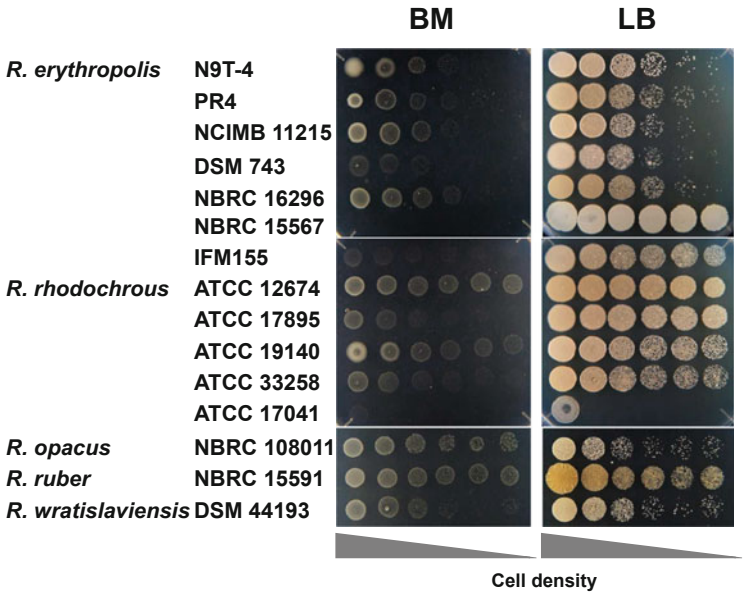

Fig. 2 Super oligotrophs in various *Rhodococcus* spp.

1995) and an NAD-dependent aldehyde dehydrogenase (ALDH) from *R. erythropolis* UPV-1, respectively. *R. erythropolis* UPV-1 could remove phenol and formaldehyde in industrial wastewater, and the ALDH had a broad substrate specificity for aliphatic aldehydes from formaldehyde to *n*-octanal (Jaureguibeitia et al. 2007). MNO is a nicotinoprotein, which was found in gram-positive methylotrophic bacteria, containing a tightly but noncovalently bound NADP (H) per subunit (Bystrykh et al. 1993). MNO catalyzes the oxidation of methanol by using NDMA as an artificial electron acceptor, whereas the enzyme has a remarkable dismutase activity for formaldehyde to produce methanol and formate without NDMA. ALDH and MNO from N9T-4 also had activities of NAD-dependent formaldehyde dehydrogenase and formaldehyde dismutase, respectively, and the enzyme activities and mRNA expression of these enzymes from the cells grown oligotrophically were higher than those from the cells grown on LB (Ohhata et al. 2007; Yoshida et al. 2011). From these results, it was suggested that CO_2 was converted to formaldehyde by an unknown reaction, which was followed by formaldehyde metabolism found in methylotrophs. However, currently, this hypothesis has been denied since the conversion of CO_2 to formaldehyde in the N9T-4 cells has not been detected and the genes involved in formaldehyde assimilation, such as the ribulose monophosphate pathway (Mitsui et al. 2000) and serine pathway (Vorholt 2002) in methylotrophic bacteria, were not found in the N9T-4 genome. These are in accordance with the fact that N9T-4 could not utilize methanol as the sole carbon source. However, intriguingly, N9T-4 has a methylotrophic formaldehyde dehydrogenase (FADH) besides oligotrophic ALDH, which is

probably dependent on NAD and mycothiol as the cofactors. Mycothiol is a thiol compound found in *Mycobacterium* and *Rhodococcus* spp., and its functions correspond to glutathione in gram-negative bacteria, plants, and animals. In the formaldehyde-dissimilation pathway in gram-positive bacteria, *S*-hydroxymethylmycothiol is formed from formaldehyde and mycothiol, and then the NAD-dependent dehydrogenation occurs to produce *S*-formyl mycothiol. It is of interest that the gene encoding the methylotrophic FADH was not upregulated under the oligotrophic conditions but expressed remarkably when formaldehyde was added to the growth medium, whereas that oligotrophic *aldA* was regulated in the opposite manner (Yoshida et al. 2011).

Another approach to elucidate the oligotrophic metabolism in N9T-4 was random mutagenesis to obtain oligotrophy-deficient mutants. Random mutation library is still a powerful tool to clarify the unknown metabolisms of various microorganisms, and it was attempted to obtain mutants that could not grow on BM but form their colonies on LB (nutrient-rich conditions) from a random mutation library constructed using transposons (Yano et al. 2015). Intriguingly, transposase and its recognition sequences were not necessary, and only linear DNA fragments containing a selection marker were enough to construct the random mutation library for wild-type N9T-4. Furthermore, not insertion mutation but deletion mutation occurred in all of the mutants, suggesting that the mechanism of nonhomologous end joining is involved in the formation of the mutants. From nearly 10,000 independent mutants, 3 types of deletion mutants were obtained, in which *aceA*, *aceB*, and *pckG* encoding isocitrate lyase, malate synthase, and phosphoenolpyruvate, respectively, were deleted. The results indicated that glyoxylate shunt and gluconeogenesis play important roles in the oligotrophy of N9T-4. Although the deletion mutants of *aldA* and *mnoA* encoding ALDH and MNO described above, respectively, could not be obtained from the random mutation library, a Δ*aldA* strain constructed by homologous recombination did not grow under the oligotrophic conditions, whereas a Δ*mnoA* strain showed the same level of oligotrophic growth as the wild-type strain.

Since it was revealed that the glyoxylate shunt was essential for the oligotrophic growth, the analysis of the TCA cycle in N9T-4 is of interest. Most of the activities of the TCA cycle were clearly observed in the cell extract prepared from the oligotrophically grown N9T-4 cells, except for the α-ketoglutarate dehydrogenase (KGDH) (Yano et al. 2015). No KGDH activity was detected in the cell extracts from the cells grown on BM and LB. It is known that *Mycobacterium tuberculosis*, which is phylogenetically closed to the *Rhodococcus* sp., also lacks KGDH activity and has the remarkable α-ketoglutarate decarboxylase (KGD) activity to produce succinic semialdehyde and CO_2 from α-ketoglutarate (Tian et al. 2005a, b). It was elucidated that protein–protein interaction between E1 (SucA) and E2 (DlaT) components of KGDH is weak and SucA catalyzes the KGD reaction without E2 and E3 components. Subsequently, succinic semialdehyde is converted to succinate by succinic semialdehyde dehydrogenase in the TCA cycle in *M. tuberculosis*. Such a TCA bypass route via succinic semialdehyde was also found in cyanobacteria (Steinhauser et al. 2012) and links to the γ-aminobutyric acid shunt via glutamate.

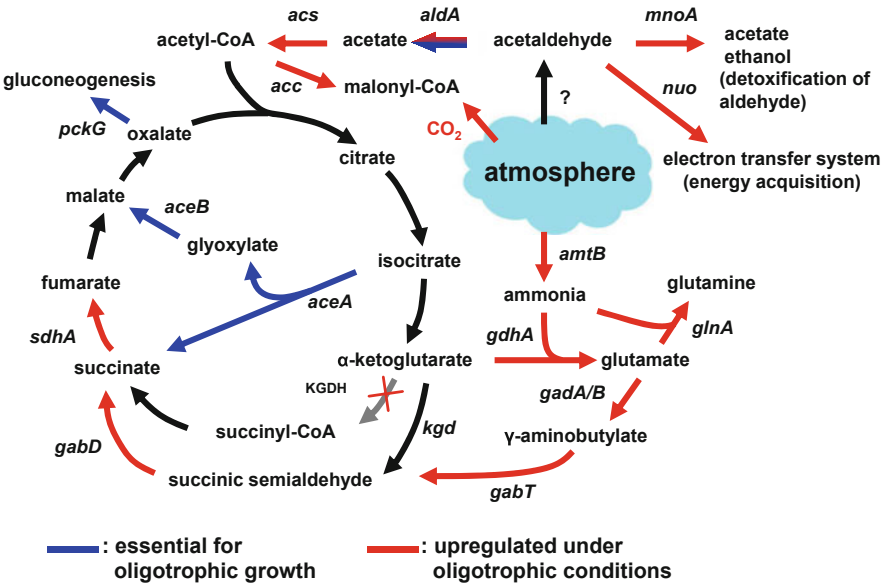

Fig. 3 Postulated oligotrophic carbon and nitrogen metabolisms in N9T-4

It is suggested that the TCA cycle in N9T-4 has similar features to those in *Mycobacterium* and cyanobacteria (Fig. 3). The physiological role of the TCA bypass routes is still unclear, but Steinhauser suggested that the bypass route is associated with low oxygen conditions, which lead to α-ketoglutarate and glutamate via the oxidative branch and succinate via the reductive branch in the TCA cycle in cyanobacteria. One presumed role of the variant TCA cycle in N9T-4 is that the bypass does not proceed via succinyl-CoA and may facilitate the CoA supply to help to maintain sufficient levels of acetyl-CoA.

Figure 3 shows carbon and nitrogen metabolisms in N9T-4 drawn based on the growth of the gene-deletion mutants and the transcriptome analysis. It should be noted that *acs* encoding acetyl-CoA synthetase was also highly expressed under the oligotrophic conditions. Since it was revealed that ALDH and MNO showed the remarkable activity toward acetaldehyde at the same levels toward formaldehyde in N9T-4, the following hypothesis can be built. Trace amounts of acetaldehyde, probably from the atmosphere, may convert to acetyl-CoA via acetate produced by ALDH and MNO, and then acetyl-CoA flows into the TCA cycle. Thus, C2 metabolism may be the key to the oligotrophic carbon metabolism in N9T-4, which is consistent with the fact that the glyoxylate shunt is essential for its oligotrophic growth. Furthermore, NADH produced from dehydrogenation of acetaldehyde by ALDH may be led to the electron transfer system to produce ATP that is used for oligotrophic growth, which was speculated based on upregulation of the genes encoding NADH oxidoreductase subunits (*nuo* genes). The function of MNO in this hypothesis is equivocal, but high concentrations of acetaldehyde are very

toxic against cells and N9T-4 may have such different types of aldehyde-utilizing and aldehyde-detoxifying systems. As described above, MNO also catalyzes aldehyde dismutation reaction, and aldehyde dismutases were found in some bacteria that can remove high concentrations of formaldehyde in industrial wastewater (Kato et al. 1986; Adroer et al. 1990; Yonemitsu and Kikuchi 2018). In spite of extensive genetic and biochemical analyses, no remarkable expression of any genes or enzymes involved in CO_2 fixation was observed. It has thus far been concluded that CO_2 is not main carbon source for the super oligotrophs and the CO_2 absorbent that has been used to construct the CO_2-limiting conditions may also remove CO_2 molecules that are essential for carboxylase reactions in the general biological metabolic pathways. This may be one of the reasons why most carbohydrates could not restore growth under CO_2-limiting conditions. A large amount of acetyl-CoA, which should be produced from *n*-alkanes such as *n*-tetradecane and *n*-hexadecane via β-oxidation, may be enough to accelerate the C2 metabolism and overcome a deficit of CO_2 under CO_2-limiting conditions.

4 Mystery in the Super Oligotroph

Some researchers have leveled the criticism that oligotrophs only utilize various volatile compounds in the laboratory in which numerous other microorganisms are cultivated in the same room or the same incubators. Another has suggested that oligotrophs could use gaseous hydrocarbons produced from laboratory wares made of synthetic resin such as polypropylene. Even if these suggestions are possible, it is certain that super oligotrophs can utilize an extremely trace amount of organic compounds, that is, oligotrophy. Furthermore, N9T-4 could grow outside of the laboratory on BM prepared in glassware, suggesting that the oligotrophy of N9T-4 is not due to such artifacts. However, it is hard to consider that N9T-4 utilizes a trace amount of acetaldehyde in the atmosphere, since the concentrations of acetaldehyde and formaldehyde in the atmosphere are at ppb levels.

There is another interesting fact that may be important to infer the carbon oligotrophic metabolism. N9T-4 shows oligotrophy not only for carbon source but also for nitrogen source, and N9T-4 could grow on BM without $NaNO_3$ (BM-N) (Yoshida et al. 2014). When the BM-N plate inoculated with N9T-4 was sealed in a plastic bag, no colony was observed (Fig. 4). Diazotrophic growth was first expected to account for the nitrogen oligotrophy, but nitrogenase activity was not detected in the cells and the putative gene encoding nitrogenase was not found in the N9T-4 genome. Transcriptome analysis revealed that one of the ammonium transporter genes (*amtB*) was strongly upregulated under oligotrophic conditions, and Δ*amtB* could not grow under nitrogen-limiting conditions. There results indicated that N9T-4 could utilize ammonia in the atmosphere as the nitrogen source. Actually, injection of ammonia vapor enhanced the growth of N9T-4 on BM-N in the closed system prepared by using a 9-L desiccator. The minimum concentration of ammonia that sustained the growth under the nitrogen-limiting conditions was 4 ppb, which is

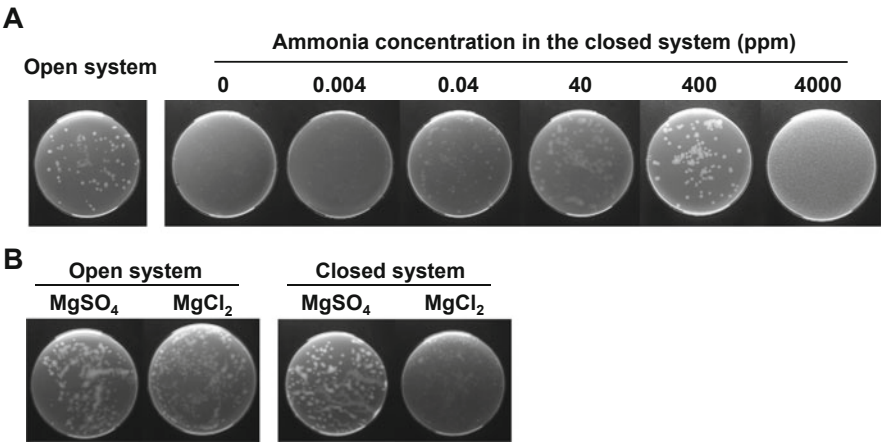

Fig. 4 Nitrogen and sulfur oligotrophy of N9T-4. (**a**) Injection of ammonium vapor restored the oligotrophic growth of N9T-4 on BM without nitrogen source in a closed culture system using 9-L desiccator. The concentrations of ammonia indicated in the figure were those estimated, if ammonia water added in the desiccator was completely volatilized. (**b**) N9T-4 could also grow on BM containing $MgCl_2$ instead of $MgSO_4$. The sulfur oligotrophy was also repressed in the closed system in a similar way as nitrogen oligotrophy

comparable to the atmospheric ammonia concentration in the place where the experiments were carried out (Matsumoto et al. 1998). In this experiment, N9T-4 cells could utilize only the amount of gaseous ammonia in 9 L of the air, whereas a constant concentration of gaseous ammonia would be supplied in the open culture system, suggesting that nitrogen oligotrophy involving incorporation of ammonia is a possible feature in nature. Recently, in environmental microbiology, attention has been drawn to the fact that some actinomycetes can uptake and oxidize atmospheric molecular hydrogen (0.53 ppm/0.40 nM) using their high-affinity [NiFe]-hydrogenase (Constant et al. 2010; Greening et al. 2014). These findings may help validate the utilization of atmospheric acetaldehyde by N9T-4.

Intriguingly, preliminary examination showed that N9T-4 could also grow on BM without a sulfur source ($MgSO_4$ was replaced with $MgCl_2$ in BM) (Yoshida et al. 2014). Similar to nitrogen oligotrophic growth, sulfur oligotrophic growth was repressed in the closed system, suggesting that gaseous sulfur molecules such as sulfur oxides and dimethylsulfide are candidates for the sulfur source for N9T-4. Furthermore, carbon monoxide (CO) could also be the carbon source for N9T-4, but further examination is necessary since a putative gene encoding CO dehydrogenase was not essential for CO utilization (Yano et al. 2012). Thus, N9T-4 can utilize the three biologically principal elements, carbon, nitrogen, and sulfur, from the atmosphere, lending it the name of "air-utilizing bacterium."

5 A Unique Intracellular Compartment in Super Oligotroph

Given that a super oligotroph, N9T-4, shows unique oligotrophic growth as described above, the intracellular structure of the cells grown under oligotrophic conditions is of interest. Transmission electron microscopic observation revealed that a relatively large and spherical compartment (named oligobody) was observed in N9T-4 cells grown on BM (Yoshida et al. 2017). Approximately 30% of the oligotrophic cells had the oligobodies, and each was around 150 nm in diameter (Fig. 5a, b). In most cases, only one oligobody was observed per cell, but in some cases they were localized at each pole of the cell with a septum at the center, suggesting that they separate at cell division (Fig. 5g). The oligobody was not observed or rarely observed (6–7% of total cells) in small sizes (approximately 70 nm in diameter) in the cells under nutrient-rich conditions (LB). The formation frequency and the size of the oligobody on BM containing glucose or *n*-tetradecane as the carbon source were at the same levels as those on BM without carbon source (Fig. 5e and f). The cells grown on BM containing *n*-tetradecane formed the other icicle-like inclusions, which were similar to wax bodies found in various alkane-degrading bacteria (Ishige et al. 2002).

To determine the elemental composition of the oligobody, energy dispersive X-ray analysis was performed. The results showed remarkable peaks corresponding phosphorous and potassium in an oligobody, whereas these peaks in the cytoplasm were very weak. The remarkable phosphorous content indicated accumulation of inorganic polyphosphate (polyP) in the oligobodies. It is known that polyphosphate kinases catalyze the reversible transfer of the terminal phosphate of ATP as the substrate to form a polyP chain. These kinases are divided into two categories, PPK1 and PPK2, which tend to catalyze the elongation and degradation of a polyP chain, respectively (Zhang et al. 2002). PolyP granules in microorganisms are well known, as they were formerly called volutin granules (Meyer 1904) and have recently been called acidocalcisomes (Docampo et al. 2005). Docampo said that acidocalcisomes are the only "organella" conserved from bacteria to man. The genes encoding PPK1 and PPK2 (*ppk1* and *ppk2*, respectively) were found in the N9T-4 genome, and as expected, Δ*ppk1* showed lower polyP content and oligobody formation than the wild type, whereas those of Δ*ppk2* were not affected. However, Δ*ppk1* and Δ*ppk2* showed the same levels of oligotrophic growth as the wild type, suggesting that polyP accumulation and oligobody formation are not involved in the oligotrophy of N9T-4 (Yoshida et al. 2017).

As described above, polyP synthesis requires ATP and was not essential for oligotrophic growth; nevertheless, why does N9T-4 accumulate polyP and form oligobodies under low carbon and energy conditions? This phenomenon is also one of the mysteries of N9T-4, which is interesting and should be clarified. It is known that *Escherichia coli* accumulates polyP under nutrient starvation conditions and polyP activates Lon protease, which helps to facilitate ribosomal protein degradation and supply amino acids to the cells (stringent response) (Kuroda et al. 2001). Indeed,

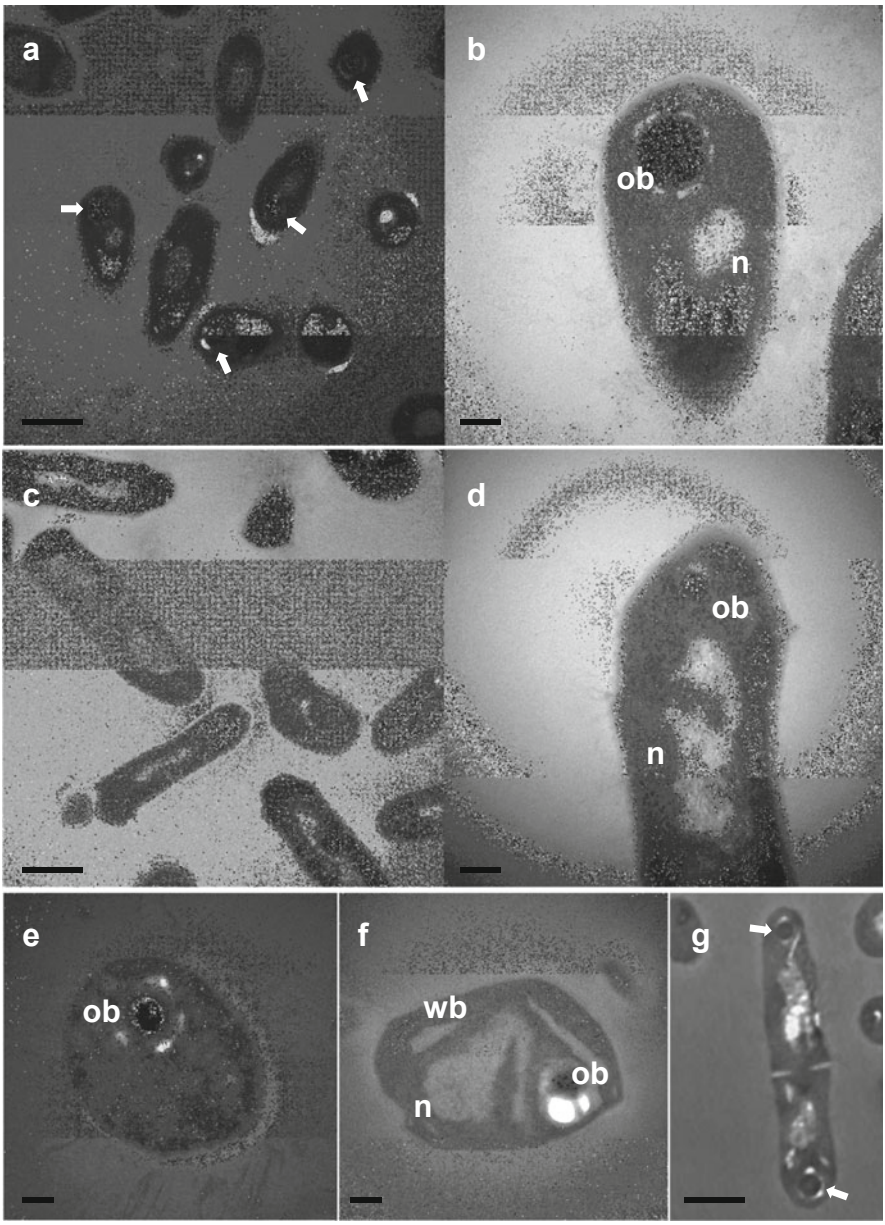

Fig. 5 Oligobodies found in N9T-4 cells. (**a**) and (**b**) BM-grown cells. (**c**) and (**d**) LB-grown cells. (**e**) BMG-grown cells. (**f**) BMT-grown cells. (**g**) a BM-grown cell during cell division. (**a/b**) and (**c/d**) shows images at two different magnifications focused on a single cell or multiple cells. All cultured cells were cultivated at 30 °C for 3 days (LB-grown cells) or 4 days (all others). Structures are labeled as follows: n, nucleoid; ob, oligobody; wb, lipid body. Arrows also indicate oligobody. Scale bars are 500 nm in **a, c,** and **g** and 100 nm in **b** and **d–f**

the conditions for polyP accumulation are quite different between *E. coli* and the super oligotrophy, and polyP was accumulated when cultivation was shifted to starvation from nutrient-rich conditions in *E. coli* and during oligotrophic conditions in N9T-4. However, it may be possible that oligotrophic conditions can be starvation or stress signals in N9T-4 to synthesize polyP in the cells.

6 Future and Biotechnological Application of Super Oligotrophs

As described above, there are many unsolved mysteries to the super oligotroph, N9T-4. In particular, the carbon metabolism should be clarified for the biotechnological application of super oligotrophs. Is it really possible that super oligotrophs utilize atmospheric acetaldehyde as carbon and energy sources? Cultivation in a closed system using a plastic bag was attempted to elucidate the carbon utilization, which was used to clarify ammonia utilization from the atmosphere, but N9T-4 could grow on BM in the closed system. A rapid concentration system for acetaldehyde may exist in N9T-4, considering that mRNAs for *aldA* and *mnoA* were expressed at levels of 20–100-fold after 30-min induction under the oligotrophic conditions (Yoshida et al. 2011). It was confirmed that ALDHs were also highly expressed in the other super oligotrophs isolated until now, so it is certain that aldehyde metabolism is the key in the carbon metabolism in the super oligotrophs in nature.

As the authors in the other chapters mention, *Rhodococcus* spp. bacteria have a great potential for biotechnological application through production of useful compounds (Yamada and Kobayashi 1996; Tajparast and Frigon 2015; Castro et al. 2016) and bioremediation (Soleimani et al. 2007; Kim et al. 2018). N9T-4, a super oligotrophic *Rhodococcus* sp., accumulates polyP under oligotrophic conditions, and recently, it was found that N9T-4 accumulated trehalose and glycogen when grown on BM, with the amount of trehalose being relatively high (0.28 g/g dry cell weight) while that of glycogen was very low (6.7 mg/g dry cell weight) (Yano et al. 2016). Thus, carbon metabolism in this super oligotrophy is still powerful even under oligotrophic conditions, so that it may be feasible to apply it to produce useful compounds at low cost by metabolic engineering.

One of the problems for the biotechnological application of the super oligotrophs is that super oligotrophs cannot grow well in a liquid culture medium ($OD_{660} < 0.1$). This growth defect in liquid culture may be due to their preference for air and/or necessity of cell scaffolding during cultivation. For its industrial application, an effective cultivation system should be established to obtain a large amount of the cells. Recently, submerged cultivation of N9T-4 was attempted using a polyurethane foam sponge with a supporting material to achieve approximately ten times the oligotrophic growth of the bacterium in the liquid culture medium (Matsuoka and Yoshida 2018). This method also facilitated the collection of bacterial cells, and a

high concentration of bacterial cells can be obtained simply by squeezing the sponge. It is expected that the physiology of oligotrophy in N9T-4 will be elucidated completely using the sponge.

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Adaptation of *Rhodococcus* to Organic Solvents

Carla C. C. R. de Carvalho

Contents

Abstract Most of the commercially interesting compounds and those affecting the environment are poor water soluble. Bacteria able to carry out the bioconversion or bioremediation of such compounds in systems using organic solvents as substrate and/or product reservoir are valuable. Strains of *Rhodococcus* have been reported to be particularly solvent tolerant whilst presenting a broad array of enzymes with potential for the production of industrially relevant compounds and/or for the metabolism of recalcitrant organic solvents. Under stressful conditions, 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. The adaptability and versatility of *Rhodococcus* cells can further broaden their application scope.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_5

1 Introduction

Sound and successful biocatalytic and bioremediation processes require bacterial strains tolerant or adapted to organic solvents. These cells should work in the presence of organic solvents necessary to overcoming the low water solubility of hydrophobic substrates and/or products in biocatalytic processes (de Carvalho and da Fonseca 2002a; Daugulis 2001; Heipieper et al. 2007) or be able to biodegrade organic solvents released into the environment (Aislabie et al. 2006; de Carvalho and da Fonseca 2007; Leisinge 1996; Parales and Haddock 2004). The use of an organic phase in bioreactors enhances productivity and facilitates downstream processing: (1) by controlled delivery of a toxic substrate from the organic to the aqueous phase containing the biocatalyst, or (2) by continuous extraction of a byproduct or of an inhibitory product. The group of extremophilic bacteria able to tolerate organic solvents retains their enzymatic activity in the presence of these compounds (Inoue 2011; Sardessai and Bhosle 2004).

The first report of a bacterium tolerant to organic solvents was made by Inoue and Horikoshi (1989) who discovered that *Pseudomonas putida* IH-2000 was 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 mainly 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 2005; 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 allows them to adhere directly to organic solvents containing the substrate of interest, which improves mass transfer between reservoir and biocatalyst (de Carvalho et al. 2007, 2009). The production of surface-active compounds, which decrease the surface tension of water and increase the solubility of low water-soluble compounds, further enhances the biocatalytic/degradation abilities of *Rhodococcus* cells. However, the ability of these cells to adapt the lipid composition and surface properties of their cell envelop is probably the most remarkable feature observed during adaptation to organic solvents (de Carvalho et al. 2009, 2016; Heipieper et al. 2007).

1.1 Predicting Solvent Toxicity

Organic solvents are, in general, toxic to bacterial cells even at low concentrations. The toxicity of water-immiscible solvents may result from (1) phase toxicity by direct contact between solvents and cells, and/or (2) molecular toxicity caused by the molecules of solvent dissolved in the aqueous phase. These compounds partition to cell membranes, increasing their fluidity and disrupting the phospholipid bilayer and interfering with membrane-bound proteins and ultimately causing cell death (Heipieper et al. 1991, 1994; Sikkema et al. 1995; Vermuë et al. 1993). 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 methods to predict a solvent's toxicity based on its physicochemical properties. In 1985, Brink and Tramper proposed the use of the solvent polarity and its molecular size to predict the suitability of a solvent to be used in a multiphasic biocatalytic system. Solvent polarity could be determined by the Hildebrand solubility parameter, δ, whilst the latter could be expressed as molecular weight or molar volume. In this system, high biocatalytic rates should be obtained by choosing organic solvents with low polarity ($\delta < \sim 8$) and high molecular weight ($M > \sim 150$). A few years later, the hydrophobicity of a solvent measured as the logarithm of the octanol-water partition coefficient, log $P_{O/W}$, was proposed as a better predictor of the antimicrobial action of a solvent (Laane et al. 1987; Osborne et al. 1990; Sikkema et al. 1994). According to the log $P_{O/W}$ 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 co-workers 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 cellular membranes 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 whilst 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 coefficients 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 values of 4–5 was observed for microorganisms (Laane et al. 1985; Vermuë et al. 1993), above which the solvents do not

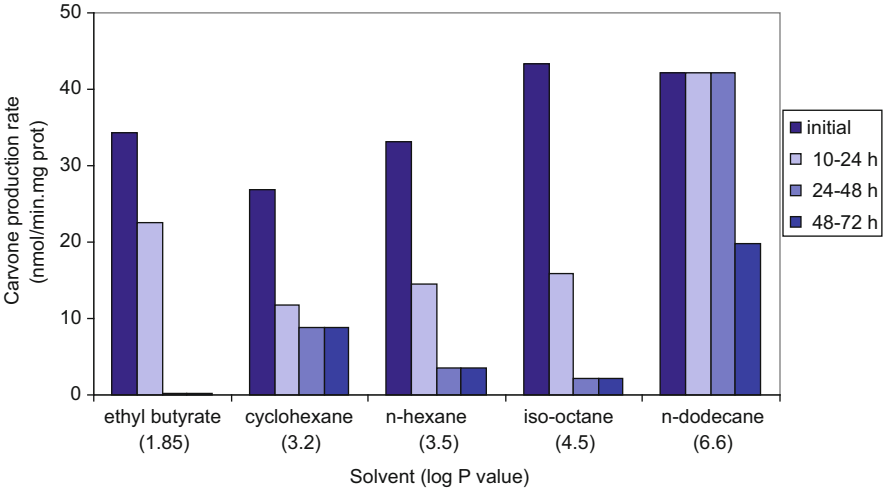

Fig. 1 Effect of the log *P* value of the solvent on *R. erythropolis* DCL14 cells carrying out the biotransformation of carveol into carvone in an organic-aqueous system (adapt. from de Carvalho and da Fonseca 2002a)

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. The author however noted 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 favourable solvents are often low (Cassells and Halling 1990). The transition between toxic and biocompatible solvents depends on the type of compound and also on the concentration of the solvent used in the test, and thus the concentration in the aqueous phase which causes loss of half of the metabolic activity of the bacterial cells should be determined (Vermuë et al. 1993). Nevertheless, the log *P* values could be used as an indication of the biocatalyst behaviour, even in processes requiring cofactor regeneration by viable cells (Fig. 1).

Hamada and co-workers (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, whilst the BATH assays were better to predict the behaviour of bacterial cells in organic-aqueous two-phase systems. The differences in the obtained results using the two techniques were ascribed to the effect of electrostatic interactions between bacteria and oil droplets.

As shown later by de Carvalho et al., *R. erythropolis* cells presented apparent contradictory results in the BATH and CAM when grown in C5–C16 alkanes: cell surface hydrophobicity, estimated by BATH, increased with the chain length of the alkane, but decreasing contact angles were observed (de Carvalho et al. 2009). These results could be explained by the fact that these cells could increase their zeta potential with the number of carbons in the alkane molecule: from −35.8 to +4.7 mV when the cells grew on pentane and hexadecane, respectively. The surprising change of polarity observed in *R. erythropolis* cells grown on long-chain-length alkanes could explain the strong cell adhesion to hexadecane observed during the BATH assay (where both hydrophobic and electrostatic forces are involved) and the contact angle measurements (resulting from van der Waals forces).

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 showing that the accumulation of these compounds occurs at varying 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, whilst 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 should 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 microorganisms' ability 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 but mainly from changes in the 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 in order 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 saturation-unsaturated and in the long-chain-short-chain fatty acid ratios can be seen as long-term changes to regulate membrane fluidity since 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 initially agreed that without an outer membrane, Gram-positive bacteria should be less tolerant to organic solvents. However, recent studies indicate that Gram-positive cells share tolerance mechanisms with their Gram-negative counterparts, including efflux pumps systems, isomerization of unsaturated fatty acids and modifications of the membrane phospholipids headgroups, formation of vesicles where toxic compounds may be stored and repair processes involving increased rate of phospholipids biosynthesis (de Carvalho et al. 2014a; Pacífico et al. 2018; Torres et al. 2011).

The permeable cell wall of Gram-positive bacteria does not usually restrict the penetration of antimicrobial agents, but vancomycin-intermediate-resistant *Staphylococcus aureus* strains present a significant thickened cell wall (Lambert 2002). Bacteria containing mycolic acids, such as mycobacteria and *Rhodococcus*, have cells 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 property of being able 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, whilst 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 because the cell is highly hydrophobic due to the mycolarabinogalactan-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 non-mucoid 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 water-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 was expected to 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 and co-workers (2007) also found the Gram-positive *Bacillus subtilis* to be more tolerant than *P. putida* towards 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 (Abe et al. 1995), could play an important role in decreasing solvent toxicity.

Rhodococcus strains have been found to endure considerably high concentrations of known toxic compounds, e.g. phenol, benzene and toluene (Table 1). The work of Gutiérrez and co-workers (2003) with *Rhodococcus* sp. showed that 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. Gutiérrez et al. (2009) studied the constitutive processes that confer resistance to benzene. Non-adapted *Rhodococcus* sp. 33 cells were able to endure shock concentrations

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)
	Oleyl alcohol, bis (2-ethylhexyl) phthalate	100%	Yamashita et al. (2007)
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 Diesel oil Phenol	34%[c] 27%[c] 12.5 mM[c]	Margesin et al. (2005)
Rhodococcus sp. NO20-3	*n*-Hexadecane Diesel oil Phenol	54%[c] 37%[c] 12.5 mM[c]	Margesin et al. (2005)
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	1000 mg/L	Prieto et al. (2002)
	Ethanol Butanol Dimethylformamide Dodecane bis(2-Ethylhexyl) phthalate	40% 20% 50% 5% 5% 1%	de Carvalho et al. (2004)
	Toluene	20%[d]	de Carvalho et al. (2007)
R. erythropolis DCL14	Methanol Ethanol Butanol Cyclohexanol Dodecanol	15%[a] 20%[a] 2%[a] 1%[a] 5%[a]	de Carvalho et al. (2005)
	Iso-octane	99.99%	de Carvalho et al. (2000)
	C5–C16 *n*-alkanes Motor oils Fuel oil	0.25%[a] 2%[a] 1.6%[a]	de Carvalho and da Fonseca (2007)
R. erythropolis 17S	Phenol	100 mg/L	Korobov et al. (2017)

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

of up to 1000 mg/L of pre-solubilized 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 is 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). The tolerance of *R. erythropolis* IBB_{Po1}, isolated from Poeni crude oil-contaminated soil, to alkanes and aromatics was ascribed to the presence of catabolic (*alkB*, *alkB1*, *todC1*, *todM*, *xylM*), transporter (HAE1) and trehalose-6-phosphate synthase (*otsA1*, KF059973.1) genes in the large genome (Stancu 2014). Following 1 and 24 h of exposure to 1% to alkanes and aromatics, the cells presented changes in cell growth pattern, morphology, hydrophobicity and also on the metabolic fingerprinting and *otsA1* gene sequence. Trehalose-6-phosphate synthases, TPSs and OtsAs, are involved in the production of surface-active trehalose lipids during *Rhodococcus* growth on medium with long-chained *n*-alkanes (Kim et al. 1990; Rapp et al. 1979). In strain *R. opacus* 1CP, OtsA1 was found to be involved in the overproduction of trehalose lipids, whilst OstA2 presented a major role in trehalose metabolism and trehalose lipid formation for the cell wall (Tischler et al. 2013). The role of OtsA2 was further supported by the presence of ORFs with putative function in cell envelope biogenesis next to the *ostA2* gene in this strain.

In 1942, Withell observed an exponential relation between the duration of a stress episode and bacterial death, which could be explained by the presence of 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 and co-workers (2007), indicate 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; Torres et al. 2011). 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, and (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, the number of studies on the adaptation of non-pathogenic strains to nonantibiotic compounds is still limited.

In a study carried out to assess the effects of organic solvents in organic-aqueous systems on *R. erythropolis*, *Xanthobacter* Py2, *Arthrobacter simplex* and *Mycobacterium* sp. NRRL B-3805, principal component 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 such as 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. erythropolis* 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 behaviour could also be ascribed to solvent toxicity (de Carvalho et al. 2003).

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 to diverse stresses are induced, and the cells become simultaneously resistant to, e.g. 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). Isolates from equine samples of *R. equi*, a facultative intracellular pathogen, able to produce biofilms presented higher resistance to macrolide antibiotics (Gressler et al. 2015). The same could be true regarding organic solvents as cells inside aggregates and biofilms are less exposed to the deleterious action of these compounds. *Rhodococcus* adhesion to both metallic and nonmetallic surfaces is promoted by a modulation of the cell lipid composition in response to both the type of surface and nutrients available (Rodrigues and de Carvalho 2015). Although *Mycobacterium vaccae* cells adapted to organic solvents presented an increased tolerance towards efflux pump inhibitors but not towards antibiotics (Pacífico et al. 2018), *R. erythropolis* cells adapted to toluene presented an increased resistance against 50% ethanol and Betadine® and Micropur® tablets used for surface disinfection and water purification (de Carvalho et al. 2007). The ability of *Rhodococcus* cells to form biofilms on the surface of indwelling central venous catheters led to the suggestion of adding them to the list of biofilm-forming organisms in immunocompromised hosts such as cancer patients (Al Akhrass et al. 2012). Since several rhodococcal species, including *R. opacus*, *R. erythropolis* and *R. jostii*, present genes allowing them to transport many different substrates including solvents and drugs, not only their biodegradation

potential may be increased but also their pathogenicity. The rationale to study the adaptation mechanisms to organic solvents thus presents a further motivation.

3.1 Adaptation of the Cell Wall and of the Cellular Membrane

When challenged by organic solvents, the most important adaptive response shown by tolerant bacterial cells is probably the mechanism that allows them to control the fluidity and viscosity of the membrane which is termed "homeoviscous adaptation" (Sinensky 1974). This mechanism, initially shown for cells responding to changes in the growth temperature, is based on the phase transition temperature which reflects the temperature that causes a change in the physical state of the phospholipid bilayer from an ordered gel phase to a disordered liquid-crystalline phase (Sinensky 1974; Eze 1991). By changing the length of the fatty acids and their configuration, the cells alter the phase transition temperature of lipids and thus membrane fluidity (de Carvalho and Caramujo 2018). For example, whilst the saturated fatty acid C16:0 (palmitic acid) presents a melting temperature of 63 °C, the monounsaturated fatty acid in the *trans* conformation (*trans*-C16:1, palmitelaidic acid) and *cis* conformation (*cis*-C16:1, palmitoleic acid) has a melting temperature of 33 and 0 °C, respectively (Heipieper et al. 2007). Parameters such as the degree of saturation, *cis*/*trans* ratio and *iso*/*anteiso* ratio thus allow the assessment of the changes in membrane fluidity promoted by the cells.

Several papers have reported that, when exposed to toxic organic compounds, tolerant bacterial strains change the fatty acid profile of their membrane (e.g. de Carvalho et al. 2009; Heipieper et al. 1994; Isken and de Bont 1998; Pacífico et al. 2018; Sardessai and Bhosle 2002; Sikkema et al. 1995; Torres et al. 2011). The existence of an outer membrane allows Gram-negative bacteria to quickly modify and adapt the lipopolysaccharides, efflux pumps and the fatty acid composition of the cellular membrane (Ramos et al. 2002). 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, whilst the remaining changes are long-term responses (Junker and Ramos 1999). As previously mentioned, the mechanisms involved in the adaptation of Gram-positive strains have been less studied, but it has been suggested that the mechanisms should be similar to those presented by Gram-negative bacteria (Fang et al. 2007; Nielsen et al. 2005; Ramos et al. 2002; Torres et al. 2011).

When the effect of organic solvents was studied in 29 strains belonging to 6 species of the *Rhodococcus* genus, namely, *R. erythropolis*, *R. fascians*, *R. longus*, *R. opacus*, *R. rhodochrous* and *R. ruber*, it was found that composition of the medium, solvent concentration and individual traits of each strain strongly affected bacterial resistance (Korshunova et al. 2016). The cells resistant to solvents increased the relative surface area and reduced the rigidity of the bonds between the cell wall components.

Responses in *R. erythropolis* DSM 1069 occurring in the few minutes following osmotic stress caused by sodium chloride were observed in the lipid composition and net surface charge of the cells (de Carvalho et al. 2014b). The doubling time of strain DSM 1069, under the most favourable conditions of the study, was 3.5 h, but salt-induced changes in the total lipid composition of the cells could be observed after only 6 min. The most surprising result was the production of polyunsaturated fatty acids (PUFAs), which reached 36.3% of the total lipids in bacteria exposed to 5.5% NaCL for 35 min. The production of PUFAs is unusual in bacteria, and until the 1990s, it was thought that bacteria, with the exception of certain cyanobacteria, could not produce them. In the case of strain *R. erythropolis* DSM 2016, the production of PUFAs was accompanied by a concomitant decrease in the percentage of monounsaturated fatty acids, suggesting the action of fatty acid desaturases which have been found in *R. erythropolis* strains. Production of PUFAs was also observed in *R. erythropolis* DCL14 during adaptation to extreme conditions (de Carvalho 2012). A stepwise adaptation strategy allowed the growth of these cells at 4–37 °C, pH 3–11 and in the presence of up to 7.5% sodium chloride and 1% copper sulphate. The adapted cells adjusted the relative proportion of straight, methyl and cyclopropyl saturated, unsaturated and hydroxyl-substituted fatty acids, produced PUFAs and regulated their net surface charge. Furthermore, the adapted *R. erythropolis* cells could degrade C6 to C16 *n*-alkanes and alcohols under conditions that did not allow the growth of non-adapted cells.

Metz et al. have also described a pathway for the synthesis of long PUFA which uses a polyketide synthase-like gene cluster instead of multiple desaturases and elongases (Metz et al. 2001). Seven polyketide synthase genes were identified in whole genome of *Rhodococcus* sp. RHA1 (McLeod et al. 2006). Polyketide synthases are also involved in the production of secondary metabolites (McLeod et al. 2006) and mycolic acid synthesis (Portevin et al. 2004).

Mycolic acids of rhodococci 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. Organic solvent extracts of *R. equi* cells allowed the identification of two channel-forming proteins: protein PorA(Req) is cation selective, has a single-channel conductance of 4 nS in 1 M KCl and is highly permeable to positively charged solutes and porB(Req) prefers the passage of anions, is not voltage gated and presents a conductance of 300 pS in 1 M KCl (Rieβ et al. 2003).

n-Alkane droplets have negative zeta potentials, e.g. 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; de Carvalho et al. 2007). Bouchez-Naïtali and co-workers (2001) also observed a direct uptake of *n*-hexadecane by four *R. equi* strains, which did not produce biosurfactants. *R. erythropolis* S+14He is able to select and transport, by an energy-driven transport system, *n*-hexadecane from mixtures of *n*-alkanes into intracellular inclusions (Kim et al. 2002). *Rhodococcus* sp. BAP-1 transports fluoranthene across the cell membrane by a process requiring energy and concurrent catabolism with significant production of CO_2 (Li et al. 2014). *R. erythropolis* NTU degrades long-chain *n*-alkanes at concentrations up to 20%, and a correlation between the amount of H^+ ions in the culture medium and biodegradation of C14–C16 alkanes could be found (Liu et al. 2012). This may be used to follow alkane consumption by pH monitoring.

Yoneda et al. adaptively evolved *R. opacus* PD630 to phenol (to improve the tolerance of the strain to lignin-derived phenolic compounds) over 40 passages using phenol as sole carbon source (Yoneda et al. 2016). Two strains isolated from passages 33 and 40 showed increased phenol consumption rates, and a twofold increase in the production of lipids from phenol was observed when compared to the wild-type strain. Whole-genome sequencing and comparative transcriptomics indicated that both adapted strains presented highly upregulated degradation pathways and putative transporters for phenol. As pointed out by the authors, this suggests a new mechanism of bacterial aromatic tolerance related to the transport of toxic phenolic compounds into the cell which requires a proper balance between transport and degradation rates to prevent accumulation of toxic compounds inside the cell.

Kurosawa et al. studied the tolerance and adaptive evolution of an engineered triacylglycerol producing strain, *R. opacus* MITXM-61, to lignocellulose-derived inhibitors such as phenols, furans and organic acids (Kurosawa et al. 2015). Levulinic acid affected lipid biosynthesis and altered the principal fatty acid component of *Rhodococcus* TAGs from even-numbered C16–C18 fatty acids to odd-numbered C15–C17 fatty acids. An evolutionary adaptation strategy using short-term serial transfers of the cell cultures in inhibitor-containing liquid medium resulted in an evolved strain with improved tolerance to lignin, 4-hydroxybenzaldehyde and syringaldehyde.

When comparing the effect of low and high 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 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

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	Gutiérrez et al. (1999)
R. opacus GM-14, GM-29, and 1CP	Benzene, phenol, 4-chlorophenol, chlorobenzene or toluene	Increase content of branched (10-methyl) fatty acids	Tsitko et al. (1999)
R. opacus PDW4	4-Chlorophenol	Adjustments in the average chain length and unsaturation index of mycolic acids	de Carvalho et al. (2016)
R. opacus MITXM-61	Phenols, furans and organic acids	Altered TAGs from even-numbered to odd-numbered fatty acids	Kurosawa et al. (2015)
R. erythropolis DCL14	Short-chain alcohols (methanol, ethanol, butanol) n-Alkanes and n-alkanols	Decrease of degree of saturation Increase in degree of saturation	de Carvalho et al. (2005)
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 da Fonseca (2007)
R. erythropolis DCL14	Toluene	C14:0 and C16:0 increased whilst C18:0 decreased; increased percentage of *iso*-branched fatty acids when compared to straight-chain	de Carvalho et al. (2007)
R. erythropolis CCM 2595	Phenol	Changes in the chain length and unsaturation level of mycolic acids	Kolouchová et al. (2012)
R. erythropolis E1	C2–C7 *n*-alkanoic acid salts C9–C15 *n*-alkanes	MA profile according to the even-odd nature of the carbon chain of substrate Changed cell wall permeability	Sokolovská et al. (2003)
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)

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 and 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 triacylglycerols and wax esters under nitrogen starvation (Alvarez et al. 1996; Voss and Steinbüchel 2001).

Kolouchová et al. (2012) studied the mycolic acids of two *R. erythropolis* strains able to degrade phenol. One of the strains, *R. erythropolis* CCM 2595, was adapted to phenol as sole carbon source for 6 months, whilst *R. erythropolis* CCM 2595 *pSKRK 21 phe* contains a plasmid allowing the cells to present higher phenol hydroxylase activity. Soluble humic acids were added to the growth media to help the transport of organic substances into the cells as result of their interaction with components of the cell surface envelop.

R. opacus PDW4 challenged with 4-chlorophenol, a standard monoaromatic chemical used to investigate cellular toxicity and adaptation, changed their mycolic acid content according to the xenobiotic concentration (de Carvalho et al. 2016). Both the average chain length and unsaturation index of the mycolic acids decreased up to a concentration of 4-chlorophenol of 150 mg/L but increased for higher concentrations. Curiously, Solyanikova and co-workers showed that *R. opacus* 1 cp, after storage in a dormant state, could degrade chlorinated phenols that had not been degradable before dormancy (Solyanikova et al. 2011). During the dormant period, the cells were incubated at 20 °C under static conditions for 4 months, and it was not clear if this process induced the appearance of a novel metabolic pathway or favoured an existing one. Energy and carbon starvation or nutrient-induced stationary phase of *R. erythropolis* SQ1 cells also promoted higher resistance to heat shock and oxidative damage than that observed in exponentially grown cells (Fanget and Foley 2011).

The traditional way of favouring the growth of variants more adapted within a population uses repeated passages of the culture under selective conditions. As mentioned previously, using a stepwise strategy to adapt *R. erythropolis* DCL14 cells to extreme conditions of temperature, pH, salt and copper concentrations, it was possible to degrade C6–C16 *n*-alkanes and alcohols under conditions that previously did not allow the growth of this bacterium (de Carvalho 2012). The cells made the necessary adjustments to maintain the polarization of the membrane, changing the fatty acid composition of the cellular membrane and the net surface charge. The same strategy resulted in *R. erythropolis* DCL14 cells adapted to 20–65% toluene concentrations (de Carvalho et al. 2007). The cells adapted by increasing the percentage of tetradecanoic and hexadecenoic acids whilst decreasing the percentage of octadecanoic acid. The proportion of saturated *iso*-branched fatty acids also increased during toluene adaptation, whilst 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 most interesting physicochemical surface properties adaptation of *Rhodococcus* cells to organic solvents was observed with *R. erythropolis* DCL14 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, the cells even becoming positive when the cells 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 bacterial cells and the surface. In the case of strain DCL14, the positive surface charge contributes to the attachment of the cells to negatively charged surfaces such as *n*-hexadecane droplets (zeta potential of -46.0 ± -3.4 mM).

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.

The content of 10-methyl branched fatty acids 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 (Tsitko 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* are still unclear, but the results of Tsitko et al. (1999) suggest that the cell envelope lipids containing 10-methyl-branched fatty acids should be involved in the adaptation of *Rhodococcus* strains to compounds affecting the cellular membrane, such as aromatics. The melting temperature of methyl-branched fatty acids depends on the position of the methyl substitution which will divide the lipid chain into longer and shorter sections (de Carvalho and Caramujo 2018).

The cell surface hydrophobicity of *Rhodococcus* sp. Q15, which is able to mineralize alkanes, diesel and Bunker C crude oil at both 5 and 24 °C, was also higher after cell growth in 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 then on glucose-acetate. The cells were thus able to modulate the membrane fluidity to respond to both low temperature and hydrocarbon toxicity.

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 unsaturated 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.

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, whilst 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 2016; de Carvalho and da Fonseca 2005; 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, whilst the presence of multiple pathways and

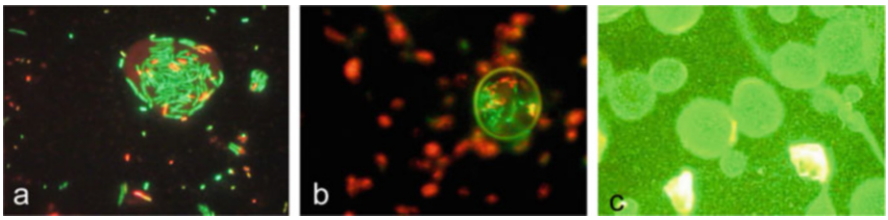

Fig. 2 *R. erythropolis* cells inside *n*-dodecane droplets. (**a**, **b**) Solvent droplets trapped between a slide and cover slip (horizontal and vertical field widths equal to 0.16 and 0.12 mm, respectively); (**c**) solvent droplets adsorbed on silica gel particles (horizontal and vertical field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

gene homologous enhance their versatility (Larkin et al. 2005, 2006; van der Geize and Dijkhuizen 2004). Studies reporting the ability of *Rhodococcus* strains to act as whole-cell biocatalyst even in anhydrous organic solvents have also been published (e.g. Sameshima et al. 2008; Yamashita et al. 2007).

Rhodococcus sp. N-774, found in 1980, was the first strain to produce acrylamide at industrial scale, but *R. rhodochrous* J1 was found to contain a much more active nitrile hydratase (Nagasawa et al. 1993). The latter strain produces 30,000 tons per year of acrylamide (Mitsubishi Rayon, pers. comm.) and 6000 tons per year of nicotinamide by Lonza Guangzhou Fine Chemicals (de Carvalho 2016).

In two-phase systems, *R. erythropolis* cells migrate towards 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 3-D solvent droplets adsorbed to solid particles, e.g. of silica gel. Cells were preferentially inside organic droplets in systems with 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 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. The use of organic solvents for in situ product removal in fermentation and biocatalytic systems is a powerful technique to increase bioprocess productivity (Dafoe and Daugulis 2014). Strains able to tolerate different

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 may provide a broader choice during bioprocess development as the best solvents to extract product(s) may be used instead of only the most biocompatible.

R. erythropolis NTU-1 degrades *n*-alkanes and branched alkanes in mineral medium and also under saline conditions, but in the latter case, cells formed aggregates (Liu et al. 2009). The biofloccules trapped significant amount of the substrate, enhancing removal of the alkane in addition to bioremediation. During the bioremediation of contaminated sea water, the biofloccules may allow a physical separation and a relatively fast removal of hydrocarbon compounds.

Pospíšilová et al. reported an increased growth and degradation of phenol by *R. erythropolis* cells stimulated by a homogenous electromagnetic field (Pospíšilová et al. 2015). The electromagnetic field stimulated biofilm formation although cells presented a lower hydrophobicity, suggesting the possibility of using a biofilm reactor for phenol degradation. Enhanced aggregation and production of extracellular polymeric substances was also observed in *R. jostii* exposed to toluene and a mixture of perfluoroalkyl acids (Weathers et al. 2015). Furthermore, the results suggested that flocculation was a biological response rather than the result of chemical interactions of surfactant and perfluoroalkyl acids.

R. erythropolis IEGM 267 and *R. rhodochrous* IEGM 107 pre-grown in the presence of 0.1% (v/v) *n*-hexadecane were able to partially and completely degrade 500 mg/L of dehydroabietic acid, a toxic tricyclic diterpenoid (Cheremnykh et al. 2018). Pregrowth in the presence of the C16 alkane was essential for the degradation of the compound. In the presence of *n*-hexadecane, the cells formed 10–50 μm aggregates with more than ten viable cells, and when exposed to dehydroabietic acid, strain IEGM 107 promoted cell aggregates with up to 0.5 mm in size. Strain IEGM 265 formed aggregates with up to 5 mm of diameter. The cells also decreased the zeta potential during the biodegradation and increased their length and width. An increase in the ratio of the cell surface area to its volume promoted a higher cell-substrate contact.

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 which are time dependent and associated to changes in the physiological state, protein synthesis, or degradation. When in contact with a toxic compound, the cells may express an enzyme(s), or use alternative pathways to catabolize and decrease the concentration of the biocide in the microenvironment surrounding the cells. For economical reasons, the cells usually express only the required enzymes after being exposed to the toxic compound.

R. pyridinivorans NT2 was found able to degrade 4-nitrotoluene, recognized as priority pollutant, at a concentration of 400 mg/L, through partial reduction of the amino group (Kundu et al. 2013). The strain was isolated from an effluent sediment of a pesticide manufacturing facility in Gujarat, India. The cells produced trehalose-based biosurfactants which reduced the surface tension of the media to 29 mN/m. Strain NT2 was also able to degrade 2,6-dinitrotoluene which is a common isomer produced during the synthesis of 2,4,6-trinitrotoluene (TNT) and used in the production of herbicides, dyes and foams (Kundu et al. 2016). The cells were able to use

2,6-dinitrotoluene as sole carbon and nitrogen source. The major byproduct of TNT synthesis, 2,4-dinitrotoluene, which is used as waterproofing and plasticizing agent in explosives and propellants, is also degraded by strain NT2 (Kundu et al. 2015). Besides the ability to degrade the intermediates of the TNT biodegradation pathway, some *R. erythropolis* strains are able to degrade TNT through the formation of Meisenheimer complexes (Vorbeck et al. 1998). The complex is further reduced to a yellow metabolite identified as the protonated 3,5-dihydride complex of TNT. The 1000 tons of TNT produced annually and the numerous contaminated sites resulting from military and industrial activities worldwide make bioremediation of TNT and its derivatives of paramount importance (Serrano-González et al. 2018).

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 non-adapted 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 non-adapted 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 throughout the 35 days of the experiment. The fatty acid composition of the adapted cells presented a higher amount of branched fatty acids and a 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 non-adapted 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 co-metabolism of *o*-, *m*- and *p*-xylene further enhanced the degradation of toluene (de Carvalho et al. 2007). Leneva and co-workers (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 whilst 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 pre-exposed 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, whilst the non-adapted 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. On the contrary, other studies done with *R. opacus* B-4 suggested that benzene tolerance is not related to the utilization or degradation of this organic compound (Na et al. 2005). A deletion-insertion mutant defective in the benzene dioxygenase genes, strain B-4KSN01, grew in 10% benzene as well as the wild strain B-4.

A *R. rhodochrous* isolated from a bacterial consortium enriched on toluene from a sample collected on a gasoline-contaminated aquifer was able to degrade benzene, toluene, ethylbenzene and xylene as primary carbon source (Deeb, Alvarez-Cohen 1999). When different combinations were tested, it was found that benzene and toluene degradation rates were enhanced in the consortium by the presence of *o*-xylene, whilst toluene, benzene or ethylbenzene inhibited xylene degradation rates. In the case of *R. rhodochrous*, ethylbenzene degradation decreased toluene degradation, benzene induced a slight inhibitory effect and *o*-xylene had no significant effect on toluene degradation.

Two mutants of *Rhodococcus* sp. 33 unable to degrade benzene were still tolerant to 500–800 mg/L of benzene (non-adapted strain 33 cells were able to tolerate ca. 1000 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 biodesulphurization of petroleum occurs in the presence of high concentrations of hydrocarbons. Many of the described competent bacteria to perform desulphurizations are *R. erythropolis* strains, e.g. 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 sulphur source and to 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 sulphur 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 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 non-desulphurizing *Pseudomonas* strains, which could explain why transference of biodesulphurizing genes into Gram-negative strains did not promote biodesulphurization activity. An increased DBT desulphurization 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.

R. erythropolis ATCC 4277 is able to carry out the biodesulphurization of heavy gas oil (HGO) at a rate of 5 g sulphur/(kg HGO.h) when grown in cheap media such as residues (cassava wastewater) and byproducts (trub) (Porto et al. 2017). Kawaguchi et al. made a recombinant strain from the organic-tolerant strain *R. opacus* B-4 to express the genes *dszA*, *dszB* and *dszC* from *R. erythropolis* IGTS8 to prevent inhibition by the sulphate end product of the 4S pathway of dibenzothiophene observed in this strain (Kawaguchi et al. 2012). Unlike the wild-type B-4 strain, the recombinant strain could grow in dibenzothiophene as sole sulphur source. Besides, the reaction could take place in biphasic systems with *n*-hexadecane as organic solvent, which allowed higher concentrations of substrate and thus higher desulphurization activities. *R. erythropolis* PD1 is also able to carry out the biodesulphurization of dibenzothiophene in both aqueous and biphasic systems (Derikvand et al. 2015). Nevertheless, total biodesulphurization of fossil fuel at industrial scale is not expected to occur in the near future (Soleimani et al. 2007). There are two main reasons that hamper the industrial application of biodesulphurization processes: failure to explore the full range of applications of biodesulphurization biocatalysts (e.g. to detoxify warfare agents or to produce fine chemicals) and failure to develop biocatalysts with activities high enough for a commercial petroleum desulphurization process (e.g. able to overcome inhibition caused by the product 2-hydroxybiphenyl (Kilbane 2017)).

3.3 Other Mechanisms of Protection

Iwabuchi and co-workers (2000) reported an association between colony morphotypes and oil tolerance in *R. rhodochrous*. The mucoidal strain was resistant to 10% (v/v) *n*-hexadecane, whilst the rough derivatives were sensitive to this concentration. Furthermore, when the extracellular polymeric 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 defence 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) plays an important role in hydrocarbon tolerance.

Cells of *R. erythropolis* DCL14 present non-EPS and EPS producer variants. When exposed to the terpenes carveol and carvone and to organic solvents, the degree of saturation of the membrane phospholipids decreased, whilst 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, whilst the non-stressed 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 due to 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* (Lang and Philp 1998), *R. erythropolis* 51T7 (Marqués et al. 2009), 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. Kuyukina et al. proposed the use of methyl *tert*-butyl ether (MTBE) for the extraction of biosurfactants from *R. ruber*. The EPS produced contained ca. 10% polar lipids and 45% non-polar lipids and reduced the surface tension of water to 29.2 mN/m. Pen et al. studied the adhesion forces between *Rhodococcus* RC291 and a silicon oxide surface by force spectroscopy (Pen et al. 2015). They showed that cells in the late growth phase have a greater adhesion to the surface than those in the early growth stage. The EPS molecules from late exponential phase are less densely bound but have chains able to extend which could result in contraction and extension of the EPS and to changes in the density of the binding sites. On the other hand, the denser EPS of the late stationary phase provides shelter for the cells. Besides it could endure higher compression and retained higher amounts of water.

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 2005; 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, whilst 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).

Coaggregation studies demonstrated interactions between *Rhodococcus* sp. strain MF3727, *Rhodococcus* sp. strain MF3803 and two strains of *Acinetobacter calcoaceticus* (MF3293, MF3627), all isolated from food processing surfaces (Møretrø et al. 2015). Coaggregation may contribute to biofilm formation whilst providing protection against cleaning and disinfection agents.

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. Membrane transport systems are involved in several cellular processes, including uptake of essential nutrients, secretion of metabolites, extrusion of toxic byproducts and maintenance of cell homeostasis (Saier 2000). A recent review discussing membrane transport system in *Rhodococcus* shows that cells of this genus show a plastic genome coding for numerous efflux pumps (de Carvalho et al. 2014a). These cells specialized in transporting substrates such as solvents and drugs, and some species such as *R. opacus*, *R. erythropolis* and *R. jostii*, present genes for the transport of several different substrates. The association of efflux systems and adapted phenotypes clearly indicate that the cells have the equipment to respond to challenging compounds and conditions.

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 desulphurizations. 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 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), makes these cells ideal candidates to in situ bioremediation of hydrocarbon-contaminated sites.

Acknowledgements The author acknowledges Fundação para a Ciência e a Tecnologia, I.P. (FCT), Portugal, for financial support under programme "FCT Investigator 2013" (IF/01203/2013/CP1163/CT0002).

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Degradation of Alkanes in *Rhodococcus*

Martina Cappelletti, Stefano Fedi, and Davide Zannoni

Contents

Abstract Alkanes are widely distributed in the environment as they not only constitute the large fraction of crude oil but are also produced by many living organisms. They are saturated hydrocarbons of different sizes and structures, which pose a variety of challenges to degradative microorganisms due to their physicochemical properties, i.e., the extremely limited solubility and the high energy required for activation. The hydrophobic cell surface of *Rhodococcus* spp., the ability to produce biosurfactants, and the possession of a wide range of oxygenases allow coping with such challenges. In particular, monooxygenase enzymes are involved in the activation of alkanes by converting them into alcohols, which undergo a series of oxidation steps before being converted to fatty acids. *Rhodococcus* alkane monooxygenases belong to different families (i.e., AlkB-like monooxygenase, soluble di-iron monooxygenase, cytochrome P450), have different genetic organization, and are subject to different regulatory mechanisms, which are poorly known. Because of their long-term survival capacity, broad catabolic abilities, and effective contact mechanisms with hydrocarbon molecules, alkanotrophic *Rhodococcus* strains have biotechnology applications and potential in bioremediation and biotransformation reactions.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_6

1 Introduction

Alkanes are saturated hydrocarbons with the general formula C_nH_{2n+2} consisting of carbon and hydrogen atoms; they can be linear (*n*-alkanes), cyclic (cycloalkanes), or branched (*iso*-alkanes). The *n*-alkanes, methane (C_1), ethane (C_2), propane (C_3), and butane (C_4) are gaseous at room temperature, while *n*-alkanes ranging between C_5 (pentane) and C_{17} (heptadecane) are liquid, and those $>C_{18}$ (octadecane) are solid (the subindex indicates the number of carbon atoms of the *n*-alkane molecule).

Alkanes are the most abundant hydrocarbons (with an estimated abundance of 20–50%) in crude oil. They are also naturally produced by living organisms (plants, algae, and bacteria) as waste products and chemoattractants or for structural and defense purposes (van Beilen et al. 2003). In relation to this, alkanes have been detected in most soil and water systems; bacterial oxidation of alkanes is a very common phenomenon in natural environments and a major process in geochemical terms (van Beilen et al. 2003).

As alkanes are apolar and chemically inert compounds, their utilization by microorganisms faces significant challenges in relation to the energy required to activate them, their limited solubility, and their tendency to accumulate in cell membranes (Rojo 2009). Due to this, microorganisms with the capacity to degrade alkanes are of great interest in addition to the fact that microbial degradation of long-chain *n*-alkanes remains the main process for the remediation of oil-contaminated areas (Leahy and Colwell 1990; Binazadeh et al. 2009).

The solubility of alkanes decreases as molecular weight increases and aliphatic linear alkanes are normally biodegraded more rapidly as compared to multiple-branched alkanes such as pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane) (Leahy and Colwell 1990).

Rhodococci are frequently isolated from petroleum hydrocarbon-contaminated environments where they play an important role in the degradation of aliphatic hydrocarbons including alkanes (Hamamura et al. 2006; Yuste et al. 2000). In this respect, many *Rhodococcus* species have been described to be able to use a wide range of alkanes as carbon and energy sources with high efficiency and growth rate (Table 1) (Binazadeh et al. 2009). Molecular methods, targeting 16S rRNA and key functional genes, further highlighted the wide distribution of *Rhodococcus* spp. in diverse hydrocarbon-contaminated environments (Hamamura et al. 2006, 2008), including oil-contaminated soils, rhizosphere, wastewater, coastal sediments, and seawater (Hassanshahian et al. 2013; Acosta-González et al. 2016; Viggor et al. 2015; Mikolasch et al. 2015). Despite the low temperatures generally limit hydrocarbon biodegradation, alkanotrophic *Rhodococcus* spp. were found in cold petroleum-polluted ecosystems (antarctic and alpine soils) (Margesin et al. 2003; Luz et al. 2004; Habib et al. 2018), and many *Rhodococcus* strains were able to metabolize hydrocarbons at $T < 5$ °C (Whyte et al. 1998, 1999). A large diversity can be observed among different *Rhodococcus* strains in terms of substrate specificity, enzymatic systems, and physiological response. The wide distribution and high presence of *Rhodococcus* spp. in oil-contaminated environments have been

Table 1 List of the main alkanotrophic *Rhodococcus* strains described in the literature along with their genetic and functional characteristics

Strain	Alkanes used as growth substrate[a]	Microbial features related to alkane oxidation		References
		Genetic data[a]	Functional data	
R. jostii RHA1	C_3	One *alkB* gene One *prm* gene cluster	RHA1 *prm* gene is transcriptionally induced during the growth on C_3 and cometabolizes NDMA	Sharp et al. (2007) and Ekprasert (2014)
R. ruber SP2B	C_5–C_{21}	Two *alkB* genes	The *n*-hexane degradation capacity of SP2B is inducible	Amouric et al. (2010)
R. qingshengii Q15	C_8–C_{32}, branched alkanes, substituted cyclohexane	Four *alkB* genes Two CYP153 genes	Q15 possesses multiple *alkB* genes with quite sequence divergence; it performs both terminal and subterminal oxidation of alkanes; it has low temperature alkane degradation range	Whyte et al. (1998, 2002a, b)
R. erythropolis NRRL B-16531	C_6–C_{36}	Four *alkB* genes Two CYP153 genes	NRRL B-16531 is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes with quite sequence divergence	Whyte et al. (2002b) and van Beilen et al. (2002b)
R. erythropolis T7-2	C_{12}–C_{36}	ND	T7-2 is able to use diesel oil as its sole carbon and energy source at 15 °C	Huang et al. (2008)
Rhodococcus sp. strain SoD	C_{12}–C_{28}	One *alkB* gene	SoD is able to use diesel oil as sole carbon and energy source	Quatrini et al. (2008)
R. aetherivorans BCP1	C_2–C_{28}	Two *alkB* genes One *smo* gene cluster One *prm* gene cluster	BCP1 has at least three different alkane monooxygenases involved in the oxidation of *n*-alkanes C_3-C_{22}; it performs terminal and subterminal oxidation of propane and terminal oxidation of butane	Cappelletti et al. (2011, 2015)
R. opacus R7	C_{12}, C_{14}, C_{20}, C_{24}	One *alkB* gene One *prm*-like gene	R7 *alkB* gene is involved in the oxidation of medium- and long-chain *n*-alkanes	Di Gennaro et al. (2014) and Zampolli et al. (2014)
R. erythropolis PR4	C_8, C_{12}, C_{19}, C_{16}	Four *alkB* genes Two CYP153 genes and other mono- and di-oxygenase genes	The multiple monooxygenase genes of PR4 are differently induced after the growth on diesel oil, hexadecane, or hydrocarbon mixtures	Sekine et al. (2006) and van Beilen et al. (2006)

(continued)

Table 1 (continued)

Strain	Alkanes used as growth substrate[a]	Microbial features related to alkane oxidation		References
		Genetic data[a]	Functional data	
R. rhodochrous NCIMB 12566	C_6–C_{16}	One CYP450 gene	NCIMB 12566 produces a cytochrome P450 able to hydroxylate *n*-octane	Cardini and Jurtshuk (1970)
Rhodococcus sp. strain 1BN	C_6, C_8, C_{16}–C_{28}	One *alkB* gene	1BN degrades medium- (C_6) and long-chain alkanes (C_{16}–C_{28})	Andreoni et al. (2000)
Rhodococcus sp. strain T12	Pristane	ND	T12 degrades pristane at low temperatures	Kunihiro et al. (2005)
Rhodococcus sp. strain TMP2	C_9–C_{24}	Five *alkB* genes	Three *alkB* genes in TMP2 are transcriptionally induced by *n*-alkanes and pristane. TMP2 strain is able to degrade pristane at low temperatures	Takei et al. (2008)
Rhodococcus sp. strain RR12	C_{14}–C_{34}	ND	RR12 is able to grow on high molecular mass hydrocarbon mixture	Yuste et al. (2000)
Rhodococcus sp. strain RR14	C_{14}–C_{34}, pristane	ND	RR14 is able to grow on high molecular mass hydrocarbon mixture	Yuste et al. (2000)
Rhodococcus sp. strain NCIM 5126	C_{13}–C_{17}, pristane	ND	NCIM 5126 metabolizes *n*-alkanes through both bi-terminal and mono-terminal oxidation pathway while it metabolizes pristane only through mono-terminal oxidation pathway	Sharma and Pant (2000)
R. fascians 154-S	C_6–C_{24}	Four *alkB* genes	154-S is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)
R. fascians 115-H	C_6–C_{32}	Three *alkB* genes	Growth on long-chain *n*-alkanes	Van Beilen et al. (2003)
R. erythropolis 42-O	C_6–C_{32}	Five *alkB* genes	42-O is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)
R. erythropolis 50-V	C_6–C_{32}	Four *alkB* genes	50-V is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)
R. erythropolis NRRL B-16531	C_6–C_{36}	Four *alkB* genes	NRRL B-16531 is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)

(continued)

Table 1 (continued)

Strain	Alkanes used as growth substrate[a]	Microbial features related to alkane oxidation		References
		Genetic data[a]	Functional data	
R. erythropolis 23-D	C_6–C_{36}	Five *alkB* genes	23-D is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)
R. erythropolis 35-O	C_6–C_{16}	Five *alkB* genes	35-O is able to grow on medium- to long-chain *n*-alkanes and possesses multiple *alkB* genes	Van Beilen et al. (2003)
R. erythropolis S+14He	C_9–C_{30}, pristane	ND	S+14He is able to degrade specific *n*-alkanes within a mixture; *n*-alkane molecules seem to be accumulated inside the S+14He cell	Kim et al. (2002)
R. opacus B4	C_8, C_{10}	Two *alkB* genes	The activity of the promoter of the two B4 *alkB* genes was investigated in anhydrous organic solvents	Sameshima et al. (2008)
Rhodococcus sp. strain p52	C_{14}, C_{24}, C_{32}, pristine	Two *alkB* genes; One CYP185 gene	p52 *CYP*185 gene is transcriptionally induced on long-chain *n*-alkanes and pristine	Yang et al. (2014)
R. rhodochrous PNKb1	C_3	ND	PNKb1 performs both terminal and subterminal propane oxidation	Woods and Murrell (1989)
R. erythropolis SK121	ND	Six *alkB* genes Five CYP153 genes	SK121 possesses multiple *alkB* genes	Nie et al. (2014)
R. erythropolis ATCC 4277	C_5–C_{16}	ND	ATCC 4277 performs only subterminal *n*-alkane oxidation	Ludwig et al. (1995)
Rhodococcus sp. strain EH831	C_6	ND	EH831 performs only subterminal *n*-alkane oxidation	Lee et al. (2010)
Rhodococcus sp. strain Moj-3449	C_{10}–C_{35}	ND	Moj-3449 performs only subterminal *n*-alkane oxidation	Binazadeh et al. (2009)
Rhodococcus sp. strain BPM 1613	Pristane ($C_{19}H_{40}$)	ND	BPM 1613 converts pristane to terminal oxidation metabolic intermediates which are then degraded through either β- or ω-oxidation.	Nakajima et al. (1985)

(continued)

Table 1 (continued)

Strain	Alkanes used as growth substrate[a]	Microbial features related to alkane oxidation		References
		Genetic data[a]	Functional data	
R. ruber SBUG 82	Pristane ($C_{19}H_{40}$)	ND	SBUG 82 performs both terminal and subterminal oxidation of pristane	Nhi-Cong et al. (2009)

[a]The subindex indicates the number of carbon atoms of the *n*-alkane molecule; *NDMA* N-nitrosodimethylamine; pristine = $C_{19}H_{40}$

associated not only to their wide catabolic abilities but also to their hydrophobic cell surfaces and their capacity to respond to various stress conditions for long-term survival in harsh environments, i.e., metal resistance, high salinity, desiccation, high concentration of organic solvents, and biosurfactant production (de Carvalho et al. 2005; Quatrini et al. 2008; Sameshima et al. 2008; Cappelletti et al. 2016). Further, in some cases (mainly with alkanes $>C_5$), it was shown that *Rhodococcus* alkane degradation pathways were not affected by catabolic repression, so that alkanes were efficiently degraded also in the presence of alternative carbon sources (Warhust and Fewson 1994). Several *Rhodococcus* strains were also shown to produce high value-added compounds during the growth on *n*-alkanes, i.e., triacylglycerols and wax esters. Taken together, these features identify *Rhodococcus* genus as an ideal candidate for bioremediation, microbial-enhanced oil recovery, and hydrocarbon bioconversion to valuable products. Considerable interest has therefore been devoted to the study of the physiology of alkanotrophic rhodococci and their enzymatic systems involved in alkane transformation.

2 Physical Interaction Between *Rhodococcus* Cells and Alkanes

To be metabolized, alkanes must cross first the outer hydrophilic cell wall of Gram-positive bacteria and then penetrate the periplasmic cell membrane to get access to cytosol. The alkane bioavailability is therefore strongly limited by the hydrophobic character of these compounds so that the cell surface needs to favor the contact between these water-insoluble molecules for an efficient biodegradation process (Stroud et al. 2007). Apparently, all microorganisms able to utilize hydrocarbons, like *Rhodococcus* spp., have overcome this bottleneck through several adaptive mechanisms, here briefly outlined and summarized in Fig. 1.

While the entry into cells of alkane molecules from the water phase is thought to be possible only for low-molecular-weight alkanes that are partly soluble in water, e.g., propane and butane, *Rhodococcus* cells gain access to medium- and long-chain *n*-alkanes by adhering to alkane droplets and/or by surfactant-facilitated access

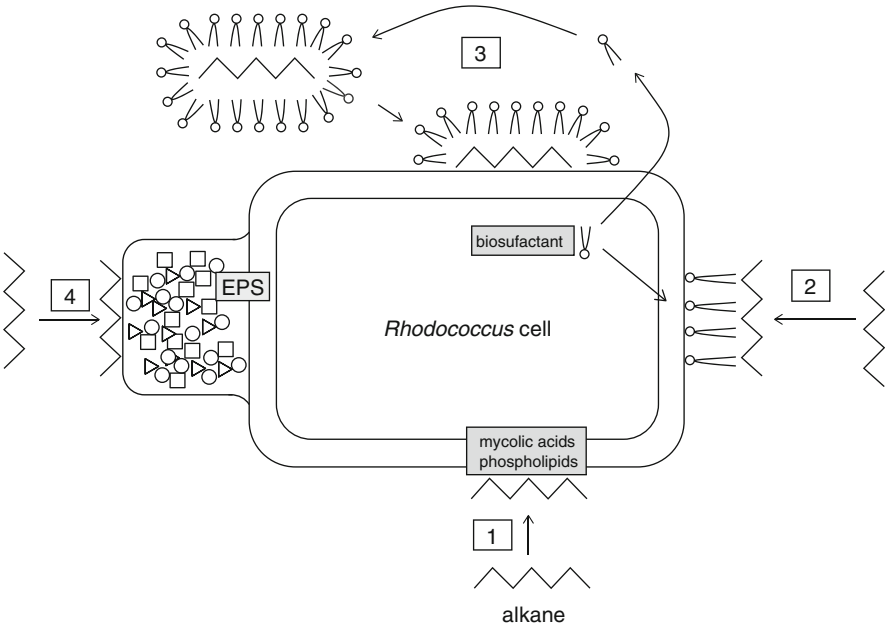

Fig. 1 Mechanisms of *Rhodococcus* cell contact with alkanes. (1) The contact is associated to an increase of cell membrane hydrophobicity due to the change of mycolic acids and phospholipids composition. (2) The contact is mediated by biosurfactants that after being produced remain associated to the cell wall. (3) The biosurfactants are excreted from the cell and allow the emulsification of the alkane molecules. (4) The production of extracellular polymeric substances (EPS) enhances the alkane-cell contact

through alkane emulsification. In these latter cases, a first mechanism would imply the direct contact of cells with large hydrocarbon droplets; alternatively, the biosurfactant activity would allow the entry of alkanes into cells by supporting the cell contact with fine oil droplets formed through emulsification process (Hua and Wang 2014).

Growth of *Rhodococcus* cells on alkanes has frequently been reported to induce the formation of surface-active compounds of diverse chemical nature, which improve the utilization of these hydrophobic compounds as growth substrates (Lang and Philp 1998; Yakimov et al. 1999; Rapp and Gabriel-Jürgens 2003; Peng et al. 2007). Rhodococci typically produce trehalose-containing glycolipid biosurfactants as extracellular or cell wall-associated compounds. In those *Rhodococcus* spp. where the biosurfactants are excreted from the cell, emulsification of the alkane with aqueous culture medium results in a very large surface area of contact between cell and hydrocarbon. More frequently, however, molecules with biosurfactant properties are retained by the *Rhodococcus* cell and localized on the outer cell surface, so that the cell surface increases its hydrophobic character facilitating the attachment and subsequent entry of alkanes to be used as growth substrate (Kretschmer et al. 1982; Bredholt et al. 1998; Lang and Philp 1998). On the

other hand, the direct contact between bacterial cells and hydrocarbons depends on the interfacial area extent, which in turn is strictly related to cell hydrophobicity. For example, it has been reported that in *Rhodococcus* sp. strain TMP2, a strain unable to produce biosurfactants, the direct contact of its cell surface with pristane was fundamental for an effective assimilation and growth on this branched alkane (Takei et al. 2008). The high hydrophobicity of the cell surface has been related to (1) the aggregation of *Rhodococcus* cells in flocs, which controls the interfacial alkane entry mechanisms (Bouchez-Naïtali et al. 2001), and (2) the ability of some *Rhodococcus* strains to get into the oil phase when they are grown in aqueous/organic biphasic systems, the organic phase being composed of long-chain liquid alkanes, e.g., *n*-hexadecane (Yamashita et al. 2007; Iwabuchi et al. 2009). An increase of the cell surface hydrophobicity has been widely reported in *Rhodococcus* spp. cultivated on alkanes, and an important role in this change has been assigned to the cell wall lipids and mycolic acids (Stratton et al. 2003; Kolouchová et al. 2012). In particular, both mycolic acids and phospholipids were proposed to affect the membrane permeability as well as the passive entry and/or active transport of hydrophobic substrates (Sokolovská et al. 2003). Several studies have demonstrated that the membrane composition and cell surface properties differed after feeding the cells with different alkanes (de Carvalho et al. 2005; Iwabuchi et al. 2009; Cortes and de Carvalho 2015). The growth on alkanes and change of the cell envelope permeability were also observed in parallel with an increase of the antibiotic resistance in cells of *R. ruber* and *R. erythropolis* (Kuyukina et al. 2000; Sokolovská et al. 2003). This effect was mainly linked to a decreased penetration of hydrophilic antibiotic molecules into *Rhodococcus* cells after they were grown on alkanes (Sokolovská et al. 2003).

In addition to cell wall components, the production of extracellular polymeric substances (EPS) with surface-active properties was shown to play an important role in the ability of *Rhodococcus* cells to adhere to hydrocarbons (Neu 1996; Whyte et al. 1999). EPS is known to increase the bacterial tolerance toward organic solvents and, more in general, toward various stress conditions, i.e., metals and antimicrobials (Iwabuchi et al. 2009). Growth on diesel fuel of *Rhodococcus* sp. strain Q15 [recently affiliated to *R. qingshengii* (Táncsics et al. 2015)] was linked to production of EPS clusters surrounding and connecting cells, so to enhance their contact with hydrophobic substrates (Whyte et al. 1999). In *R. erythropolis* PR4, the expression of genes involved in EPS biosynthesis was upregulated during the growth on alkanes and hydrocarbon mixtures (Laczi et al. 2015).

Hydrocarbons that are adsorbed on the surface of microbial cells can go across the cytoplasmic membrane through the use of three main mechanisms, namely: (1) passive diffusion; (2) passive-facilitated diffusion; or (3) energy-dependent uptake (Hua and Wang 2014). To date, very few studies have addressed the problem linked to the entry of hydrocarbons into *Rhodococcus* cells. Most of the studies have reported that the increase of cell surface hydrophobicity (induced by biosurfactants, mycolic acid, phospholipid, EPS production) might facilitate the attachment and subsequent transport of alkanes into the cell without the need of energy and specialized membrane component (Hommel 1990; Lang and Philps 1998). Only one study suggested the presence of a respiratory energy-driven selective transport of *n*-hexadecane (C_{16}) into

R. erythropolis strain S+14He cells, this process being repressed by the cytochrome oxidase inhibitor azide (N_3^-) when used at 30 mM (Kim et al. 2002). Further details and studies are needed to clarify the mechanisms of possible respiratory-dependent hydrocarbon uptake mechanisms in *Rhodococcus* spp.

3 Enzymes Responsible for Alkane Oxidation in *Rhodococcus*

Hydroxylation of alkanes by alkane monooxygenases (MOs) represents the first and most critical step in aerobic alkane metabolism. Oxygen acts as a reactant to activate alkane molecules so that alkanes are converted to alkanols, which can be further metabolized providing both carbon and an energy source to cells. In particular, the oxidation of *n*-alkanes ($>C_2$) occurs at the terminal and/or subterminal carbon of the aliphatic chain resulting in the production of a primary alcohol, of a secondary alcohol, or of a mixture of the two isomers, depending on the enzymatic activity and selectivity of the alkane MOs involved in the first alkane oxidation step.

Based on the number and type of cofactors, protein subunits, redox partners, and cellular location, different MO superfamilies can be distinguished in bacteria (Coleman et al. 2006, 2011). Among these, three MO superfamilies are typically involved in alkane oxidation in *Rhodococcus* spp., namely: (1) alkane hydroxylases (AlkB), (2) soluble di-iron monooxygenases (SDIMO), and (3) cytochromes P450 of the CYP153 ($CY_{tochrome}\ P_{450}$) group. While AlkB-type MOs and CYP153 enzymes are typically involved in oxidation of liquid alkanes (C_5–C_{16}), SDIMO act on short-chain hydrocarbons [gaseous and short-chain liquid alkanes and alkanes ($<C_8$)] (Coleman et al. 2011). For the only gaseous alkane metabolism, an additional enzyme was proposed to be involved in the initial oxidation step of propane and isobutane in *R. rhodochrous* ATCC 21198. This enzyme was suggested to be an intermolecular dioxygenase catalyzing the reaction of two molecules of alkane with one molecule of oxygen (see Sect. 3.4) (Babu and Brown 1984); however, no further studies were performed on the genetics and biochemistry of this putative dioxygenase.

In recent years, to assess the cell biodegradation potential and activity in situ, different molecular methods, based on the use of degenerate primers or hybridization probes targeting the different types of alkane monooxygenase, have been largely exploited (Smits et al. 1999; Vomberg and Klinner 2000; Kohno et al. 2002). Through these methods, the high abundance and activity of *Rhodococcus* spp. in diverse oil-contaminated environments was revealed. Additionally, advances in genome sequencing procedures along with the parallel increase in the amount of genomic data related to *Rhodococcus* strains have exponentially extended our knowledge on the alkane MO enzymes in this genus.

3.1 Membrane Non-heme Di-iron Alkane Hydroxylases (AlkB)

AlkB is a membrane alkane monooxygenase, which contains a conserved non-heme di-iron cluster in the active site catalyzing the oxidation of *n*-alkanes to the corresponding alkanols. While one atom of molecular dioxygen is introduced in the substrate by the AlkB MO, the second oxygen atom is reduced to water using two electrons from $NADH+H^+$, these reducing equivalents delivered to MO through the activity of the electron transfer components rubredoxin and rubredoxin reductase (van Beilen et al. 2002a; van Beilen and Funhoff 2007). Together, the three components, AlkB MO, rubredoxin, and rubredoxin reductase, form the integral membrane non-heme iron alkane hydroxylase. In this respect, the primary amino acid sequences of AlkB proteins present six hydrophobic membrane-spanning regions along with eight highly conserved histidine residues, the latter being essential for the iron coordination in the active site of MO (Shanklin et al. 1994, 1997).

AlkB hydroxylase is encoded by *alkB* gene, which has been described as the most common and widely distributed gene among all the alkane degraders (Smits et al. 1999; Kohno et al. 2002; Kloos et al. 2006; Amouric et al. 2010; Viggor et al. 2015). The *alkB* gene has been therefore used as favorite target for the description of alkane-degrading community composition, determination of the abundance of oil-degrading bacteria, and evaluation of the alkane biodegradation potential (Smits et al. 1999, 2002; van Beilen et al. 2002b; Kloos et al. 2006; Hamamura et al. 2008; Nie et al. 2014; Cappelletti et al. 2017a).

In the literature, *Rhodococcus* is among the most cited bacterial genera in relation to *alkB* gene identification, and *Rhodococcus alkB* sequences have been often described as the most abundant in studies describing the diversity of alkane hydroxylase genes in environments contaminated with crude oil and related pollutants (Whyte et al. 2002a; Hamamura et al. 2008; Luz et al. 2004; Quatrini et al. 2008; Jurelevicius et al. 2013; Cappelletti et al. 2017a) and also in pristine soils (Kloos et al. 2006).

Starting from *Rhodococcus* strains containing more *alkB* homologous genes, AlkB MO have been distinguished into different groups (AlkB1–AlkB7) based on the phylogenetic analysis of their sequences, often being related to similar role in hydrocarbon assimilation in respect of preferred sizes of alkanes. Panicker et al. (2010) reported that the distribution of different *alkB* gene groups in rhodococcal population was correlated with the types of alkanes present in the soil.

Also based on the above reported observations, several authors concluded that the *alkB* gene belongs to *Rhodococcus* core genome, which includes those genes present in all rhodococci (Nie et al. 2014; Orro et al. 2015). The study by Táncsics et al. (2015) suggested that *alkB* gene can be used as phylogenetic marker for *Rhodococcus* taxonomy resolution. The possible use of *alkB* as a tool for *Rhodococcus* systematics highlights the importance and conservation of this gene during the evolution of *Rhodococcus* spp. and the wide distribution of the *alkB* gene within this genus.

3.1.1 Organization and Expression of *alkB* Genes in *Rhodococcus*

In *Rhodococcus s*pp., the *alkB* gene is typically arranged in a cluster with genes coding for two separate rubredoxins (*alkG1*, *alkG2* or *rubA1*, *rubA2*) and a rubredoxin reductase (*alkT* or *rubB*) (Fig. 2a). These genes are organized in a head-to-tail module, and the ORFs have overlapping stop and start codons. The organization of the genes within the *alk* cluster suggested their translational coupling aimed at the production of stoichiometric amounts of proteins and at the coupled regulation of their activity (Whyte et al. 2002b).

Many *Rhodococcus* spp. possessing one or more *alk* gene cluster(s) showed additional *alkB* genes not comprised within an *alk* gene cluster. For instance, four homologous alkane monooxygenase genes (*alkB1*, *alkB2*, *alkB3*, and *alkB4*) were initially identified in *R. qingshengii* Q15 and *R. erythropolis* NRRL B-16531. Among these, *alkB1* and *alkB2* were parts of clusters containing both rubredoxin and rubredoxin reductase genes, whereas *alkB3* and *alkB4* were found as isolated genes in the genome (Whyte et al. 2002b). In *R. aetherivorans* BCP1, while a single *alkB* gene was initially identified within an orthodox *alk* gene cluster, an additional *alkB* gene, which was outside the rubredoxin/rubredoxin reductase gene cluster, was detected by genome analysis (Cappelletti et al. 2011, 2013). The presence of isolated *alkB* genes in *Rhodococcus* genomes was suggested to be functionally related to electron transfer redox components encoded by other *alk* gene clusters (Whyte et al. 2002b; van Beilen et al. 2002a) or, alternatively, to result from horizontal gene transfer events (Laczi et al. 2015).

Most of the expression studies concerning *alkB* gene clustered with rubredoxin and the rubredoxin reductase genes demonstrated the *alkB* inducibility by alkanes (Sameshima et al. 2008; Cappelletti et al. 2011). Some studies reported the expression of all the genes included in the *alkB* gene cluster (Laczi et al. 2015), and, when tested, the co-transcription of these genes within a single polycistronic mRNA was reported (Cappelletti et al. 2011). In *R. aetherivorans* BCP1, both *n*-alkanes ranging from C_6 to C_{22} and 1-alkanols were shown to be inducers of the *alkB* promoter (P_{alkB}), and the P_{alkB} induction level was proportional to the alkane concentration added to the growth medium (Cappelletti et al. 2011).

On the other hand, not always the expression of *alkB* gene was induced by alkanes in *Rhodococcus* strains. Indeed, Takei et al. (2008) have shown that only three out the five *alkB* genes detected in *Rhodococcus* sp. strain TMP2 were transcriptionally induced by alkanes, such as hexadecane (C_{16}) and pristane. In *Rhodococcus ruber* SP2B, despite the hexane degradation ability was inducible, the *alkB* gene expression was detected after cell growth on glucose, and this was associated with the absence of an alkane-inducible promoter (Amouric et al. 2010). This might be related to the occurrence of subtle regulatory mechanisms controlling the activity of some rhodococcal AlkB enzymes.

Putative regulatory binding sites were described upstream of *alkB* gene in *R. opacus* B4, *R. ruber* SP2B, and *R. aetherivorans* BCP1 (Sameshima et al. 2008; Amouric et al. 2010; Cappelletti et al. 2011). These binding sites had low

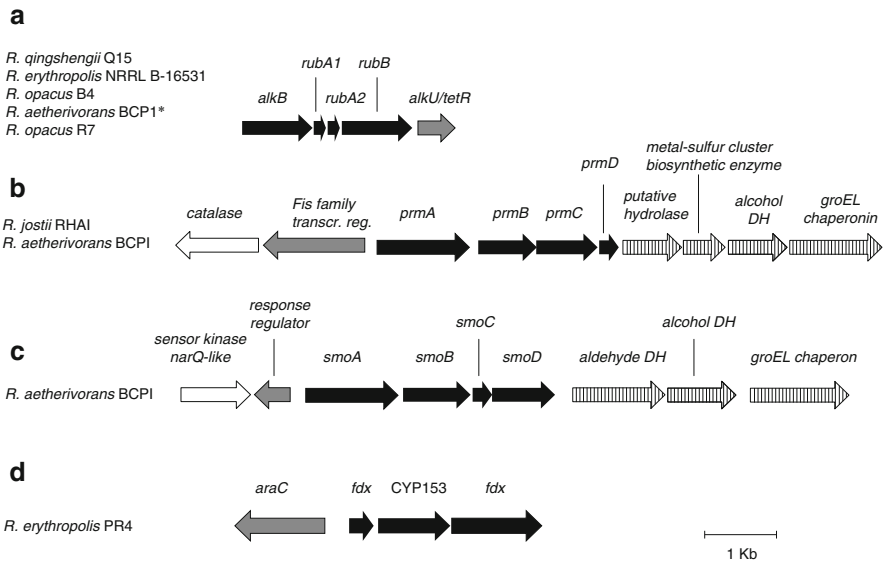

Fig. 2 Organization of the gene clusters encoding the alkane monooxygenases involved in alkane activation molecule in *Rhodococcus*. (**a**) *alkB* gene cluster encoding the membrane di-iron monooxygenase AlkB; (**b**) *prmABCD* gene cluster encoding the soluble di-iron monooxygenase Prm, standing for propane monooxygenase; (**c**) *smoABCD* gene cluster encoding the soluble di-iron monooxygenase Smo; and (**d**) the gene cluster encoding the heme-containing cytochrome P450 of CYP153 family. For each alkane monooxygenase gene cluster, the most representative *Rhodococcus* strains characterized in the literature are shown. Additionally, the flanking regions are displayed if they are conserved in *Rhodococcus* strains. Genes are represented by arrows, and the following colors/patterns were used: black, alkane monooxygenase gene clusters; gray, transcriptional regulator; stripes, genes involved in degradation reaction downstream of the initial alkane oxidation. The asterisk indicates that in BCP1 strain the *alkU/tetR* gene is transcribed in the opposite direction from that displayed

similarity with the target sequence recognized by AlkS which is the transcriptional regulator of the very well-known *alkB* gene cluster described in *P. putida* GPo1 (van Beilen et al. 2001).

A gene (*alkU* or *tetR*) encoding a putative TetR family transcriptional regulator (TetR-FTR) was found immediately downstream of the inducible *alk* gene cluster of a large number of *Rhodococcus* spp. and actinomycetes (Whyte et al. 2002b; Smits et al. 2002; Amouric et al. 2010; Liang et al. 2016a) (Fig. 2a). The conservation of the relative position of a TetR-FTR gene in proximity of the *alkB* gene cluster was taken as evidence of a role for TetR in the alkane-induced response (Cappelletti et al. 2011). The only functional study on the involvement of TetR-FTR on *alkB* gene expression in actinomycetes was reported in *Dietzia* sp. strain DQ12-45-1b (Liang et al. 2016a). In this strain, the TetR-FTR AlkX was found to repress the transcription of the *alkB*-like gene by binding to *alkB* promoter (Liang et al. 2016a). The repression of the transcription of the *alkB*-like gene was released when AlkX interacted with fatty acids, which are the intermediates in *n*-alkane degradation.

This was indicative of a product positive feedback mechanism regulating the *alkB* expression. Bioinformatic analyses revealed some conservation of the *Dietzia* AlkX binding site sequence in *Rhodococcus alkB* promoters (Liang et al. 2016a). Further, in *R. erythropolis* PR4, the *n*-hexadecane-induced transcriptome included both the transcripts belonging to an *alkB* gene cluster and the proximal TetR-FTR coding gene (Laczi et al. 2015). On the other hand, not in all *Rhodococcus* spp., TetR-FTR is transcribed in the same direction of *alkB* gene cluster, e.g., *R. aetherivorans* BCP1 (Cappelletti et al. 2011). These aspects, taken together, suggest that *Rhodococcus* probably shares with *Dietzia* the involvement of the TetR-FTR in *alkB* expression control, although possible divergences may exist in the regulation of TetR-FTR gene expression.

3.2 Soluble Di-iron Monooxygenases (SDIMO)

SDIMO are multicomponent enzymes able to hydroxylate different substrates ranging from methane, short-chain alkanes and alkenes, to phenol and toluene. SDIMO are found in phylogenetically and physiologically diverse bacteria and are classified into different groups (1–6) based on gene organization, substrate specificity, and sequence similarity (Leahy et al. 2003; Coleman et al. 2006, 2011; Holmes and Coleman 2008). In this respect, groups 3, 5, and 6 include SDIMO involved in alkane oxidation, and among these, the soluble methane monooxygenase from methanotrophic bacteria and the butane monooxygenase from *Thauera butanivorans* (formerly *Pseudomonas butanovora*) are the best characterized. These SDIMO, catalyzing the hydroxylation of methane and butane, show substrate unspecificity being able to co-oxidize a large range of short-chain hydrocarbons and structural analogous compounds (McDonald et al. 1997; Sluis et al. 2002; Coleman et al. 2006).

SDIMO are largely distributed throughout actinomycetes, mainly of *Mycobacterium* and *Rhodococcus* genera, and they were reported to be involved in the metabolism low-molecular-weight alkane and alkenes (Shennan 2006). The *Rhodococcus* SDIMO that have been functionally characterized are the alkene monooxygenase (AMO) from *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) strain B-276 (Saeki and Furuhashi 1994), two propane monooxygenases encoded by *prmABCD* gene cluster from *Rhodococcus jostii* RHA1 (Sharp et al. 2007), and the SDIMO encoded by *smoABCD* cluster in *R. aetherivorans* BCP1 (Cappelletti et al. 2015). Except for AMO, the other SDIMO were found to be involved in the degradation of short-chain *n*-alkanes and in the co-oxidation of different contaminants.

3.2.1 The Organization and Expression of SDIMO Gene Clusters *prmABCD* and *smoABCD*

In *Rhodococcus*, the SDIMO gene clusters contain four genes coding for (1) the catalytic large and the structural small subunits of the oxygenase component, (2) the

reductase containing both flavin and iron-sulfur clusters, and (3) a small "coupling protein" (Fig. 2b, c). According to the common biochemical features described for all SDIMO, the substrate oxidation is catalyzed by the oxygenase component, which contains the binuclear iron active site able to activate the molecular oxygen. The reductase component mediates the electron transfer from NADH to the active site of the oxygenase. The catalytic activity seems to be supported by a small "coupling protein," which is thought to facilitate the correct protein folding and di-iron center assembly (McCarl et al. 2018).

The different SDIMO gene clusters showed different substrate specificity and transcriptional inducer range. The *prmABCD* gene cluster (SDIMO group 5) in *Rhodococcus* spp. was induced by gaseous *n*-alkanes, mainly propane and, at a lower level, butane (Sharp et al. 2007; Cappelletti et al. 2015). Because of the initial correlation of this gene cluster with propane oxidation, the gene cluster was named *prmABCD*, and the encoded monooxygenase was the propane monooxygenase (Prm MO). In *R. jostii* RHA1, studies on the *prmA* gene mutant demonstrated the key role of Prm MO in the utilization of propane as sole source of carbon and energy and in the transformation of water contaminant *N*-nitrosodimethylamine (NDMA) (Sharp et al. 2007). Similar *prmABCD* gene clusters were described in other taxonomically related genera, i.e., *Gordonia*, *Mycobacterium*, and *Pseudonocardia* (Kotani et al. 2003, 2006; Furuya et al. 2011). Like in *Rhodococcus* spp., *prm* gene clusters were induced by gaseous *n*-alkanes, and they were found to play a key role in propane metabolism (Kotani et al. 2003; Furuya et al. 2011). Notably, in all the *prmABCD* expression studies, the genes within the *prm* cluster were induced as polycistronic operons (Kotani et al. 2003; Sharp et al. 2007; Cappelletti et al. 2015).

The flanking regions of the *prm* gene clusters were conserved among *Rhodococcus* spp. and other actinomycetes (Fig. 2b) (Cappelletti et al. 2015). Some of these genes were induced in *R. jostii* RHA1 cells grown on propane (Sharp et al. 2007). A gene encoding a FisR family transcriptional regulator (FisR-TR) was conserved upstream of *prm* gene cluster in different *Rhodococcus* spp. (Fig. 2b). The conservation of a FisR-like transcriptional regulator in the proximity of *prmABCD* gene suggested the involvement of this regulator in Prm MO expression. Accordingly, Cappelletti et al. (2015) described a palindromic sequence, overlapping the −35 region of the *prm* promoter, which shares similarities with FisR-type regulator binding site in *E. coli* (Cappelletti et al. 2015).

In *R. aetherivorans* BCP1, an additional SDIMO gene cluster, named *smoABCD*, was identified on the basis of sequence similarity in the database, and it was affiliated to SDIMO group 6. Unlike the *prmABCD* cluster that has a chromosomal localization in strain BCP1, the *smoABCD* gene cluster is harbored by the endogenous plasmid pBMC2 (Cappelletti et al. 2013). Further, *smoA* homologous genes (with amino acid sequence similarity >60%) are rare in the database (only 13 homologs are revealed by an updated pBLAST search running the BCP1 SmoA as a query sequence—July 2018), and they all belong to *Mycobacterium* spp. and few *Rhodococcus* spp. such as *R. aetherivorans*, *R. wratislaviensis*, and *R. ruber*. In particular, *R. aetherivorans* BCP1 *smoABCD* showed a high similarity (from 85% for *smoD* to 94% for *smoA*) with the *smo* gene cluster of *Mycobacterium chubuense*

(Coleman et al. 2011). This latter strain showed a peculiar level of SDIMO diversity because four distinct types of SDIMO (*smoXYB1C1Z*, *etnABCD*, *pmoABCD*, and *smoABCD*) were identified in its genome in addition to a membrane alkane MO of the AlkB family and a P450 cytochrome of the CYP153 family (Coleman et al. 2011). In BCP1 strain, the *smo* gene cluster expression was induced by short-chain ($<C_8$) *n*-alkanes and not by succinate (Cappelletti et al. 2015). The *smoA* homolog in *Mycobacterium chubuensis* NBB4 (Gene ID: *Mycch*_5395) was induced during the cell growth on acetate, ethene, and C_2–C_4 alkanes (Coleman et al. 2011). The apparent nonspecific inducer range for *smo* gene clusters in BCP1 (all the tested alkanes were inducers) and NBB4 (non-hydrocarbon substrates were inducers in addition to gaseous alkanes) might suggest sophisticated regulatory controls still unexplored. Recently, the disrupting mutation of *smoABCD* in *R. aetherivorans* BCP1 hampered the capacity of this strain to grow on short-chain *n*-alkanes and to degrade chlorinated *n*-alkanes indicating its key role in alkane metabolism and related cometabolic processes (data not published) (See Sect. 5).

A sensor kinase and a LuxR-like response regulator were coded by two genes immediately upstream of the *smoABCD* clusters described in both *R. aetherivorans* BCP1 and *M. chubuense* NBB4 (Fig. 2c). The tail-to-tail organization of the two genes was also conserved which suggest that these two genes might code for a two-component regulatory system involved in the control of *smoABCD* expression.

In strain BCP1, Cappelletti et al. (2015) have shown that analogously to the *prmABCD* gene cluster also *smoABCD* is transcribed as a single operon. Further, both the SDIMO gene clusters showed putative CRP (catabolite repression protein) binding sites, which were conserved among homologous regions. This was in line with the catabolite repression effect observed in the presence of glucose, succinate, and rich growth medium, i.e., LB, on the transcriptional induction of both the SDIMO gene clusters in BCP1 (Cappelletti et al. 2015).

Lastly, the heterologous expression of both Prm and Smo was supported by the co-expression of a chaperonin in the host strain. This consideration finds a connection with the conservation of a chaperonin-like gene downstream of each different SDIMO gene cluster in *Rhodococcus* (Fig. 2c). Chaperonins are supposed to have an essential role in the correct folding of the hydroxylase subunit of SDIMO system and are therefore necessary for the oxygenase activity (Furuya et al. 2013).

3.3 Cytochrome P450 of CYP153 Family

Cytochromes P450 form a superfamily of heme-thiolate proteins, which are ubiquitous among all life kingdoms and catalyze a wide range of oxidative reactions usually acting as terminal monooxygenases (Hannemann et al. 2007). It is noteworthy that in relation to the remarkable variety of chemical reactions catalyzed and the enormous number of substrates attacked, the sequence conservation among P450 proteins of different families can be less than 20% (Werck-Reichhart and Feyereisen 2000). Depending on the sequence similarity and redox partner, P450 cytochromes

are categorized in families typically named as CYP ($CY_{tochome}$ P_{450}) followed by a number (Werck-Reichhart and Feyereisen 2000).

The genomes of environmental *Rhodococcus* strains generally possess many ORFs predicted to encode P450 cytochromes. *R. jostii* RHA1 possess 25 ORFs predicted to encode for P450 cytochromes (McLeod et al. 2006). *R. opacus* R7 genome, which consists of 10.1 Mb, is one of the largest bacterial genome described up to date and possesses 45 ORFs predicted to code for P450 monooxygenases (Di Gennaro et al. 2014).

However, the number of cytochrome P450 families involved in alkane degradation is limited. Cytochromes P450 of the CYP153 family function as soluble alkane hydroxylases involved in the degradation of short- and medium-chain-length *n*-alkanes (C_5–C_{10} alkanes) (Kubota et al. 2005; van Beilen et al. 2006). CYP153 enzymes require a ferredoxin and a ferredoxin reductase that transfer electrons from NAD(P)H to the cytochrome P450 in order to oxidize the alkane, enabling their host organisms to utilize it as carbon source (Koch et al. 2009). The gene cluster typically encoding the CYP153 enzyme in *Rhodococcus* and the accessory proteins are shown in Fig. 2d.

Members of CYP153 were reported to be commonly present in alkane-degrading bacteria which do not possess any AlkB MO or which have AlkB MO that do not act on medium-chain alkanes (Sekine et al. 2006; van Beilen et al. 2006). *R. rhodochrous* NCIMB 12566 was first described as having a cytochrome P450 enzyme able to hydroxylate *n*-octane (Cardini and Jurtshuk 1970). *R. erythropolis* NRRL B-16531 and *R. erythropolis* PR4 possess two related CYP153 and four AlkB (van Beilen et al. 2006). A *CYP153* gene located on a plasmid was overexpressed in *R. erythropolis* PR4 cells grown on C_{16} and on a mixture of hexadecane and branched alkanes (Laczi et al. 2015). In addition to CYP153, the study on PR4 cells grown on hexadecane hypothesized the involvement in hydrocarbon degradation of alternative families of cytochrome P450, i.e., CYP105 and CYP123 (Laczi et al. 2015). A gene encoding a P450 cytochrome of CYP185 family was transcriptionally induced in cells of *Rhodococcus* sp. strain p52 grown on the long-chain alkanes, C_{14}, C_{24}, C_{32}, and the branched alkane, pristine (Yang et al. 2014). These transcriptomic studies indicated the possible involvement of members of cytochrome P450 families different from CYP153 in *Rhodococcus* alkane metabolism, although functional studies are needed to define their role in alkane degradation pathway.

Sequence analysis revealed the presence of a putative AraC family transcriptional regulator (AFR) encoding gene located upstream of the *CYP153* gene cluster in *Proteobacteria* and *Actinobacteria*, including *Rhodococcus erythropolis* PR4 (Sekine et al. 2006) (Fig. 2d). The *AFR* gene was described to code for the CypR regulator in *Dietzia* strain DQ-12-45-1b. In this strain, the activator role of CypR in *CYP153* gene expression was identified (Liang et al. 2016b). The conservation of both gene arrangement of *AFR* and *CYP153* clusters and *CYP153* promoter region suggests that CypR may play similar roles in the *CYP153* gene regulation of *Dietzia* and *Rhodococcus* strains.

3.4 *Multiple Alkane Hydroxylase Systems in* Rhodococcus

In *Rhodococcus* genus, the presence of multiple alkane hydroxylase systems has been described as a widespread feature (Whyte et al. 2002b). Alkane hydroxylases sometimes exhibit overlapping substrate ranges, mainly when AlkB and cytochrome P450 enzymes are considered (van Beilen et al. 2006). A recent genomic and metagenomic survey targeting *alkB* and CYP153 genes in different environments reported that the simultaneous presence of *alkB* and CYP153 genes occurred only in selected bacterial genera including *Rhodococcus* and *Mycobacterium* and other specialized alkane degraders such as *Alcanivorax* (Nie et al. 2014). Further, in many cases, single *Rhodococcus* strains showed the presence of multiple genes encoding alkane MO of the same family, which in some cases showed quite divergent sequences. The coexistence of multiple homologous genes involved in catabolic pathways was proposed to contribute to the wide substrate range and environmental adaptation of *Rhodococcus*, and in some cases, this was associated to horizontal gene transfer events (van Beilen et al. 2003). Indeed, genes encoding transposases were often found in the vicinity of alkane hydroxylase-coding genes/operons (Cappelletti et al. 2015).

Typically, *Rhodococcus* strains are known to harbor multiple *alkB* genes. Indeed, most of the *Rhodococcus* isolates described by van Beilen et al. (2002b) were endowed with three to five *alkB* genes with quite divergent sequences. Each *alkB* homologous gene in *Rhodococcus* spp. was found to have different substrate ranges and induction styles, and the enzymes were suggested to belong to different AlkB groups. *R. qingshengii* Q15 and *R. erythropolis* NRRL B-16531 contained four AlkB (AlkB1-4) with quite sequence divergence (Whyte et al. 2002b; van Beilen et al. 2006). While AlkB3 and AlkB4 showed sequence similarity with *Acinetobacter* AlkB sequences, AlkB1 and AlkB2 were affiliated with AlkB sequence of other actinomycetes like *Mycobacterium*. Only genes *alkB*-1 and *alkB*-2 were located in clusters coding for the redox partner rubredoxins and rubredoxin reductase. Functional expression of AlkB2 showed the implication of this homolog in the initial oxidation of alkanes C_{12}–C_{16}, while AlkB3 and AlkB4 were associated to the degradation of very-long-chain alkanes (>C_{20}). Four of the five *alkB* genes found in *R. erythropolis* TMP2 showed the closest homology to the corresponding *alkB* genes of NRRL B-16531 and Q15. Among these genes, only *alkB*-1 and *alkB*-2 were functionally assigned to pristine metabolism, while *alkB*-3,4,5 were constitutively expressed (Takei et al. 2008). *R. aetherivorans* BCP1 possess two *alkB* genes, of which one is organized in cluster with *rubA1/2* and *rubB*, and two SDIMO gene clusters (*prmABCD* and *smoABCD*). While *alkB* gene cluster expression is induced during BCP1 growth on *n*-alkanes ranging from C_6 to C_{22}, *prmABCD* is transcriptionally induced during the growth on propane and at a lower extent on butane. SmoABCD results to be involved in the growth on short-chain alkanes (C_2–C_7) (Cappelletti et al. 2011, 2015).

R. erythropolis PR4 showed the ability to grow on a wide range of hydrocarbons, and the analysis of its genome revealed the presence of 4 *alkB* genes, 2 *CYP153*

genes, 14 additional cytochrome P450 of different families, 3 multicopper oxidase (MCO), and many other mono- and dioxygenase genes. Transcriptomic analyses indicated that the expression of four *alkB* genes, two CYP153 genes, and other nine genes encoding P450 cytochromes of different families were differently induced after the growth on diesel oil, hexadecane, or hydrocarbon mixtures. In particular, two *alkB* genes were transcriptionally induced during the growth on C_{16} and diesel oil, while the other two *alkB* genes were not expressed under any tested conditions. One *CYP153* was induced during the growth on all the hydrocarbons under analysis, while the other *CYP153* was induced on diesel oil (Laczi et al. 2015). Similar to strain PR4, up to six *alkB* homologous genes and five *CYP153* homologous genes were found in *R. erythropolis* SK121 (Nie et al. 2014). Recently new isolates closely related to *R. erythropolis* species showed the presence of multiple copies of *alkB* genes that are probably involved in the terminal oxidation of long-chain alkanes (C_{12}–C_{29}) (Likhoshvay et al. 2014).

The coexistence of multiple alkane hydroxylase genes in *Rhodococcus* spp. is thought to be the result of an evolution process aimed at differentiating and widening the catabolic processes of members of this genus in response to environmental pressure, i.e., the presence of specific classes of hydrocarbons in the initial isolation source. Further functional studies are needed to get deep into the different selectivity, substrate specificity, and transcriptional regulation featuring the diverse alkane hydroxylase activities underlying the unique alkane degradation capacities of the members of *Rhodococcus* genus.

4 Alkane Metabolism After the Initial Oxidation

Depending on the enzymatic activity and selectivity of the alkane monooxygenase/s involved in the first oxidation step, alkanes are oxidized to a primary alcohol, a secondary alcohol, or a mixture of the two isomers. SDIMO enzymes have been reported to catalyze both the alkane oxidation reactions, whereas AlkB and CYP153 alkane MOs have been mainly described to catalyze terminal oxidations (van Beilen et al. 2006). However, the subterminal alkane oxidation ability was suggested for at least one of the AlkB MOs found in *Rhodococcus qingshengii* Q15, and the mutant cytochrome P450 from *Bacillus megaterium* was able to hydroxylate alkanes at the 2-position (Ji et al. 2013). Only a few numbers of studies have investigated the catalytic properties of the different AlkB and P450 enzymes in *Rhodococcus* spp.; in this regard, the high sequence divergence featuring the different alkane MOs might be associated to still unexplored substrate ranges and enzymatic activities.

After the initial oxidation step, the metabolism of medium- and long-chain alkanes by *Rhodococcus* spp. typically follows the terminal, the subterminal, or the bi-terminal oxidation pathway (Fig. 3). In the terminal oxidation pathway, the alkane is oxidized to the corresponding primary alcohol, which is further oxidized to the corresponding aldehyde by alcohol dehydrogenases and then to the corresponding fatty acid by aldehyde dehydrogenases (Rojo 2009). In the bi-terminal oxidation

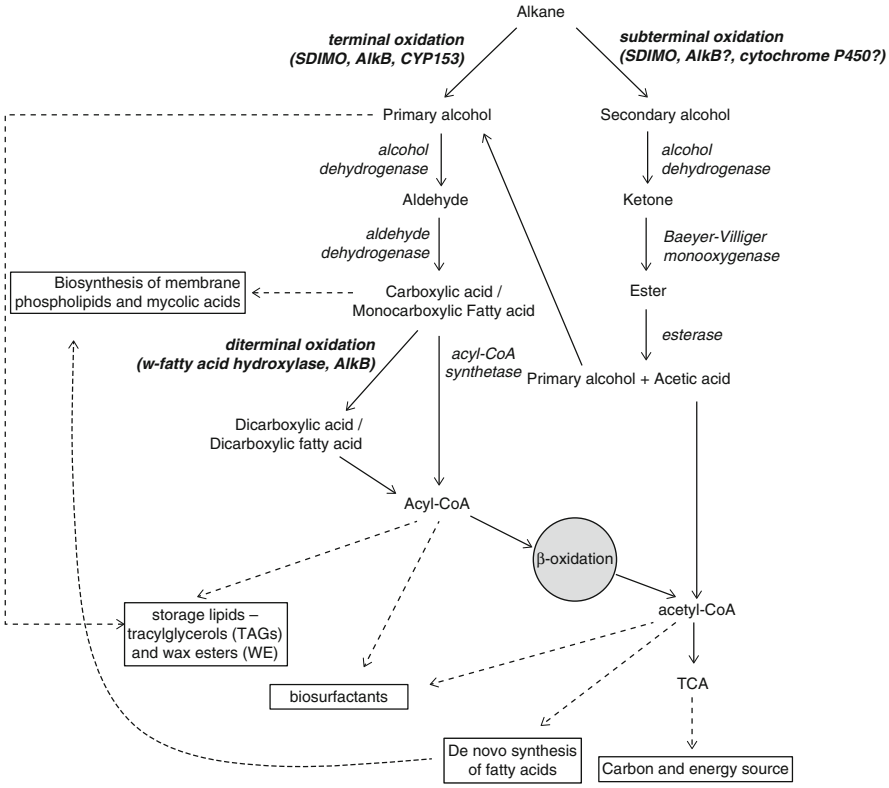

Fig. 3 The different alkane degradation pathways described in *Rhodococcus* spp. (terminal, subterminal, and bi-terminal oxidation pathways)

pathway, the fatty acid produced via terminal oxidation is further oxygenated by a ω-fatty acid monooxygenase to form dicarboxylic acid (Ji et al. 2013). In the subterminal pathway, the alkane is oxidized to the corresponding secondary alcohol, which is converted to the corresponding ketone and then oxidized to the ester by a Baeyer-Villiger monooxygenase. The ester is then hydrolyzed to an alcohol and a fatty acid molecule (Van Beilen et al. 2003). In all cases, the alkane oxidation pathways lead to the formation of CoA-activated fatty acids which enter the β-oxidation process that generates the tricarboxylic acid cycle (TCA) intermediates to be used as carbon and energy sources. Alternatively, the different metabolic intermediates produced during the alkane oxidation process can (1) be assimilated as membrane components, in this case the alkane chain can be incorporated in fatty acids composing phospholipids or can be used as precursors in the mycolic acid synthesis, and (2) be converted into storage lipids such as triacylglycerols (TAGs) and wax esters (WE) (Alvarez et al. 2013; Alvarez 2003) (Fig. 3).

Different alkanotrophic *Rhodococcus* strains typically possess a variety of alkane catabolic pathways. Several *Rhodococcus* strains showed the capacity to degrade

alkanes following either the terminal or the subterminal oxidation pathways; further, the coexistence of the two oxidation pathways was also demonstrated in some *Rhodococcus* spp. metabolizing both short- and medium-/long-chain alkanes. Whyte et al. (1998) reported both terminal and subterminal oxidation pathways of *n*-C_{12} (dodecane) and *n*-C_{16} (hexadecane) in *R. qingshengii* Q15 cells producing 1-dodecanol and 2-dodecanone and 1-hexadecanol and 2-hexadecanol, respectively. *R. erythropolis* ATCC 4277 was described to degrade C_5–C_{16} alkanes using only the subterminal pathway (Ludwig et al. 1995) similarly to *Rhodococcus* sp. strain EH831 and *Rhodococcus* sp. strain Moj-3449 grown on C_6 and C_{12} (Binazadeh et al. 2009; Lee et al. 2010). *Rhodococcus* sp. strain BPM 1613 was described to convert the methyl-branched alkane pristane to terminal oxidation metabolic intermediates which were further degraded through either β- or ω-oxidation (via bi-terminal oxidation pathway) (Nakajima et al. 1985). *Rhodococcus ruber* sp. strain SBUG 82 could also metabolize pristane through the subterminal oxidation pathway (Nhi-Cong et al. 2009). Alkanotrophic *Rhodococcus* sp. strain NCIM 5126 produced primary alcohols that were converted into monocarboxylic acids when odd carbon number alkanes (C_{13} and C_{17}) were used as growth substrates. Conversely, when NCIM 5126 was grown on even carbon number *n*-alkanes (C_{14} and C_{16}), the primary alcohols were converted only to dicarboxylic acids (Sharma and Pant 2000). These data suggested the presence of both a bi-terminal and a mono-terminal oxidation pathway of *n*-alkanes in NCIM 5126. On the other hand, this strain catabolized pristane only through mono-terminal oxidation pathway (Sharma and Pant 2000).

A peculiar capability of members of *Rhodococcus* genus and of few other taxonomically related genera (e.g., *Mycobacterium*, *Nocardia*) concerns the capacity to metabolize gaseous alkanes (Shennan 2006). Like liquid alkanes, gaseous alkanes, i.e., ethane, propane, and butane, are oxidized to the primary and/or secondary alcohols that are further converted by the activities of alcohol and aldehyde DHs to short-chain carboxylic acids (e.g., propionic acid, butyric acid). These carboxylic acids can be directly converted to intermediates of TCA cycle (Fig. 4). While ethane metabolism is assumed to be oxidized to ethanol, then to acetaldehyde and acetate, which enter the central metabolic pathways in bacterial cells, butane oxidation has been mainly described to proceed via the terminal carbon atom oxidation pathway (1-butanol, butyraldehyde, butyrate) (Ashraf et al. 1994; Cappelletti et al. 2015). Conversely, propane can be oxidized via terminal, subterminal, or a mixture of the two pathways, depending on the *Rhodococcus* strain under analysis (Ashraf et al. 1994). In *R. rhodochrous* ATCC 21198, propane is oxidized to 1-propanol, propanal, and propanoate, which is carboxylated to methylmalonyl CoA and then converted to succinyl-CoA (MacMichael and Brown 1987). The subterminal oxidation pathway is more complicated with the possibility of three variations, two of which involve the metabolism of propane via 2-propanol to acetone, which can be converted to methyl acetate or to acetol. *R. rhodochrous* PNKb1 and *R. aetherivorans* BCP1 were shown to metabolize propane via the two oxidation pathways, terminal and subterminal (Woods and Murrell 1989; Ashraf et al. 1994; Cappelletti et al. 2015). In the latter case, the propane monooxygenase was suggested not to have preference in the insertion of oxygen into the propane

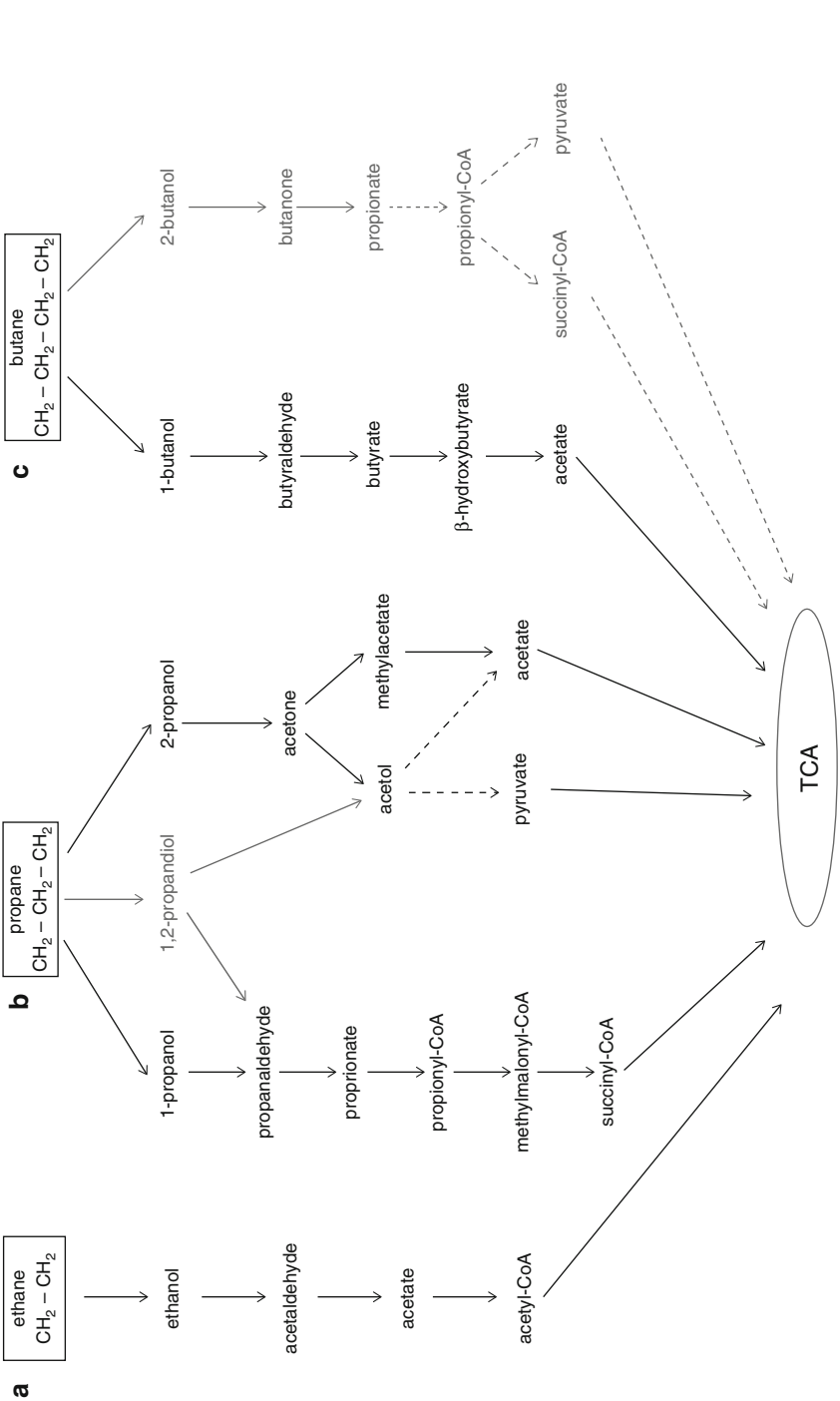

Fig. 4 Gaseous *n*-alkane [(**a**) ethane, (**b**) propane, (**c**) butane] oxidation pathways described in *Rhodococcus* spp. In gray, degradation pathways are displayed that have been only hypothesized or that have not been further characterized in *Rhodococcus*. Dashed lines correspond to more reactions. *TCA* tricarboxylic acid cycle

molecule (Ashraf and Murrell 1992), while specificity was observed at the stage of the alcohol dehydrogenase oxidizing either 1- or 2-propanol. An alternative pathway for propane oxidation was proposed to occur via 1,2-propandiol production through the activity of an intermolecular dioxygenase system (Babu and Brown 1984; Ashraf et al. 1994). The diol was proposed to be further converted to acetol or to propanal (Ashraf et al. 1994).

Several alcohol dehydrogenases (ADHs) have been described to be involved in the oxidation of the alcohol/s derived from alkane oxidation into the corresponding aldehyde or ketone in *Rhodococcus* spp. Among these, alcohol dehydrogenases involved in the alkanol oxidation were a NAD-dependent 2-propanol dehydrogenase from *R. rhodochrous* PNKb1 (Ashraf and Murrell 1990) and a NAD-dependent long-chain secondary alcohol dehydrogenase from *R. erythropolis* ATCC 4277 (Ludwig et al. 1995). NAD-dependent dehydrogenases showed secondary alcohols as preferential substrates, while primary alcohols are the preferred substrates for *N, N*-dimethyl-4-nitrosoaniline (NDMA)-dependent ADHs (Schenkels and Duine 2000). NDMA was used as an artificial cofactor for the latter alcohol dehydrogenase in *R. erythropolis* DSM 1069 incubated with alkanol derived from short-chain (both gaseous and liquid) alkane metabolism (Schenkels and Duine 2000), while both NAD^+- and NDsMA-dependent ADHs were found to be highly active during ethanol growth by *R. erythropolis* EK-1 (Pirog et al. 2008). Further, in *R. jostii* RHA1, several Baeyer-Villiger monooxygenases were shown to have a substrate range including intermediates of subterminal oxidation pathways, e.g., acetone, 2-butanone, and 2-octanone (Riebel et al. 2012). These enzymes are possibly involved in the oxidation steps downstream of the secondary alcohol production from alkane subterminal oxidation.

The overexpression of specific alcohol and aldehyde dehydrogenases (DHs) was seen in studies reporting transcriptomic and proteomic analyses of *Rhodococcus* spp. grown on alkanes as compared to orthodox carbon sources (e.g., glucose, succinate) (Sharp et al. 2007; Cappelletti et al. 2015). In these studies, the alkane-induced cells showed the overexpression of genes coding for alcohol and aldehyde DHs that were arranged in clusters with those genes encoding for alkane-induced monooxygenases (Sharp et al. 2007; Cappelletti et al. 2015). In particular, the gene encoding a secondary alcohol dehydrogenase in cluster with the *prmABCD* gene was transcriptionally induced during the growth *R. jostii* RHA1 cells on propane (Sharp et al. 2007). The proteomic profile of *R. aetherivorans* BCP1 cells grown on *n*-butane and *n*-hexane showed the upregulation of three alcohol dehydrogenases (DHs) and three aldehyde DHs which are likely to be involved in the oxidation steps occurring downstream of the monooxygenase hydroxylation (Cappelletti et al. 2015). Among these, two genes (one for alcohol DH and one for aldehyde DH) were consecutive to the *smoABCD* gene cluster encoding the alkane MO involved in the initial short-chain alkane oxidation in *R. aetherivorans* BCP1 (Cappelletti et al. 2015) (Fig. 2c).

5 Biotechnological Applications of Alkanotrophic Rhodococci

Alkanotrophic *Rhodococcus* are able to transform hydrocarbon contaminants into chemicals relevant for biotechnological and industrial applications (Larkin et al. 2005). In addition to regio- and stereoselective oxidations performed by some alkanotrophic *Rhodococcus* strains, these bacteria can also produce high-value compounds; storage lipids, biosurfactants, and carotenoids are some of the most significant biotechnological products that can be produced by *Rhodococcus* strains utilizing alkanes.

Several *Rhodococcus* strains produce biosurfactants in response to the presence of alkanes as growth substrate. They mainly consist of glycolipids containing trehalose as the carbohydrate. The physiological role of these glycolipids is involved in the uptake of hydrophobic and water-insoluble alkanes (see Sect. 2) (Lang and Philp 1998). Biosurfactants are surface-active, amphiphilic compounds characterized by a hydrophilic and a hydrophobic molecule part. In addition to the unique interfacial properties, trehalose-based biosurfactants produced by rhodococci also possess immunostimulating and cell-differentiating features along with low cytotoxicity (Kuyukina et al. 2015).

Recently, in *Rhodococcus erythropolis* SD-74, three genes were described to be involved in the biosynthesis of succinoyl trehalose lipids, including a putative acyl coenzyme A transferase, a fructose-bisphosphate aldolase, and an alkane monooxygenase (Inaba et al. 2013). Interestingly, the upregulation of the expression of the acyl coenzyme A transferase was shown to enhance the production of succinoyl trehalose lipids in this strain (Inaba et al. 2013). Further, *R. erythropolis* SD-74 cells produced succinoyl trehalolipids with acyl groups of the same carbon chain length as the *n*-alkanes used as growth substrate, suggesting the possibility to direct the synthesis of specific biosurfactants by supplying alkanes with specific chemical nature (Lang and Philp 1998; Inaba et al. 2013). This property is highly useful for the commercial production, as most biosurfactant producers do not produce specifically defined derivatives.

Triacylglycerol (TAG, triesters of glycerol and long-chain fatty acids) and wax esters (WE, esters of primary long-chain fatty acids and primary long-chain fatty alcohols) are in addition to polyhydroxyalkanoates (PHA), the most important lipids produced by bacteria, and they have relevant applications in the production of food additives, cosmetics, lubricants, oleo-chemicals, candles, and biofuels (Holder et al. 2011). PHAs are the main storage compounds produced by bacteria, while the production of TAGs and WEs has been documented in a few bacterial genera including strains of the genus *Rhodococcus* (Hernández et al. 2008; Alvarez et al. 2013). Indeed, several strains of rhodococci are able to store significant amount of TAGs, which can reach more than 70% of their biomass (Alvarez et al. 1996). TAG storage inside the cells is enhanced under nitrogen-limiting conditions when cells are grown with different carbon sources such as sugars and organic acids, but evidence that also alkane-utilizing species of *Rhodococcus* can accumulate TAGs has also

been obtained in the last two decades (Alvarez 2003). The possibility to combine TAG production with hydrocarbon degradation would couple the environmental remediation with the production of high-value compounds.

R. opacus strain PD630 is taken as a model bacterial system regarding TAG production and storage (Alvarez et al. 1996). Several studies with strain PD630 showed that the use of alkanes as carbon source influenced the type of the predominant fatty acids present in the inclusions, since their chain lengths resembled that of the substrate, suggesting that alkanes were not completely metabolized to acetate when they were accumulated as cytosolic lipid inclusions. Furthermore, *R. opacus* strain B4 was described to be able to produce and accumulate high amounts of lipids using C_{16} as sole carbon source (Castro et al. 2016). Interestingly, while strains PD630 and B4 cultivated on C_{16} displayed similar fatty acid profiles in their cell membrane fractions, *R. opacus* B4 showed a higher variability in fatty acid composition as compared to *R. opacus* PD630 in the TAG fractions (Castro et al. 2016). In this respect, the biosynthesis of lipids with a higher variety of fatty acids can be an interesting advantage since they can be used in different types of industrial and commercial applications such as in personal care, food, and beverage industries (Castro et al. 2016). The effects of carbon sources on the composition of fatty acids, synthesized by two other *Rhodococcus* strains, *R. erythropolis* DCL14 and *R. opacus* PWD4, were recently reported by Cortes and Carvalho (2015). The chain length of the different *n*-alkanes used for growth by these two rhodococci influenced the percentage of each type of fatty acids stored inside the cells. Some *Rhodococcus* strains were able to produce wax esters, in addition to TAG, during growth on *n*-alkanes and phenylalkanes using fatty alcohols produced as intermediates during their degradation (Alvarez et al. 1996; Hernández et al. 2008). For instance, in addition to TAG, wax esters were detected in hexadecane- and hexadecane/hexadecanol-grown cells of *R. jostii* RHA1 (Hernández et al. 2008). Wax esters and fatty alcohols provide diverse industrial uses, such as additives for biolubricants and cosmetic and pharmaceutical products (Lanfranconi and Alvarez 2017).

Carotenoids form another important category of organic compounds produced by rhodococci. Carotenoids have antioxidant and anticancer properties; they can also activate the immune system and promote other beneficial effects on human health (Krinsky and Johnson 2005). For these reasons the use of carotenoids as natural food additives has greatly increased in the last decade (Krinsky and Johnson 2005). Many *Rhodococcus* strains can produce carotenoid pigments such as β-carotene, γ-carotene, and chlorobactene (Tao et al. 2006). Recently, it was reported that in a *R. erythropolis* strain able to use different alkanes such as cycloalkane, *n*-hexane, and *n*-decane, as sole carbon source, the exposure to these different hydrocarbons determined specific modifications of the cell carotenoid production. In particular, higher levels of lycopene, an important precursor involved in the biosynthetic pathway of γ-carotene and β-carotene, were found in extracts of cells grown on *n*-alkanes (Stancu 2015). The ability to form aggregates and to develop biofilms has also been documented to significantly influence carotenoid production in *Rhodococcus* spp. (Zheng et al. 2013).

Another extensively studied application of alkanotrophic rhodococci is their use in bioremediation of halogenated organic compounds through oxidative cometabolism. Halogenated volatile organic compounds, including chlorinated aliphatic hydrocarbons (CAHs), are widely applied in a variety of industrial processes and are among the most frequently occurring soil and groundwater contaminants released into the environment through accidents and/or non-correct industrial management (Frascari et al. 2008; Cappelletti et al. 2012). CAHs can be extremely harmful to human health, with some of them classified as either known or suspected carcinogens (McDermott and Heffron 2013).

Various *Rhodococcus* strains have been reported to aerobically degrade different CAHs via cometabolic degradation using several *n*-alkanes as primary carbon source mainly gaseous *n*-alkanes such as propane and butane (Table 2). TCE, VC, 1,1-dichloroethane, 1,1,1-trichloroethane, and 1,2,3-trichloropropane along with CF are the most frequently cometabolized CAHs by alkanotrophic rhodococci (Table 2). The aerobic mineralization of trichloroethylene (TCE) and vinyl chloride (VC) by two alkanotrophic *Rhodococcus* strains (Sm-1 and Wrink) was first described by Malachowsky et al. (1994).

R. aetherivorans BCP1 (formerly *Rhodococcus* sp. strain BCP1) was isolated from a microbial consortium that was subject to a prolonged chloroform exposure in bioreactors (Frascari et al. 2006). Strain BCP1 was able to grow on a wide range of *n*-alkanes (gaseous, liquid, and solid) (Cappelletti et al. 2011) and when BCP1 cells were grown on *n*-hexane (C_6) or *n*-butane (C_4) had the ability to degrade efficiently CF as well as VC and 1,1,2-TCA via cometabolism (Table 2) (Frascari et al. 2006; Cappelletti et al. 2012). The BCP1 cell growth on these *n*-alkanes induced the expression of alkane monooxygenase genes that are thought to be involved in the alkane/CAH cometabolism (Cappelletti et al. 2011, 2015). Further, CF was efficiently degraded by BCP1 cells grown on *n*-butane in a continuous-flow biofilm reactor, which has a series of operational advantages (Ciavarelli et al. 2012). Other *Rhodococcus* strains able to grow on gaseous alkanes and cometabolize CAHs were *Rhodococcus* sp. strain PB1 and *R. aetherivorans* TPA (Table 2). In particular, *R. aetherivorans* strain TPA, was able to cometabolically degrade 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) under propanotrophic growth conditions. This is the first isolate described to be able to degrade a high-chlorinated CAH like 1,1,2,2-TeCA under aerobic conditions, whereas most of the successful bioremediation methods of these compounds needed to be performed under anaerobic conditions and in the presence of microbial consortia (Cappelletti et al. 2017b).

6 Summary

During the last 20 years, many studies have shown that members of *Rhodococcus* genus are broadly distributed in hydrocarbon-contaminated sites and have shown to play a key role in oil biodegradation processes, even in cold temperature environments. Among hydrocarbons, alkanes are ubiquitous compounds released in the

Table 2 Main *Rhodococcus* strains characterized for their ability to grow on *n*-alkanes and to cometabolize chlorinated aliphatic hydrocarbons (CAHs) or other xenobiotics

Strain	Primary growth substrate	Cometabolized CAHs[a] or other xenobiotics	References
Rhodococcus sp. strain Sm-1 (ATCC 51239)	Propane	1,1-DCE, *cis*-1,2-DCE, CF, TCE, 1,1,2-TCA, 1,1,1-TCA and VC	Malachowsky et al. (1994)
Rhodococcus sp. strain Wrink (ATCC 51240)	Propane	TCE and VC	Malachowsky et al. (1994)
R. rhodochrous ATCC 21197	Propane	1,1-DCE, *cis*-1,2-DCE, CF, VC, CF, 1,1,2-TCA, 1,1,1-TCA	Malachowsky et al. (1994)
Rhodococcus sp. strain PB1	Propane	VC, *cis*-DCE, 1,1,2-TCA and CF	Frascari et al. (2008)
Rhodococcus sp. strain 179 BP *Rhodococcus* sp. strain 183 BP	*n*-Butane	1,1-DCA and 1,1,1-TCA	Semprini et al. (2009)
R. ruber ENV 425	Propane	NDMA and 1,2,3-TCP	Fournier et al. (2009) and Wang and Chu (2017)
R. jostii RHA1	Propane	NDMA and 1,2,3-TCP	Sharp et al. (2007) and Wang and Chu (2017)
R. aetherivorans BCP1	*n*-Butane, *n*-hexane	CF, VC, 1,1,2-TCA	Frascari et al. (2006)
R. aetherivorans TPA	Propane	1,1,2,2-TeCA	Cappelletti et al. (2017b)

[a]*CF* chloroform, *DCA* dichloroethane, *DCE* dichloroethylene, *TCA* trichloroethane, *TCE* trichloroethylene, *TeCA* tetrachloroethane, *TCP* trichloropropane, *NDMA* *N*-nitrosodimethylamine, *VC* vinyl chloride

environments from natural and anthropogenic sources. Members of *Rhodococcus* genus have been described for their unique degradation abilities toward a wide range of alkanes and have been indicated as ideal candidates for bioremediation and biotransformation strategies. This is associated to peculiar physiological and genetic aspects: (1) the ability to resist harsh environmental conditions and to tolerate the presence of high concentration of toxic compounds; (2) the hydrophobic *Rhodococcus* cell surface and the ability to produce biosurfactants, which facilitate the contact between the cell and the hydrophobic alkane and its subsequent internalization; and (3) several *Rhodococcus* spp. contain many genes encoding alkane hydroxylases which are involved in the alkane molecule activation through an initial oxidation reaction. The genes encoding these key enzymes showed in some cases high sequence divergence in *Rhodococcus*, and each expressed monooxygenase was commonly featured by distinct substrate specificity and selectivity. Further, although only few aspects concerning alkane metabolism regulatory mechanisms have been described in

Rhodococcus, expression studies have shown that the different alkane hydroxylase genes are induced under different conditions of growth and/or in the presence of different primary substrates, e.g., alkane with specific chain length.

In terms of potential industrial applications, alkanotrophic *Rhodococcus* spp. can be exploited as microbial cell factories able to convert alkanes into lipids and carotenoids, to produce biosurfactants, and to degrade chlorinated aliphatic hydrocarbons through alkane-induced cometabolic processes.

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Biodegradation of Nitriles by *Rhodococcus*

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Contents

Abstract Nitriles occur naturally in the environment, are produced by the metabolic pathways of organisms or are released by the chemical and pharmaceutical industries, from agricultural applications or from the processing of fossil fuels. Therefore, a variety of nitrile-converting bacterial species are used to alleviate this toxic effect. Among these bacteria, *Rhodococcus* species have proven to be a superior group for

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_7

the clean-up of pollutants. Nitriles are converted into the less toxic carboxylic acid either by nitrilases or by nitrile hydratase (NHase)/amidase systems. Although NHases, nitrilases and amidases produced by different strains exhibit different catalytic characteristics towards aliphatic nitriles and aromatic nitriles, these enzymes exhibit considerable homology in amino acid sequence or structure. In contrast, the enzymes with different origins present different types of gene organization and regulatory patterns, although the amidase gene is always linked to the NHase gene. Due to the advantage of being resistant to toxic compounds, applications of *Rhodococcus* in pollutant biodegradation and biocatalytic processes are very promising. While studies on the biodegradation of nitrile pollutants focus on the screening and discovery of strains, the industrial application of these enzymes as biocatalysts focuses on engineering combined with immobilization of both *Rhodococcus* cells and enzymes to improve their performance under the adverse conditions in the catalytic process.

1 Introduction

Nitriles are widely distributed in nature and can be divided into three general categories: aliphatic nitriles, aromatic nitriles and heterocyclic nitriles. Nitriles may be produced by a variety of microorganisms and plants and exist as 3-indolylacetonitrile (an auxin precursor), 3-phenylpropionitrile (a growth hormone) and so on. In living organisms, these naturally occurring nitriles may be the product of the following pathway: amino acid → aldoxime → nitriles. Aldoxime dehydratase participates in the formation of nitrile from aldoxime. Nitriles are also products of the agricultural, pharmaceutical and chemical industries (Bhalla et al. 2012). These nitriles are important intermediates for the production of amides, acids and other compounds. Despite the importance of nitriles in organic synthesis, large amounts of nitrile compounds are released to the environment, including to soil, air and water, and are harmful and toxic to human beings; therefore, remediation of the polluted environment is an urgent issue.

Due to the widespread occurrence of nitriles in metabolic pathways and natural environments, nitrile-converting enzymes occur in a wide variety of bacterial species, such as *Pseudomonas* sp., *Bacillus*, *Corynebacterium*, *Nocardia*, *Micrococcus* and *Rhodococcus*. In microorganisms, nitriles are primarily degraded to carboxylic acids, which are then converted to other metabolites. The conversion of nitriles to carboxylic acids occurs via two types of pathways: (1) direct hydrolysis of nitriles to carboxylic acids by nitrilases and (2) cascade catalysis of nitriles by NHases and amidases via the pathway nitriles → amides → carboxylic acids. While some identified nitrile-converting strains have only one of the two pathways, others harbour both the pathways. Nitrilases and NHases produced by different strains also exhibit different catalytic preferences for aliphatic and aromatic nitriles. While

some nitrilases are capable of catalysing aliphatic nitriles efficiently, the enzymes are not efficient at catalysing aromatic nitriles. A similar phenomenon has been observed for different types of NHases. In particular, *Rhodococcus rhodochrous* J1 can produce two types of NHases, namely, high-molecular-mass NHase and low-molecular-mass NHase, which exhibit different specificities for aliphatic nitriles (Komeda et al. 1996b, c).

The diverse catalytic properties of various NHases and nitrilases enable extensive application of these enzymes to transform a vast number of man-made nitriles for the production of many important bulk and fine chemicals or to remove pollutants from waste materials and environment. NHases, which catalyse the hydration of nitriles to the corresponding amides, have been widely used in the chemical industry for the production of acrylamide, nicotinamide and 5-cyanovaleramide. In addition, nitrilases are attractive biocatalysts for the production of various commodity chemicals and pharmaceutical intermediates, such as nicotinic acid, isonicotinic acid, mandelic acid, acrylic acid, glycolic acid and benzoic acid. Among nitrile-converting strains, *Rhodococcus* strains have proven to be the most powerful and successful in the industrial transformation of various nitriles. However, there are some bottlenecks in the application of these enzymes in the transformation of nitriles to the corresponding amides or carboxylic acids, such as activity inhibition at high concentrations of substrates and products, thermal susceptibility and low nitrilase activity. As a result, genetic engineering strategies combined with immobilization were applied to enhance the stability and activities of the *Rhodococcus* cells and enzymes to broaden the industrial application of these enzymes.

2 Nitrile-Degrading Enzymes

Nitrile compounds, which have a $-C\equiv N$ functional group, are ubiquitous in natural environments as intermediates synthesized by chemical processes or biological systems. Nitrile compounds have application in many areas, for example, 2,6-dichlorobenzonitrile is used as an herbicide (Tao et al. 2016), nitrile rubber is used in medical gloves, polyacrylonitrile fibres are used to manufacture clothing, acetonitrile is used in analytical chemistry, and citalopram is used as a pharmaceutical (Fleming et al. 2010). The widespread use of nitrile compounds inevitably causes water pollution, soil contamination and accumulation of nitrile-containing waste. Over the past few decades, nitrile degradation has become a cause for concern worldwide. Biodegradation is more promising than chemical and physical methods due to the complete conversion, low cost and low level of secondary pollution associated with this method (Alexander 2001). Usually, biodegradation is carried out by enzymatic catalysis. The key enzymes used for nitrile degradation are NHase, amidase and nitrilase (Gong et al. 2017). NHase catalyses the hydration of nitriles to the corresponding amides (Ma et al. 2010); amidase catalyses the hydrolysis of amides to the corresponding acids; and nitrilase catalyses the one-step hydrolysis of nitriles to the corresponding acids (Fig. 1).

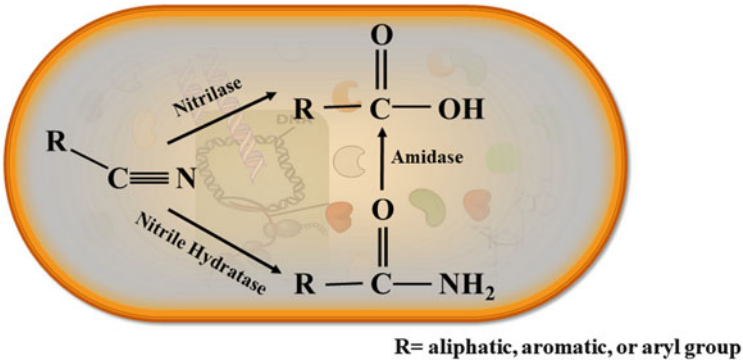

Fig. 1 Nitrile-biodegrading enzymes

2.1 NHase

NHase was first discovered in 1980 (Asano et al. 1980), and over the last few decades, many NHases have been identified and characterized. Various natural NHases are synthesized by plants and bacteria; these enzymes vary widely in length and exhibit structural diversity. However, microbial NHases usually exhibit considerable homology in amino acid sequence or structure (Cramp and Cowan 1999). Microbial NHase components are hetero-oligomers (αnβn), and typically, NHases exist as αβ dimers or tetramers (Cowan et al. 1998). Each αβ unit has a low-spin metal ion at the active centre (Huang et al. 1997), and NHases are classified into Co-type and Fe-type NHases according to the metal ions associated with the enzyme. Co-type NHase exhibits superior thermostability to Fe-type NHase in industrial biochemical synthetic processes (Cowan et al. 1998). Basically, structure determines function. As shown in Figs. 2 and 3, the sequence and structure characteristics of the α and β subunits of both classical Co-type NHases [PDB ID 1IRE from *Pseudonocardia thermophila* (Miyanaga et al. 2001) and PDB ID 1V29 from *Bacillus smithii* (Hourai et al. 2003)] and two Fe-type NHases [PDB ID 2AHJ from *Rhodococcus erythropolis* (Nagashima et al. 1998) and PDB 4FM4 from *Comamonas testosteroni* (Kuhn et al. 2012)] are illustrated.

The α subunits of Co-type and Fe-type NHases have highly homologous amino acid sequences and structures (Fig. 2). The size of the α subunit is 24–28 kDa. Structurally, three cysteine residues and one serine residue (-C-X-X-C-S-C-) in the cysteine cluster region constitute a fully conserved sequence in the α subunit of all Co- and Fe-type NHases (Fig. 2a). The three cysteine thiolates and the two main-chain amide nitrogens form a square-pyramidal geometry as ligands that co-ordinate the Fe or Co ion. The structures of the Co- and Fe-type NHase active centres are the same, except at one site. The sixth site is occupied by a water/hydroxide molecule as the active form in Co-type NHase, whereas a photolabile NO molecule is present at this site in Fe-type NHase (Endo et al. 2001).

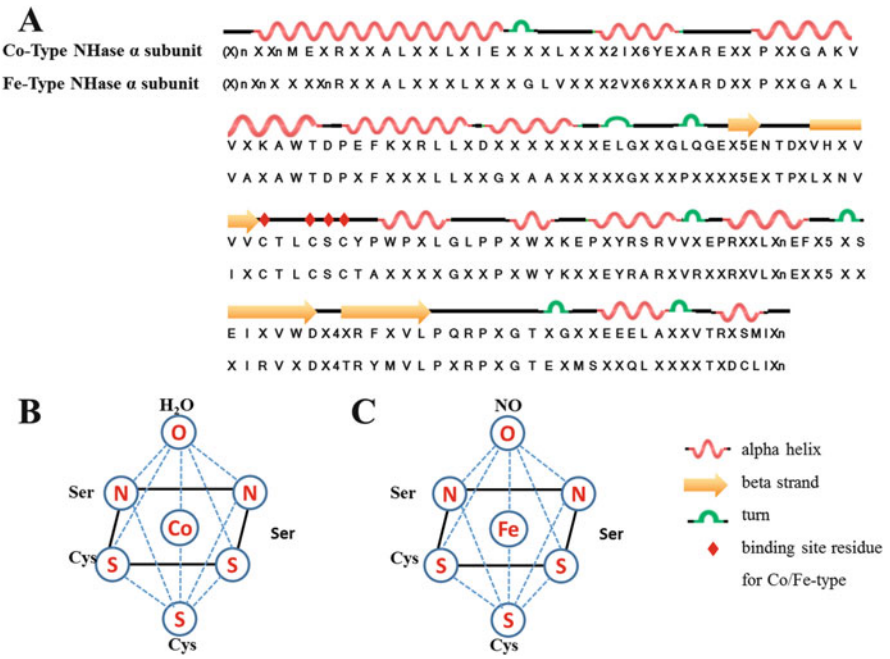

Fig. 2 Partial amino acid sequences and structure of Co- and Fe-type NHase α subunits. Co-type NHase: 1IRE (Miyanaga et al. 2001) and 1V29 (Hourai et al. 2003). Fe-type: 2AHJ (Kuhn et al. 2012) and 4FM4 (Endo et al. 2001). (**a**) Amino acid sequence alignment of Co- and Fe-type NHase α subunits. (**b**) Cobalt active centre of Co-type NHase. (**c**) Structure of the iron active centre of Fe-type NHase in the inactive state

The β subunits of NHases, ranging from 25 to 39 kDa in size, have lower amino acid sequence homology than the α subunits. Two Arg residues in the β subunit are highly conserved in both Co-type and Fe-type NHases (Fig. 3a, b). The guanidine groups of these two Arg residues react with the sulphur atoms of Cys in the α subunit and form a salt bridge network with electrostatic interactions (Fig. 3c). The formation of a salt bridge around the metal ion ensures the binding of the α and β subunits to stabilize the subunit interface. In addition, there exist several structural differences between Co-type and Fe-type NHases (Fig. 3). In particular, one α helix (blue in Fig. 3c, β111–β125) in Co-type NHase is replaced by a long loop (β95–β138 shown in Fig. 3c) in Fe-type NHase. This helix, which is composed in part of these residues (β111–β125), interacted with another helix belonging to the α subunit. This additional interaction may contribute to the better thermostability of Co-type NHase than Fe-type NHase (Miyanaga et al. 2001).

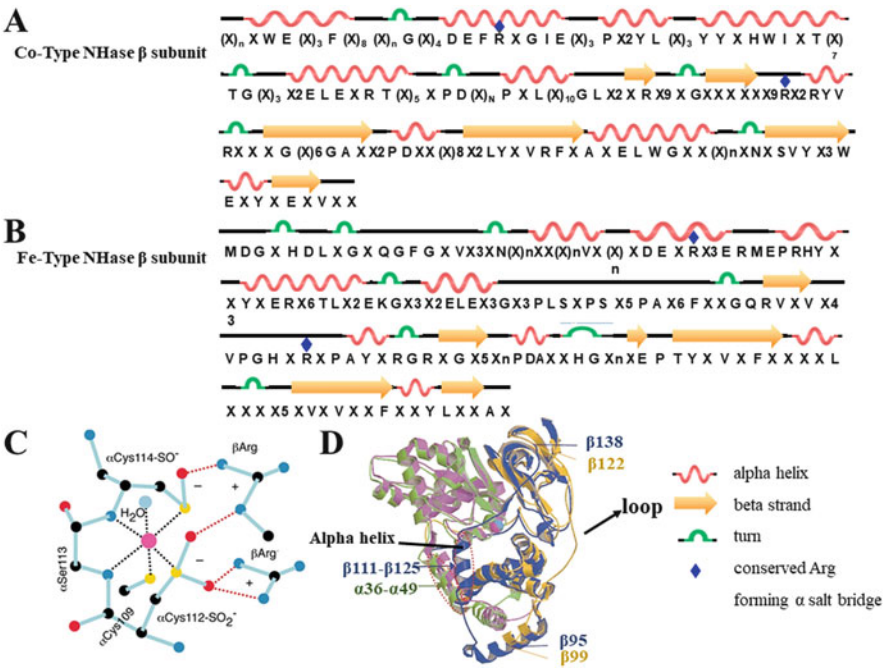

Fig. 3 Amino acid sequences and structures of Co- and Fe-type NHase β subunits. (**a**) Amino acid sequence alignment of the β subunit of Co-type NHase. Co-type NHase: 1IRE (Miyanaga et al. 2001) and 1V29 (Hourai et al. 2003). (**b**) Amino acid sequence alignment of the β subunit of Fe-type NHase. Fe-type NHase: 2AHJ (Kuhn et al. 2012) and 4FM4 (Endo et al. 2001). (**c**) Model of the noncorrin cobalt centre of NHase. Atoms are shown in different colours: pink for Co, black for C, red for O, yellow for S and blue for N. The salt bridge networks formed between the cysteines of the α subunit and the arginines of the β subunit are shown as red dotted lines (Zhou et al. 2008). (**d**) Ribbon diagram of Co-type and Fe-type NHases (Miyanaga et al. 2001)

2.2 *Amidase*

Amidase (EC 3.5.1.4) is widespread in nature and catalyses the hydrolysis of amides into the corresponding carboxylic acids and ammonia. Coupled with NHase, amidase has great potential in the degradation of toxic nitrile compounds. Based on amino acid sequence and structural homology, amidases have been classified into two groups: the nitrilase superfamily and the amidase signature (AS) family (Ohtaki et al. 2010).

The first group of amidases belongs to nitrilase superfamily. These enzymes have low amino acid sequence homology but high structural homology (Andrade et al. 2007; Kimani et al. 2007; Makhongela et al. 2007). Being members of the branch 2 nitrilase superfamily, amidases have the conserved catalytic Glu-Lys-Cys triad, and the amidase monomers have the typical nitrilase superfamily αββα sandwich fold, similar to nitrilase.

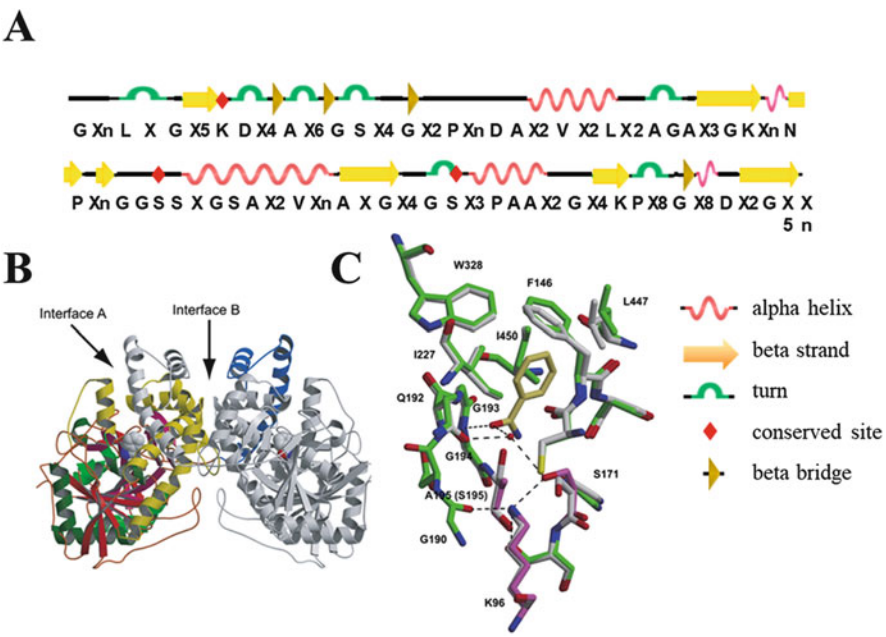

Fig. 4 Amino acid sequence and structure alignment of amidase. (**a**) Structural sequence alignment of the amidase signature (AS) region. The secondary structural elements identified in 3A1I (Ohtaki et al. 2010) are indicated in the top line. (**b**) Dimer structure of RhAmidase (Ohtaki et al. 2010). A monomer is shown in colour. (**c**) Structure of the active site of RhAmidase (Ohtaki et al. 2010)

The other group of amidases belongs to the AS family. AS family members have a highly conserved amino acid sequence, named the AS sequence, and a conserved Ser-Ser-Lys catalytic triad, and these proteins exist as homodimeric or homo-octameric complexes (Fig. 4). The amidase from *Rhodococcus* sp. N-771, namely, RhAmidase, can be taken as an example that has an active homodimeric structure and the active catalytic triad. A single monomer is shown in colour in Fig. 4b, and the two monomers binding crosswise. All helices of the small domain form interfaces A and B to participate in the formation of the homodimeric structure, and these interactions contribute to the formation of a closely packed dimer structure. The conserved catalytic Ser-Ser-Lys triad is located in the large domain. The first Lys96 can be deprotonated and acts as the catalytic base, and this residue also forms hydrogen bonds with Ser171 and Gly190; the second Ser171 is in an unusual *cis* conformation; and the third Ser195 forms a covalent bond with the substrate (Ohtaki et al. 2010; Lee et al. 2015; Valiña et al. 2004).

2.3 Nitrilase

Nitrilase was first described by Thimann and Mahadevan in 1964, and since then, over 200 nitrilases belonging to the nitrilase superfamily have been discovered and sequenced (Thuku et al. 2009). The nitrilase superfamily is characterized by a homodimeric building block with an αββα-αββα sandwich fold and is classified into 13 branches based on amino acid sequence similarity and the presence of additional domains (Thuku et al. 2009; Pace and Brenner 2001).

The nitrilases in branch 1 of the nitrilase superfamily hydrolyse nitrile (RCN) to ammonia and the corresponding carboxylic acid. Moreover, nitrilases are further categorized into aliphatic nitrilases, aromatic nitrilases and arylacetonitrilases according to substrate specificity, although some nitrilases exhibit broad substrate specificity, and the substrate specificity can also be changed by mutating key amino acid residues of nitrilases (Nigam et al. 2017). Natural nitrilases can be synthesized by plants, animals, fungi and bacteria. Among the nitrilases from different sources, microbial nitrilases are often exploited for biochemical syntheses and environmental remediation (Pace and Brenner 2001). The most well-known branch 1 nitrilases are from the most abundant nitrilase source, namely, bacteria of the *Rhodococcus* genus. To better depict their characteristics and structural homology, the sequences of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2007) and the nitrilase-related enzymes from *Agrobacterium* sp. KNK712 (PDB ID 1ERZ) (Nakai et al. 2000) and *Pyrococcus horikoshii* (PDB ID 1J31) (Sakai et al. 2004) are aligned in Fig. 5.

The majority of microbial nitrilases have a subunit size of 30–45 kDa and homomultimers with native compositions such as α_n (with n ranging from 4 to 22) (Thuku et al. 2009). The amino acid sequences of different nitrilases vary greatly, but all members exhibit secondary and tertiary homology upon alignment of their crystal structures (Thuku et al. 2007, 2009; Park et al. 2017) (Fig. 5a). Take the best characterized nitrilase from *R. rhodochrous* J1 as an example. The nitrilase in J1 is known to be inactive as a dimer in solution but to be active as an oligomeric complex (with 10–12 subunits) when subjected to heat treatment or in the presence of nitrile, ammonium sulphate or organic solvents (Thuku et al. 2009; Park et al. 2017). The dimer model of *R. rhodochrous* J1 nitrilase shown in Fig. 5b is built based on structural homology to nitrilase-related enzymes whose crystal structures have been solved (Thuku et al. 2007). The monomer association occurs via two interfaces, namely, the 'A' and 'C' surfaces, and leads to an eight-layered αββα-αββα dimer (Fig. 5b). As seen in Fig. 5a, the length of the amino acid sequence that forms 'C' surface varies much more than that of the sequence that forms the 'A' or 'D' surface (Thuku et al. 2007). The C surface is responsible for spiral elongation. All nitrilase superfamily enzymes have a conserved Glu-Lys-Cys catalytic triad, whereas the active site of the nitrilase in J1 has an extra glutamic acid and comprises the residues C165, K131, E48 and E138 (shown in Fig. 5c). C165 is speculated to initiate a nucleophilic attack on the substrate to form a tetrahedral intermediate, and K131 stabilizes the tetrahedral intermediate (Thuku et al. 2009). The two Glu residues are speculated to play an important role in positioning the substrate. E48 increases the

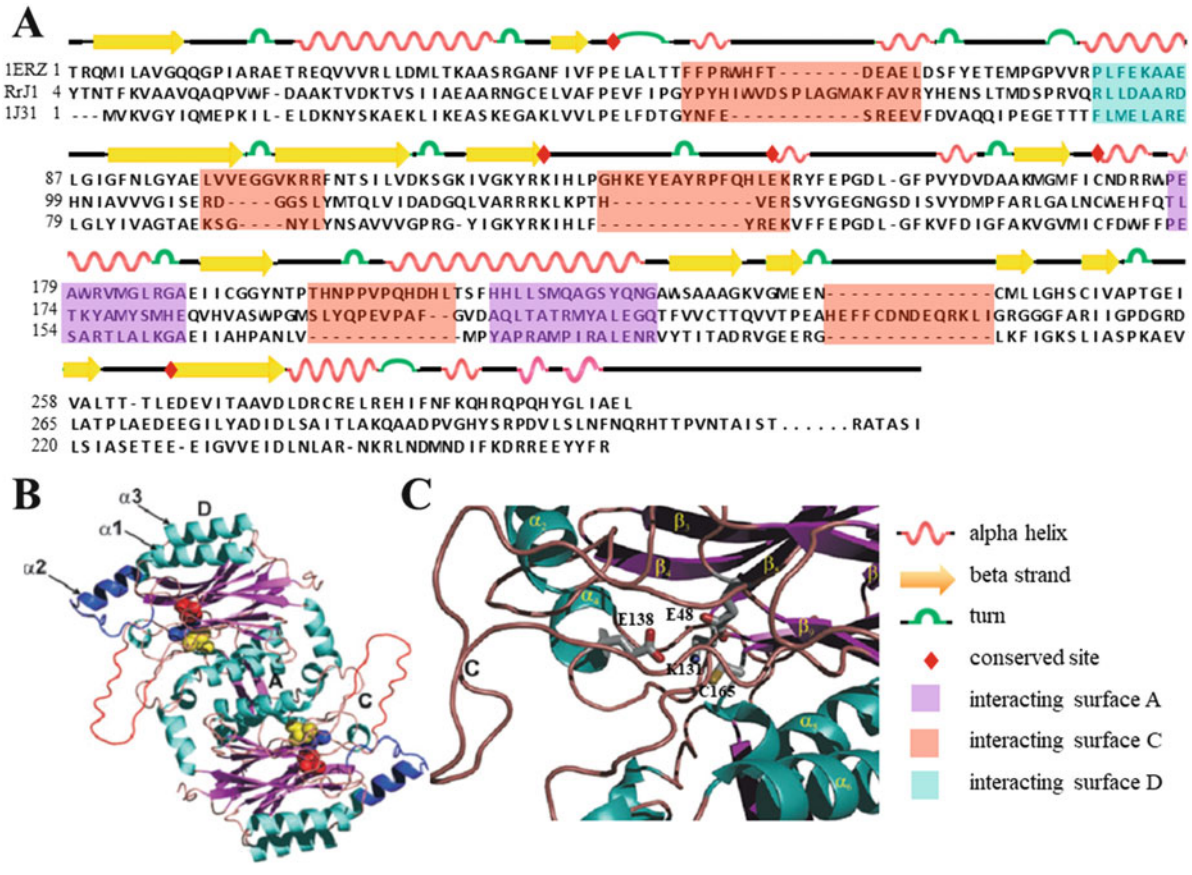

Fig. 5 Amino acid sequence and structural alignment of nitrilase. (**a**) Sequence alignment of the nitrilase from *Rhodococcus rhodochrous* J1 (RrJ1) with two nitrilase-related enzyme homologues [1ERZ (Nakai et al. 2000) and 1J31 (Sakai et al. 2004)]. The symbol in the top line indicates the position in the

nucleophilicity of the cysteine, and E138 is located in a 'C' surface loop, leading to the possibility that association of dimers moves this residue into the correct position for catalytic activity (Thuku et al. 2009).

3 Cluster Arrangement and Expression Regulation of Nitrile-Converting Genes

3.1 *Nitrile-Converting* Rhodococcus *Strains*

Nitrile-converting enzymes (NHases, nitrilases) exist in many *Rhodococcus* strains. Previous studies have reported that Fe-type and Co-type NHases were found in *R. erythropolis* and *R. rhodochrous* species and nitrilase-producing strains mainly belong to the *R. rhodochrous* species (Martínková et al. 2010). However, with the rapid development of next-generation sequencing technology, various *Rhodococcus* species have been revealed to be capable of producing NHase and nitrilase.

A genome-wide search of 283 *Rhodococcus* strains showed that all the *Rhodococcus* strains could produce amidase; 57% of the *Rhodococcus* strains harbour genes encoding NHases; and only 18% of the strains were identified as harbouring genes encoding nitrilases (Fig. 6a). Twenty-five of these strains exhibited NHase, amidase and nitrilase activities (Fig. 6b).

Except for a large number of strains not classified at the species level (designated *Rhodococcus* sp.), NHase could be produced by all the 36 strains of *R. equi*, 19 strains of *R. erythropolis* species, 8 strains of *R. opacus* and 7 strains of *R. qingshengii*. However, most of the NHases from these species have never been reported, except a number of NHases from *R. erythropolis* strains with high similarity and one from *R. equi* TG328-2, all of which bear the Fe^{3+} cofactor (Rzeznicka et al. 2010; Martínková et al. 2010). Co^{3+}-type NHases have been reported in *R. rhodochrous* J1 (Komeda et al. 1996b, c), *R. rhodochrous* M8 (Pogorelova et al. 1996; Pertsovich et al. 2005) and *R. ruber* TH (Ma et al. 2010). Specifically, in *R. rhodochrous* J1, two NHase subtypes were identified, i.e. high-molecular-mass NHase (H-NHase) and low-molecular-mass NHase (L-NHase), which exhibit different substrate specificities (Komeda et al. 1996b, c). In contrast, only H-NHase was identified in *R. ruber* TH, exhibiting 98% identity with the H-NHase from *R. rhodochrous* J1. A new type of NHase containing three types of metal ions (Co, Cu and Zn) was discovered and characterized in *Rhodococcus jostii* RHA1, which, however, shares no homology with the well-known Fe- and Co-type NHases (Okamoto and Eltis 2007).

Fig. 5 (continued) homologues. The approximate regions of the interacting surfaces A, C and D are indicated in purple, red and blue, respectively. The secondary structural elements identified in 1ERZ (Nakai et al. 2000) are indicated in the top line. (**b**) Stereo view of a dimer model of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2007). (**c**) Close-up view of the 'C' surface loop and the active site of the model of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2009)

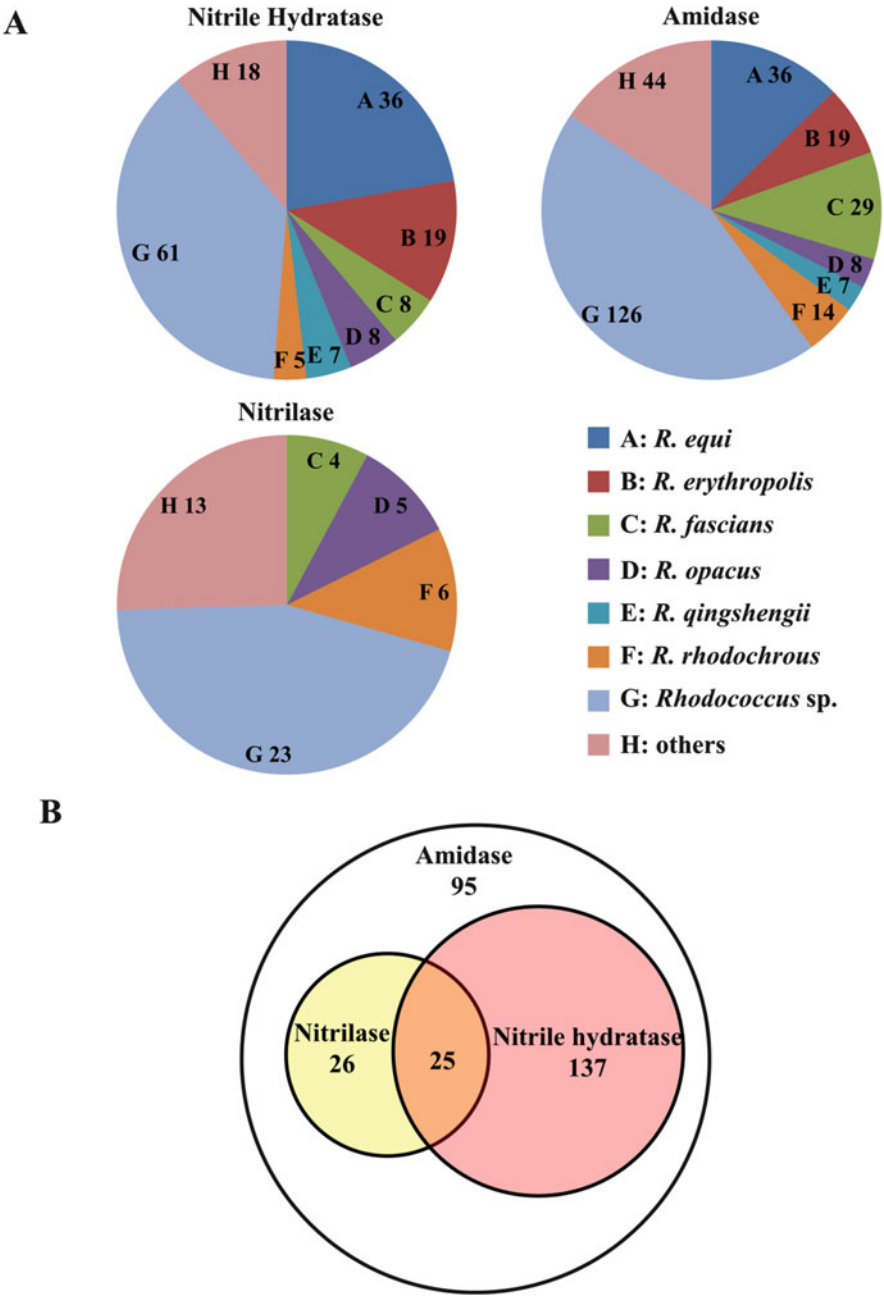

Fig. 6 *Rhodococcus* strains harbouring nitrile-converting enzymes. (**a**) NHase, amidase and nitrilase distribution in different *Rhodococcus* species. (**b**) Relationship of NHase-, amidase- and nitrilase-producing *Rhodococcus* strains

Unlike NHases, which can be produced by many *Rhodococcus* species, nitrilase-coding genes were detected in only a few species, mainly *R. fascians*, *R. opacus* and *R. rhodochrous*. In addition, many other nitrilase-containing strains have not been classified at the species level (*Rhodococcus* sp.). Nitrilases from different *Rhodococcus* strains possess individual preferences for aromatic or aliphatic nitriles. While most nitrilases prefer aromatic nitriles as substrates, the nitrilase from *R. rhodochrous* K22 exhibits significant activity towards aliphatic nitriles (Gong et al. 2012; Martínková et al. 2010).

3.2 NHase-Amidase Gene Organization and Regulation

Generally, genes that share a generalized function are often located in a gene cluster. In the metabolic pathway of nitrile degradation, nitrile is converted to amide by NHase and then transformed to carboxylic acid by amidase, so generally, the amidase gene is linked to the NHase gene, which has been detected in the majority of *Rhodococcus* strains regardless of whether the NHase is a Fe- or Co-type NHase. Despite this characteristic, Fe- and Co-type NHases represent different types of gene organization and regulation patterns.

3.2.1 Fe-Type NHase

The genes involved in the aldoxime-nitrile-amide-carboxylic acid pathway containing the Fe-type NHase are typically organized as shown in Fig. 7a, as seen in the *R. erythropolis* PR4 genome (GenBank: AP008957.1). NHase is expressed by *nha1* and *nha2*, coding for the α and β subunits, which are linked to the amidase gene (*ami*). The *nhr3* gene codes for an NHase activator, which activates the NHase via the oxidation of the iron centre and incorporation of an Fe ion into the protein (Nojiri et al. 2000). This activator gene is also essential for the function of NHase when expressing *nha1* and *nha2* genes in *E. coli*. The expression of NHase is probably regulated by *nhr4*, *nhr2* and *nhr1*, which encode regulators; however, to date, there has been no report regarding the exact functions of these genes. The gene coding for aldoxime dehydratase (*oxd*) was also found to be close to the *nha2*, *nha1* and *ami* genes in *R. erythropolis* PR4. Similar gene structures and organizations were also observed in *R. erythropolis* A4 (GenBank: AM946017.1), *R. globerulus* A-4 (GenBank: AB105912.1), *Rhodococcus* sp. N-771 (GenBank: AB016078.1) (Kato et al. 2004; Endo et al. 2001) and *Rhodococcus* sp. N-774 (Martínková et al. 2010). The NHase-amidase gene cluster was also identified in the *R. erythropolis* strains AJ270 and AJ300, whereas a copy of the insertion sequence IS1166 was present within the *nhr2* gene (O'mahony et al. 2005). In *R. jostii* RHA1, the genes coding for amidase and NHase share 79% and 90% homology, respectively, with those in *R. erythropolis* PR4; however, the regulatory genes *nhr1* and *nhr2* are absent, while the genes *oxd* and *nhr4* were detected (GenBank: CP000431.1).

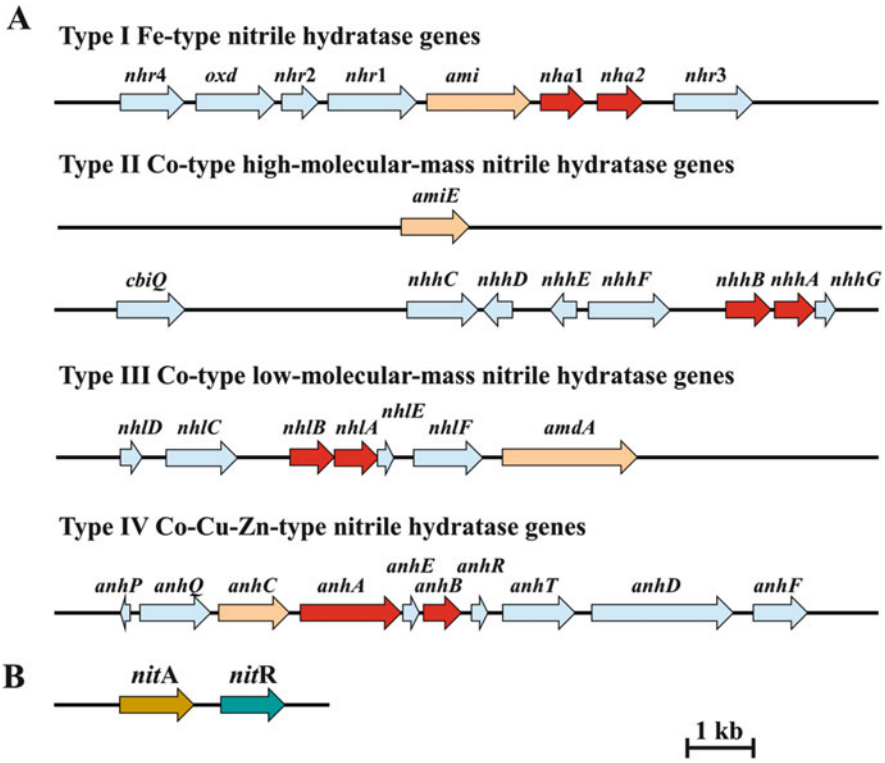

Fig. 7 Organization of nitrile-converting gene clusters from various *Rhodococcus* strains. (**a**) Four types of NHase-amidase gene cluster organizations. (**b**) Nitrilase gene cluster organization

In most cases, the NHase and amidase genes are inducible by amides (reaction products) instead of nitriles (reaction substrates) (Kobayashi and Shimizu 1998), which has been observed mostly with acetamide (Rucká et al. 2014; O'mahony et al. 2005). However, constitutive expression patterns occur in some other strains, such as *Rhodococcus* sp. N-771 and *Rhodococcus* sp. R312 (Prasad and Bhalla 2010).

3.2.2 Co-Type NHase

In contrast to the Fe-type NHase, two types of NHases, i.e. H-NHase and L-NHase, exist in *Rhodococcus* species and were found to coexist in *R. rhodochrous* J1.

The typical gene cluster of H-NHase contains *nhhC*, *nhhD*, *nhhE*, *nhhF*, *nhhB*, *nhhA* and *nhhG*, as determined in *R. rhodochrous* J1 (Fig. 7a) (GenBank: D67027.1) (Komeda et al. 1996b). *nhhB* and *nhhA* code for the subunits of NHase and are positively regulated by the essential *nhhC* and *nhhD* genes. The *nhhG* gene is homologous to *nhhB*, although the exact function of this gene has not been determined. *nhhF* encodes an insertion sequence IS1164, suggesting that horizontal gene

transfer or genetic rearrangement of the H-NHase gene cluster occurred over the course of evolution. *nhhE* encodes a 14.6-kDa protein that does not share significant similarity with any reported protein and is not indispensable for the expression of the H-NHase gene. Genes homologous to *nhhC*, *nhhD*, *nhhB*, *nhhA* and *nhhG* are present in *Rhodococcus* sp. M8 (NCBI Reference Sequence: NZ_MLYX02000005.1), whereas *nhhE* and *nhhF* are absent. Despite the absence of *nhhF*, a gene coding for another IS481 family transposase has been identified immediately upstream of *nhhB*. In addition, the whole-genome sequence of *Rhodococcus* sp. M8 reveals a gene encoding amidase that is located 15.8 kb upstream of the H-NHase gene, which is consistent with the observation that amidase is always found together with NHase (Fig. 7a). As a result, whether amidase is present in the gene cluster of *R. rhodochrous* J1 H-NHase should be re-evaluated by cloning a sequence longer than the current 6555 bp. A gene encoding the cobalt ECF transporter T component (CbiQ) has also been identified upstream of *nhhBA* in *Rhodococcus* sp. M8 and may be involved in cobalt ion transport (Fig. 7a). Homologous H-NHase genes were also found in *Rhodococcus pyridinivorans* (Kohyama et al. 2006).

The subunits of L-NHase are encoded by *nhlB* and *nhlA*, which are clustered with *nhlD*, *nhlC*, *nhlE*, *nhlF* and *amdA* (amidase). *nhlC* and *nhlD* are located upstream of the *nhlBA* genes and code for negative and positive regulators, respectively. *nhlC* may function as an activator to inhibit the repression effect of *nhlD* in the presence of the inducer amide. *nhlE* codes for a protein that is homologous with the β subunit of L-NHase. *nhlF* encodes a markedly hydrophobic protein with eight hydrophobic putative membrane-spanning domains and is considered to be involved in cobalt uptake.

Expression of H-NHase and L-NHase also exhibits different preferences for various types of amide inducers. While H-NHase and L-NHase can both be induced by acetamide, propionamide, acrylamide and methacrylamide, H-NHase can be selectively induced by urea, and L-NHase can be selectively induced by cyclohexanecarboxamide (Komeda et al. 1996b).

3.2.3 Co-Cu-Zn-Type NHase

Other than the classical Fe- and Co-type NHases, a novel NHase that requires Co, Cu and Zn ions for its activity was discovered in the *R. jostii* RHA1 plasmid (Okamoto and Eltis 2007). Although the subunits of this NHase are encoded by *anhA* and *anhB*, similar to the Fe- and Co-type NHases, a gene, named *anhE*, with unknown function is present between *anhA* and *anhB*, and this gene has never been reported in any other *Rhodococcus* strains. The *anhC* gene encoding amidase is located upstream of *anhAB*. Three regulatory genes, namely, *anhP*, *anhQ* and *anhR*, and a possible cobalt transporter gene, namely, *anhT*, are clustered with the *anhAB* and *anhC* genes. A similar gene cluster, containing *anhP*, *anhQ*, *anhC*, *anhA*, *anhE*, *anhB*, *anhR* and *anhT*, has been identified in *R. opacus* 1CP (GenBank: CP009111.1). However, *anhD* is not present in this cluster, and *anhF* is missing in the genome. The expression of NHase and amidase is induced by acetamide.

Comparing the gene clusters of the three types of NHases, NHase genes are always associated with the amidase gene, although the gene arrangement differs; some amidase genes are adjacent to the NHase, while others may be 15.8 kb away from the NHase gene. Expression of NHase and amidase is always regulated by some genes present in the cluster.

3.3 Nitrilase Gene Organization and Regulation

Compared with the complex NHase-amidase gene cluster, the nitrilase gene cluster has been reported in only *R. rhodochrous* J1, which contains a *nitA* gene, encoding nitrilase, and a *nitR* gene, encoding a positive regulator of *nitA* expression (Fig. 7b) (Komeda et al. 1996a). These two genes are co-transcribed as a single mRNA and induced by isovaleronitrile. The nitrilase gene from *R. rhodochrous* K22 has also been found to be inducible by isovaleronitrile, but isobutyronitrile and fumaronitrile are the most favourable inducers for nitrilases from *R. rhodochrous* PA-34 and *R. rhodochrous* NCIMB11216 (Bhalla et al. 1992; Hoyle et al. 1998).

4 Biodegradation of Nitrile Pollutants

Nitriles are important compounds that are widely manufactured and used in the chemical industry and in agriculture. For example, acetonitrile is a commonly used solvent, and acrylonitrile is a precursor of acrylic fibres and plastics. Nitrile herbicides, such as dichlobenil, ioxynil and bromoxynil, are widely used for rice, corn, wheat and berry crops (Kobayashi and Shimizu 2000). These compounds, however, have been extensively released into our environments through industrial waste water or agricultural chemicals. Most nitriles are highly toxic and harmful to humans and the environment, resulting in an urgent need for remediation of nitrile-contaminated soil and water.

Chemical hydrolysis of nitriles requires harsh conditions such as extreme pH and elevated temperatures and leads to the creation of large amounts of by-products and secondary pollutants (Mukram et al. 2016). Bioremediation is a cost-effective technology to restore polluted environments by degrading these compounds into harmless intermediates by using microorganisms. A number of microorganisms, such as *Rhodococcus*, *Nocardia*, *Bacillus*, *Pseudomonas* and *Arthrobacter*, have been reported for the degradation of nitriles (Gong et al. 2012). Among these microorganisms, *Rhodococcus* species harbouring nitrilase or NHase are the most frequently used species. Nitrilase catalyses the hydrolysis of nitriles into their corresponding acids (ammonium salts), while NHase catalyses the hydration of nitriles to the corresponding amides. Mostly, NHase coexists in *Rhodococcus* with amidase, which converts amides into acids (ammonium salts) (Martínková et al. 2010). Nitrile pollutants include aliphatic nitriles and benzonitrile herbicides. The

former, including acrylonitrile, acetonitrile, butyronitrile and adiponitrile, are primarily released by the chemical industry, while the latter are found in the residues of agricultural chemicals. The strains capable of degrading these compounds are summarized in Table 1.

Aliphatic nitrile pollutants, such as acrylonitrile and acetonitrile, can be degraded by *Rhodococcus* effectively via the NHase/amidase pathway or nitrilase pathway (Martínková et al. 2010). Strains harbouring NHases used for the industrial

Table 1 *Rhodococcus* species involved in the biodegradation of nitrile pollutants

Substrate	Organism	Enzyme involved	Product detected	References
Acrylonitrile vapour	*R. ruber* NCIMB 40757	Nitrilase	Ammonium acrylate	Roach et al. (2004)
Acrylonitrile	*R. erythropolis* AJ270	NHase	Amide	Baxter et al. (2006)
Acetonitrile	*R. erythropolis* BL1	NHase/ amidase	Acid; ammonium	Langdahl et al. (1996)
Acetonitrile	*R. pyridinivorans* S85-2; *B. diminuta* AM10-C-1	NHase/ amidase	Acid and amide	Kohyama et al. (2006)
Acetonitrile	*R. pyridinivorans* S85-2; *Rhodococcus* sp. S13-4	NHase/ amidase	Acid and amide	Kohyama et al. (2007)
Propionitrile, butyronitrile, valeronitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Acid and amide	Mukram et al. (2015)
Acetonitrile; acrylonitrile; crotononitrile	*R. rhodochrous* BX2	NHase/amidase (dominant); nitrilase	Ammonium	Fang et al. (2015) and An et al. (2018)
Butyronitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Acid and amide	Mukram et al. (2016)
Bromoxynil; ioxynil	*R. rhodochrous* NCIMB 11215	Nitrilase	Acid	Harper (1985)
Dichlobenil	*R. erythropolis* 9675; *R. erythropolis* 9685	NHase	Amide	Holtze et al. (2006)
Dichlobenil	*R. erythropolis* AJ270	NHase	Amide	Meth-Cohn and Wang (1997)
Bromoxynil; chloroxynil; ioxynil	*R. rhodochrous* PA-34; *Rhodococcus* sp. NDB 1165	Nitrilase	Acid	Veselá et al. (2010)
Chloroxynil; bromoxynil; ioxynil; dichlobenil	*R. erythropolis* A4; *R. rhodochrous* PA-34	NHase/ amidase	Amide; acid	Veselá et al. (2012)
Benzonitrile; indole-3-acetonitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Amide; acid	Mukram et al. (2015)

bioproduction of acrylamide can be used for the degradation of acrylonitrile in contaminated environments and waste waters (see Table 1). However, the corresponding amides can be highly toxic, for example, acrylamide is a neurotoxic compound with an LD_{50} (p.o.) of 107–203 mg/kg in rats (Martínková et al. 2010). As a result, amidases are required for further degradation of amides into the corresponding acids (ammonium salts). For the most part, the catalytic efficiencies of NHase and amidase in the same microorganism do not match, necessitating the combination of two different microorganisms for increased degradation efficiency. For example, *R. pyridinivorans* S85-2, with high NHase activity, and *Brevundimonas diminuta* AM10-C-1, with high amidase activity, were combined together for the biodegradation of acetonitrile. With the cascade reaction, 6 M acetonitrile could be converted to acetic acid with a conversion rate of over 90% in 10 h (Kohyama et al. 2006). In addition to the NHase/amidase pathway, nitrilases, which catalyse nitrile into acid (ammonium salt) in one step, also have great potential for the biodegradation of nitrile pollutants. Using *R. ruber* NCIMB 40757 immobilized in synthetic silicone polymer rings, Roach et al. achieved an acrylonitrile elimination capacity of over 7.2 kg/m^3/h with a removal efficiency of 90%. The nongrowing biocatalysts could work as long as 70 days, which showed the excellent tolerance of *Rhodococcus* against the toxic nitrile (Roach et al. 2004).

Benzonitrile herbicides include 3,5-diiodo-4-hydroxybenzonitrile (ioxynil), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) and 2,6-dichlorobenzonitrile (dichlobenil) and their ammonium salts and ester analogues (Holtze et al. 2008; Martínková et al. 2010). The utilization of benzonitrile herbicides in agriculture has caused long-lasting pollution in groundwater. Chloroxynil, bromoxynil and ioxynil can be degraded into the corresponding amide or acid by several strains (Table 1). However, the biodegradation of dichlobenil to acid is much more difficult, and 2,6-dichlorobenzamide was considered to be the dead-end product. Though the use of dichlobenil was banned in Denmark in 1997, 2,6-dichlorobenzamide was still the most frequently detected contaminant in the groundwater in 2006 (Holtze et al. 2008). Recently, *R. erythropolis* A4 was reported to degrade 2,6-dichlorobenzamide to 2,6-dichlorobenzoic acid (Veselá et al. 2012). Using *R. erythropolis* A4 resting cells, 0.5 mM dichlobenil was degraded to amide with a conversion rate of 40% and to acid with a conversion rate of 41% in 3 days.

The biodegradation of nitrile pollutants focuses on the screening and discovery of strains, but a few studies have also focused on protein and strain engineering. The activity, substrate specificity and stability of functional enzymes involved in biodegradation can be enhanced by directed evolution and rational design. *Rhodococcus* strains with high tolerance against toxic nitriles and amides can be engineered as efficient and robust hosts for overexpression of NHase/amidase or nitrilase. It is expected that the combination of protein engineering and strain engineering will help improve the performance of *Rhodococcus* in the biodegradation of nitrile pollutants.

5 Bioconversion of Nitriles for Industrial Applications

Bioconversion of nitriles to the corresponding amides or carboxylic acids catalysed by NHase, amidase or nitrilase has been successfully applied in industrial scale. Among these, the most successful case is the application of NHase for production of amides, such as acrylamide and nicotinamide. Under the harsh industrial conditions, however, the natural state biocatalysts in terms of no matter whole cells or enzymes are not stable enough to maintain high productivity and multiple reuses. Hence, diverse cell engineering and enzyme engineering strategies have been applied to enhance the stability/activity/specificity performances of biocatalysts. We can expect that the industrial applications of *Rhodococcus* in bioconversion of nitriles will be further improved and accelerated by various new biotechnologies such as synthetic biology, enzyme rational design and genome editing tools.

5.1 Amide Production Using NHase

Amides are important chemicals due to their wide applications in enhanced oil recovery, nutritional supplements, medical treatment and so on. In particular, acrylamide and nicotinamide are manufactured at the kiloton scale. Owing to the advantages of this process, such as high productivity, high product purity and environmental friendliness, the biotransformation of nitriles to the corresponding amides with NHase is considered to be superior to the conventional chemical process. Currently, NHase is used to synthesize various amides, including acrylamide, nicotinamide, picolinamide, benzamide, indole-3-acetamide and 3-indolacetamide (Prasad and Bhalla 2010). Of these processes, NHase-mediated catalysis of acrylonitrile to acrylamide is the most successful and was the first case in which biotechnology was applied in the petrochemical industry (Kobayashi and Shimizu 1998). Using bioproduction of acrylamide as a typical example, the synthesis of amides with NHase will be discussed here.

Because of the considerable merits of the process, such as simplicity and ease of manipulation, biocatalysis using free resting cells harbouring NHase as the biocatalyst is the main method for the production of acrylamide to date. Many *Rhodococcus* strains have been industrialized to produce acrylamide, including *Rhodococcus* sp. N-774, *R. rhodochrous* J1 and *R. ruber* TH (Yamada and Kobayashi 1996). At the laboratory scale, the highest acrylamide content was obtained by fed-batch biotransformation of acrylonitrile at 10 °C for 10 h with wild-type *R. rhodochrous* J1, which could produce as high as 650 g/L acrylamide (Nagasawa et al. 1993).

However, many problems have emerged in the application of NHase. First, NHases are thermolabile, and their optimal operation temperature ranges from 20 to 35 °C. In contrast, the strong exothermal heat generated upon the conversion of acrylonitrile to acrylamide inhibits NHase activity. Second, NHase is susceptible

to attack by polar organic solvents, i.e. the product acrylamide and substrate acrylonitrile, which hampers the accumulation of high concentrations of acrylamide. The NHase inactivation is enhanced with increasing reaction temperature and acrylamide concentration. Third, cell flocculation and sedimentation are common phenomena in most *Rhodococcus* species, resulting in very low cell density and in the failure of large-scale fermentation for preparation of *Rhodococcus* catalysts (Jiao et al. 2017). In addition, as mentioned above, in almost all *Rhodococcus* strains, inherent amidases are co-expressed with NHase, thus hydrolysing the amide product into carboxylic acid and ammonia, which not only leads to wastage of amides but also increases the cost of the subsequent purification process (Prasad and Bhalla 2010).

To meet the urgent demand for the production of acrylamide at high concentrations, various strategies have been proposed to evolve engineered strains at both the cell level and the enzyme level (Fig. 8). To solve the problem of by-product production, NHase may be heterologously expressed in a host lacking amidase activity; however, this solution is not ideal as amidases exist in all the known superior *Rhodococcus* strains (Prasad and Bhalla 2010). Therefore, amidase activity is generally eliminated via a gene knockout in the parent organism (Ma et al. 2010). In light of the hydrophobic properties of various *Rhodococcus* cells, cell flocculation and sedimentation are associated with the smooth-rough colony dimorphism phenomenon, and the smooth-type cells are preferred for industrial application to prevent cell flocculation in large-scale fermentation (Jiao et al. 2017).

Additional efforts have focused on how to enhance the thermal stability and acrylamide tolerance of in vivo NHases. Chaperones are a large family of proteins that have indispensable functions, including aiding the folding of newly synthesized polypeptide chains, oligomeric assembly, responding to the stress denaturation of proteins, assisting proteolytic degradation and transcription factor activation. Therefore, the *E.coli* chaperones GroEL-GroES were introduced to *R. ruber* TH3 to aid NHase folding and stabilize the NHase. Recently, it was discovered that the stress tolerance and cell integrity of *R. ruber* TH3 could be improved by overexpressing the small heat shock protein Hsp16 of *Rhodococcus*, thereby increasing the viability of *R. ruber* cells under high concentrations of acrylamide in the solution. Overexpression of the *Rhodococcus* chaperone GroEL2 was also reported to enhance the organic solvent tolerance of *Rhodococcus* (Takihara et al. 2014). In contrast to rational design with chaperones, a transcriptional regulation strategy by sigma factor random mutation and directed evolution selection was applied to increase the bioconversion productivity of acrylamide (Ma and Yu 2012).

The catalytic performance of the resting cells is ascribed to the activity and stability of the enzyme itself. Compared with the laborious random mutation method, rational design is suitable for enzymes whose crystal structures have been revealed, such as NHase. In addition, the identification of the thermosensitive regions and introduction of stable interactions have been highlighted. By RMSF calculations for thermophilic and mesophilic NHases, three deformation-prone thermosensitive regions were identified and stabilized by the introduction of salt bridges (Chen et al. 2013), and the stabilized C-terminus of the β subunit was the most powerful. In light of the instability of the C-terminus, a salt bridge-based design

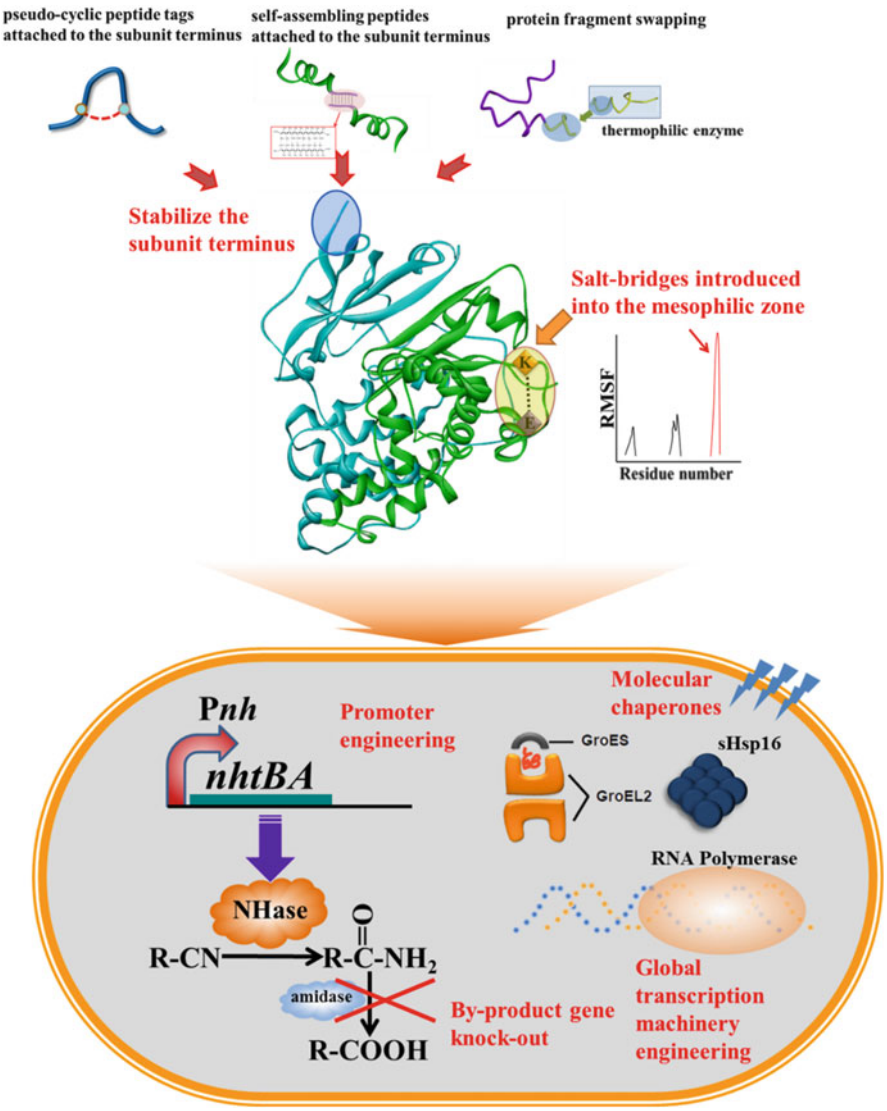

Fig. 8 Strategies used to evolve the engineered NHase-producing *Rhodococcus* strains at both the cell level and the enzyme level

of pseudocyclic peptide tags attached to the subunit terminus was recently applied to the NHase from *R. ruber* TH3, improving the NHase stability significantly without compromising activity. Using STAR (site-targeted amino recombination) software and molecular dynamics to determine the crossover sites for fragment recombination, a homologous protein fragment swapping strategy, involving the swapping of

the mesophilic NHase fragments for the thermophilic NHase fragments, was proposed by Cui et al. (Cui et al. 2014). Similarly, Sun et al. constructed a chimeric NHase by swapping the corresponding C-domains of the β subunit from thermosensitive BpNHase and thermostable PtNHase (Sun et al. 2016b). Regardless of enzyme structure, Liu et al. attached the terminus of the NHase with two self-assembling peptides and obtained enzymes with enhanced stability (Liu et al. 2014). In total, all these efforts involve rigidifying flexible sites to enhance the stability of NHase (Yu and Huang 2014).

Finally, a synergistic evolution strategy for *Rhodococcus* cells and in-cell NHases promises to accomplish the recycling of recombinant *Rhodococcus* cells for production of high concentration acrylamide. Recently, some molecular toolkits for gene expression in *Rhodococcus* strains were developed (Jiao et al. 2018; DeLorenzo et al. 2018), and these may be potentially applied to NHase-producing strains as further modifications to improve catalytic performance.

The successful application of free cells as catalysts notwithstanding, immobilized cells and enzymes confer several benefits and are being used, as previously reviewed in detail (Velankar et al. 2010).

5.2 Carboxylic Acid Production Using Amidase

In addition to its widespread application in amide production, *Rhodococcus* has also been utilized for the synthesis of important carboxylic acids via the NHase/amidase pathway (Ismailsab et al. 2017; Ramteke et al. 2013; Maksimova et al. 2017). For example, whole cells of *R. equi* A4, with NHase and amidase activities, were utilized in the biotransformation of benzonitrile, 3-cyanopyridine, (*R*,*S*)-3-hydroxy-2-methylenebutanenitrile and (*R*,*S*)-3-hydroxy-2-methylene-3-phenylpropanenitrile to the corresponding acids (Kubáč et al. 2006). *R. erythropolis* ZJB-09149, harbouring NHase and amidase, was used to transform 2-chloro-3-cyanopyridine to 2-chloronicotinic acid (Jin et al. 2011). *Rhodococcus* sp. G20 was used for the transformation of β-aminopropionitrile to β-alanine (Liang et al. 2008). The enantioselectivity of NHase and amidase is also of great interest to organic chemists. For example, *R. erythropolis* NCIMB 11540 was found to have a highly active NHase/amidase enzyme system, which can be used to transform α-hydroxynitriles (cyanohydrins) to enantiopure α-hydroxy carboxylic acids. (*R*)-2-Chloromandelic acid and (*R*)-2-hydroxy-4-phenylbutyric acid were prepared at the gram scale with high optical (e.e. >99 and 98%, respectively) and chemical (98%) yields (Osprian et al. 2003). *R. erythropolis* AJ270, harbouring amidase, has been applied in the enantioselective desymmetrization of functionalized prochiral malonamides to afford carbamoylacetic acids with high yield and excellent enantioselectivity (Zhang et al. 2011). Biotransformation of nitrile with *R. erythropolis* AJ270 has been utilized for the enantioselective syntheses of diverse polyfunctionalized organic compounds that are not readily achievable by other methods (Wang 2005).

5.3 Carboxylic Acid Production Using Nitrilase

Recently, a variety of nitrilases from different microorganisms have been discovered to have applications in the fields of biosensing (Roach et al. 2003), bioremediation (Fang et al. 2015; Li et al. 2013) and biocatalysis. In particular, the high substrate specificity, enantioselectivity and regioselectivity of nitrilases make these enzymes attractive biocatalysts for the production of various commodity chemicals and pharmaceutical intermediates at the laboratory scale, such as nicotinic acid, isonicotinic acid, mandelic acid, acrylic acid, glycolic acid and benzoic acid (Bhalla et al. 2018). The biocatalytic reactions that use nitrilases and can be performed in aqueous solutions at moderate temperatures and pH are more efficient, economical and eco-friendly than traditional chemical synthesis methods. To date, *Rhodococcus* species harbouring nitrilase, which is considered to be a potential biocatalyst to hydrolyse nitriles for industrial applications, has been widely investigated (Table 2).

5.3.1 Important Aromatic and Aliphatic Carboxylic Acids

Aromatic acids, including nicotinic acid, isonicotinic acid, benzoic acid and hydroxybenzoic acid, have been synthesized using nitrilase as a biocatalyst. Among these syntheses, the biosynthesis of nicotinic acid (Lonza, China) has been successfully applied at the industrial scale (Gong et al. 2012). Free cells of *R. rhodochrous* J1 and *Rhodococcus* sp. NDB1165 were reported as biocatalysts for the conversion of 3-cyanopyridine to nicotinic acid with productivity values of 172 g/L and 196.8 g/L, respectively (Mathew et al. 1988; Prasad et al. 2007).

Among the aliphatic and aryl aliphatic acids, acrylic acid, glycolic acid, 3-hydroxyvaleric acid and mandelic acid have been widely investigated for their nitrilase-based biotransformation processes. Acrylic acid and its derivatives are commonly applied in surface coatings, textiles, adhesives, paper treatment, polymeric flocculants, dispersants and so on (Xiaobo et al. 2006). Currently, most commercial acrylic acid is produced by partial oxidation of propylene, which leads to energy wastage and the formation of by-products. To date, many subspecies of *Rhodococcus* have been isolated from the biosphere with high substrate specificity for acrylonitrile, including *R. rhodochrous* J1 (Nagasawa et al. 1990) and *R. ruber* AKSH-84 (Kamal et al. 2011). Moreover, *R. rhodochrous* tg1-A6, which was obtained after treatment with UV light combined with lithium chlorinate, was used to synthesize acrylic acid. The results exhibited that the highest concentration of acrylic acid obtained was 414.5 g/L during a 10-h continuous reaction (Luo et al. 2006).

However, most of the novel wild-type nitrilases have been found to not be suitable as potential biocatalysts for industrial applications due to their relatively low activity and stability. Immobilization has been considered to be an effective way to enhance the stability and reusability of biocatalysts. Yucai He et al. immobilized the cells of *Rhodococcus* sp. CCZU10-1 using calcium alginate, achieving efficient biocatalyst recycling with a product-to-biocatalyst ratio of 776 g terephthalic acid/g

Table 2 *Rhodococcus* harbouring nitrilase used for conversion of nitriles to the corresponding acids

Product	Substrate	Organism	Catalyst	Conversion (%); volumetric productivity (g/L/days); e.e. (%)	References
Nicotinic acid	3-Cyanopyridine	*R. rhodochrous* J1	Whole cells	100; 159; n.a.	Mathew et al. (1988)
Nicotinic acid	3-Cyanopyridine	*Rhodococcus* sp. NDB1165	Whole cells	94; 430; n.a.	Prasad et al. (2007)
Acrylic acid/ methacrylic acid	Acrylonitrile/ methacrylonitrile	*R. rhodochrous* J1	Whole cells	98.5; 392; n.a. 100; 260; n.a.	Nagasawa et al. (1990)
Acrylic acid	Acrylonitrile	*R. ruber* AKSH-84	Whole cells	63; 109; n.a.	Kamal et al. (2011)
Acrylic acid	Acrylonitrile	*R. rhodochrous* tg1-A6	Whole cells	n.a.; 994.8; n.a.	(Luo et al. 2006)
Acrylic acid	Acrylonitrile	Recombinant *R. ruber TH3dAdN*(*nit*)	Whole cells	n.a.; 2069.4; n.a.	Sun et al. (2016a)
Glycolic acid	Glycolonitrile	*R. rhodochrous* tg1-A6	Whole cells	n.a.; 14; n.a.	Luo et al. (2016)
Benzoylformic acid	Benzoyl cyanide	*Rhodococcus* sp. CCZU10-1	Immobilized cells	>91; 28.32; n.a.	He et al. (2012)
Terephthalic acid/ isophthalic acid	Tetrachloroterephthalonitrile/ isophthalonitrile	*Rhodococcus* sp. CCZU10-1	Immobilized cells	93.5; 64.8; n.a. 92.7; 62.4; n.a.	He et al. (2014)
(*R*)-Ethyl-3-hydroxyyglutarate	Ethyl 4-cyano-3-hydroxybutyate	*R. erythropolis* ZJB-0910	Whole cells	46.2; n.a.; 99	Dong et al. (2010)
(*S*)-2-Cyano-2-methylpentanoic acid	2-Methyl-2-propylmalononitrile	*R. rhodochrous* J1	Whole cells	97; 80; 96	Yoshida et al. (2013)

DCW and 630 g isophthalic acid/g DCW (He et al. 2014). In addition to immobilization, the use of efficient heterologous expression systems and desired variants generated by directed evolution or site-directed mutagenesis are also predominant strategies. Recently, the recombinant strain *R. ruber* TH3dAdN(Nit), overexpressing the nitrilase from *R. rhodochrous* tg1-A6, showed the highest activity to date (187.0 U/mL) and converted the acrylonitrile to acrylic acid with a volumetric productivity of 344.9 g/L/h (Sun et al. 2016a).

5.3.2 Synthesis of Enantiopure Carboxylic Acids

Nitrilases are attractive biocatalysts for the synthesis of chiral intermediates of various pharmaceuticals due to their inherent superiority in terms of enantioselectivity and regioselectivity. (*R*)-(−)-Mandelic acid (Mitsubishi Rayon, Japan; BASF, Germany) has been successfully produced at the industrial scale using nitrilase as a biocatalyst (Schmid et al. 2001). (*R*)-(−)-Mandelic acid is widely used as an optical resolving agent and as an intermediate in the preparation of various pharmaceuticals, such as semisynthetic penicillin, cephalosporin, antiobesity drugs and antitumour agents (Bhalla et al. 2018). Various bacteria have been reported to produce (*R*)-(−)-mandelic acid, such as *Pseudomonas putida* MTCC 5110, *Alcaligenes faecalis* ECU0401 and *Alcaligenes* sp. MTCC 10675.

Many research studies have focused on exploiting the biotransformation process of valuable compounds whose chemical synthesis is difficult. The nitrilase of *R. rhodochrous* J1, when expressed in *Escherichia coli*, enantioselectively hydrolysed 2-methyl-2-propylmalononitrile to form (*S*)-2-cyano-2-methylpentanoic acid (CMPA) with 96% e.e. in fed-batch mode (Yoshida et al. 2013). Optically pure (*R*)-ethyl-3-hydroxyyglutarate is a key precursor for the synthesis of the chiral side chain of rosuvastatin. Whole cells of *R. erythropolis* ZJB-0910 were used to produce (*R*)-ethyl-3-hydroxyyglutarate with a yield of 46.2% (e.e. >99%) (Dong et al. 2010).

In fact, the paucity of available enzymes and the relatively low enzyme activity and stability remain the main limitations of nitrilase-catalysed biotransformation for industrial applications. Virtually, all nitriles are water-insoluble and destructive to cells. In most studies, the reaction is often carried out in fed-batch mode. Nevertheless, this type of operating mode always has several challenges, which arise due to substrate inhibition, production inhibition and a severe scale-up effect in industrial applications. Therefore, the exploration of effective biocatalysts and design of novel bioreactors and downstream processes are two main areas to consider to exploit the full industrial potential of nitrilases.

6 Conclusions

Aerobic and nonsporulating *Rhodococcus* is a genus of Gram-positive bacteria with a relatively fast growth rate (http://en.wikipedia.org/wiki/Rhodococcus). *Rhodococcus* species are of great environmental, chemical and pharmaceutical importance owing to their powerful ability to degrade diverse pollutants in the environment and to produce valuable chemicals such as acrylamide. The level of nitriles in the environment is increasing due to their widespread use in diverse fields such as organic synthesis. Biodegradation of nitriles by *Rhodococcus* has thus become a focus of research over the years. The three major nitrile-degrading enzymes in *Rhodococcus*, i.e. NHase, amidase and nitrilase, with various reaction optima and substrate specificities, have been well described in terms of not only the organization and regulation of gene clusters but also the modification and evolution of enzyme structures and functions.

In comparison with other nitrile degraders, *Rhodococcus* species possess a number of advantages. For example, the species have the advantages of the varied fatty acid composition of their membrane lipids, the existence of several molecular chaperones, the presence of mycolic acids in their cell envelope and their sufficient extracellular polysaccharides (EPS), which lead to an improved resistance to toxic compounds, simple uptake of hydrophobic nitriles and large-scale high-cell-density fermentation without cell flocculation, respectively. Therefore, applications of *Rhodococcus* in pollutant biodegradation and biocatalytic processes are very promising. In particular, the use of nitrile-converting enzymes to produce some valuable chemicals and pharmaceuticals such as acrylamide has been commercialized, and further applications of nitrile biotransformation also have the potential to be scaled up.

The role of *Rhodococcus* in biocatalysis has been increasingly highlighted. These bacteria have been increasingly developed as advantageous biocatalysts, such as for the bioproduction of high-value chiral chemicals. The use of *Rhodococcus* in synthetic biology and metabolic engineering to obtain various interesting compounds and enzymes is also highly desirable. Therefore, new methods and tools for the genetic engineering of *Rhodococcus* are of great interest in the future.

Acknowledgements Support via the projects NSFC-21776157/21476126 (the National Natural Science Foundation of China) and 973-2013CB733600 (the National Key Basic Research Project 973) is gratefully acknowledged.

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The Desulfurization Pathway in *Rhodococcus*

Shanshan Li and 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 upgrading the fossil fuels on sulfur content limitation with their gentle desulfurization and high desulfurizing competence, without lowering the calorific value of the fuel. Recent advances have demonstrated the desulfurization pathway called "4S" pathway, including four enzymes, and the molecular mechanism for biodesulfurization has also been described. In addition, genetic

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_8

manipulations, such as co-expression of flavin reductases, promoter modification, increasing the expression of key enzymes, expressing of desulfurization enzymes in heterologous hosts, and rearranging the *dsz* gene cluster were also used to improve sulfur removal efficiencies. In this chapter, we summarize the mechanism of biodesulfurization in *Rhodococcus*.

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 gases 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. The concentrations of BT and DBT in fossil fuels are prominently decreased by hydrodesulfurization (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

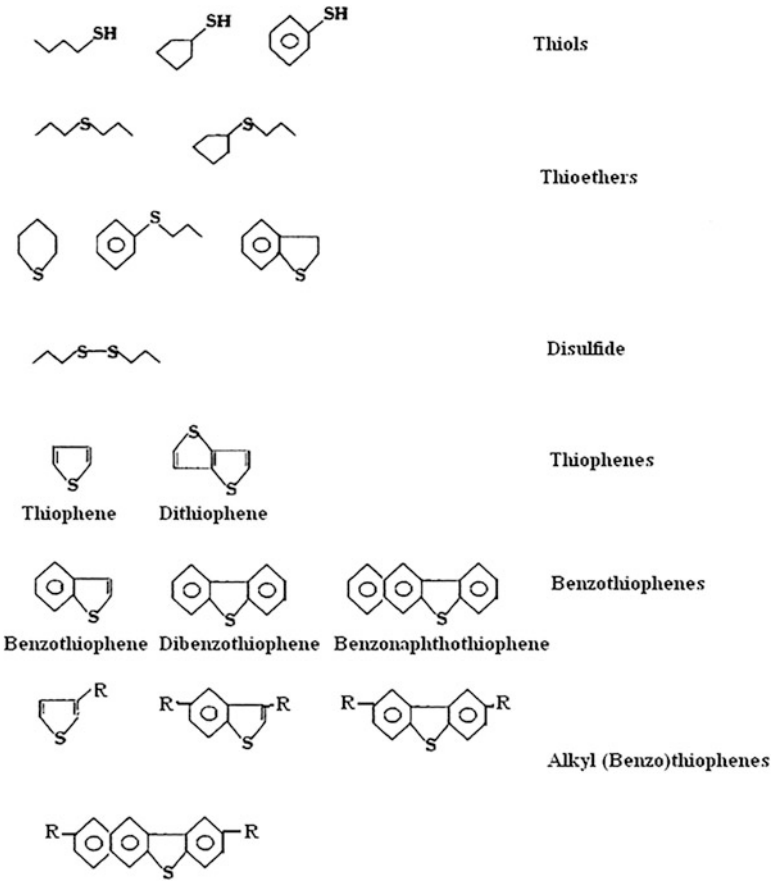

Fig. 1 Chemical structure of typical organic sulfur compounds in fossil fuel. The alkylated DBTs are different in type of substituents, number of substituents, and their bond position on benzene ring

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. 2005a), *Gordonia* (Rhee et al. 1998; Li et al. 2006a, b), *Mycobacterium* (Li et al. 2003, 2005b, 2007a, b; Chen et al. 2008), *Pseudomonas* (Gupta et al. 2005; Shan 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 and Bassi 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.

The "4S" pathway for sulfur removal was first reported for *Rhodococcus erythropolis* IGTS8 in 1993 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* ECRD1 (Grossman et al. 1999), *Rhodococcus* B1 (Denis-Larose et al. 1997), *Rhodococcus* SY1 (Omori et al. 1992), *Rhodococcus* UM3 (Purdy et al. 1993), *Rhodococcus* sp. KT462, 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

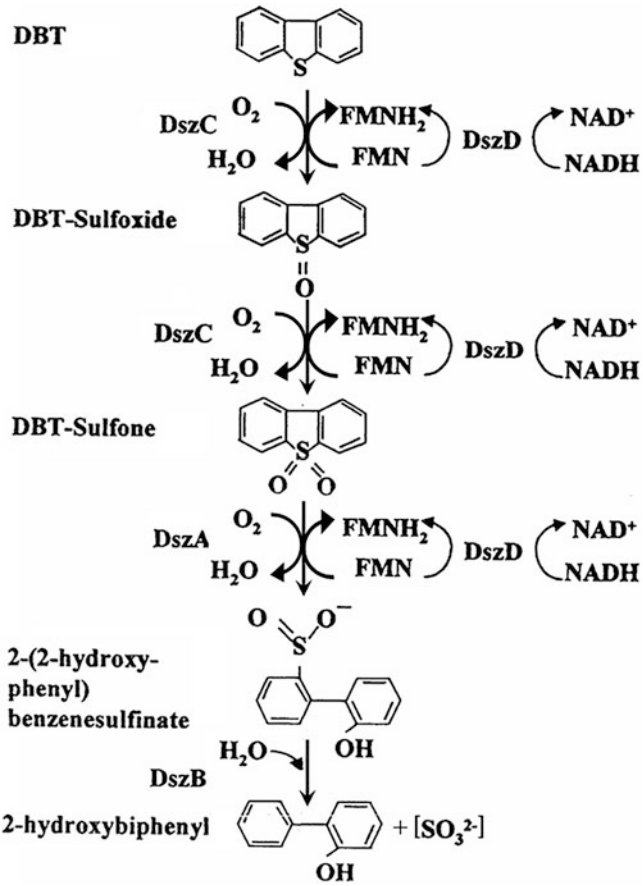

Fig. 2 "4S" pathway of microbial DBT desulfurization and involvement of relative enzymes and flavin reductase

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.

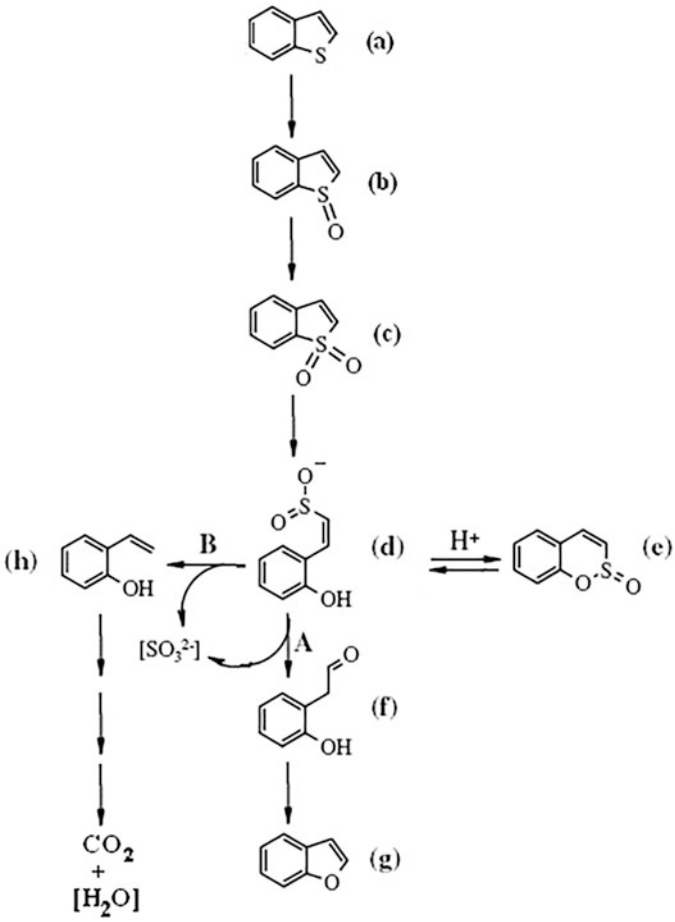

Fig. 3 Two possible degradation pathways of Benzothiophene (BT). (A) *G. desulfuricans* strain 213E (B) *Paenibacillus* sp. strain A11-2: (a) BT; (b) BT sulfoxide; (c) BT sulfone; (d) 2-(2′-hydroxyphenyl) ethen 1-sulfinate; (e) benzo[*e*][1,2]oxathiin *S*-oxide (sultine); (f) 2-(2′-hydroxyphenyl)ethan-1-al; (g) Benzofuran; (h) *o*-hydroxystyrene

Based on the mass spectral data, two different pathways of desulfurization of sulfur from benzothiophene were proposed, and each end product has been identified (Fig. 3). In both of the pathways, BT is firstly converted to BT sulfoxide, then BT sulfone, and following 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).

3 Enzymes Involved in Specific Desulfurization

The enzymes involved in specific DBT-desulfurizing pathways have been purified and characterized; their optimum reacting conditions and activities have also been studied. Compared to DBT-desulfurizing enzymes, the enzymes in BT-desulfurizing pathway were rarely known.

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 and 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→DBTO2). 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 et al. 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 sulfonic 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 (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 interference of substrate binding or catalysis.

A Cys residue must be the catalytic center of DszB because SH reagents inhibited the enzyme activity. 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 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, which were 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 overcomes these drawbacks. 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 compound. 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.

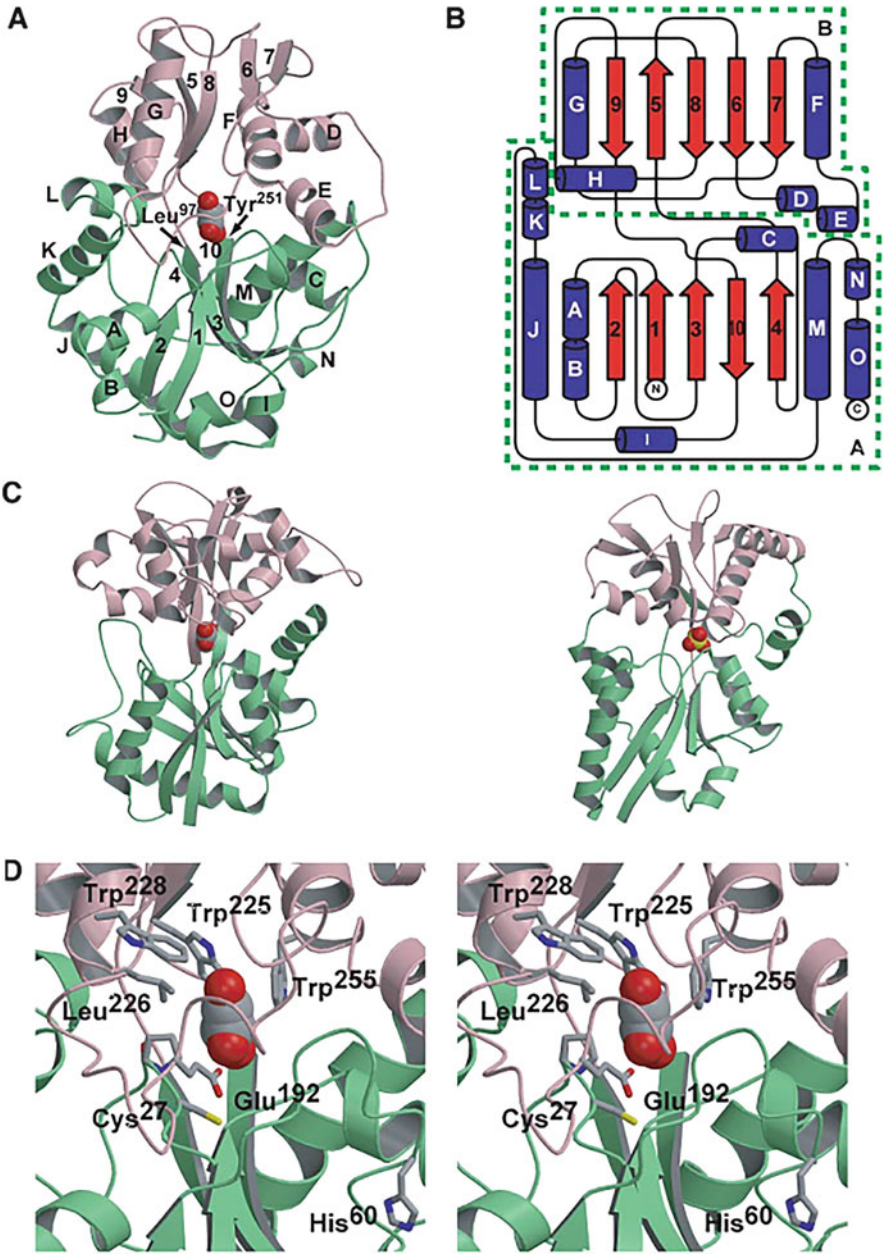

Fig. 4 The overall structure of DszB. (**a**) ribbon model of DszB. Domain A and B are colored in light green and pink, respectively. Two crossover residues that define the domains are labeled. (**b**) Topology diagram of DszB. Helices and strands are colored in blue and red, respectively. Dotted green 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

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 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, *dsz*C) transcribed in the same direction, coding for three proteins Dsz A, Dsz B, and Dsz C, respectively (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.

Fig. 4 (continued) the active site. A glycerol molecule is depicted in space-filling model. Residues mentioned in the text are depicted as sticks

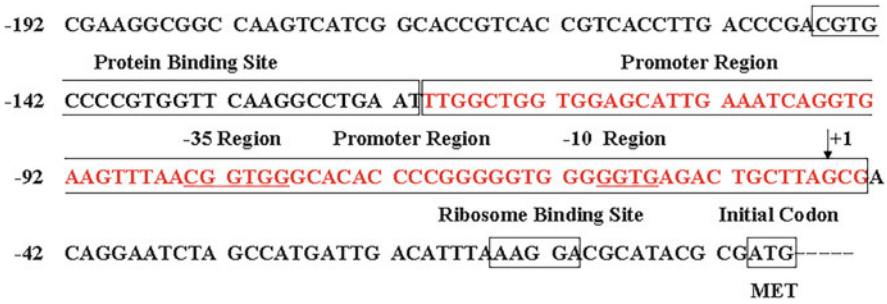

Fig. 5 Sequence of the 385-bp *dsz* promoter-containing fragment that starts immediately after the *Hin*dIII site. Nucleotides below the line indicate the positions of mutations, including the deletion of a C at 210. Boxed sequences are the protein-binding domain and the promoter region deduced from 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

The termination codon of *dsz*A and the initiation codon of *dsz*B overlap (ATGA, Fig. 5), 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 severalfold less in the cytoplasm, as compared with Dsz A and Dsz C (Li et al. 1996).

These genes, when cloned into a non-desulfurized 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. 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

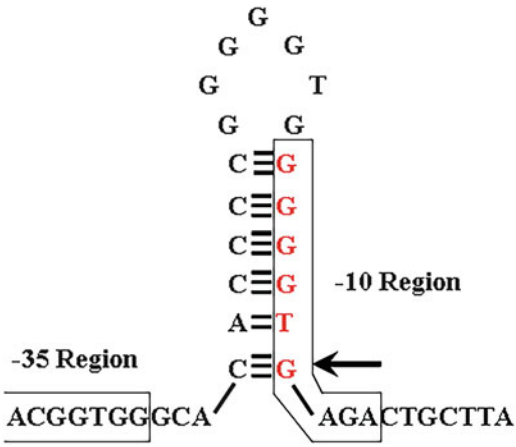

Fig. 6 Potential hairpin structure located in the *dsz* promoter. The hairpin is between −75 and −57 and has a free energy value of −15.4 kcal (1 cal = 4.184 J). Deletion of the G at −57 (indicated by the arrow), as in mutant R4, would reduce this to −13.2 kcal

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 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 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 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 Co-expression 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 harveyi* 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, which 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 16S 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 16S 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 reaction 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 *dszA*, *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 *dszA* 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. (2007a, b) 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 4S pathway. Hirasawa et al. (2001) constructed an *Escherichia coli-Rhodococcus* shuttle vector, and the desulfurization gene cluster, *dsz*ABC, and the related reductase gene, *dsz*D, 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 of *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 bacteria were 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 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 transposon is 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 5 times faster than that of the native *dsz* operon (Li et al. 2007a, b), 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. 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 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. 2006a, b). 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.

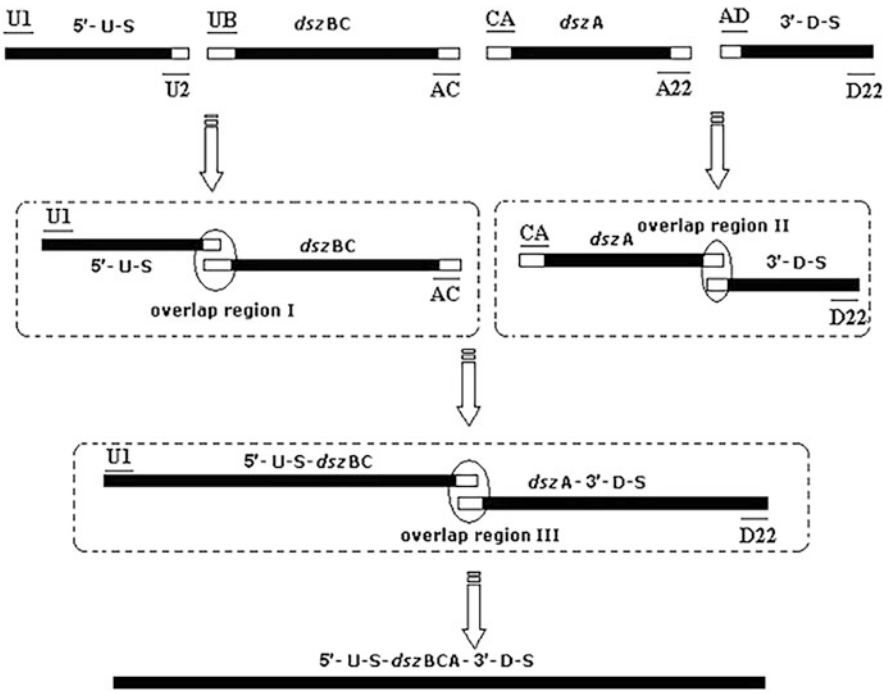

Fig. 7 Rearrangement of the *dsz* operon by overlap PCR. Fragments of the 400-bp 5′ upstream segment (5′-U-S) and the 400-bp 3′ downstream segment (3′-D-S) of *dsz*ABC and the *dsz*A and *dsz*BC segments, including the overlap regions, were amplified by PCR, then the ligated 5′ upstream-*dsz*BC segment (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

6 Process Engineering for BDS with *Rhodococcus*

To apply the BDS process from laboratory to industrial level, it is necessary to improve the BDS process engineering. In this section, we summarized the main advances in process design for BDS with *Rhodococcus* strains from two parts: the application of immobilized cells during BDS process and the design of the novelty bioreactor for BDS.

6.1 The Application of Immobilized Cells

Though biodesulfurization with growing cells is a simple and widely accepted methods with different *Rhodococcus* strains, the application of immobilized cells

during BDS process could help improve the desulfurization of DBT with a higher concentration, increase desulfurization efficiency, and enhance the separation of cells from the final products.

Rhodococcus spp. strains were immobilized for BDS process on silica (Si), alumina (Al), and sepiolite (Sep) or encapsulated in polymeric materials such as alginates with the advantages of biocompatibility and low-cost. However, the immobilization leads to the reduction mass transference of oil to the bacterial cells. One considerable solution to address this problem is the use of surfactants. The synthetic surfactants such as Tween 80, Span 80, and Triton X-100 have been confirmed to enhance biodesulfurization effectively. Derikvand and Etemadifar (2014) demonstrated that the addition of (Tween 80 and Span 80) increased the dissolved DBT concentration in the aqueous phase and facilitate its close to *R. erythropolis* R1 in the alginate beads and resulted in the increase of biodesulfurization. Taking into account the toxicity of synthetic surfactants, biological surfactants (biosurfactants) with the advantages of non-toxicity, biodegradation, and adaption to extreme pH and temperature were also studied. The biosurfactant from a marine bacteria strain was purified and added in the BDS system of *R. erythropolis*. The results showed that adding biosurfactants increased the higher BDS activity when compared with synthetic surfactant Tween 80. It may be due to the formation of micelles increases the solubilization of DBT (Dinamarca et al. 2014).

Generally, the bacterial surface was negatively charged which will lead to the adsorption with positively charged nano-ɣ Al2O3. The production of 2-HBP increased twofolds after 24 h in the *Rhodococcus* spp. strains alginate beads containing nano-ɣ Al2O3 when compared with the controls. The combination of nano-ɣ Al2O3 and alginate encapsulated cells could be an effective approach to enhance BDS process (Derikvand et al. 2014).

Hassan et al. (2013) synthesized the Fe3O4 magnetite nanoparticles, which could be magnetically separated from oil/water biphasic system conveniently. The magnetite nanoparticle–coated *R. erythropolis* FMF and *R. erythropolis* IGTS8 cells exhibited similar desulfurization activity with the free cells (67 ± 3 and 69 ± 4, respectively). Furthermore, the coated Fe3O4 nanoparticles facilitate the recovery of cells from the biodesulfurization systems and then increase the recycled times of the immobilized cells.

6.2 Bioreactor Design

A vertical rotating immobilized cell reactor (VRICR) was designed and investigated for its BDS activity with the *R. erythropolis* (Amin 2011). The maximum desulfurization rate was up to 167 mM 2-HBP/Kg/h, and 100% of sulfur could be removed from the model oil (dibenzothiophene in hexadecane) within a 120 h period. Another research studied the influence of bed lengths and support particle size on the desulfurization efficiency of immobilized *R. rhodochrous* cells in a catalytic bed

reactor packed with silica. The results suggested that longer bed, lower substrate flow, and large particle size would be benefited for the desulfurization (Alejandro et al. 2014).

To increase the utilizing efficiency of bacteria cells for desulfurization, a new aqueous–organic two-layer partitioning and continuous process was designed. Different from the batch and fed-batch processes, the biphasic bioreactor showed the efficient biodesulfurization activity for a long period (Yang et al. 2007). In this bioreactor, the oil and the cells were kept in organic and aqueous phase, respectively. DBT transferred from the oil phase to the aqueous phase and desulfurized by the bacterial cells. And then the produced 2-HBP was washed out of the bioreactor to reduce the inhibition.

7 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.

Acknowledgements We gratefully acknowledge the support for our research from the Natural Science Foundation of Tianjin, China (grant numbers 05YFJMJC00700, 09JCZDJC03200). We also thank the publisher of the American Society of Microbiology and the American Society for Biochemistry and Molecular Biology to offer kindly help with useful pictures.

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Bioremediation of Contaminated Environments Using *Rhodococcus*

Maria S. Kuyukina and Irena B. Ivshina

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 stimulated by nutrient and oxygen amendments. Laboratory and field-scale studies on *Rhodococcus* application in

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_9

cleanup technologies are reviewed relating to in situ subsurface and groundwater remediation, on-site treatments of contaminated soils, sludges, wastewaters, and gaseous emissions.

1 Introduction

Environmental pollution with anthropogenic organic compounds has become one of the most urgent problems worldwide. This negatively impacts economical and social developments and 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, and 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; Ivshina et al. 2015a). 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 the optimization of limiting conditions (Alexander 1999). Currently, methods of biological remediation of contaminated sites gain ever increasing popularity due 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

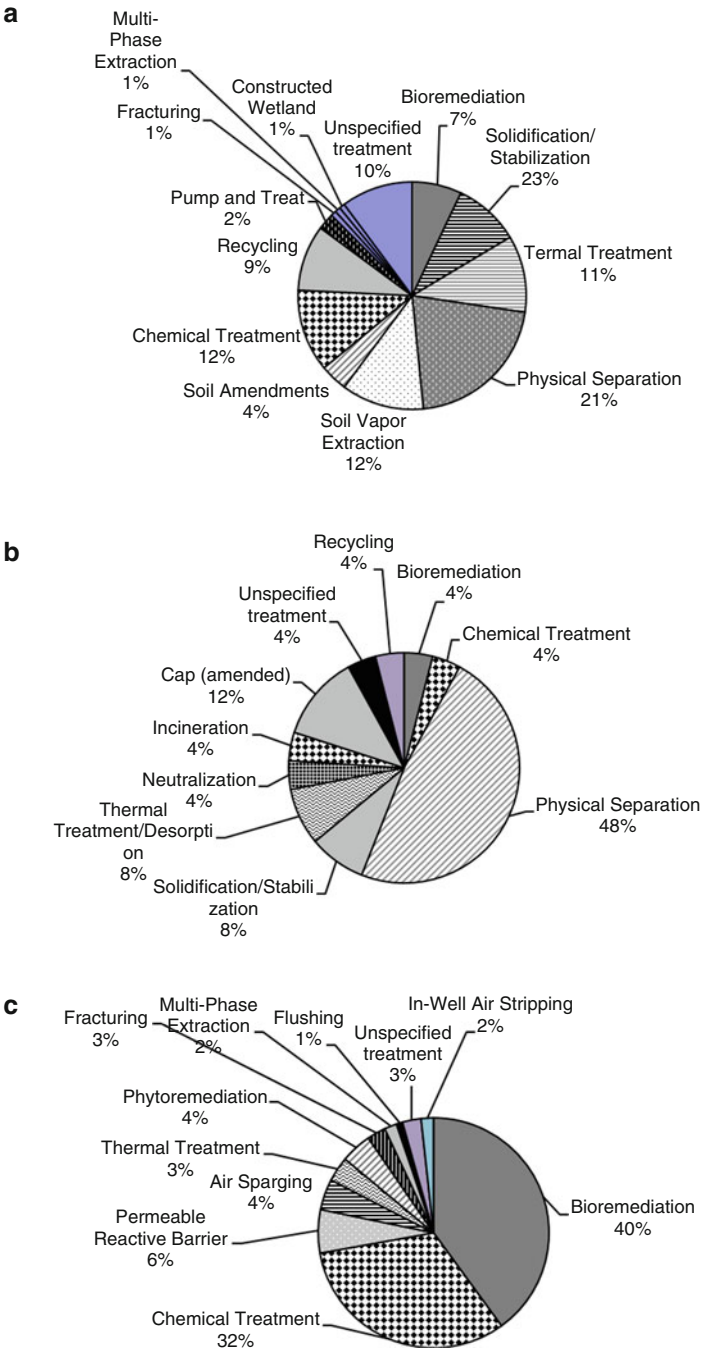

Fig. 1 Proportion of bioremediation among remediation technologies reported in the US EPA Superfund Remedy Report (15th Edition) for 2012–2014. (**a**) Source [soil, sludge, sediment, solid waste, debris, drummed waste, leachate, and any nonaqueous phase liquid both light (LNAPL) and

contaminants into less harmful, nonhazardous substances, which are further integrated into natural biogeochemical cycles (Alexander 1999; 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 groundwaters) 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

Fig. 1 (continued) dense (DNAPL)] treatment. (**b**) Sediment treatment. (**c**) In situ groundwater treatment. Figures generated from US EPA (2017)

(Van der Geize and Dijkhuizen 2004; Martínková et al. 2009; Ivshina et al. 2017). 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, various pharmaceuticals, and 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 early 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, and 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 groundwaters, and 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 sub-zero 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}–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–C_{12}) as well as a broad range of long-chain alkenes (C_{19}–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. A recent study of Sinha et al. (2017) revealed a seasonal dominance of *R. fascians* in Arctic fjord heterotrophic bacterial communities. 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. However, in a recent study of Nie et al. (2014), only a few *alkB* sequences retrieved from freshwater, marine, and terrestrial metagenomic databases were closely related to the sequences found in previously identified microbial genomes, suggesting the presence of numerous novel *alkB* genes, including those from *Rhodococcus*, in different environments. Since rhodococci are known to harbor multiple *alkB* genes for different alkane 1-monooxygenases, similarity levels of *alkB* genes with the unique nucleotide sequence encoding a conserved amino acid motif:WLG(I/V/L)D(G/D)GL can be useful for revealing the evolution and improving systematic of this genus (Táncsics et al. 2015).

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 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 *Rhodococcus 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, ethylbenzene, 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). 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 outcompeted other aromatic-degrading bacteria, such as *Pseudomonas*.

Soils, sediments, and waters contaminated with chlorinated hydrocarbons, nitroaromatic and complex heterocyclic 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; Ito et al. 2016). 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 outcompeted 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). An endosulfan sulfate-degrading *R. koreensis* strain S1-1 with a new metabolic pathway was isolated from soil long-term contaminated with this pesticide (Ito et al. 2016).

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. Masy et al. (2016a) demonstrated *R. erythropolis* biofilm formation on gravel and such biofilm was stable during 15-month experiment in real car parking conditions and it was able to limit hydrocarbon leaching from artificial rainfall.

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 2018).

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. 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 have 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, b; 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, b; Larkin et al. 2005; Lee and Cho 2008), transformations of nitrogen and sulfur compounds (Xu et al. 2006; Shen et al. 2009a, b), and the

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

Characteristics	Description	Advantage in bioremediation	References
High catabolic diversity	Degradation of wide range of chemicals, including aliphatic and aromatic hydrocarbons and their nitro- and halogenated derivatives, 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), and 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), and 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 groundwaters	Ivshina et al. (1981), Elo et al. (2000), Mergaert et al. (2001), Priestley et al. (2006), and 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., wastewaters	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) and 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), and 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), and Fahy et al. (2008b)
Ecological behavior	*K*-strategy and high competitiveness in communities under crowded, substrate-limited conditions	Survival in bioaugmentation inoculaand indigenous communities	Juteau et al. (1999), Margesin et al. (2003), and Borin et al. (2006)

(continued)

Table 1 (continued)

Characteristics	Description	Advantage in bioremediation	References
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), and Aoshima et al. (2007)

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), sulfonated azo dyes (Joshi et al. 2008), pesticides (Nagy et al. 1995; Ito et al. 2016), pharmaceuticals (Ivshina et al. 2015b; Homklin et al. 2012), cyanides (Baxter and Cummings 2006; An et al. 2018), benzothiazoles (Besse et al. 2001), hydrofurans (Daye et al. 2003; Tajima et al. 2012), 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; Ivshina et al. 2017). It is assumed that prolonged persistence of hydrophobic organic compounds in the environment is mainly determined by their solubilization-limited bioavailability for microorganisms. Many bacteria can assimilate hydrophobic substances, such as 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 and PAHs from soil, thus facilitating its biodegradation by soil microorganisms (Kuyukina et al. 2005; Ivshina et al. 2016). It should be noted that many hydrocarbons, for example, low-molecular-weight alkanes, monoaromatics, and chlorinated aliphatic compounds, are toxic to microorganisms primarily due 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 (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 14 azo dyes individually as well as in simulated mixed wastewater, suggesting its possible application in industrial wastewater treatment. Although the abovementioned 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 resource-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

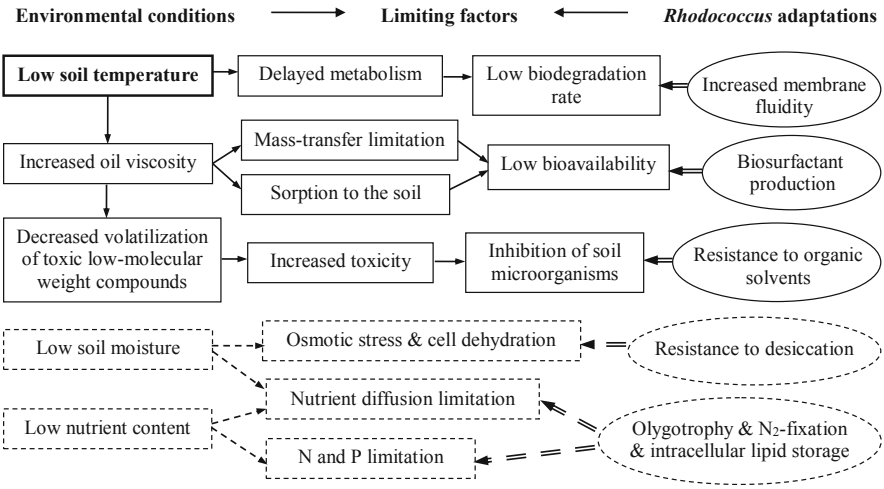

Fig. 2 Adaptations of *Rhodococcus* to cold soil conditions. Optional environmental conditions, factors, and corresponding adaptations are shown by dotted lines

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, leads 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 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, such as 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, 2013). 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 (Kuyukina et al. 2017).

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, 2017; Hatzinger et al. 2017). 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 (chapters "Genetics of the Virulence of *Rhodococcus equi*" by Vázquez-Boland and "Phytopathogenic Strategies of *Rhodococcus fascians*" by Vereecke). 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. There are only two reports by Kitagawa and Tamura (2008a, b) on *R. erythropolis* producing antibiotics active against Gram-positive bacteria, including *Rhodococcus* and related genera. 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; Hongming et al. 2015) could be used in phytoremediation projects for contaminated agricultural soils. Additionally, Shagol et al. (2014) described an arsenic-tolerant plant-growth-promoting *R. aetherivorans* strain isolated from smelter-polluted soil, which consistently increased root length of maize in the presence of 100 and 200 μM As(V) and can therefore enhance the efficiency of phytoremediation in As-polluted 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 pollutants since the most well-known cleanup 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

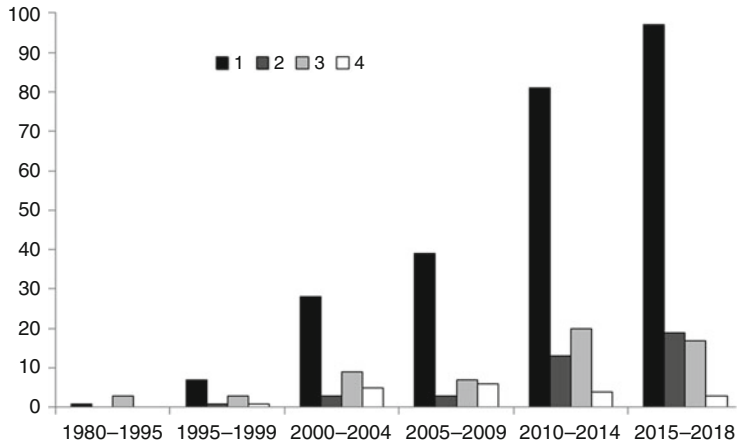

Fig. 3 Number of research articles concerned to *Rhodococcus* applications in environment bioremediation (according to http://www.scopus.com). Queries: Title/Abstract/Keywords: *Rhodococcus* and (1) soil bioremediation; (2) groundwater bioremediation; (3) bioreactor degradation; (4) air biofilter. Nonrelevant papers 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 non-inoculated 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 non-sterile 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. In our experiments, poly(vinyl alcohol) cryogel-immobilized *R. ruber* and *R. erythropolis* survived successively in oil-contaminated

Table 2 Selected examples of *Rhodococcus* application for bioremediation of contaminated environments

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	References
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 co-culture 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)
Soil contaminated with crude oil	Laboratory systems with preheated and non-heated soils	Poly(vinyl alcohol) cryogel-immobilized culture of *R. ruber* and *R. erythropolis*	+	Kuyukina et al. (2013)
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.	+	Rodrigues et al. (2006)
Groundwater artificially contaminated with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	Repacked aquifer sediment columns operated at 15 °C	Liquid culture of *Gordonia* sp., *Pseudomonas fluorescens*, and *R. jostii*	+	Fuller et al. (2015)
Tap water artificially contaminated with gasoline	Laboratory aerobic biobarriers filled with volcanic pumice	Liquid culture of *Rhodococcus* sp. and *Methylibium petroleiphilum*	±[a]	Daghio et al. (2015)

(continued)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	References
Benzene-contaminated groundwater	Laboratory groundwater microcosms	Indigenous bacterial community containing *Rhodococcus*	+	Fahy et al. (2006, 2008a)
In situ treatment—field studies				
Shoreline 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. (2000)
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)
Petroleum hydrocarbon polluted groundwater	Closed bipolar system (one extraction and two injection wells)	Pre-grown consortium of zymogenous microorganisms containing *Rhodococcus*	+	Beškoski et al. (2017)
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*	+	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)
Crude oil-contaminated soil	Composted inoculated biopiles with addition of mineral nutrients and straw as bulking agent	Liquid culture of biosurfactant-producing *R. 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)

(continued)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	References
Crude oil-contaminated soil	Landfarming cells with addition of woodchips 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 cultures of *Pseudomonas putida*, *Acinetobacter johnsonii*, and *R. erythropolis*	+	Genovese et al. (2008)
Soil artificially contaminated with diesel oil and fuel oil	Inoculated biopiles with addition of rhamnolipid biosurfactant	Liquid cultures of *Gordonia alkanivorans*, *R. erythropolis*, *Acinetobacter junii*, and *Exiguobacterium aurantiacum*	+	Lin et al. (2010)
Olive mill waste (humid husk)	Pilot biopile amended with waste wool, olive leaves and twigs, wheat straw, and pigeon manure	Indigenous tannin-degrading bacterial community containing *R. rhodochrous*	+	Federici et al. (2011)
PAH-contaminated soil	On-site landfarming plots added with nutrients	Liquid culture of *R. ruber*	+	Sun et al. (2012)
Aged fuel-contaminated soil	Pilot tank mimicking groundwater flow conditions and two bioreactors	Liquid culture of *R. erythropolis*	+	Masy et al. (2016b)
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)
Groundnut cake naturally contaminated with aflatoxin B1	Shake-flask bioreactors	Liquid culture of *R. erythropolis*	+	Dogan et al. (2017)
Sediment contaminated with polycyclic aromatic hydrocarbons	Aerobic slurry-phase bioreactors with mineral nutrient addition	Indigenous community containing *Rhodococcus*	+	Ringelberg et al. (2001)
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)
Phenol-contaminated water	Stirred-tank bioreactor	Liquid culture of *Rhodococcus* sp.	+	Yaacob et al. (2016)
Ammonium- and 2-fluorophenol-contaminated synthetic wastewater	Sequencing batch reactor	Aerobic sludge granules and liquid culture of *Rhodococcus* sp.	+	Ramos et al. (2017)
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)
Di-*n*-octyl phthalate-contaminated water	Sequencing batch reactor	Liquid cultures of *Arthrobacter* sp. and *Rhodococcus* sp.	+	Zhang et al. (2018)
1,1,2,2-Tetrachloroethane-contaminated water	Continuous stirred-tank reactor	Liquid cultures of *R. aetherivorans*	+	Cappelletti et al. (2018)
Simulated azo dye- contaminated wastewater	Laboratory flask experiments under microaerophilic condition, with addition of yeast extract and glucose	Mixed culture from dye-contaminated soil and sludge and containing *R. globerulus*	+	Joshi et al. (2008)

(continued)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	References
Model petroleum- contaminated water	Fluidized-bed column bioreactor	Sawdust-immobilized culture of *R. ruber* and *R. opacus*	+	Kuyukina et al. (2009)
Oil-field wastewater	Fluidized-bed column bioreactor	Sawdust- and polyvinyl alcohol cryogel- immobilized cultures of *R. ruber* and *R. opacus*	+	Kuyukina et al. (2017) and Serebrennikova et al. (2017)
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)
Artificial groundwater contaminated with *N*-nitrosodimethylamine	Propane-fed membrane bioreactor	Liquid cultures of *R. ruber*	+	Hatzinger et al. (2011)
Organic cyanide-contaminated groundwater	Fluidized-bed reactors	Coconut granular activated carbon immobilized *R. rhodochrous* and *Bacillus mojavensis*	+	An et al. (2018)
Synthetic pharmaceutical wastewater contaminated with ibuprofen and ketoprofen	Aerobic suspension-sequencing batch reactor	Indigenous agricultural community containing *R. ruber*	+	Hasan et al. (2016)
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)
Artificial waste air containing 2-chlorotoluene	Biotrickling filter	Polyurethane foam-immobilized culture of *Rhodococcus* sp.	+	Dobslaw and Engesser (2018)
Air polluted with benzene	Compost-packed biofilter	Indigenous compost community containing *Rhodococcus*	+	Borin et al. (2006)
Air polluted with isopropylbenzene	Two-phase partitioning bioreactor with addition of ethanol	Liquid culture of *R. erythropolis*	+	Aldric and Thonart (2008)

Volatile compounds of tobacco leaves	Polyamide fiber-packed microbioreactors	Mixed consortium of *Pseudomonas*, *Rhodococcus*, *Nocardia*, and *Micrococcus*	+	Zagustina et al. (2012)
Gas-phase mixture of methanol, α-pinene and H_2S	Biotrickling filter	Autotrophic H_2S-degrading culture, *Candida boidinii*, *R. erythropolis*, and *Ophiostoma stenoceras*	+	López et al. (2013)
Air polluted with toluene, benzo(*a*) pyrene, and formaldehyde	Vermiculite-packed column biofilter	Consortium of *R. erythropolis* and *Fusarium solani*	+	Vergara-Fernández et al. (2018)
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)
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)
Model wastewater containing 2,4,6-trinitrophenol	Pilot biological aerated filter with mineral nutrient addition	Liquid culture of *Rhodococcus* sp.	+	Shen et al. (2009a)
Industrial wastewater contaminated with hydrocarbons, surfactants, and heavy metals	Pilot biotreatment installation	Immobilized algal-bacterial co-culture containing *Rhodococcus* sp.	+	Safonova et al. (2004)
Groundwater polluted with ethyl *t*-butyl ether and gasoline	On-site pilot bioreactor	Liquid culture of *R. wratislaviensis*, *R. aetherivorans*, and *Aquincola tertiaricarbonis*	+	Fayolle-Guichard et al. (2012)

(continued)

Table 2 (continued)

Contaminated substrate	Bioremediation method	*Rhodococcus* application mode	Effect of treatment	References
Groundwater polluted with *N*-nitrosodimethylamine and *N*-nitrodimethylamine	Field-scale propane-fed fluidized-bed bioreactor	Coconut granular activated carbon immobilized *R. ruber*	+	Hatzinger et al. (2017)

[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. *ND* not determined

soil under drought conditions, contributing to efficient alkane biodegradation (Kuyukina et al. 2013). Several treatability studies showed positive effects of *Rhodococcus* co-cultures 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 *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. In a recent field study of Beškoski et al. (2017), groundwater contaminated with petroleum hydrocarbons from an underground storage tank was treated using a closed bipolar system (one extraction and two injection wells) by bioaugmentation with pre-grown indigenous hydrocarbon-degrading consortia containing *Rhodococcus* and biostimulation with nutrients. After 60 days of bioremediation, more than 95% of *n*-alkanes, terpanes, and steranes were biodegraded; phenanthrene and its methyl-, dimethyl-, and trimethyl-isomers were removed completely, suggesting the applicability of this technology for the in situ treatment of PAH-contaminated groundwater.

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 (Van Hamme et al. 2003), it is still being used in many countries. 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 woodchips. 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; Kuyukina et al. 2013) temperatures. Thus, mineralization of ^{14}C-labelled hexadecane at 5 °C was significantly greater in both crude oil-contaminated and pristine soil microcosms seeded with *Rhodococcus* sp. Q15 cells compared to non-inoculated 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). Taheri et al. (2008) performed a feasibility study for the soil polluted with disulfide oil, a waste product of liquefied petroleum gas desulfurization, 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. More recently Sun et al. (2012) applied a liquid *R. ruber* culture for on-site landfarming of heavily PAH-contaminated soil at the abandoned cooking plant site. They found out that a combination of biostimulation and bioaugmentation significantly enhanced the removal of PAHs from the contaminated soil. 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*, *Pseudomonas 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. Masy et al. (2016b) applied electrical resistivity tomography to detect soil heterogeneities and to monitor *R. erythropolis* biodegradation activity during a pilot-scale bioremediation of aged fuel-contaminated clay loam soil. 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, and 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 and 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 landfarming 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, 2017). 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 non-bioaugmented reactors, although identification of indigenous bacterial populations of the non-bioaugmented reactor by 16S rDNA sequencing revealed *Rhodococcus* members among other biphenyl-degrading bacteria. Liquid and immobilized *Rhodococcus* spp. cultures were efficiently applied to different type bioreactors treating water contaminated with phenol, di- and trinitrophenol, fluorophenol, phthalic acid ester, nitrosodimethylamine, and organic cyanide (Kitova et al. 2004; Weidhaas et al. 2007; Shen et al. 2009a; Hatzinger et al. 2011; Yaacob et al. 2016; Ramos et al. 2017; An et al. 2018; Zhang et al. 2018). Moreover, complex emergent pollutants, such as aflatoxin and pharmaceuticals, can be efficiently degraded by *Rhodococcus* in bioreactors (Hasan et al. 2016; Dogan et al. 2017). As a promising approach to mitigate biofouling in membrane bioreactors, the abovementioned quorum quenching activity of *Rhodococcus* was used

alone or in combination with chlorination, resulting in reduced levels of *N*-acyl homoserine lactones and extracellular biopolymers in the biofilm (Maqbool et al. 2015; Weerasekara et al. 2016).

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; Fayolle-Guichard et al. 2012; Hatzinger et al. 2017). Particularly, pilot membrane bioreactors inoculated with *Rhodococcus*-containing mixed cultures were used to treat tetrahydrofuran and dichloropropene waste streams (Katsivela et al. 1999; Daye et al. 2003). Industrial wastewater inoculation with an algal-bacterial co-culture 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). Groundwater polluted with ethyl *t*-butyl ether (ETBE) and gasoline was treated in the on-site bioreactor inoculated with *R. wratislaviensis*, *R. aetherivorans*, and *Aquincola tertiaricarbonis* cultures, and a 100-fold increase in the abundance of the *ethB* gene encoding a cytochrome P450 involved in ETBE biodegradation was detected by *q*-PCR, thus reflecting the groundwater colonization by the relevant microorganism (*R. aetherivorans*) (Fayolle-Guichard et al. 2012). However, more recently Hatzinger et al. (2017) carried out a pilot propane-fed bioreactor treatment of groundwater contaminated with *N*-nitrosodimethylamine and *N*-nitrodimethylamine left from the use of liquid rocket propellant, and they demonstrated the replacement of initially inoculated propane-oxidizing *R. ruber* ENV425 with native propanotrophs along with the significant increase in microbial diversity and propane monooxygenase abundance over the year of experiment resulted in the efficient contaminant biodegradation.

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 physicochemical methods (Vinage and von Rohr 2003). A modified rotating biological contactor inoculated with a suspension of *Pseudomonas 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. Bacterial-fungal consortia containing *Rhodococcus* were successfully tested in column biofilters to treat most common industrial and residential air pollutants, such as volatile organic compounds, PAHs, and hydrogen sulfide (López et al. 2013; Vergara-Fernández et al. 2018). These studies also revealed the ability of rhodococci to survive for a long time in two-component and complex microbial populations of biofilters and to contribute considerably to the contaminant degradation. However, in earlier study of Borràs et al. (2010), a co-culture of *Pseudomonas aeruginosa* CCM 1960 and *R. erythropolis* CCM 2595 inhibited both laccase production and PAH degradation by white-rot fungi in the case of removal of acenaphthylene, benzo[*a*]pyrene, dibenzo[*a*,*h*]anthracene, and benzo[*g*,*h*,*i*]perylene from contaminated soil, thus indicating the need for further research of rhodococcal-fungal interactions during bioremediation.

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 "Diversity and Plasticity of Rhodococcus Genomes" by Cappelletti and Di Gennaro), which could be used in constructing recombinant strains for improved bioremediation inocula. Indeed, the complete nucleotide sequences of numerous *Rhodococcus* genomes provided new insights that could facilitate biotechnological exploitation of this genus. For example, amidase genes from *R. erythropolis* MP50 and *Agrobacterium tumefaciens* D3 co-expressed along with nitrile hydratase from *Bradyrhizobium japonicum* USDA 110 were used for biodegradation of dihalogenated benzonitrile herbicides by recombinant *Escherichia coli* (Pei et al. 2017). Furthermore, *Arabidopsis thaliana* (*Arabidopsis*) was transformed with the hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-degrading *xplA* and associated reductase *xplB* from *R. rhodochrous* 11Y in combination with the 2,4,6-trinitrotoluene (TNT)-detoxifying nitroreductase *nfsI* from *Enterobacter cloacae*, resulting in transgenic plants beneficial for remediating RDX- and TNT-contaminated soil and groundwater. 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. 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; Coffey et al. 2010; Táncsics et al. 2015). 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.

Acknowledgments This research was funded by the Ministry of Science and Higher Education of the Russian Federation (State Task Registration No. 01201353247 for IEGM) and the Russian Science Foundation grant 18-14-00140 for PSU.

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Production of Trehalolipid Biosurfactants by *Rhodococcus*

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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, antitumor, and anti-adhesive 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, improved material construction, and biomedicine. The present chapter summarizes recent research on *Rhodococcus* biosurfactants and focuses on biosynthesis features, physicochemical and bioactive properties, and their potential applications.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_10

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 hydrophobic substrate uptake, cell adhesion, and surface colonization to microbial antagonism and pathogenesis (Neu 1996; Ron and Rosenberg 2001; Van Hamme et al. 2006). 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.

Fields of application or potential use of biosurfactants are diverse and include not only oil, cleaning, food, and cosmetic industries and environmental protection but also agriculture and medicine (Marchant and Banat 2012; Sachdev and Cameotra 2013). The global biosurfactant market is estimated to grow at a compound annual growth rate (CAGR) of 5.6% from 2017 to 2022. Based on application, the agricultural chemical segment is projected to grow at highest CAGR due to increasing demand for green surfactants to improve soil quality and promote plant growth and plant-microbe interactions (Biosurfactants market 2018).

The most promising applications of biosurfactants are the environmental remediation technologies since product purity is of less concern (Lang and Philp 1998; Christofi and Ivshina 2002; Banat et al. 2010). 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; Gudina et al. 2013; Kuyukina et al. 2015). 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. Thereby, possible optimization of a biosurfactant synthesis process would allow increasing the product yield at relatively low material and energy expenditures. 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 or agro-industrial wastes) and versatile biochemical properties compared to other types of biosurfactants (Kitamoto et al. 2002; Lang 2002; Franzetti et al. 2010). 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; Paulino et al. 2016). Members of *Rhodococcus* species possessing unique biological properties and wide catabolic abilities are perspective candidates for biosurfactant producers (Van der Geize and Dijkhuizen 2004; Ivshina et al. 2017).

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; Franzetti et al. 2010), while other *Rhodococcus* species are also reported as active biosurfactant producers (Table 1). Comparing the data on surface-active properties of the presented *Rhodococcus* species (*R. erythropolis*, *R. fascians*, "*R. longus*," *R. opacus*, *R. ruber*, and *R. wratislaviensis*), it can be suggested that biosurfactant production is generalized throughout this genus. 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

Table 1 Biosurfactant-producing *Rhodococcus* species and their product characteristics

Microorganism	Surfactant product	Surface tension (mNm^{-1})	Interfacial tension (mNm^{-1})	CMC (mgl^{-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) and 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. erythropolis P6-4P	Cell-free culture broth	28	n.d.	10*	Cai et al. (2014)
R. erythropolis Z25	Crude organic extract	29.5	4.5	70	Xia et al. (2011)
R. fascians A3	Cell-free culture broth	27	n.d.	n.d.	Gesheva et al. (2010)
"*R. longus*" (3 strains)	Whole culture broth	27.2 (0.40)	1.8 (0.1)	90*	Ivshina et al. (1998) and Philp et al. (2002)
R. opacus (3 strains)	Whole culture broth	26.5 (0.61)	3.0 (1.75)	95*	Ivshina et al. (1998) and Philp et al. (2002)
R. ruber (15 strains)	Whole culture broth	27.4 (0.57)	2.7 (1.61)	72*	Ivshina et al. (1998) and 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. ruber Z25	Crude organic extract	29.5	1.0	57–133	Zheng et al. (2012)
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)
R. wratislaviensis P1-5P	Cell-free culture broth	28	n.d.	11*	Cai et al. (2014)
Rhodococcus sp. (6 strains)	Cell-free culture broth	27.3–28.6	n.d.	n.d.	Malavenda et al. (2015)
Rhodococcus sp. 51T7	Cell-free culture broth	30	n.d.	n.d.	Espuny et al. (1995)
Rhodococcus sp. BS-15	Tri-glucose lipid	29.5	n.d.	2.4 (2.3×10^{-6} M)	Konishi et al. (2014)
Rhodococcus sp. H13-A	Octaacyl-trehalose	n.d.	0.02	1.5	Singer and Finnerty (1990)
Rhodococcus sp. PML026	Trehalolipid	29	n.d.	250	White et al. (2013)
Rhodococcus sp. SD-74	Succinoyl trehalolipid	19	n.d.	5.6×10^{-6} M	Tokumoto et al. (2009)
Rhodococcus sp. TA6	Whole culture broth	29.8	n.d.	30*	Shavandi et al. (2011)

CMC critical micelle concentration, *CMD* critical micelle dilution, *n.d.* none determined
[a]Standard deviations

Corynebacterium spp., which were 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). A cold-adapted Antarctic soil isolate *R. fascians* A3 was found to produce rhamnose-containing biosurfactant when grown on glucose and kerosene (Gesheva et al. 2010). Recently, biosurfactant production of alkanothrophic strains *Rhodococcus wratislaviensis* BN38 and P1-5P has been characterized (Tuleva et al. 2008; Cai et al. 2014). It should be noted that among 53 valid *Rhodococcus* species, only 6 species (and 1 invalid species—"*R. longus*") are reported to produce biosurfactants so far. However, members of these species (*R. equi*, *R. erythropolis*, *R. opacus*, *R. rhodochrous*, *R. ruber*, and *R. wratislaviensis*) are the most common environmental isolates, and they represent the major portion of *Rhodococcus* holdings in culture collections. Nevertheless, many biosurfactant-producing *Rhodococcus* sp. strains are still waiting for species identification. Biosurfactant production is also described in *Rhodococcus*-related genera, namely, *Nocardia* (Kim et al. 2000), *Gordonia* (Arenskötter et al. 2004), *Dietzia* (Kavyanifard et al. 2016), *Corynebacterium* (Cooper et al. 1979), *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. Trehalolipids are produced by members of closely related actinobacterial genera, including *Rhodococcus*, *Nocardia*, *Gordonia*, *Dietzia*, *Corynebacterium*, *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; Konishi et al. 2014) and polysaccharides (Iwabuchi et al. 2000), and the existence of additional chemical structures could be expected.

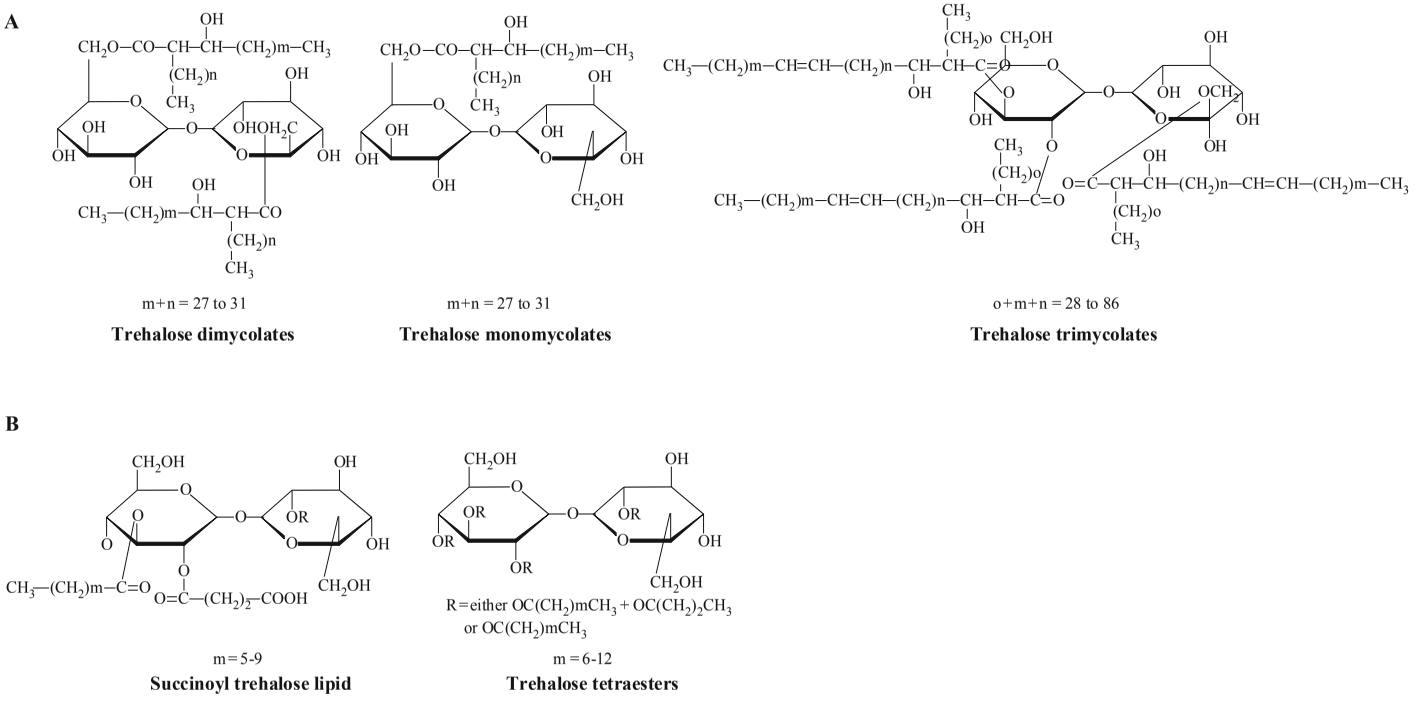

Fig. 1 Chemical structures of mostly known trehalolipids from *Rhodococcus*. (**a**) Nonionic and (**b**) anionic trehalolipids

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; Zheng et al. 2012; Konishi et al. 2014). Considering surface tension, interfacial tension, and CMC values, *Rhodococcus* biosurfactants compare well with other microbial and some synthetic surfactants. Another essential feature of biosurfactants is their emulsifying activity, i.e., the ability to emulsify various hydrocarbons and oils. 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. Cold-adapted *Rhodococcus* sp. strains isolated from Arctic and Antarctic shoreline sediments produced stable emulsions (E_{24} = 50–67%) with kerosene (Malavenda et al. 2015). A *Rhodococcus* sp. strain TA6 emulsified efficiently (E_{24} = 50–70%) kerosene, gas, and motor oils but not *n*-pentane, toluene, or light crude oil, suggesting its specificity toward long-chain hydrocarbons (Shavandi et al. 2011). 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 isopropyl myristate (Marqués et al. 2009). Thermal, osmotic, and pH stabilities of *Rhodococcus* biosurfactants were also reported (Kuyukina et al. 2006; Marqués et al. 2009; Shavandi et al. 2011; Kundu et al. 2013; White et al. 2013). However, there is still very little information available on physicochemical properties of *Rhodococcus* biosurfactants, thereby hindering their introduction to industrial fields.

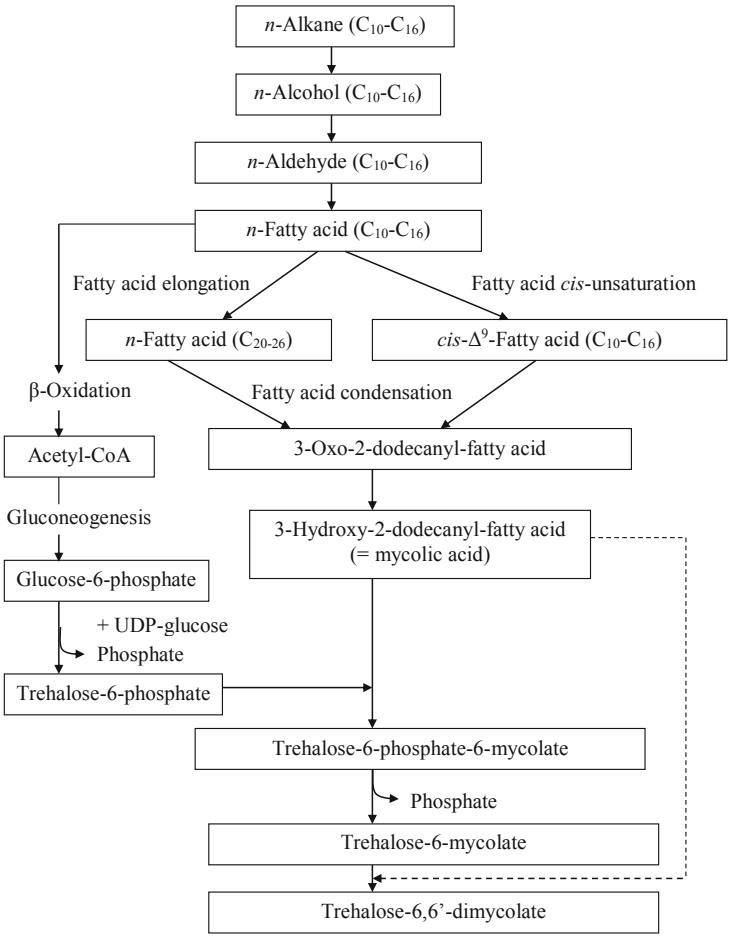

Fig. 2 Scheme of trehalose mono- and dimycolate synthesis from *n*-alkanes. (Modified from Lang and Philp 1998)

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-coenzyme A 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 Alvarez and Steinbüchel, this volume). Intermediates of the fatty acid ß-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, corynebacteria (Kacem et al. 2004), and rhodococci (Sydor et al. 2008; Ivshina et al. 2014). Three novel types of genes involved in the biosynthesis of succinoyl trehalolipids, including a putative acyl-coenzyme A (acyl-CoA) transferase (*tls*A), fructose-bisphosphate aldolase (*fda*), and alkane monooxygenase (*alk*B), were identified in *Rhodococcus* sp. SD-74. An overexpression of *tls*A, but not *fda*, resulted in twofold increase in the glycolipid production, thus suggesting that this final conversion of acyl-CoA, succinate, and trehalose is the rate-limiting step of succinoyl trehalolipid biosynthesis (Inaba et al. 2013). However, precise biochemical mechanisms of acylation leading to the formation of anionic trehalose tetraesters 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 *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 the 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 upward 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

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

Growth substrate	Biomass, gl^{-1}	Surface tension, mNm^{-1}	Surfactant concentration, gl^{-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)

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 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). Since the acyl group composition of *Rhodococcus* trehalolipids is affected by the *n*-alkane used as the substrate, this opens possibilities to obtain specifically defined derivatives for commercial applications (Inaba et al. 2013).

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 non-limiting 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 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.

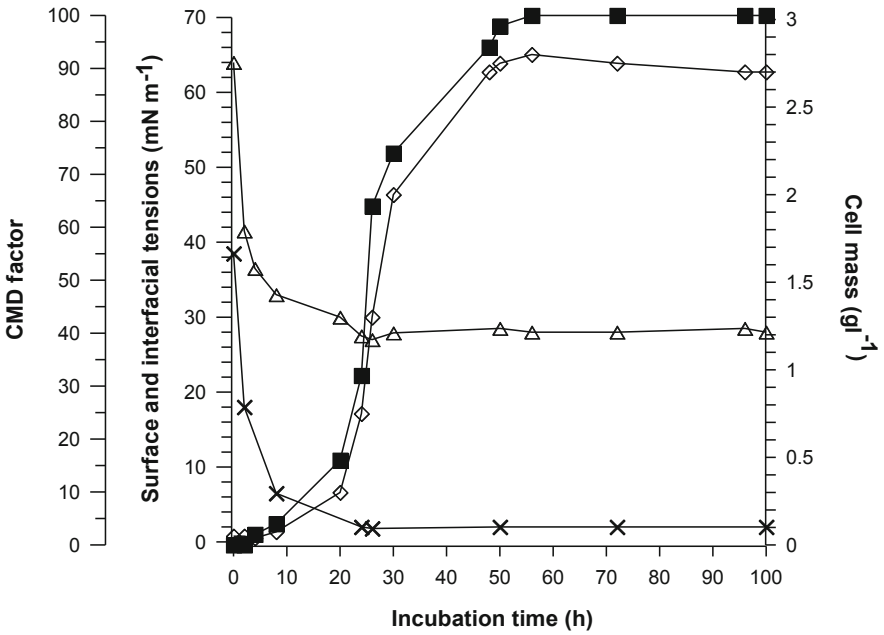

Fig. 3 Surfactant properties of *Rhodococcus ruber* IEGM 231 during batch culture growth on *n*-hexadecane. ■, CMD factor; Δ, surface tension; x, interfacial tension; ◊, cell mass. (From Philp et al. 2002)

Importantly, cheap renewable substrates, such as technical glycerol (waste product of biodiesel production) and sunflower and rapeseed oils, were tested successfully for the biosurfactant synthesis by *Rhodococcus*, thus suggesting sustainable alternatives to petroleum-derived hydrocarbons (Ruggeri et al. 2009; Pirog et al. 2015; White et al. 2013; Malavenda et al. 2015).

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,

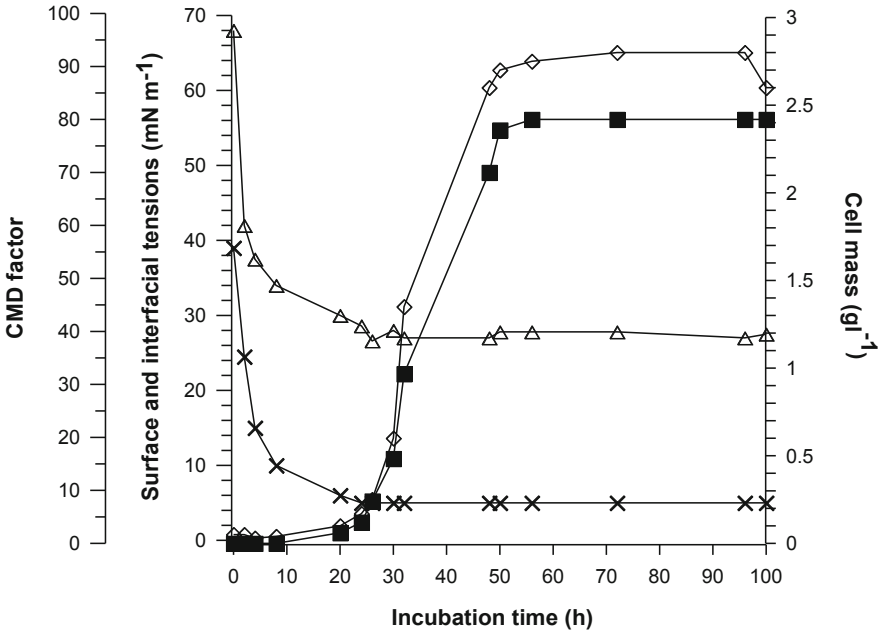

Fig. 4 Surfactant properties of *Rhodococcus erythropolis* IEGM 20 during batch culture growth on *n*-hexadecane. ■, CMD factor; Δ, surface tension; x, interfacial tension; ◊, cell mass. (From Philp et al. 2002)

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).

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 their 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, and

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

Solvent system	Surfactant concentration, gl^{-1}	Surface tension, mNm^{-1}	Interfacial tension, mNm^{-1}	CMC, mgl^{-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)

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; Ivshina et al. 2017). Cell-associated biosurfactants promote the adhesion of rhodococcal cells not only to liquid hydrocarbons but also to hydrophobic solid surfaces (Neu 1996; Ivshina et al. 2013b), 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; de Carvalho, this volume), as well as to physical factors, for example, high temperature and desiccation (Sung et al. 2004). 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 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, and 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 *GlomerelIa cingulata* (Kitamoto et al. 2002). Trehalose dimycolate from *M. tuberculosis* potentiated non-specific 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-hydrophobic interfaces. The studies of Aranda et al. (2007) and Ortiz et al. (2008, 2009) showed that *Rhodococcus* trehalolipid interactions with cellular membranes result in the altered structural and rheological membrane properties, which might contribute to the molecular mechanisms of trehalolipid biological activity. More recent studies of the same group (Zaragoza et al. 2013; Teruel et al. 2014) revealed particular mechanisms of trehalolipid interactions with membrane phospholipids and enzymes, demonstrating their destabilizing and inhibitory effects on enzyme activities.

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 asteroides*, *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. 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, IL-6, IL-10) and chemokines (MCP-1, MIP-1α, IL-8) (for review, see Ryll et al. 2001; Kuyukina et al. 2015). Biological activity of

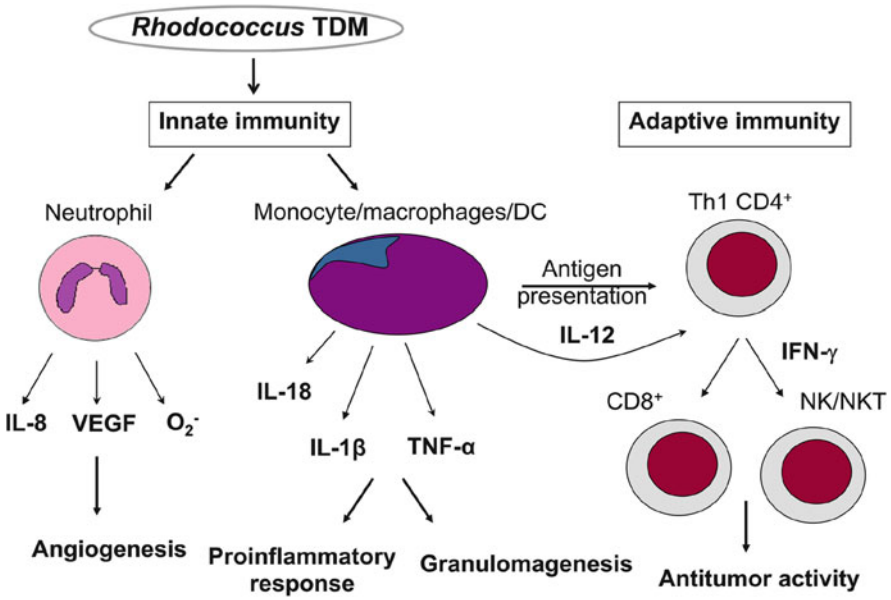

Fig. 5 Immunomoregulating properties of *Rhodococcus* trehalolipids. *DC* dendritic cell, *IL-1b* interleukin-1 beta, *IL-18* interleukin-18, *TNF-a* tumor necrosis factor alpha, *IL-12* interleukin-12, *IL-8* interleukin-8, *NK* natural killer cell, *NKT* natural killer T cell, *IFN-g* interferon gamma, *VEGF* vascular endothelial growth factor, *Th1* type 1 T-helper, O_2^- superoxide anion. (From Kuyukina et al. 2015)

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* IEGM 231 consisting of trehalose dimycolate, diacyltrehalose, and monoacyltrehalose stimulated interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) production of human monocytes (Kuyukina et al. 2007; Gein et al. 2011). At the same time, it stimulated the production of anti-inflammatory cytokines IL-12 and IL-18 by monocytes and mononuclear cells, as well as the secretion of IL-8 by neutrophils (Chereshnev et al. 2010; Baeva et al. 2014). Interestingly, TLB exhibited cytokine-stimulating activity only when applied as an ultrasonic o/w emulsion, but not as an oleophilic film, presumably due to its inhibitory effect on the monocyte adhesion (Gein et al. 2011). Additionally, a trehalolipid from *R. ruber* displayed no cytotoxicity against human lymphocytes and therefore could be proposed as a potential immunomodulating and antitumor agent (Fig. 5) (Kuyukina et al. 2015).

6 Industrial Potential

Current and potential application fields for microbial surfactants, including oil, pharmaceutical, food, and cosmetic industries as well as agriculture and environmental technologies, are reviewed by Christofi and Ivshina (2002), Kitamoto et al. (2002), Lang (2002), Mulligan (2005), Singh et al. (2007), Banat et al. (2010), Marchant and Banat (2012), Sachdev and Cameotra (2013), and Paulino et al. (2016). Biosurfactants can be used as emulsifiers, de-emulsifiers, wetting and foaming agents, functional food ingredients, agriculture chemicals, detergents, and flocculating agents. In particular, biosurfactants from cold-adapted microorganisms, active at low and freezing temperatures, were reviewed recently as promising candidates for sustainable and low-energy impact products and processes, such as gas hydrate technologies, cold-active detergents, and biodiesel (Perfumo et al. (2018). However, despite 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 a few percent; 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 cleanup of oil contaminations (Singh et al. 2007; Banat et al. 2010; Marchant and Banat 2012). Enhanced oil recovery methods utilizing microorganisms and/or their metabolites for the residual oil extraction were developed in the late 1970s, when Rapp et al. (1977) reported 30% increase in oil recovery from sandy rocks using trehalolipids from *Rhodococcus* (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 non-bioavailable 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. 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; Banat et al. 2010). 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 was 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 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). Considering their low toxicity and biodegradability, crude glycolipids from *R. ruber* can be used in soil washing technologies for simultaneous removal of PAHs and sulfur heterocycles (PASHs) (Ivshina et al. 2016).

Pacwa-Płociniczak et al. (2016) revealed plant growth-promoting traits (1-aminocyclopropane-1-carboxylic acid deaminase activity, phytohormone and siderophore production, and phosphate solubilization) coupled with biosurfactant production in two *R. erythropolis* strains (CD 106 and CD 111) isolated from soil heavily contaminated with petroleum hydrocarbons, suggesting their applications in microbe-assisted phytoremediation. However, despite of the promising laboratory results, only limited field studies have been performed so far on application of

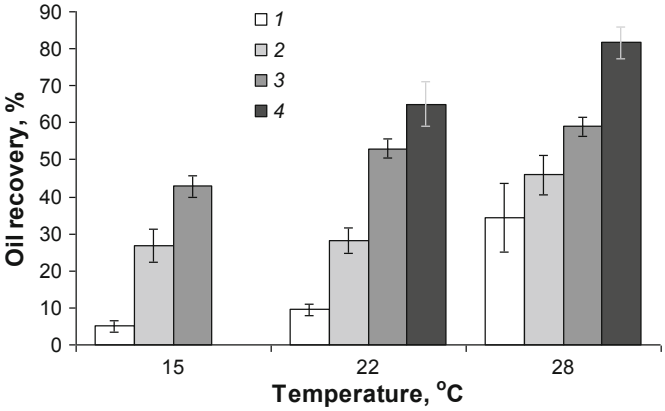

Fig. 6 (Bio)surfactant-enhanced oil recovery from the model soil at different temperatures. Surfactants used: 1, water (control); 2, Tween 60 (synthetic surfactant); 3, *Rhodococcus* biosurfactant produced on *n*-dodecane; 4, *Rhodococcus* biosurfactant produced on *n*-hexadecane. (From Kuyukina et al.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

Table 4 The toxicity of biosurfactants compared to synthetic surfactants

Surfactant	IC_{50} of *Vibrio fischeri* (mgl^{-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 IC_{50}—inhibition concentration leading to a 50% light decrease measured in Microtox toxicity test. (From Ivshina et al. 1998)

microbially produced biosurfactants in soil remediation processes (Christofi and Ivshina 2002; Ławniczak et al. 2013).

Microbial surfactants were also used to remove heavy metals from soil (Mulligan 2005; Ławniczak et al. 2013), although this potential for *Rhodococcus* biosurfactants is still to be revealed. In our experiments, non-specific resistance of *Rhodococcus* cultures to heavy metals correlated positively with their growth on hydrocarbons and biosurfactant production (estimated as emulsifying activity) (Ivshina et al. 2013a). Furthermore, the biosurfactant produced by *R. ruber* IEGM 231 was found to form complexes with Ni^{2+} ions, demonstrating conditional stability constants comparable to those determined for rhamnolipids, most intensively used in biosurfactant-assisted bioremediation studies (Ochoa-Loza et al. 2001; Mulligan 2005; Banat et al. 2010).

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; Paulino et al. 2016). Additionally, several *Rhodococcus* (*R. globerulus*, *Rhodococcus* sp.) were shown to possess de-emulsification 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, and food and fermentation technologies (Singh et al. 2007). Our study revealed anti-adhesive effects of the *R. ruber* IEGM 231 biosurfactant against Gram-positive and Gram-negative bacteria with different percentages of inhibition (30–76%) (Kuyukina et al. 2016). Interestingly, the biosurfactant was more active against growing bacteria rather than resting cells, thus showing biofilm-preventing properties potentially useful for food processing applications (Nitschke and Silva 2016). But herein, 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 dodecyl sulfate (SDS) and cetyl trimethylammonium bromide (CTAB), revealed weakening effects of surfactant additives on PVA cryogels (data not shown). It was also found 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

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)

various industrial and biotechnological applications. Moreover, biosurfactants were recently suggested as useful chemical aids in the synthesis of nanostructured materials with tunable pore size and surface hydrophilicity (Boffa et al. 2014).

Singh et al. (2007) reviewed biotechnological applications of microbial 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 abovementioned studies of *Rhodococcus* trehalolipid interactions with phospholipid membranes and enzymes (Zaragoza et al. 2013; Teruel et al. 2014), would lead to the biosurfactant penetration 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 the introduction of nontoxic trehalolipids produced by nonpathogenic *Rhodococcus* species (Sakaguchi et al. 2000; Kuyukina et al. 2015). 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 being currently developed for *Rhodococcus* surfactants. Several studies showed that rhodococci are able to grow on vegetable oils and glycerol waste, forming stable emulsions due to intensive surfactant production (Haba et al. 2000; Sadouk et al. 2008; Ruggeri et al. 2009; Pirog et al. 2015; White et al. 2013; Malavenda et al. 2015). 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 these alternative 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 Cappelletti et al., this volume). 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 properties. For example, 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 genetically engineered strains would make them economically attractive and competitive with synthetic surfactants.

Acknowledgments This research was funded by the Ministry of Science and Higher Education of the Russian Federation (State Task Registration No. 01201353246 for IEGM and State Task 6.3330.2017/4.6 for PSU).

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Biology of Triacylglycerol Accumulation by *Rhodococcus*

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_11

Abstract Members of the genus *Rhodococcus* are specialist in the accumulation of triacylglycerols (TAG). Some of them can be considered oleaginous microorganisms since they are able to produce significant amounts of those lipids under certain conditions. In this context, *R. opacus* strain PD630 and *R. jostii* RHA1 became models among prokaryotes in this research area. The basic knowledge generated for rhodococci could be also extrapolated to related microorganisms with clinical importance, such as mycobacteria. The biosynthesis and accumulation of TAG by species of the genus *Rhodococcus* 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 TAG can be controlled by the composition of the carbon source used. The biosynthesis and accumulation of novel TAG containing unusual components, such as aromatic and isoprenoid fatty acids, by members of *Rhodococcus* and related genera has been reported. The low specificity of wax ester synthase/diacylglycerol acyltransferase enzymes (WS/DGAT), 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 an important role of these lipids in the physiology of these microorganisms. Genomic, transcriptomic, and proteomic data from TAG-accumulating rhodococci are now available, and some genes coding for enzymes of the central metabolism, the Kennedy pathway, lipid transporter proteins, structural lipid inclusion body-associated proteins, and transcriptional regulatory proteins have been identified and characterized. This article aims to summarize the most relevant achievements of basic research in this field, including the most recent knowledge emerged from studies on TAG accumulation by rhodococci.

1 Introduction

Triacylglycerols (TAG) 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 (PHA) mainly function as a carbon and energy-reserve material in most bacteria (Anderson and Dawes 1990; Steinbüchel 1991). PHA 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 PHA (Steinbüchel 1991; Steinbüchel and Valentin 1995). Despite the wide occurrence of PHA among prokaryotes, TAG also occurs as storage lipids in several groups of prokaryotes (Alvarez and Steinbüchel 2002; Alvarez 2006).

Within the last decades, the reports on new TAG-accumulating bacteria have been considerable increased. Gram-negative bacteria are able to accumulate neutral

lipids composed of wax esters (WS) as main lipids and TAG only as minor components. WS and TAG 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 non-sporulating 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 TAG as intracellular inclusions. The majority of the published research on basic aspects of bacterial TAG 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 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 for environmental biotechnology purposes, respectively.

In this review, we will summarize the current knowledge on the TAG metabolism, physiology, and molecular biology in members of the *Rhodococcus* genus.

2 Triacylglycerol Accumulation by Species of the Genus *Rhodococcus*

The ability to accumulate TAG is a widespread feature among 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 TAG in the cells after cultivation on gluconate and other substrates (Alvarez et al. 1996). Figure 1 shows a cell of strain PD630 containing several TAG granules in the cytoplasm. Voss and Steinbüchel (2001) used *R. opacus* strain PD630 for high cell density cultivation to obtain high concentrations of TAG 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 the TAG accumulation by *R. opacus* and *Gordonia* sp. from agro-industrial wastes, such as carob and orange wastes and sugar cane molasses. *R. opacus* was also able to produce high yields of cell biomass and lipids from whey, which is a waste of the

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*	
	Whey	45.1	Herrero and Alvarez (2016)
	Lactose	38.0	
	Galactose	36.7	
R. opacus MR22 (DSMZ 3346)	Gluconate	48.0	Alvarez et al. (1997b)
	Hexadecane	43.0	
	Valerate	42.5	
	Whey	46.1	Herrero and Alvarez (2016)
	Lactose	36.5	
	Galactose	35.2	
R. jostii RHA1	Gluconate	56.9	Hernández et al. (2008)
	Glucose	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. fascians F7	Glycerol	44.6	Herrero et al. (2016)
R. erythropolis DSMZ 43060	Gluconate	21.0	Alvarez et al. (1997b)
	Hexadecane	17.6	
	Valerate	15.1	

(continued)

Table 1 (continued)

Bacterial strains	Carbon source	TAG content[a]	References
R. erythropolis 17	Gluconate	7.7	Alvarez (2003)
	Pentadecane	56.8	
	Hexadecane	43.4	
R. aetherivorans IAR1	Toluene	24.0	
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	
Rhodococcus sp. A27	Fructose	30.0	Röttig et al. (2016)
R. rhodochrous	Glucose	50.0	Shields-Menard et al. (2015)
R. corynebacterioides DSM 20151	Glucose	9.2	Bequer Urbano et al. (2013)
	Hexadecane	17.9	
Rhodococcus sp. A5	Glucose	11.5	Bequer Urbano et al. (2013)
	Hexadecane	35.3	
R. opacus B4	Hexadecane	140.0*	Castro et al. (2016)

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

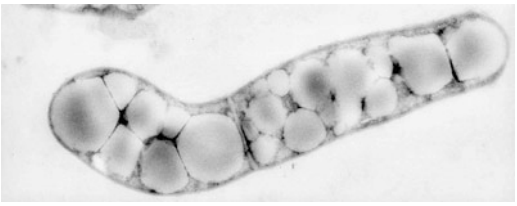

Fig. 1 Micrograph showing a cell of *Rhodococcus opacus* PD630 containing several TAG granules during growth on gluconate as sole carbon and energy source. Picture: F. Mayer from the Georg-August University of Göttingen, Germany

dairy industry generated worldwide in enormous quantities (Herrero and Alvarez 2016). On the other hand, lubricant-based wastewater was successfully used as sole carbon source by *R. opacus* PD630 for its conversion into TAG (Da Silva et al. 2016). All these studies demonstrated that cultivation of rhodococci on a cheap residual carbon source from agricultural or industrial products could be applied to the biotechnological production of interesting single cell oils and probably other lipid-derived products as well.

In addition to TAG, 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). Rhodococci are able to accumulate PHA containing short-chain length monomer units, such as 3-hydroxybutyric acid (C_4) (3HB) and/or 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 accumulated by most rhodococci, with the exception of *R. ruber* and the related *Nocardia corallina*, which produced large amounts of both storage lipids, TAG 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).

Hernández et al. (2008) reported the occurrence of glycogen in *R. jostii* cells during growth on gluconate, in addition to TAG and PHA. The accumulation of glycogen seems to be a usual feature among rhodococci, since this material has been also identified in cells of *R. erythropolis*, *R. fascians*, *R. opacus*, and *R. equi* (Hernández and Alvarez 2010). These rhodococcal species were able to produce glycogen up to 0.2–5.6% of cellular dry weight principally during exponential growth phase.

3 Composition and Structure of Rhodococcal Triacylglycerols

Rhodococci are able to produce a diversity of TAG with a high variability of fatty acid composition depending of the carbon source used for cell cultivation. Chemical analyses of TAG 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 non-related 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 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 TAG compared to lipids occurring in cells cultivated on glucose or gluconate (Alvarez et al. 1997b; Alvarez 2003). The mentioned strains possess 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 TAG 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 TAG 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 TAG or wax esters. *Nocardia globerula* strain 432 accumulated TAG 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 *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 sole carbon source. After 6 days of incubation, a mixture of novel TAG 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 be also 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 TAG during cultivation on gluconate, thus producing a cocoa butter-like oil containing about 74% saturated fatty acids with a relative high content of stearic acid (>18%) (Wältermann and Steinbüchel 2000). All these results demonstrated that the content and composition of rhodococcal TAG can be influenced by the carbon source used for 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 TAG 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 TAG (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 TAG 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 TAG 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 N source is 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 TAG principally during the stationary growth phase. This is logic 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, which 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 TAG (Alvarez et al. 2000). This indicated that TAG 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 and Alvarez 2018). 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 non-replicative drug-resistance 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, TAG 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 knowledge obtained on the biochemistry of TAG biosynthesis in rhodococci is still fragmentary, some generalizations can be made in this section based on experimental and genomic data. In this section, we subdivide the biosynthesis of TAG 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 TAG requires an efficient metabolic network able to produce 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 ATP. For more

detailed information on the central metabolism of rhodococci see Chapter "Central Metabolism of Species of *Rhodococcus* Genus" by Hernández et al. in this volume. However, many bacteria, which are not able to accumulate TAG, 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 produce 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, *R. opacus* PD630, and *R. opacus* B4, may be involved in the maintenance of the intracellular pools of acetyl-CoA and acetyl-P in these microorganisms. 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 acid containing odd number of carbon atoms may account 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 glycolytic routes, among other possible ATP-generating reactions. One of the sources of reducing equivalents for fatty acid biosynthesis in rhodococci (at least in *R. jostii* RHA1) is the Entner-Doudoroff (ED) pathway, which was significantly up-regulated during TAG-accumulation

conditions (Dávila Costa et al. 2015; Juarez et al. 2017). Enzymatic analyses also demonstrated the induction of ED enzymes in strain RHA1 during lipogenesis (Juarez et al. 2017), suggesting that ED pathway might be one potential source of NADPH. On the other hand, MacEachran and Sinskey (2013) demonstrated that the NADPH-depending reaction catalyzed by the non-phosphorylative glyceraldehyde dehydrogenase enzyme (GAPN) provides reducing equivalents for fatty acid biosynthesis in *R. opacus* PD630. GAPN catalyzes the reversible oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate with the production of NADPH, which can be used for lipid synthesis. Malic enzyme might also be involved in the generation of NADPH in oleaginous rhodococci (Table 2).

Proteomic studies performed in *R. jostii* RHA1 revealed an intensive metabolic reorganization to generate an oleaginous physiological state, which includes the activation of the ED pathway, glycogen mobilization, induction of glyceroneogenesis to generate glycerol-3-phosphate precursor, degradation of amino acids to produce acetyl-CoA, propionyl-CoA, and NADPH, and inhibition of L-ectoine biosynthesis, which consumes acetyl-CoA and reducing equivalents, among other changes (Dávila Costa et al. 2015).

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 PHA and TAG 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 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 TAG 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 ACC 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 complex is formed by three functional components, such as biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase (Cronan and Waldrop 2002). In general, the ACC complex 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. Interestingly, *R. jostii* RHA1 possesses a eukaryotic-like ACC with multiple domains (RHA1_RS20530), which increased its abundance 1.9-fold during cultivation under TAG-accumulating conditions as revealed by proteomic analyses

Table 2 Enzymes/proteins related to TAG accumulation identified and characterized in TAG-accumulating rhodococci

Strain and protein ID	Protein name	Function	Source
Central metabolism			
Rhodococcus opacus PD630			
OPAG_03892 (TadD)	Non-phosphorylative glyceraldehyde dehydrogenase	Oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate	MacEachran and Sinskey (2013)
Rhodococcus jostii RHA1			
RHA1_RS44255	NADPH-dependent malic enzyme	Decarboxylation of malate to pyruvate	Hernández and Alvarez (2018)
Rhodococcus fascians F7			
ACG96_05175 (GlpK1)	Glycerol kinase	Transfer of a phosphate from ATP to glycerol	Herrero et al. (2016)
ACG96_05170 (GlpD1)	Glycerol-3-phosphate dehydrogenase	Reversible conversion of dihydroxyacetone-P to glycerol-3-P	Herrero et al. (2016)
Fatty acid synthesis			
Rhodococcus opacus PD630			
OPAG_00508	Thioesterase	Release of fatty acid from acyl-ACP	Huang et al. (2016)
Kennedy pathway (TAG synthesis)			
Rhodococcus opacus PD630			
OPAG_07257 (Atf1)	WS/DGAT	Acylation of DAG	Alvarez et al. (2008)
OPAG_00138 (Atf2)	WS/DGAT	Acylation of DAG	Hernández et al. (2013)
Rhodococcus jostii RHA1			
RHA1_RS00400 (PAP2)	Phosphatidic acid phosphatase	Synthesis of DAG from phosphatidic acid	Hernández et al. (2015)
RHA1_RS26160 (Atf8)	WS/DGAT	Acylation of DAG	Amara et al. (2016)
Lipid transporters			
Rhodococcus jostii RHA1			
RHA1_RS27545 (Ltp1)	ABC transporter protein	Importer of fatty acids	Villalba and Alvarez (2014)
Lipid inclusion body-associated proteins			
Rhodococcus opacus PD630			
OPAG_00658 (TadA)	Structural Protein	TAG body-associated protein	MacEachran et al. (2010)
Rhodococcus jostii RHA1			
RHA1_RS10270 (TadA/MLDS)	Structural protein	TAG body-associated protein	Ding et al. (2012)

(continued)

Table 2 (continued)

Strain and protein ID	Protein name	Function	Source
Transcriptional regulator proteins			
Rhodococcus jostii RHA1			
RHA1_RS31140 (NlpR)	Regulatory protein	Modulate gene expression	Hernández et al. (2017a)
RHA1_RS10275 (MLDSR)	Regulatory protein	Modulate gene expression	Zhang et al. (2017)
Rhodococcus opacus PD630			
OPAG_03371 (NlpR)	Regulatory protein	Modulate gene expression	Hernández et al. (2017a)

WE wax esters, *TAG* triacylglycerols, *DAG* diacylglycerols, *WS* wax ester synthase, *DGAT* diacylglycerol acyltransferase, *P* phosphate

(Dávila Costa et al. 2015). The activity of this ACC, which may be involved in fatty acid biosynthesis in strain RHA1 during TAG accumulation, seemed to be controlled at the transcriptional level. However, redox proteomic analyses suggested that the activity of this eukaryotic-like ACC may also be finely modulated by redox status of the cell (Dávila Costa et al. 2015). The role of this ACC in fatty acid and TAG synthesis and accumulation in *R. jostii* RHA1 should be investigated in the future.

The biosynthesis of fatty acids is performed by a multienzymatic complex known as fatty acid synthase (FAS). This complex catalyzes the successive reaction of condensation, dehydration, and reduction. Two alternative FAS complexes exist in organisms. The FAS type II is present in most prokaryotes, and some eukaryote organelles, such as mitochondria and chloroplasts, consist of independent proteins encoded by different genes (Bloch 1977). In contrast, the FAS type I consists in a unique large protein with the different catalytic activities. FASI enzymes are found in the cytoplasm of eukaryotic cells and in a subgroup of actinobacteria. FASI 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 FASII (Bloch 1977; Zimhony et al. 2004). FASII uses medium-chain length fatty acids (C_{16}–C_{24}) as primers for synthesizing long-chain length mycolic acids (Shweizer and Hofmann 2004). The FASI multienzyme gene of mycobacteria and rhodococci seems to be structurally very similar. All rhodococcal enzymes are similar in size and amino acid sequences, comprising 3128 amino acids in *R. jostii* RHA1 (RHA1_RS06915), 3107 in *R. opacus* B4 (*ROP_11350*), 3100 in *R. erythropolis* PR4 (*RER_38730*), and 3103 in *R. erythropolis* SK121 (*RHOER0001_5412*), among others. The main products of rhodococcal FASI may be C_{16}–C_{18} fatty acids, which may be utilized for phospholipids and TAG biosynthesis. Proteomic analyses indicated that the FASI system increased 3.9-fold its abundance in *R. jostii* RHA1 cells under TAG-accumulation conditions (Dávila Costa et al. 2015). In addition, three components of the FASII system, such as malonyl-CoA-[acyl-carrier protein] (ACP) transferase, β-ketoacyl-[ACP] reductase,

and β-ketoacyl-[ACP] synthase, also increased their abundances during lipogenesis. The contribution of FASII to TAG accumulation by strain RHA1 and other rhodococci must be investigated in the future.

Thioesterases (TE) are enzymes that play an essential role in fatty acid biosynthesis, hydrolyzing the thioester bond between a carbonyl group and a sulfur atom of acyl-ACP to release the fatty acids. They are then converted to fatty acyl-CoAs that are further transformed to TAG and other lipids in cells. Thus, production and composition of fatty acids are determined by acyl-ACP TEs. Huang et al. (2016) reported the occurrence of four genes coding for putative TE enzymes in *R. opacus* PD630. After a molecular and physiological characterization of these genes/enzymes, the authors concluded that the putative acyl-ACP TE2 (OPAG_00508) and TE4 (WP_012687673.1) contribute to the production of total fatty acids and also have a specific influence on the fatty acid composition in *R. opacus* PD630 (Huang et al. 2016). However, the role of TE1 (EHI47208.1) and TE3 (WP_005241865.1) in the lipid content and fatty acid composition could not be determined in strain PD630.

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). 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, thus, the final acyl composition of TAG. 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 enzyme (DGAT). Kalscheuer and Steinbüchel (2003) identified as 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 TAG 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

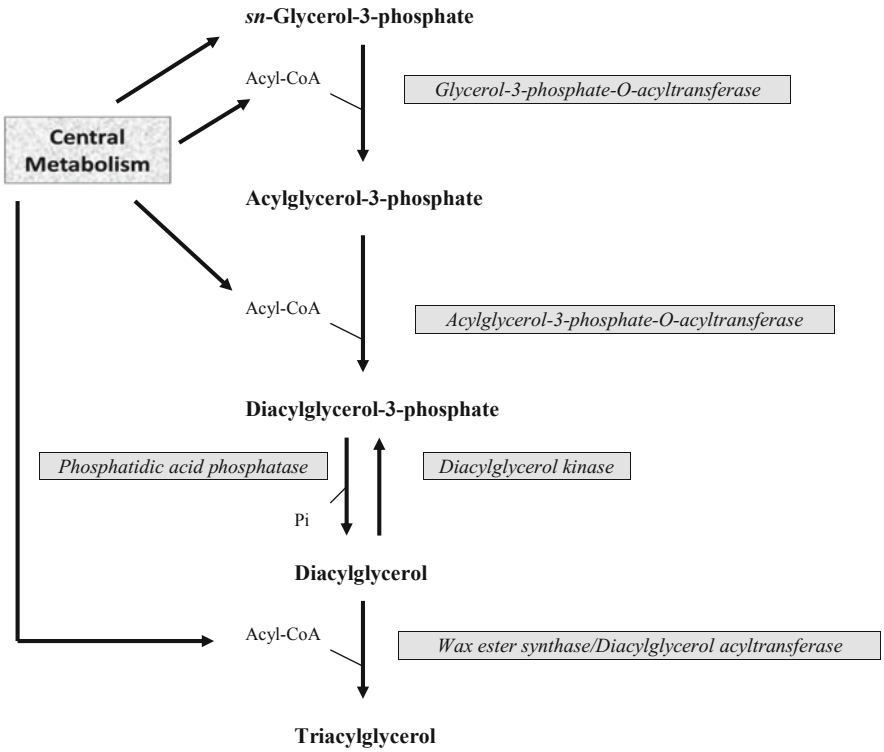

Fig. 2 Pathway for TAG biosynthesis (Kennedy pathway) in rhodococci

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 able to produce 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 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 3948 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 is mainly responsible for TAG biosynthesis in *R. opacus* PD630, it was clear that additional WS/DGAT contributes to the total DGAT activity and TAG content in this strain. Interestingly, TAG accumulated by the *atf1*-disrupted mutant showed a significant reduction of oleic acid content in comparison to TAG 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 TAG (Alvarez et al. 2008). When the genome database of *R. jostii* RHA1 was publicly available, nine additional *atf*-homologous genes were identified in strain PD630 (*atf2*–*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). 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*–*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). The gene coding for Atf2 from *R. opacus* PD630 was also cloned and characterized. The disruption of *atf2* gene resulted in a decrease of approximately 30% of TAG content and in any evident modification in the fatty acid composition of lipids in comparison to the wild-type PD630 (Hernández et al. 2013). The results of that study demonstrated an active role of Atf2 in the TAG accumulation process in *R. opacus* PD630 (Table 2).

R. jostii RHA1 is also able to accumulate significant amounts of TAG, in addition to other storage compounds, such as PHA, glycogen, and polyphosphate (Hernández et al. 2008). This strain possesses all necessary genes/enzymes for TAG biosynthesis via the Kennedy pathway. Amara et al. (2016) reported the occurrence of 16 *atf* genes potentially encoding DGAT enzymes in *R. jostii* RHA1. Transcriptomic analyses revealed that the *atf8* transcripts were the most abundant during cultivation of cells under lipid-accumulating conditions. Interestingly, the disruption of *atf8* promoted a 70% decrease in TAG accumulation when compared to the wild-type strain (Amara et al. 2016). These results suggested that Atf8 was the main DGAT enzyme involved in TAG accumulation by *R. jostii* RHA1 under the conditions used in the study. The WS/DGAT genes of strain RHA1 are not located in 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; Amara et al. 2016). However, some of the 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 varying numbers of putative WS/DGAT genes in the genome of different species, such as *R. opacus*, *R. equi*, *R. jostii*, *R. fascians*, and *R. erythropolis* (Villalba et al. 2013). The WS/DGAT gene number found in the rhodococcal genomes seems to be a strain-dependent feature.

Phosphatidic acid phosphatase (PAP) is other key enzyme involved in the Kennedy pathway for phospholipids and TAG synthesis. Bioinformatic analysis of the *R. jostii* RHA1 genome showed the occurrence of several genes coding for putative PAP proteins (Hernández et al. 2015). A similar situation was observed in the genomes of *R. opacus* and *R. erythropolis*. The high diversity of PAP enzymes in rhodococci suggests differing or specialized functions of these enzymes within lipid metabolism for adapting cells to diverse environmental conditions. The functional role of RHA1_RS00400 as a PAP enzyme was analyzed by cloning and expressing its gene in *E.coli* and *R. jostii* RHA1. Results of this study demonstrated that RHA1_RS00400 play an active role in TAG biosynthesis and accumulation in strain RHA1 catalyzing the desphosphorylation of phosphatidic acid to yield diacylglycerol (DAG), which is the direct precursor for TAG synthesis in rhodococci (Fig. 2). Interestingly, Amara et al. (2016) suggested that three genes encoding phosphatases of the haloacid dehalogenase superfamily, which were significantly expressed during lipid accumulation, can catalyze this enzymatic step in TAG biosynthesis pathway in *R. jostii* RHA1.

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

6 Transporter Proteins Related with TAG Accumulation

Lipid transporters may play a key role in the maintenance of the lipid homeostasis of oleaginous rhodococci. Villalba and Alvarez (2014) identified and characterized a novel ATP-binding cassette transporter involved in long-chain fatty acid import in *R. jostii* RHA1 named as Ltp1. Interestingly, *ltp1* gene was clustered with other genes coding for the three putative acyltransferase enzymes of the Kennedy pathway for TAG synthesis. Results of this study suggested that Ltp1 transporter plays a role in lipid homeostasis in rhodococcal cells and in the distribution of fatty acids between different metabolic pathways and lipid species, since overexpression of *ltp1* in the RHA1 promoted a significant increase of TAG and cellular biomass formation (Villalba and Alvarez 2014). The study demonstrated the relevance of lipid transporters as molecular tools for improving TAG accumulation through genetic engineering in rhodococci (Table 2).

7 TAG Inclusion Bodies and Their Associated Proteins

Detailed studies on the formation of lipid inclusions in bacteria have been made in *Acinetobacter 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 had 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 beside the other necessary compounds (substrates, etc.) used in combination with a quartz crystal microbalance in combination with scanning force microscopy. The changes of the frequency of the quartz crystal and the changes at the surface, respectively, could be interpreted as similar steps occurring in vitro (Wältermann et al. 2005).

There are evidences that lipid bodies are surrounded by a half-unit membrane of phospholipids with several proteins associated. In this context, Chen et al. (2014) performed an integrated omic study including genome and transcriptomic analyses and a proteome of isolated lipid inclusions from *R. opacus* PD630. They identified 177 proteins involved in lipid metabolism and lipid body dynamics. Among several enzymes and proteins, the authors identified a dominant structure-like protein (LPD06283) and several dynamin and SNARE-like proteins probably involved in lipid body dynamics. LPD06283 is identical to TadA of strain PD630 and its

orthologue RHA1_RS10270 from *R. jostii* RHA1. TadA had been previously identified and characterized by MacEachran et al. (2010) as a lipid body-associated protein in *R. opacus* PD630. Gene disruption or overexpression promoted alterations in the size of the inclusion bodies and in TAG content in cells. Its orthologue in *R. jostii* RHA1 (RHA1_RS10270) was identified as one of the major proteins co-purified with the lipid inclusion bodies (Ding et al. 2012) (Table 2). Deletion of this gene induced the formation of super-sized lipid bodies in strain RHA1. Proteomic analyses performed with this strain revealed that RHA1_RS10270 was one of the three most abundant proteins during TAG accumulation (Dávila Costa et al. 2015). Interestingly, Zhang et al. (2017) proposed that this inclusion body-associated protein (TadA) increases survival rate of cells under nutritional and genotoxic stress. The authors renamed TadA protein as MLDS (microorganism lipid droplet small protein) (Table 2). The study demonstrated that TadA/MLDS binds DNA and recruits DNA to lipid inclusion bodies (Zhang et al. 2017). Protein binding to DNA occurs without sequence specificity through its positively charged C-terminus domain. The authors suggested that the association of DNA to lipid inclusion bodies mediated by the TadA/MLDS protein contributes to protection against environmental stresses, such as UV radiation.

More studies are necessary to understand the biogenesis and the structural and functional dynamics of TAG inclusion bodies in rhodococci.

8 Regulation of Lipid Accumulation in *Rhodococcus*

Several genes and proteins are required for the transition from vegetative growth to oleaginous phenotype. The control of metabolic transition between these physiological states might require a complex regulatory network involving pleiotropic global regulators and other regulatory proteins working at different hierarchical levels. The first regulatory protein leading to activation of lipogenesis and TAG accumulation was identified and characterized in *R. jostii* RHA1 (Hernández et al. 2017a). The transcriptional regulator NlpR (Nitrogen Lipid Regulator) participates in the modulation of nitrogen and lipid metabolisms in response to nitrogen limitation in the environment. More specifically, NlpR contributes in *R. jostii* RHA1 and also in *R. opacus* PD630 to the allocation of carbon into the different lipid fractions, such as TAG, DAG, fatty acids, and phospholipids, in response to nitrogen levels, increasing the rate of carbon flux into lipid metabolism (Hernández et al. 2017b). NlpR, which seems to be part of GlnR regulon, is significantly up-regulated during cultivation of cells under nitrogen-limiting conditions (Hernández et al. 2017a). This regulatory protein is a global regulator that controls the expression of several genes involved in nitrogen and lipid metabolisms, such as those genes implicated in the NO_3^-/NO_2^- assimilation, fatty acid synthesis (FASI and FASII), the Kennedy pathway for TAG, and phospholipid synthesis, among others. Thus, NlpR modulates large modules of lipid metabolism controlling the carbon flux within the module in response to nitrogen limitation. However, *nlpR* gene is not essential for lipid synthesis and

accumulation since *nlpR*-disrupted mutant is still able to accumulate significant amounts of TAG (Hernández et al. 2017a, b).

These results suggested that additional regulatory components are simultaneously involved in TAG accumulation during nitrogen-limiting conditions. In this context, Zhang et al. (2017) reported the characterization of a transcriptional regulatory protein (MLDSR) in *R. jostii* RHA1, which regulates the expression of gene coding for the MLDS protein described above (Sect. 7) as a lipid inclusion body-associated protein. MLDS seems to participate in the TAG accumulating dynamics and lipid inclusion body biogenesis in rhodococcal cells. The MLDSR protein is induced at low nitrogen conditions, stimulating the expression of its own gene and *mlds* gene. Nevertheless, MLDSR is able to regulate transcription both positively and negatively (Zhang et al. 2017). At low concentration of MLDSR, it positively regulates expression of *mlds* gene and itself, whereas when MLDSR becomes high, this protein represses the expression of these genes. MLDSR concentration in cellular cytosol seems to be controlled by the lipid inclusion body recruitment, thus modulating MLDS expression.

Juarez et al. (2017) suggested the involvement of a regulatory mechanism mediated by the cAMP-dependent CRP regulator for TAG accumulation in *R. jostii* RHA1. They identified putative CRP-binding sites in some genes significantly up-regulated during TAG accumulation, such as those of the Entner-Doudoroff pathway and the WS/DGAT Atf8, which is the main WS/DGAT enzyme involved in TAG accumulation in *R. jostii* RHA1 according to a previous study (Amara et al. 2016). These results suggested that TAG accumulation in strain RHA1 is probably driven by an increase of cAMP concentration in cells that activates the expression of genes involved in lipogenesis. However, this hypothesis should be experimentally confirmed.

Proteome analyses revealed differential expression of proteins involved in TAG accumulation in *R. jostii* RHA1 after addition of methyl viologen (MV), a potent prooxidant (Dávila Costa et al. 2017). The presence of MV in the culture medium promoted a significant decrease in the abundance of key proteins of fatty acid synthesis and the Kennedy pathway for TAG biosynthesis under nitrogen-limiting conditions in comparison to those cells cultivated at the same conditions in the absence of MV. These results suggested the occurrence of a NADPH-mediated process regulating TAG accumulation in *R. jostii* RHA1, since the antioxidant response against MV competes with lipogenesis for NADPH pools. Finally, redox proteomic analyses suggested that the activities of some key enzymes probably involved in lipogenesis, such as the eukaryotic-like acetyl-CoA carboxylase, FabF, and fructose 1,6-biphosphatase, are regulated posttranscriptionally by reversible thiol modifications in response to changes in the redox status of rhodococcal cells (Dávila Costa et al. 2015). These results suggested that TAG accumulation by oleaginous rhodococci could be regulated not only at transcriptional level but also by posttranscriptional mechanisms (Fig. 3). Indeed, we still have a very limited understanding on how TAG accumulation is controlled at molecular and metabolic level in rhodococci.

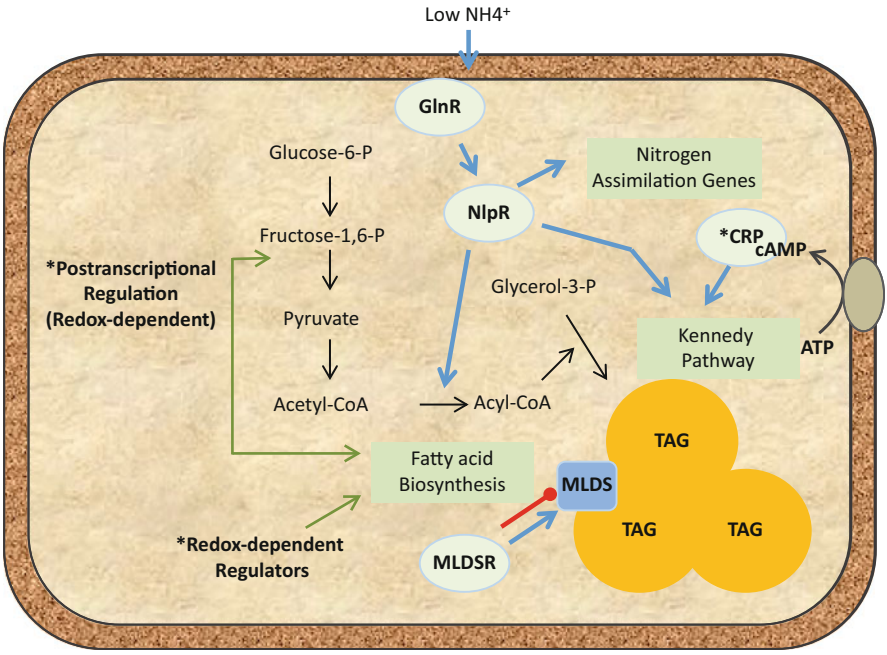

Fig. 3 Some components of the regulatory network controlling TAG biosynthesis and accumulation in oleaginous rhodococci. Blue arrows indicate activation, red bars indicate repression, and green arrows indicate control of gene expression or enzyme activities by unknown mechanisms (activation or repression). During growth under nitrogen-limiting conditions, the global regulator NlpR is probably activated via GlnR. NlpR positively modulates the expression of genes involved in nitrogen assimilation and lipid metabolism. The transcriptional regulator MLDSR activates the expression of *mlds* gene, which codes for a lipid inclusion body-associated protein and itself. At high concentrations of MLDSR in the cytosol, this protein represses expression of the respective genes. Adenylate cyclases generate cAMP which binds to CRP protein. Activated CRP probably modulates the expression of genes involved in TAG biosynthesis. Additional redox-dependent regulatory mechanisms at transcriptional and posttranscriptional level are probably involved in the control of TAG accumulation in rhodococci. (*) Only indirect evidences are available and experimental confirmation is necessary

9 Physiological Functions of TAG in *Rhodococcus*

Rhodococcus species, which are enriched in a particular class of lipids, such as TAG, may be highly dependent on these compounds and their functions, for successful survival in the environment. In this context, TAG seem to play a key role for the cells under growth-restricting conditions that frequently predominate in the environment (Fig. 4).

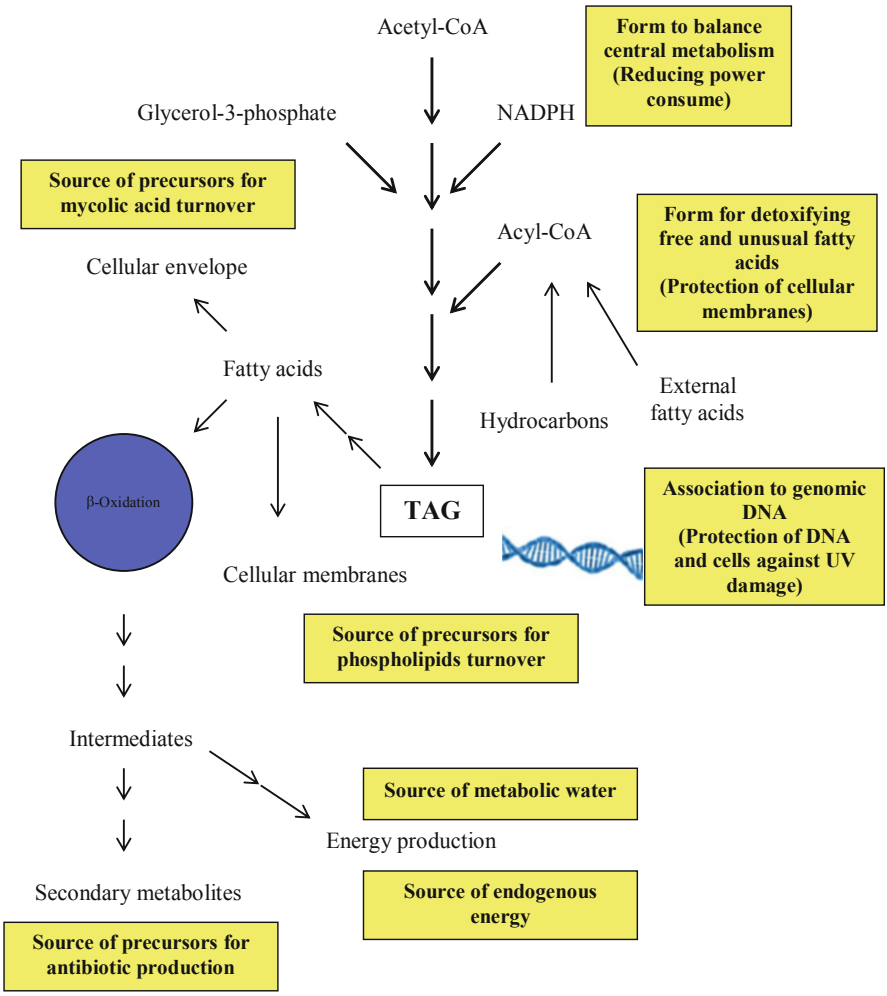

Fig. 4 Physiological functions of TAG proposed for TAG-accumulating rhodococci

9.1 TAG as Endogenous Carbon and Energy Sources

Rhodococci have been detected in different natural environments, such as tropical, arctic, and arid soils, as well as 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 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 other 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 adaptation to environments with poor nutritional conditions and tolerance to other extreme stresses. The accumulation of significant amounts of TAG by rhodococci is a carbon-intensive and energy-demanding process, which compete with cell growth. Thus, the occurrence of such storage compounds in microorganisms which habits energy-poor environments must be an important feature for their physiology. It is known that TAG are excellent reserve materials due to their extremely hydrophobic properties, which allow their accumulation in large amounts in cells without changing the osmolarity of cytoplasm. In addition, oxidation of TAG produce the maximum yields of energy in comparison with other storage compounds such as carbohydrates and PHA, since the carbon atoms of the acyl moieties of TAG are in their most reductive form (Alvarez and Steinbüchel 2002). Previous studies revealed that TAG 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 TAG 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 TAG as a storage form of energy for its long-term survival under dormancy (Daniel et al. 2004). This microorganism survives for decades within the host in a state of non-replicative, drug resistance dormancy. This state results probably in a diminution in basal metabolic rate, which facilitates survival of cells at expenses of the accumulated TAG.

In addition, TAG 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 (Hauschild et al. 2017). Different rhodococcal strains with the ability to accumulate TAG were isolated from desert soil samples (Bequer Urbano et al. 2013; Röttig et al. 2016). In this context, TAG mobilization enhanced cell survival of the indigenous *Rhodococcus* sp. A5 during cultivation under carbon starvation and desiccation conditions (Bequer Urbano et al. 2013).

9.2 TAG as Source of Precursors for Membranes and Cell Envelope

TAG 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. 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 pre-treated with isoniazid (40 μg/mL) exhibited lower survival percentages, which were approximately 18% less than those of non-treated cells after 22 days under dehydration conditions. These results suggested that mycolic acid turnover using the pre-formed fatty acids contained into TAG, contributed to cell envelope adaptation under water stress conditions in *R. opacus* PD630 (Alvarez et al. 2004).

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

So far there is only indirect evidence on the role of TAG as source of precursors, such as pre-formed 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.

9.3 TAG 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 TAG may be a form to detoxify 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 considering 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 TAG 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 TAG containing medium-chain length fatty acids (C_8–C_{12}) after cultivation on naphthyl-1-dodecanoate as sole carbon source (Silva et al. 2010). All these results suggest that TAG 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.

9.4 TAG as a Form to Balance Central Metabolism

The biosynthesis of TAG by rhodococci may be also a form 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 2018). When the terminal electron acceptor is not sufficiently supplied during cultivation of cells under conditions of limited aeration, TAG may serve as a sink for reducing equivalents in cells. Under oxygen-limiting conditions, the excess of 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 TAG allows cells to balance their metabolism according to the changes of environmental conditions (Alvarez and Steinbüchel 2002; Alvarez 2006).

9.5 TAG as Source of Intermediates for Secondary Metabolism

There is some evidence that TAG may serve as source of intermediates for the synthesis of compounds, which are not essential for growth but for survival of cells in the environment. Some authors demonstrated that TAG 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, TAG may serve as a source of intermediates for the biosynthesis of the extracellular polymeric substance (EPS) produced as response of 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 any external carbon source was available, cells must produce the protective EPS using an endogenous carbon and energy source, such as TAG, among other possible.

9.6 TAG Seem to Protect Cells Against UV Radiation

There are evidences that TAG contribute to withstand the stress exerted by UV radiation in rhodococci. The inhibition of TAG mobilization by the addition of Orlistat promoted a dramatic decrease in cell survival after UV radiation of rhodococcal cells (Bequer Urbano et al. 2013). It has been proposed that the inhibition of TAG degradation by the addition of the metabolic inhibitor might generate an imbalance of the NADPH/NADH ratio affecting the functionality of defense mechanisms against oxidative stress. Interestingly, Zhang et al. (2017) demonstrated that the lipid inclusion body-associated protein (MLDS) provides a survival advantage to bacterial cells under UV-mediated stress by interaction of inclusion bodies with genomic DNA. The authors suggested that the association of lipid droplets with DNA promoted a protection of cells in *R. jostii* RHA1 from UV damage (Zhang et al. 2017). Altogether, these studies suggested that TAG metabolism influences the responses of rhodococci to UV-mediated stress.

10 Biotechnological Significance of Rhodococcal TAG

The world is currently facing a severe energy crisis. On the one side, the known and accessible sources of crude oil and other fossil resources are being slowly but continuously depleted, and on the other side, the demand for fossil resources is rising due to a continuing global industrialization in particular also in countries with large populations like 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 that is currently mainly produced from liquefied corn starch or sugar cane in particular in North and South America, respectively. TAG are currently produced

at large scale by agriculture for synthesis of fatty acid methyl esters. They are currently in Europe the preferred products from renewable resources and are referred to as "Biodiesel." Biodiesel is produced from synthetic methanol of the chemical industry and from TAG by chemical transesterification yielding beside FAME about 10% (wt/wt) glycerol as a by-product (Röttig et al. 2010). Very little amounts of biodiesel are also enzymatically produced (Adamczak et al. 2009). Biodiesel and bioethanol are currently capturing about 90% of the biofuel market (Antoni et al. 2007; Uthoff et al. 2009).

TAG for biodiesel production are currently exclusively produced by agriculture; comparably very little amounts are obtained from the use of frying oil of fast-food restaurants. The main crops for TAG production are rapeseed in Europe, palm oil trees in Southeast Asia, and Soja in North America. TAG could, however, in principle also be produced in bacteria. One of the probably most suitable candidates is *R. opacus* due to its extraordinary high lipid content and the good growth of the cells. As already outlined in other parts of 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 liter and even of 500 liter (Voss and Steinbüchel 2001). Using a mineral salts medium 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 done 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 TAG from renewable resources. Production in bacteria gives a greater flexibility in comparison to 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 toward the utilization of such carbon and energy sources for growth and lipid production. This is important in order to avoid a competition between feed and food industry on one side and chemical and energy industry on the other side and also for a sustainable production of lipids and also to avoid a further increase of emission of greenhouse gases (Searchinger et al. 2008; Fargione et al. 2008). In this context, one of the biggest challenges at present is the degradation and conversion of lignocellulosic wastes into lipids by rhodococci. Microbial degradation of lignocellulosic material is almost exclusive to fungi. Kosa and Ragauskas (2012) reported the ability of *R. opacus* strains to convert 4-hydroxybenzoic and vanillic acid as lignin model compounds, into TAG using the β-ketoadipate pathway. In order to efficiently combine lignocellulose catabolism with TAG synthesis in rhodococci, the oxidative machinery of lignocellulose material should be improved by genetic and metabolic engineering. Different studies demonstrated that the production of TAG from the main components of lignocellulosic material is feasible. Xiong et al. (2012) reported the accumulation of lipids by engineered *R. jostii* RHA1 and *R. opacus* PD630 from xylose, an important component of lignocellulose, under nitrogen-limited conditions. The heterologous

expression of two genes from *Streptomyces lividans* TK23, *xylA* encoding xylose isomerase and *xylB* encoding xylulokinase, promoted not only growth but also lipid accumulation (52.5% in strain RHA1 and 68.3% in strain PD630 of CDW, respectively). In a different study, Hetzler and Steinbüchel (2013) conferred the ability to utilize cellobiose for growth and production of TAG to *R. opacus* PD630. In this study, recombinant PD630 accumulated fatty acids up to 39.5 ± 5.7% of CDW from cellobiose. *R. opacus* PD630 is not able to utilize L-arabinose present in lignocellulosic hydrolysates as sole carbon source for growth and TAG synthesis. For this reason, Kurosawa et al. (2015) introduced *araB*, *araD*, and *araA* genes derived from a *Streptomyces* species, into strain PD630. After 3 days of cultivation, recombinant cells produced 39.7% of CDW of lipids from L-arabinose. The oleaginous bacterium *R. jostii* RHA1 has also been engineered to utilize L-arabinose derived from lignocellulosic biomass for TAG accumulation. The heterologous expression of the operon, *araBAD*, and *araFGH* genes encoding the arabinose transporter from *Escherichia coli*, as well as the additional expression of *atf1* gene encoding a WS/DGAT from *R. opacus* PD630, promoted the production of 56.8% (CDW) of lipids in the recombinant *R. jostii* RHA1 (Xiong et al. 2016).

The ability of these oleaginous bacteria to convert organic compounds into lipids of interest in biotechnology can be extended to other industrial wastes, such as whey or glycerol. Herrero and Alvarez (2016) reported the efficient production of cellular biomass (6.1–6.3 g/L) and lipids (45–48% of CDW) from whey by different strains of *R. opacus*. On the other hand, *R. fascians*, *R. erythropolis*, and engineered cells of *R. opacus* were able to produce significant amounts of TAG from glycerol, which is the main by-product from biodiesel industry (Herrero et al. 2016).

The key enzyme of triacylglycerol or wax ester biosynthesis in bacteria is a novel type of an acyltransferase which was so far not known in other groups of organisms. They occur frequently in multiple copies in bacteria, and also *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 length 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), yet, data from preliminary enzymatic studies and from physiological experiments clearly indicate also for the acyltransferases of this bacterium a low substrate specificity. This makes the enzymes from *R. opacus* and *R. jostii* also to putative candidates for the synthesis of fine chemicals or oleochemicals comprising organic alcohols and thiols to which acyl moieties were covalently attached (Stöveken and Steinbüchel 2008).

11 Concluding Remarks

The accumulation of TAG is a common feature among rhodococci. Some of them can be considered as oleaginous microorganisms because they produce significant amounts of TAG as intracellular inclusion bodies. Although the knowledge acquired during the last decade about the production of TAG in rhodococci has been considerable, many fundamental aspects remain to be clarified. The understanding of this topic in rhodococci is important because two members of this genus, *R. opacus* PD630 and *R. jostii* RHA1, have been the preferred research models 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 relevant for the survival of this microorganism in the host cells and, thus, for the development of the disease. Therefore, TAG biosynthesis may be a new target for developing drugs to prevent this important disease. Basic knowledge on 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 the better understanding of their physiology and relation with the environment. TAG may permit cells to survive under fluctuating and unfavorable conditions as occur normally in natural environments. In this context, the occurrence of TAG 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 omic data bases of TAG-accumulating strains, will permit interesting advances in our understanding of the biology of *Rhodococcus* genus. Moreover, several studies have demonstrated the feasibility of producing interesting lipids from diverse industrial wastes by genetic modification of the rhodococcal species and strains.

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Interaction of *Rhodococcus* with Metals and Biotechnological Applications

Alessandro Presentato, Elena Piacenza, Martina Cappelletti, and Raymond J. Turner

Contents

Abstract In studies of environmental stresses caused by metals, *Rhodococcus* species are routinely identified as part of a beneficial microbial rhizosphere community. These bacterial strains, inhabiting diverse ecological niches, possess a variety of enzymatic activities to carry out relevant biodegradation reactions, such as degradation of organic pollutants in some cases using them for both carbon and energy. In this context, most *Rhodococcus* strains have been found to have very high

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_12

levels of metal resistance. Thus, these microorganisms are not only capable of metabolizing various organic pollutants in the presence of co-contaminating heavy metals, but they can also bioadsorb and/or bioconvert various metals and metalloids [metal(loid)s]. Indeed, some *Rhodococcus* exploit these metal(loid) compounds to generate biogenic nanoscale materials of intriguing physical-chemical properties, which can find applications in biotechnology.

This book chapter has the focus in overviewing the biotechnological relevance of the *Rhodococcus* genus relationship with metal(loid)s, the bioprocesses elicited by these microorganisms in handling metal(loid)s' toxicity, and the importance of these actinomycetes in the context of the bioremediation and bionanotechnology fields.

1 Introduction

Metal elements such as aluminum (Al), cadmium (Cd), cesium (Cs), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), silver (Ag), strontium (Sr), zinc (Zn), and uranium (U) and the metalloids arsenic (As), selenium (Se), and tellurium (Te) are naturally occurring throughout the Earth's crust, being defined "heavy" as both their atomic weight and density are higher than water (Fergusson 1990). Although some of these elements (e.g., Fe, Se, Zn, Cu) are essential in traces for living organisms, the overdose of these and exposure to some others (e.g., As, Pb, Hg, whose toxicity is undisputed) can be severely toxic (Chang et al. 1996; Tchounwou et al. 2008). Indeed, the global human concern toward these contaminants relies mostly on their mutagenicity and carcinogenicity, negatively impacting the quality of life of both flora and fauna in different habitats (i.e., aquatic, terrestrial, and atmospheric) (Chakraborty et al. 2017). This aspect is exacerbated by the intense industrial progress in several fields (i.e., engineering, biomedicine, agriculture, metallurgy, microelectronics, mining operations, etc.), as well as unjustified waste disposal processes, which inexorably add to environmental pollution. The result is that there are sites in almost every community on the planet with a buildup of substantial amounts of recalcitrance toxic metal(loid)s in diverse ecological niches (Chakraborty et al. 2017). In this context, a prolonged exposure to metal(loid) compounds is often associated to human poisoning, as well as to their biomagnification at successively high levels in the food chain (Phillips and Rainbow 2013).

Toxicity of a metal to an organism depends on a variety of physical-chemical and biological parameters influencing the metal ion speciation and bioavailability. Chemical parameters include temperature, pH, redox potential, and presence/absence of compounds capable of chelating or binding to the metal. Some biological parameters include the metal-lipid solubility, physiological state of the organism, presence/absence of key metal interacting proteins, and any physiological adaptation of the organisms. The combination of such parameters and their interplay contribute to the environmental resiliency of metal(loid)s (Hamelink et al. 1994). Focusing the attention at the molecular level, it is generally believed that proteins are the primary

targets of metal(loid)s. Indeed, malfunctioning of proteins in their native conformation can be attributed to (1) the binding of metals to protein ligands such as free thiols (RSHs), imidazoles, amines, or carboxylates, (2) the displacement of essential metal ions in metalloenzymes, (3) the oxidation of the amino acid side chains, and (4) their interaction with non-folded proteins causing their aggregation and, therefore, impairing protein homeostasis in living organisms (Beyersmann and Hartwig 2008; Sharma et al. 2011; Lemire et al. 2013).

As dramatic as the above picture may appear, human beings have evolved the concept of *environmental remediation*, which combines physical-chemical and biological knowledge for strategies aimed to decrease the concentration of metal (loid) compounds into the environment, restoring its ecological fitness. Overall, biological methods, exploiting the natural catabolic abilities of bacteria, fungi, and plants to clean up diverse contaminated matrices in a *green* and cost-effective manner (Strong and Burgess 2008), are generally preferred over physical-chemical approaches, as the latter often have cost-prohibitive and non-efficient results at low metal(loid) environmental load (Ali et al. 2013).

Aerobic non-sporulating microorganisms belonging to the *Rhodococcus* genus are known for their environmental robustness and persistence, as they can physiologically adapt (i.e., modification of the cell membrane composition, formation of intracellular inclusions) to several stressful conditions (e.g., pH, desiccation, low nutrient availability, presence of xenobiotic compounds, etc.) (Martínková et al. 2009; Alvarez and Steinbüchel 2010; Presentato et al. 2018a). This allows members of this genus to populate diverse ecological niches. Although much less is known about the *Rhodococcus* genus potential to resist toxic metal(loid) compounds, recently the unique capacity of some rhodococci to overcome environmental stresses like the presence of antimicrobials and very high concentrations of metal(loid)s such as tellurite [Te (IV)], arsenate [As (V)], and selenite [Se (IV)] has been described (Orro et al. 2015; Cappelletti et al. 2016; Presentato et al. 2016, 2018b). Such findings highlight how these microorganisms are worth exploring as candidates for bioremediation of metal(loid)-polluted sites. Further, it has been reported that bacteria are able to convert toxic metal(loid) ion/oxyanions into less/nontoxic metal forms, generating discrete structures at the nanometer scale (1×10^{-9} m) with different shapes, i.e., nanoparticles (NPs) and nanorods (NRs) (Turner et al. 2012). To this aim, different *Rhodococcus* strains have been studied and exploited as cell factories for nanotechnological purposes, proving the specific proficiency of rhodococci to biosynthesize high-quality biogenic metal(loid) nanostructures with relevant biotechnological applications (Ahmad et al. 2003; Otari et al. 2012; Kundu et al. 2014; Presentato et al. 2016, 2018b, c). Thus, this chapter will overview the interaction between *Rhodococcus* genus and metal(loid)s in terms of bacterial resistance and strategies adopted to counteract metal(loid) toxicity, as well as their remarkable ability in generating biogenic nanomaterials.

2 Microbial Interaction with Metal(loid)s

A large number of organisms are capable of thriving in the presence of high metal (loid) load, being this feature ascribed to intrinsic and/or induced biochemical mechanisms combined with environmental factors (Gadd 1992a). In this respect, bacteria are defined to be tolerant as they are not killed by the toxicity exerted by metal(loid) compounds through their intrinsic properties yet do not grow or proliferate, while those that are resistant show the ability to live because of the induction of detoxification mechanisms implemented in response to the presence of metal(loids) (Gadd 1992b). Bacteria have evolved uptake mechanisms in order to exploit diverse nutrients, which enable them to survive even under extreme and adverse environmental conditions (Brooks et al. 2011). Because microorganisms have coevolved with the geochemistry of the planet and inhabit all possible niches, many can utilize even toxic metal(loid) compounds (i.e., As, Cd, Te, Se, etc.) (Gadd 2010). For instance, bacterial cells can encounter metal(loid) ions from either aqueous or solid matrices, using them as (1) energy source, (2) terminal electron acceptor during microbial respiration, and (3) cofactors in metalloproteins and enzymes (Ehrlich 1997; Valls and de Lorenzo, 2002). Thus, microbial scavenging and biotransformation of metal(loid) elements can be related to either detoxification processes or resistance phenomena, which are carried out as parallel events related or not with bacterial growth (Park et al. 2018).

The different mechanisms exploited by microbes to cope with metal(loid)s' toxicity can rely on their transformation by means of either redox processes or alkylation reactions (Ledin 2000). Bacterial cells can sequester metal(loid) ions onto their cell wall either in a passive and metabolism-independent manner (biosorption) or through active mechanisms (bioaccumulation), which is a metabolism-dependent process based on the actual metal(loid) uptake (Ledin 2000). Further, metal(loid) ions can be trapped outside bacterial cells through interaction with (1) surface proteins, (2) carbohydrates, (3) cell wall polymers, and (4) extracellular polymeric substance (EPS) often produced when bacteria grow as a biofilm. Finally, there may be chelation (precipitation and/or complexation) through the production of either siderophores or specific metalloproteins (Gupta and Diwan 2017). All these strategies occur in the microbial world; hence, the exploitation of the microbial catabolic abilities represents a powerful tool to abate the metal(loid) load polluting the environment.

2.1 Rhodococcus *Tolerance/Resistance Toward Metal(loid)s*

Bacterial strains belonging to the *Actinomycetales* order, which include *Rhodococcus* genus, exploit different mechanisms enabling them to highly tolerate metal(loid) compounds (Pavel et al. 2013). For instance, the reduction of cellular sensitivity, the intracellular sequestration of metal ions and oxyanions, their complexation with siderophores, the alteration of the membrane permeability, mutations,

and repairing mechanisms of the DNA responsible for both plasmid and chromosomal DNA stability are some of the mechanisms implemented by bacteria to tolerate and/or resist to metalloids' toxicity (Stillman 1995; Garbisu and Alkorta 2003; Figueira et al. 2005). A primary mechanism through which metal(loid) compounds can exert their toxicity toward bacteria relies on the alteration of the cellular thiol chemistry. Indeed, the reaction of metal(loid)s with cell thiols leads to the depletion of glutaredoxin and thioredoxin from their activity of reducing metal (loid) compounds and becoming oxidized (Turner 2001). Thus, such reactions can cause an increase in the intracellular content of reactive oxygen species (ROS), which can ultimately cause cellular death (Turner et al. 1999). This process is even more emphasized if we consider the presence of oxygen as terminal electron acceptor in the case of strictly aerobic microorganisms, as in the case of *Rhodococcus* strains. This process is even more emphasized if we consider the presence of oxygen as terminal electron acceptor in the case of strictly aerobic microorganisms, such as in the case of *Rhodococcus* strains. However, instead of having the redox buffer molecule glutathione (GSH), actinomycetes are mostly characterized by the presence of genes involved in the synthesis of mycothiols (MSHs), which are more stable and less prone to oxidation (Newton et al. 1996). Thus, the potential of actinomycetes in resisting the toxicity exerted by metal(loid)s overall derives from the *redox stability* of these microorganisms. Rhodococci investigated for metal tolerance to date are listed in Table 1 along with their resistance levels. It is noteworthy to highlight that among the metal(loid) elements listed in Table 1, tellurium in the form of oxyanion tellurite (TeO_3^{2-}) is typically the most toxic, with minimal inhibitory concentrations (MIC) as low as 1 μg mL^{-1} (Taylor 1999). This concentration is several orders of magnitude lower than other metal(loid) compounds (Nies 1999; Harrison et al. 2004), and yet *R. aetherivorans* BCP1 strain showed to be highly resistant to this oxyanion (Presentato et al. 2016, 2018c).

In nature microorganisms are found in a close relationship to each other and adhering to a surface forming a complex community known as biofilm. Within a biofilm, the bacterial cells are overprotected by a heterogeneous surrounding matrix defined as extracellular polymeric substance (EPS), which is mostly constituted by water, proteins, polysaccharides, lipids, and extracellular DNA (e-DNA) (Harrison et al. 2005). Thus, a bacterium living within a biofilm has the advantage of resisting and adapting to harsh environmental conditions (this topic has been reviewed by Harrison et al. 2007). In line with this, Adhami et al. (2017) described two different *Rhodococcus* strains with an enhanced metal resistance against Cd, Cr, Cu, Pb, and Zn supplied as salts when these bacterial cells were grown as biofilms in comparison with those grown as planktonic cultures (Adhami et al 2017).

Table 1 Minimal inhibitory concentration (MIC) of different metal(loid)s of *Rhodococcus* species

MICs of metal(loid)s of *Rhodococcus* spp. (mM)										
Strain		Cd	Cr	Cu	Hg	Pb	Se	Te	Zn	References
R. RS67		ND[a]	ND	ND	ND	0.5	ND	ND	0.2	Perelomov et al. (2018)
R. rhodochrous		8	1	4	ND	8	ND	ND	8	Adhami et al. (2017)
	MBEC[b]	16	4	8	ND	16	ND	ND	16	
	MBIC[c]	4	0.5	1	ND	4	ND	ND	2	
R. rhodnii		8	1	4	ND	8	ND	ND	8	
	MBEC	16	4	8	ND	16	ND	ND	16	
	MBIC	4	0.5	2	ND	2	ND	ND	1	
R. aetherivorans BCP1		ND	ND	ND	ND	ND	500	11.2	ND	Presentato et al. (2016, 2018b, c)
R. strain 3		8–10	ND	8–16	ND	>16	ND	ND	8–16	Vela-Cano et al. (2014)
R. ruber	AC239	ND	>1.8	3.7	0.28	ND	ND	ND	ND	Fleck et al. (2000)
	AC74	ND	>1.8	1.5	0.17	ND	ND	ND	ND	
	AC87	ND	>1.8	3.5	0.28	ND	ND	ND	ND	
R. erythropolis	AC272	ND	>1.8	3	0.21	ND	ND	ND	ND	
	AC265	ND	>1.8	0.1	0.03	ND	ND	ND	ND	

[a]Not determined
[b]Minimum biofilm eradicating concentration
[c]Minimum biofilm inhibitory concentration

3 Metal(loid) Biosorption in *Rhodococcus*

Biosorption is recognized as an emerging and *eco-friendly* approach for metal(loid)s' or radionuclides' removal from polluted environments or in biomining, as it is cost-effective, is highly efficient, and can be specific. This technique relies on the application of different microbial biomasses (i.e., microalgae or bacterial cells of the genera including *Bacillus*, *Pseudomonas*, *Streptomyces*, etc.) as biosorbents, which possess a natural affinity toward metal(loid) compounds, contributing to a feasible and economic way toward the purification of metals from contaminated matrices (Volesky 1990; Wilde and Benemann 1993). From an engineering perspective, bacteria are considered good biosorbents as their small size offer a high surface-to-volume ratio, conferring a large surface area of interaction with metal(loid)s. Several molecular mechanisms are possible for biosorption performed by bacteria (Gadd and White 1993), dependent on factors, such as capacity, affinity, and specificity of the biomass and/or biosorbent considered, as well as the physical-chemical conditions of a given environmental niche and the metal of interest.

3.1 Parameters Influencing Metal(loid) Biosorption

The biosorption of metal(loid)s by bacteria depends on the characteristics of the surface structural chemistry of the cell wall and membrane (i.e., charge and orientation of the metal(loid) binding functional groups), as well as the chemistry and speciation of the considered metal(loid) (Ledin 2000). A given bacterial cell surface can be composed of lipids, peptidoglycan, lipopolysaccharides, lipoproteins, and enzymes, being featured by carboxyl (COO^-), sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), amino (NH_2^+/NH_3^+), carbonyl (CO^-), and hydroxyl (OH^-) functional groups as well as the acyl chains of membranes. Thus, the amphoteric behavior of a cell wall depends on the presence of these anionic and cationic groups, being those anionic predominant over the cationic ones (Plette et al. 1995; van der Wal et al. 1997).

Restricting the focus onto rhodococci, it has been reported that these microorganisms have a cell wall characterized by polysaccharides, carboxylic acids, lipid groups, and mycolic acids, which are responsible for the amphoteric behavior of the cellular surface (Stratton et al. 2002; Botero et al. 2007). Moreover, Plette et al. (1995) showed that the isolated cell wall material deriving from *R. erythropolis* A177 was featured by a chemical heterogeneity due to the existence of at least three different proton group binding sites (i.e., carboxylic, phosphate, and amino types), therefore leading to a surface charge dependent on the pH and salt level (Plette et al. 1995). A reasonable explanation for the effect of the pH on the sorption capability of a given biosorbent relies on the pKa of the biosorbent molecules and the combined isoelectric point of all biosorbent molecules and the number of active sites present as well as the speciation state of the metal ion. Therefore, at pH values lower than the biosorbent isoelectric point, its surface will be positively charged because of the

association with hydronium (H_3O^+) ions, determining a repulsion of metal cations. By contrast, the biosorbent surface will be negatively charged for pH values higher than its isoelectric point; hence, it favors the adsorption of cations. Furthermore, at pH values above the pKa of a given biosorbent, its functional groups will be dissociated, enabling proton exchange with the metal(loid) in solution (Cayllahua and Torem 2010). In line with this, Cayllahua and Torem (2010) reported that the biosorption capability of a *R. opacus* strain toward Al (III) ions was optimal at pH of 5, as its isoelectric point was c.a. 3.26. Similar observations were also reported for ions of Pb (II), Cr (III), Cu (II), Cd (II), and Zn (II) (Sheng et al. 2004; Vasquez et al. 2007; Bueno et al. 2008). Thus, depending on the type of metal(loid)s to be removed (i.e., anions or cations), it must be considered the characteristics of the biosorbent and the pH of the system at which the metal(loid)s' sorption occurs.

In natural settings, a variety of different metal(loid) ions other than those targets of bioremediation can be found, leading to a competition for the available binding sites present on the surface of bacterial cells, therefore interfering with the remediation of polluted sites. Calcium [Ca (II)], for example, is known as the major bivalent ion competitor for metal(loid) sorption in soil (Doyle et al. 1980; Xue et al. 1988; Flemming et al. 1990). However, it is noteworthy to mention that the cell wall material isolated from *R. erythropolis* A177 showed an enhanced Cd (II) sorption in the presence of Ca (II) ions. Indeed, the binding of Ca (II) ions to the cell wall can cause (1) the breaking of cross-linkages that normally occurs between different groups (e.g., COO^- and NH_3^+) of the cell wall and (2) the disruption of bonds occurring between phosphate groups of the phospholipids and others present in the peptidoglycan layer. These effects cause a drastic change of the cell surface and wall morphology leading to an increased availability of the number of accessible and active binding sites for other bivalent cations, such as Cd (II). Thus, since the binding of Ca (II) to the cell wall enhances the binding of Cd (II), in this case Ca (II) can be considered as a cooperative acting ion toward Cd (II), influencing in a salt-dependent manner the sorption potential of *R. erythropolis* A177-derived cell wall (Plette et al. 1996).

The concentration of the biosorbent in a given environment is another parameter of crucial importance, as the sorption capability of a biosorbent tends to decrease when its concentration rises. This aspect might be related to the cross-linkages occurring among the functional groups as described above (Koch 1990). Indeed, the cell wall material recovered from *R. opacus* strain was capable of efficiently adsorbing Al (III), Cd (II), and Zn (II) ions, while their sorption dropped down when the biosorbent concentration was increased (Vasquez et al. 2007; Cayllahua and Torem 2010).

3.2 EPS-Mediated Extracellular Biosorption

Another modality through which microorganisms can interface with metal(loid) compounds relies on the production of biological exudates featured by a high

adsorption potential. Overall, these biologically active compounds are known as EPS (see Sect. 2), which includes polysaccharides and their derivatives, proteins, peptides, metabolites, nucleic acids, and lipids. These microbial-derived compounds showed to have an efficient adsorptive property (Jixian et al. 2015; Zheng-bo et al. 2015), due to the presence of different functional reactive groups [i.e., OH^-, COO^-, acetamido ($AcNH^+$), NH_3^+] characterizing the polymer chains, which in turn confer to EPS: (1) high hydrophilicity, (2) polarity, and (3) structural flexibility (Crini 2005). Moreover, the amount of functional binding groups featuring EPS reflects its actual biosorption potential, working also as enhancer for the metal(loid) binding to microbial cell surfaces (Liu and Fang 2002; Wei et al. 2011). Since the treatment of metal(loid)-polluted sites via EPS is based on the binding of ions, the pH values at which the sorption process occurs are of fundamental importance as discussed above with the cell surface. Indeed, the competition between protons and metal(loid) ions to bioadsorb onto the active and accessible sites present in the EPS surface should be controlled, in order to maintain the appropriate electronic state allowing for chelation or complexation (Lamelas et al. 2006; Guibaud et al. 2008).

The EPS composition will be highly dependent on the bacterial species/strain, and thus its adsorption properties might be influenced by different protocols applied, reagents utilized, and physical-chemical (e.g., sonication, ionic exchange resin) method adopted for the extraction (Liu and Fang 2002; Comte et al. 2005). This leads toward the challenge that every system will be different and optimization strategy will need to be empirically determined. This reflects the importance of fundamental microbiology research to expand the knowledge on the possible microbial factories implied for this purpose.

3.3 Parameters Influencing EPS Biosorption

As biosorption EPS-mediated appears an attractive and *green* choice to develop new eco-sustainable strategies of environmental remediation, researchers have focused their attention in exploiting a variety of microorganisms belonging to diverse families (i.e., *Cyanobacteriaceae*, *Bacillaceae*, *Pseudomonadaceae*, *Myxococcaceae*) as cell factories for the production and extraction of EPS to test against different metals (e.g., Cd, Cu, Pb, Mn, Ni, Zn, Hg) (Merroun et al. 2003; Salehizadeh and Shojaosadati, 2003; Freire-Nordi et al. 2005; Lau et al. 2005; Paperi et al. 2006). Among rhodococci, Dobrowolski et al. (2017) studied the sorption capability of EPS material derived from *R. opacus* and *R. rhodochrous*, revealing a fast adsorption rate (between 1 and 30 min) of Ni (II), Pb (II), Co (II), Cd (II), and Cr (VI) for EPS material from both strains, which supports their application in flow adsorption systems (Dobrowolski et al. 2017).

The application of microbial EPS is highly influenced by the pH values at which the metal(loid)s' sorption occurs. Indeed, the best biosorption of Ni (II) mediated by the EPS recovered from either *R. opacus* or *R. rhodochrous* strains occurred at pH values ranging from 3 to 4, as, decreasing the content of H_3O^+ ions, the EPS surfaces

resulted more negatively charged and thus more prone to bind this divalent cation. By contrast, an increase of the pH value (>4) resulted in a decreased EPS sorption potential, as the two prominent species of Ni (II) ions in solution (i.e., $Ni(OH)_2$ and $Ni(OH)_3^-$) could not be adsorbed onto the negatively charged surfaces characterizing EPS. Similar observations can be drawn for Pb (II) and Co (II), while sorption up to a pH value of 7.5 was observed in the case of Cd (II), as cadmium hydroxyl [$Cd_x(OH)_y$] was the major species of Cd present in solution at this pH (Dobrowolski et al. 2017).

Temperature is another parameter to experimentally control for the optimization of a biosorption process, as it can influence the ionization of the EPS functional groups and influence the formed biosorbent-metal(loid) complex, as well as the diffusion and equilibrium kinetics. Generally, an increase in the temperature can cause (1) a decrease of the biosorption extent in the case of an exothermal process, (2) a destruction of the biosorbent's active sites, and (3) a faster ion motion, which enhances off-rates of the metal(loid)s to bioadsorb (Dobrowolski et al. 2017).

Other than EPS, humic substances (HSs) present in the soil and aquatic environments can mitigate the toxicity of metal(loid)s and organic compounds, which is due to the complex chemistry of HSs thus providing sorption properties (Boguta and Sokołowska 2016). Further, the network generated among metal(loid) compounds, bacteria, and environment can influence (1) the speciation and bioavailability of metal(loid)s, (2) the sorption phenomenon, (3) the metabolic capacity of bacteria toward xenobiotics, and (4) the biochemical/physiological functions of bacteria (Perminova and Hatfield 2005). Although the comprehension of this process is difficult and poorly investigated, Perelomov et al. (2018) described different bacteria capable of tolerating higher amounts of Zn (II) and Pb (II) consequently to the copresence of HSs and derivative fractions (i.e., humic acids, hymatomelanic acids). Indeed, a study employing infrared spectroscopy revealed that humic acids and hymatomelanic acids possess different functional active sites represented by alcohols, phenols, amines, amides, and carboxylic acids, which can be either free or intermolecularly bonded (Rodrigues et al. 2009), enabling HSs to bind cations (Kar et al. 2011). Thus, the addition of 200 mg L^{-1} of HSs (mixture of humic and hymatomelanic acids) resulted in an enhanced tolerance to Zn (II) in the case of *Pseudomonas fluorescence* 142NF and *R.* sp. RS67, which were five- and fourfold more tolerant, respectively, as compared to the non-HSs amended cultures (Perelomov et al. 2018). This effect originates from the stable HS-Zn complex formed (Perminova et al. 2006), which is not bioavailable to the bacteria. Similar results were achieved in the case of Pb (II), therefore highlighting the importance of HSs in attenuating metal(loid)s' toxicity and bioavailability. However, this also limits the ability to remediate a site of the metal pollutant.

As discussed in this section, biosorption is a powerful biotechnological tool that can be exploited for the remediation of metal(loid)-polluted sites. The process relies on the binding, complexation, ion exchange, crystallization, and/or precipitation of the metal(loid) in the biochemical context of a given cell wall. The parameters (i.e., pH, salt level, temperature, and biosorbent concentration) described are crucial and must be optimized according to the physical-chemical characteristics of both metal

(loid) and biosorbent, particularly focusing on its amphoteric behavior, in order to take advantage of biosorbents for bioremediation purposes.

4 Metal(loid) Bioaccumulation in *Rhodococcus*

As opposed to biosorption, metal(loid) accumulation is an energy-demanding and metabolic-dependent process, as it requires an active uptake transporter by a living organism and, therefore, it occurs slower as compared to the above described biosorption phenomenon (Mowll and Gadd 1984). In this respect, Mirimanoff and Wilkinson (2000) showed that the initial Zn adsorption performed by *R. opacus* C125 occurred in less than 1 min; on the other hand, microorganisms have evolved specialized uptake systems for essential metal(loid)s that are needed for the cell biochemistry. Specific ion channels or carriers facilitate uptake for the ion down its concentration gradient and active transporters using either ATP or the electrochemical gradient as energy for transport. Such transporters are key to charge metalloenzymes that exploit transition metals as cofactors. For example, molybdenum (Mo) is actively taken up, in the form of oxyanion (MoO_4^{2-}), either by an ATP-binding cassette (ABC) transporter with high affinity or through low affinity carrier systems, which have been both evolved by those microorganisms that use Mo for nitrogenases and/or oxidoreductases featured by molybdopterin as cofactor, respectively (Pau et al. 1997).

A common route for toxic heavy metal accumulation is mediated by transporters of essential metals, which facilitate the transport of closely related elements. For example, the molybdate ABC transporter will also transport tungsten (W) as WO_4^{2-}, in this way nonessential elements can be bioaccumulated. Bacteria can take up divalent cations such as Co (II), Zn (II), and Ni (II) exploiting the magnesium [Mg (II)] transport system, although this strategy only weakly contributes to the uptake of transition metals in the presence of magnesium (Smith and Maguire 1998). Thus, bacterial strains featured by genes coding for enzymes dependent on Ni (II) as cofactor exploit specific transporters for Ni (II), which constitute a family of permeases featured by eight transmembrane segments with high affinity for this cation but a very low transport capacity, while *Escherichia coli* cells exploiting an ABC transporter represents an exception (Hausinger 1997; Eitinger and Friedrich 1997). These permeases for Ni (II) have been identified in different bacterial genera (i.e., *Bradyrhizobium*, *Alcaligenes*, *Helicobacter*) (Fu et al. 1994; Eitinger et al. 1997; Fulkerson et al. 1998). Among actinomycetes, *Mycobacterium tuberculosis* relies on this permease to accumulate Ni (II), supporting the activity of a Ni-dependent urease, which is also an important virulence factor for this microorganism (Cole et al. 1998).

Concerning rhodococci, Komeda et al. (1997) studied in *R. rhodochrous* strain J1 the NhlF transporter for Co (II), which is responsible to provide Co (II) for nitrile hydratases containing non-corrin Co as cofactor (Komeda et al. 1997). Nitrile utilization by *R. rhodochrous* strain M8 was dependent on the nitrile hydratase

activity as well, whose expression was enhanced by the presence of Co (II) in the growth medium as compared to Co-limiting conditions, suggesting that Co (II) deficiency could lead to either a weak expression of the nitrile hydratase coding gene or to a fast nitrile hydratase transcript (mRNA) degradation (Pogorelova et al. 1996). However, the NhlF transporter is highly similar at amino acid sequence level to the Ni (II) transporter HoxN of *Alcaligenes eutrophus* (Wolfram et al. 1995). Although both NhlF and HoxN transporters share similar membrane topology, Co (II) uptake NhlF-mediated was impaired by the copresence of Ni (II), thus indicating that NhlF could also transport Ni (II) (Komeda et al. 1997). Later, Degen et al. (1999) demonstrated that HoxN was able to mediate the transport of Ni (II) and incapable of taking up Co (II), while NhlF had a promiscuous binding ability toward these divalent cations (Degen et al. 1999). Indeed, NhlF transporter could mediate Ni (II) uptake, as demonstrated by the enhanced Ni-dependent urease activity of recombinant *Escherichia coli* clones expressing *nhlF* gene grown under Ni-limiting conditions, as well as cross-complementing Ni deficiency due to the deletion of the *hoxN* gene in *Alcaligenes eutrophus*. Nevertheless, the Ni (II) uptake by NhlF was markedly reduced when *R. rhodochrous* strain J1 was cultured in the presence of both nickel and cobalt salts, therefore suggesting that Co (II) was the preferred cation-binding substrate, as well as the NhlF transporter was featured by a certain level of ion selectivity toward two similar transition metals (Degen et al. 1999).

4.1 Metal(loid) Homeostasis in Microbial Bioaccumulation

In addition to metal(loid) uptake, the efflux system is a biologically relevant phenomenon responsible for the control of toxin accumulation in the cell, and examples exist to control metal(loid) accumulation, as well as its homeostasis between intra- and extracellular environment, highlighting a sort of "microbial metal toxicity care". This is the case for *R. opacus* C125 cells that accumulated at most c.a. 6 μmol g^{-1} (dry weight) of Zn (II) after 20 min exposure to 5 μM Zn (II), which with time drastically decreased. When Zn-loaded *R. opacus* C125 cells were exposed to either additional 5 μM Zn (II) or Cd (II), cellular Zn concentrations decreased, therefore suggesting both an inducible and cross-inducible efflux mechanism by Zn (II) and Cd (II), respectively, as well as the existence of Zn-binding ligands capable of mediating the accumulation of this transition metal in the first place (Mirimanoff and Wilkinson, 2000).

Overall, nonessential metal(loid)s can be taken up by microorganisms, and the amount of these compounds accumulated varies as function of the species and the type of metal(loid) considered. An example is given by cesium (Cs), which became a metal of pollutant interest after the Chernobyl accident in 1986, attracting the research interest in both the removal and the fate of radioactive Cs. In this respect, Tomioka et al. (1994) investigated two different *Rhodococcus* strains (i.e., *R. erythropolis* CS98 and *R.* sp. CS402) capable of tolerating and growing in the presence of Cs and rubidium (Rb) supplied in a mineral salt medium up to

concentrations of 1 mM. It was found that Cs uptake by both *Rhodococcus* strains was negatively influenced by the presence of monovalent cations such as K (I) and Rb (I). Reasonably, Cs (I) and Rb (I) may substitute K (I) in bacteria growing under K-limiting conditions due to the poor specificity of the K (I) transport systems (Avery et al. 1991, 1992). This idea was reported earlier in the case of other bacterial strains (i.e., *Rhodopseudomonas capsulata*, *Escherichia coli*, and *Rhodococcus* spp.; Rhoads and Epstein 1977; Jasper 1978; Tomioka et al. 1994).

If the biosorption phenomenon is the first event occurring when bacterial cells interface metal(loid)s, the active bioaccumulation of these ions within the intracellular environment would be a complementary event. To date we are still at early stages to appreciate the diversity and mechanisms for most metal uptake systems. However, the microbial potential in handling metal(loid)s' toxicity goes beyond the above depicted strategies, as metal(loid) bioconversion and/or biotransformation reactions may take place, leading to either biomineralization or production of valuable metal(loid) products. Indeed, the next section will overview this aspect of the microbial world that crosses and merges itself with the nanotechnology field.

5 Nanotechnology: A Physical-Chemical Perspective

Nanotechnology is the science related to the world of *very small material*, which implies the manipulation of matter at molecular or atomic level (Horikoshi and Serpone 2013). The prefix *nano* is referred to structures defined as intermediate states between molecules and bulk materials (Yuwen and Wang 2013). The uniqueness of nanostructures relies on the possibility to manipulate physical-chemical properties of the materials scaled down to the nanorange (1–100 nm) (Yuwen and Wang 2013), conferring them fundamental and peculiar features as high surface-to-volume ratio and large surface energy with high spatial confinement (Cao 2004a). These peculiar characteristics allow in turn innovative chemical, catalytic, mechanical, electrical, and opto-magnetic properties in the nanomaterials (Appenzeller 1991; Yuwen and Wang 2013), making them suitably manipulated for applications in multidisciplinary fields, such as biomedicine/biotechnologies, energy production, environmental engineering, material science, and optoelectronics (Cao 2004a; Horikoshi and Serpone 2013).

In the past few decades, the nanoscience field has exponentially grown in terms of study and implementation, leading to the development of new methods for nanomaterial production (Rao et al. 2004). In this context, we have witnessed the generation of nanostructures having various compositions and morphologies, of which the most exploited to date are *zero-dimensional* (0D) nanoparticles (NPs), nanocrystals and cluster quantum dots (QDs), *one-dimensional* (1D) nanorods (NRs), nanowires (NWs), nanotubes (NTs) and nanobelts (NBs) (or ribbons), nanoporous solids, *two-dimensional* (2D) arrays of NPs, thin films, and *three-dimensional* (3D) structures (superlattices) (Rao et al. 2004).

Although the physical-chemical methods to synthesize nanomaterials are efficient and available for a scaled-up production, they require the use of toxic substances that are converted during the fabrication process into hazardous waste requiring specialized disposal or are released to the environment (Zhang et al. 2006, ACS Nano 2011; CDC 2014). Moreover, several of these production procedures require expensive equipment and chemicals, affecting the production costs. Therefore, these drawbacks led to a demand for the generation of novel cost-effective and *eco-friendly* methods of nanomaterial production (Ankamwar et al. 2005).

5.1 Microbial Nanotechnology

To date, the new and *green* frontier in nanostructure synthesis is constituted by the exploitation of the natural ability of biological systems (i.e., plants, bacteria, fungi, yeasts, and algae) to bioconvert toxic metal(loid) ions into their less toxic elemental forms producing nanostructures (Suresh et al. 2004; Bhainsa and D'Souza 2006; Song and Kim 2009). Particularly, bacteria are among the organisms that have been the most explored for nanomaterial generation, due to their ability to populate different environmental niches that has led to unique biochemistry and physiology features (Li et al. 2011; Pantidos and Horsfall 2014). Indeed, in nature, the production of metal(loid), metal sulfide, and oxide nanostructures by bacterial cells is achieved through either aerobic detoxification processes or the use, under anaerobic conditions, of metal(loid) ions as terminal electron acceptors to produce energy (Li et al. 2011).

Among the diverse bacterial species that can biosynthesize nanostructures, those belonging to the *Actinomycetales* order recently gained interest in the nanoscience field, as they previously showed their proficiency to produce bioactive compounds, such as antibiotics and biocides (Duraipandiyana et al. 2010). Thus, several *Streptomyces*, *Thermoactinomyces*, and *Nocardia* strains have been investigated for the production of intra- or extracellular gold (Au), silver (Ag), zinc oxide (ZnO), copper oxide (CuO), or selenium (Se) NPs upon their growth and/or exposure to the metal(loid) precursor salts (Forootanfar et al. 2014; Manimaran and Kannabiran 2017). For example, Fig. 1 shows the different cellular localizations of metalloid nanomaterials generated by *R. aetherivorans* BCP1 as a result of metal(loid) oxyanion (i.e., SeO_3^{2-} and TeO_3^{2-}) bioconversion into their elemental forms (i.e., Se^0 and Te^0). The intracellular biosynthesis of metal(loid) NPs by actinomycetes is mainly ascribed to either (1) electrostatic interactions occurring between metal cations and $R\text{-}COO^-$ groups of enzymes associated with the bacterial cell membrane (Manimaran and Kannabiran 2017) or (2) a Painter-type reaction between RSH groups of cellular enzymes and metalloid oxyanions (Tan et al. 2016). As a result, these reactions can reduce the metal(loid) ions, leading to the formation of elemental metal(loid) nuclei, which in turn aggregate forming nanomaterials of defined shape (Ahmad et al. 2003; Sunitha et al. 2013). On the other hand, it is proposed that soluble secreted enzymes are responsible for the reduction of metal cations into their elemental forms in the case of the extracellular generation of metal-based nanomaterials (Manivasagan et al. 2016).

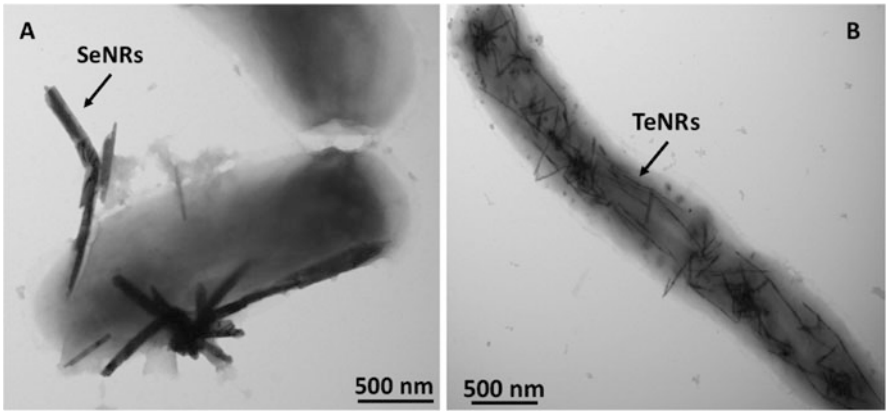

Fig. 1 Electron micrographs of *R. aetherivorans* BCP1 cells featured by the presence of (**a**) selenium nanorods (SeNRs) and (**b**) tellurium nanorods (TeNRs). Data not published, Dr. Alessandro Presentato, University of Calgary (2017)

5.2 Rhodococcus *Strains as Cell Factories for Nanotechnological Purposes*

Considering the great potential of the *Rhodococcus* genus for biotechnological purposes, recently a few research groups started to evaluate the ability of these microorganisms to produce metal(loid) nanomaterials through the bioconversion of metal(loid) salts. Although this research field is only in its infancy, various *Rhodococcus* strains revealed their capability of generating both intra- and extracellular Au-, Ag-, ZnO-, Se-, and Te-based nanostructures (Ahmad et al. 2003; Otari et al. 2012; Kundu et al. 2014; Presentato et al. 2016, 2018b, c), whose key features are summarized in Table 2, while an example of different biogenic nanomaterial shapes is shown in Fig. 2. A common property of these biogenic metal(loid) nanomaterials is their natural thermodynamic stability, which is in contrast with the behavior of those chemically synthesized (Piacenza et al. 2018). Indeed, materials at the nanoscale are generally unstable from a thermodynamic point of view, as they possess a high surface energy (Goldstein et al. 1992). To overcome their instability, nanostructures tend to either combine forming bigger aggregates (Ostwald ripening process) or agglomerate each other without altering individual particles (Cao 2004b). Since the unique properties of nanosized materials are strictly dependent on their small size, it is imperative to avoid their aggregation, making it necessary to use expensive and hazardous substances to stabilize chemically produced nanostructures prior their use (Cao 2004a; Piacenza et al. 2018). By contrast, the natural thermodynamic stability of metal(loid) nanomaterials generated by *Rhodococcus* species is a fundamental feature for the application of these nanostructures in different fields. Moreover, these biogenic nanomaterials resulted in being stabilized by biomolecules (e.g., proteins, enzymes, or surfactant-like molecules) produced by the bacterial strains themselves (Kundu et al. 2014,

Table 2 Production of metal(loid) nanomaterials by *Rhodococcus* spp. and their characterization

Strain	Core	Localization	Shape	Size (nm)	Reducing agents/ stabilizers	References
Rhodococcus sp.	Au	Intracellular	Spherical	5–15	Cellular proteins	Ahmad et al. (2003)
R. NCIM 2891	Ag	Intracellular	Spherical	ca. 100	NADH-dependent nitrate reductases	Otari et al. (2012)
R. pyridinivorans NT2	ZnO	Extracellular	Spherical porous	100–120	Extracellular proteins, heterocyclic compounds	Kundu et al. (2014)
R. aetherivorans BCP1	Te	Intracellular	Rod-shape	200–450	MSHs, biosurfactants	Presentato et al. (2016)
R. aetherivorans BCP1	Se	Intracellular	Spherical	50–90	MSHs, biosurfactants	Presentato et al. (2018a)
			Rod-shape	400–600		
R. aetherivorans BCP1	Te	Intracellular	Spherical	100–900	MSHs, surfactant-like molecules	Presentato et al. (2018b)

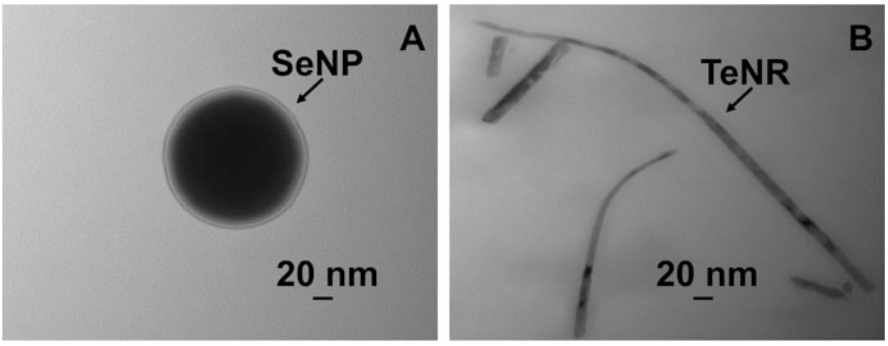

Fig. 2 Electron micrographs of (**a**) selenium nanoparticles (SeNPs) and (**b**) tellurium nanorods (TeNRs) recovered from *R. aetherivorans* BCP1 selenite- and tellurite-grown cells, respectively. Data not published, PhD Student Elena Piacenza, University of Calgary (2017)

Presentato et al. 2016, 2018b, c), therefore constituting a *greener* choice as compared to the compounds used for the stabilization of chemical nanostructures.

To date, metal(loid) nanomaterials generated by *Rhodococcus* spp. have been mainly investigated for their potential use in biomedicine. Indeed, AgNPs produced by *R.* sp. NCIM 2891 were found to be efficient as both antimicrobials toward

Escherichia coli and *Bacillus subtilis* pathogenic indicator strains and anticancer agents limiting the proliferation of A549 lung cancer cell line (Subbaiya et al. 2014). Similarly, ZnO NPs biosynthesized using *R. pyridinivorans* NT2 have been used as coating for cotton textile surfaces intended for the production of footwear (Kundu et al. 2014). As a result of this conjugation process, the cotton fibers acquired long-term UV-protective, photocatalytic, and antimicrobial properties, whose efficacy was evaluated against *Staphylococcus epidermidis* ATCC 12228, an opportunistic pathogen of the sole of the foot (Kundu et al. 2014). Moreover, ZnO NPs showed their proficiency as an in vitro drug delivery system of an anticancer compound (i.e., anthraquinone) toward HT-29 colon carcinoma cell line, without exerting cytotoxic effects on normal peripheral blood mononuclear cells (PBMCs) (Kundu et al. 2014). Finally, the potential of using metal(loid) nanomaterials generated by *Rhodococcus* strains in other fields rather than biomedicine has been recently explored (Presentato et al. 2018c). Particularly, since Te is a well-known narrow band gap *p*-type semiconductor (Araki and Tanaka 1972) exhibiting high photoconductivity, piezo, and thermoelectricity (Araki and Tanaka 1972; Tangney and Fahy 2002; Suchand Sandeep et al. 2010), TeNRs obtained from resting (not growing) cells of *R. aetherivorans* BCP1 were studied for the ability to conduct electrical current. As a result, these TeNRs revealed electrical conductivity approaching the values established for chemically produced TeNRs, highlighting the potential of these biogenic nanomaterials in the development of electrical components or devices (Presentato et al. 2018c).

6 Summary

Rhodococcus is a bacterial genus with strong resilience in handling metal(loid)s' toxicity, applying different strategies to tolerate and/or resist metal(loid) compounds (i.e., biosorption, bioaccumulation, efflux, extracellularly chelation) regardless their form (i.e., anions, cations, oxyanions). This is in part related with the rhodococci's innate and adaptive mechanisms, enabling them to survive and thrive, even under typically unfavorable conditions. The intense study and efforts devoted to comprehending how bacteria interface metal(loid)s led to understanding that *Mother Nature* can be respectfully exploited for different purposes, such as the bioremediation of metal(loid)-polluted environmental matrices and the generation of *eco-friendly* strategies to expand the nanotechnology field without negatively affecting the environment. Thus, this kind of research implies that the knowledge of both the microbiology and the physical-chemical characteristics of metal(loid)s must be used in a conscious way, as the system represented by the *real life* is complicated through a variety of different factors constantly interacting each other. Only by awareness of this complex picture it is possible to predict the biological effects occurring in natural systems.

Acknowledgments Natural Science and Engineering Research Council of Canada (NSERC) is gratefully acknowledged for the support of this study (Grant/Award Number: 216887-2010).

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Plant-Associated *Rhodococcus* Species, for Better and for Worse

Isolde M. Francis and Danny Vereecke

Contents

Abstract *Rhodococcus fascians*, causative agent of the leafy gall syndrome, produces a mixture of cytokinins to modify the hormone landscape of its broad range of plant hosts leading to tissue deformations and developmental alterations. Recent developments indicate that the pathogenic nature of these bacteria is superimposed on its plant growth-promoting effect. In the last two decades, its unique position as the only species within the genus able to interact with plants has been overthrown. Indeed, Pistachio Bushy Top Syndrome is an emerging disease linked to the presence of two *Rhodococcus* species, *R. fascians* and *R. corynebacterioides*. Both bacteria would act synergistically to cause the symptoms, giving the prospect of virulence strategies that differ from those of the leafy gall inducers. Additionally, as a result of microbiome research, it is clear that many *Rhodococcus* species live in close association with plants, and several of them exhibit plant growth-promoting

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9_13

activities. Finally, genome analyses of a collection of *R. fascians* isolates imply that the taxonomic position of this group of bacteria within the genus will have to be reevaluated, and likely a new genus consisting of several species will be proposed soon.

1 Introduction

As illustrated by most chapters in this book, members of the genus *Rhodococcus* are renowned for their specialized metabolic capacities, and most are harmless, environmental, free-living organisms (Ceniceros et al. 2017). The genus, however, also comprises two pathogens: *R. equi*, the causative agent of pneumonia in foals leading to major losses worldwide in the equine industry (von Bargen and Haas 2009; Anastasi et al. 2016; MacArthur et al. 2017; Vázquez-Boland et al. this issue), and *R. fascians*, a plant pathogen causing the leafy gall syndrome mainly affecting ornamental production facilities all over the world (Depuydt et al. 2008b). Nevertheless, based on recent developments, the monopoly of *R. fascians* within the genus to interact with plants appears to be broken.

As a result of a growing environmental awareness, the use of agrochemicals to assure optimal crop yield is now considered less favorable, and considerable efforts are made toward more integrated agricultural approaches in which crop productivity and resilience against abiotic and biotic stress can be achieved with a reduced input. In that context, the use of biostimulants has gained a lot of attention, and the search for microorganisms that can stimulate plant productivity and protect crops from adverse conditions has become a major endeavor (Calvo et al. 2014). As an interesting side effect of these studies, a much broader view is obtained on the composition of rhizospheric and phyllospheric microbiomes, and it has become clear that diverse actinobacteria, including *Rhodococcus* species, readily associate with plants (Hamedi and Mohammadipanah 2015). Additionally, a novel disease termed Pistachio Bushy Top Syndrome has been reported which appears to be caused by two *Rhodococcus* species (Stamler et al. 2015a, 2015b).

Here, we summarize the latest progress on the *R. fascians* leafy gall pathology and provide an overview of the novel insights on the capacity of other *Rhodococcus* species to beneficially or detrimentally interact with plants.

2 *Rhodococcus fascians*, Causative Agent of the Leafy Gall Syndrome

2.1 Symptoms and Host Range

R. fascians, unlike most pathogens, is a genuine biotroph that does not truly damage its host but uses subtler strategies to infect plants. It was first recognized as a plant pathogen in 1936 as the causative agent of fasciation on pea (Brown 1927; Tilford 1936). However, on most hosts, fasciations are not induced, but rather the disease is apparent as a combination of several symptoms, including deformed leaves and flowers, swollen tissues due to secondary growth of vascular tissues, loss of apical dominance, excessive shoots, witches' brooms, and/or differentiated galls covered with small shoots, collectively called the leafy gall syndrome (Stes et al. 2011b, 2013; Dolzblasz et al. 2018). The host range of *R. fascians* has reached over 150 species across 50 plant families, including primarily herbaceous dicotyledonous plants and some monocots (Putnam and Miller 2007). The extensive host range of leafy gall-inducing *R. fascians* isolates illustrates that the virulence factors of these bacteria target widely conserved pathways in plants. Since typical symptoms associated with the leafy gall syndrome lead to disfigured plants, *R. fascians* has mainly been problematic in the ornamentals industry where plants are grown for their aesthetic value (Depuydt et al. 2008b).

2.2 The Trick-with-the-Cytokinin-Mix

The genome of the leafy gall-inducing model strain *R. fascians* D188 consists of three replicons: the circular chromosome, a circular plasmid pD188 encoding cadmium resistance and not involved in pathogenicity, and a conjugative linear virulence plasmid pFiD188 (Fig. 1). Over 25 years of research on the interaction between strain D188 and the model plants *Arabidopsis thaliana* and *Nicotiana tabacum* resulted in a molecular and developmental framework bringing together the pathways of microbe and plant that contribute to the disease (reviewed by Stes et al. 2011b, 2013).

In essence, key to symptom development is the bacterial production of an array of cytokinins via genes of the *fas* operon encoded on pFiD188 (Crespi et al. 1992; Francis et al. 2012; Pertry et al. 2009, 2010) and the concomitant modification of the hormone landscape of the infected plant (Depuydt et al. 2008a, 2009a; Stes et al. 2011a, 2012, 2015; Dhandapani et al. 2018). The interaction starts with the epiphytic colonization of the plant. During this phase, no symptoms are induced, but the bacteria secrete low levels of a mixture of cytokinins and of auxin produced via chromosomal pathways that stimulate the metabolism of the plant cells resulting in the release of nutrients to the benefit of bacterial colonization (Cornelis et al. 2001; Depuydt et al. 2009b; Dhandapani et al. 2017, 2018; Francis et al. 2016). As a

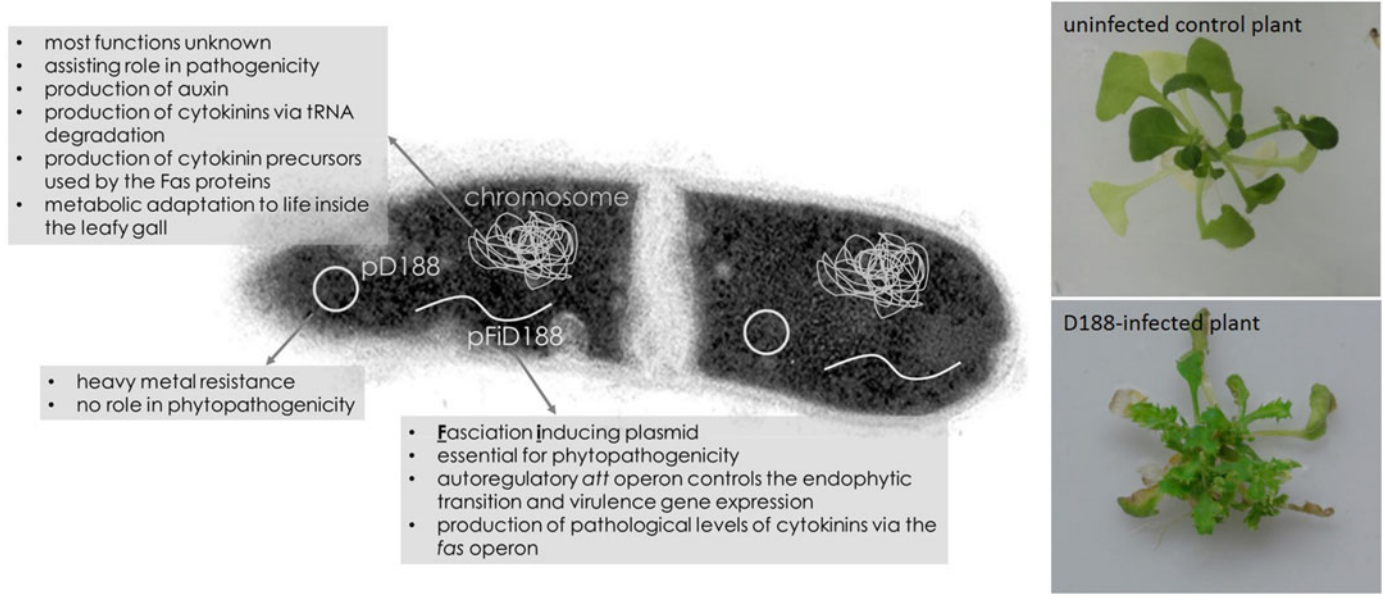

Fig. 1 Genome composition of the leafy gall-inducing model *R. fascians* strain D188 and symptoms on *Arabidopsis thaliana*

consequence of the shift in the primary metabolism of the plant upon perception of the bacteria (Depuydt et al. 2009b), a physiological change is triggered in the bacteria that will ultimately lead to the second phase of the interaction: the pathogenic endophytic phase. This phase is initiated by the production of an autoregulatory molecule encoded by the *att* locus located on pFiD188, establishing a positive regulatory loop resembling quorum sensing. The *att* system activates the transition to the endophytic phase possibly by stimulating the production of cell wall-degrading enzymes allowing active penetration of plant tissues and results in the production of higher pathological levels of a mixture of cytokinins encoded by the *fas* locus inducing the formation of an abundance of meristematic tissues in the plant that develop into shoots (Temmerman et al. 2000; Maes et al. 2001; Pertry et al. 2009; Fig. 1). The physiology of the symptomatic tissues is very different from uninfected plants, and the high concentration of meristematic cells leads to the provision of specific nutrients (Depuydt et al. 2009a, 2009b). These nutrients are believed to be metabolized via the glyoxylate shunt, since the bacteria require a functional malate synthase encoded by the chromosomal *vicA* gene for efficient survival inside the plant tissues (Vereecke et al. 2002). Hence, the success of the interaction with the host depends on the co-option of chromosomal and linear-plasmid-encoded functions, and eventually, through hormone secretion, the bacteria trigger the plant to provide it with a unique niche in terms of food and protection (Depuydt et al. 2009b; Dhandapani et al. 2017, 2018).

The virulence mechanism described above for *R. fascians* D188 is most likely similar for most other pathogenic leafy gall-inducing isolates because the *fas* and *att* genes are highly conserved (Creason et al. 2014a; Radhika et al. 2015). Nevertheless, the central position of cytokinins in the mode of action of the leafy gall inducers has been debated since their discovery because of the very low levels produced by in vitro-grown *R. fascians* cultures (Thimann and Sachs 1966; Eason et al. 1995; Pertry et al. 2009; Radhika et al. 2015). Even today, newcomers in the field question if cytokinins are essential for the leafy gall syndrome (Creason et al. 2014a; Savory et al. 2017), but until now, no valid alternative function for the *fas* operon has been proposed, and no other mode of action has been experimentally demonstrated (Vereecke, 2018). In contrast, there is a wealth of information that supports the indispensable role of *fas*-derived cytokinins in leafy gall formation:

1. The implication of cytokinins in symptom development has been recognized since 1966 (Klämbt et al. 1966; Helgeson and Leonard, 1966; Rathbone and Hall 1972; Scarbrough et al. 1973; Balázs and Sziráki 1974; Armstrong et al. 1976; Murai et al. 1980; Crespi et al. 1992; Eason et al. 1996; Dhandapani et al. 2017).
2. Mutations in the *fasA* and *fasD* genes render *R. fascians* nonpathogenic (Crespi et al. 1992, 1994; Pertry et al. 2009, 2010).
3. There is a 100% correlation between the presence of *fasD* and virulence (Eason et al. 1995, 1996; Stange et al. 1996; Nikolaeva et al. 2009; Creason et al. 2014a; Savory et al. 2017).

4. The biochemical activity of several Fas proteins in the production of cytokinins has been experimentally demonstrated (Crespi et al. 1992; Pertry et al. 2009, 2010; Radhika et al. 2015).
5. The cytokinins produced by *R. fascians* in culture are recovered in a higher amount in infected plants versus non-infected ones (Pertry et al. 2009, 2010; Radhika et al. 2015; Dhandapani et al. 2017).
6. *R. fascians*-infected plants activate cytokinin homeostasis mechanisms in the same way as if they were subjected to an excess of exogenous cytokinins (Depuydt et al. 2008a, 2009b; Motte et al. 2013).
7. The transcriptome of infected *Arabidopsis* and sweet pea plants clearly shows an enhanced cytokinin response (Depuydt et al. 2008a, 2009b; Dhandapani et al. 2017, 2018).

One challenging aspect of the trick-with-the-cytokinin-mix model was that the same mix of *fas*-dependent cytokinins was also detected in the supernatants of the plasmid-free strain D188-5. The cytokinin levels in this nonpathogenic derivative are much lower than those produced by the wild-type strain D188, and they are believed to result from tRNA degradation (Pertry et al. 2009, 2010). Because the bacteria cause symptoms only when the linear plasmid pFiD188 is present (Crespi et al. 1992), it was postulated that virulence does not depend on the exposure of the plant to one specific kind of cytokinin molecule but rather to particular concentrations and ratios of several of these plant growth regulators (Pertry et al. 2009, 2010). The higher pathological levels of cytokinins could only be produced through a co-option of chromosomal and linear plasmid genes (Francis et al. 2016). Although the cytokinin-mix production strategy used by *R. fascians* has been extended to plant-insect interactions (Giron and Glevarec 2014), Radhika et al. (2015) provided the missing link in the model. Indeed, the *mtr* genes encoding SAM-dependent methyltransferases and located upstream of the *fas* operon are involved in methylating the precursor of 2-iP used by FasD. These methylated cytokinins, which are not produced by plants, are resistant to plant cytokinin dehydrogenase-oxidases, are recognized by the plant cytokinin receptors, and have high morphogenic activities, most likely represent unique virulence-associated cytokinins (Radhika et al. 2015).

2.3 *Epidemiology of Leafy Gall Disease in Ornamental Nurseries*

As early as 1950, modes of spread of *R. fascians* within commercial nurseries of ornamental plants have been documented, and infield survival of up to 5 years in soil under favorable conditions has been reported (Baker 1950; Faivre-Amiot 1967; Oduro 1975; Miller et al. 1980), but overall information on the epidemiology of leafy gall-inducing *R. fascians* isolates remains scarce. Until recently, no in-depth information on the composition of the *R. fascians* populations on infected plants was available, and the relatedness between the bacteria on different plants within a

nursery or between nurseries had not been studied. The phylogenetic relationship of 60 isolates collected from symptomatic tissues from single plants or from different plants in the same production facility was analyzed using a genomic epidemiological approach. The data showed that single nurseries could be infected by up to seven genotypes, that single plants could be colonized by at least three genotypes, and that different nurseries harbored highly related genotypes. Altogether, it could be concluded that the pathogens had been introduced in nurseries at multiple and independent occasions. Additionally, evidence was provided for point source outbreaks likely as a result of the acquisition of diseased plants from a common provider, but the presence of reservoir populations in some nurseries was apparent as well. When the linear plasmids of these isolates were compared, two major plasmid types could be distinguished, but the distribution of these plasmids indicated that plasmid exchange between genotypes occurred frequently. Additionally, the pathogenic isolates coexisted with nonpathogenic ones (Savory et al. 2017). Thus, as in other plant-associated bacteria, including *Pantoea agglomerans* (Weinthal et al. 2007), *Streptomyces scabies* (Zhang et al. 2016; Zhang and Loria 2017), *Pseudomonas syringae* (Monteil et al. 2016), and *Pseudomonas savastanoi* pv. *savastanoi* (Buonaurio et al. 2015), also in leafy gall-associated *Rhodococcus* species, the acquisition of a virulence plasmid drives pathogen evolution.

2.4 Rhodococcus fascians*, a Troubled Species*

The taxonomic position of *R. fascians* has been troublesome since it was first isolated from diseased peas. At that time, the causative agent of the observed fasciations was named *Phytomonas fascians*, which was thought to be a specialized form of *Phytomonas (Agrobacterium) tumefaciens* (Tilford 1936). Few years later, the bacterium was renamed *Bacterium fascians*, because the generic name "*Phytomonas*" for plant pathogenic bacteria was no longer valid (Lacey 1939). Nonetheless, also this generic name led to persistent controversy, and based on post-fission movements of the bacteria and the presence of intracellular bodies, it was eventually recognized as *Corynebacterium fascians* (Dowson 1942). In the following years, it became apparent however that the leafy gall-inducing bacteria were more similar to *Rhodococcus* species than to *Corynebacterium*, and thus, more than 40 years later, in 1984, Goodfellow reclassified the pathogen as *Rhodococcus fascians* based on genetic, phenetic, and chemical characteristics (Goodfellow 1984). Now, as a consequence of the data obtained by (next-generation) sequencing techniques, once again doubt has been casted on the taxonomy of this microbe, and after 34 years, phylogenomic evidence suggests that the systematics of the genus *Rhodococcus* should be revised, and the *R. fascians* species likely belong to a distinct genus encompassing several species (Creason et al. 2014b; Sangal et al. 2016; Savory et al. 2017).

A strong indication that the genus *Rhodococcus* probably needs to be divided into more genera was based on 16S rRNA phylogenetic analysis of 217 actinomycete

isolates, which revealed the occurrence of at least four *Rhodococcus* groups that were as phylogenetically distinct from each other as from *Nocardia* and *Gordonia* (Gürtler et al. 2004). However, an example that 16S rRNA-based classification is probably not sufficient to resolve the *Rhodococcus* genus is the inclusion into the species *R. fascians* of new isolates from diverse extreme environments unrelated to plants, such as Antarctic soils, marine waters, tidal flats, sediments contaminated with nuclear waste, polychlorinated dioxins, crude oil and hydrocarbons, deep glacier ice cores, and even carp intestines (Gürtler and Seviour 2010). Genome analysis of 19 isolates obtained from diseased ornamental plants, 2 isolates from a glacial ice core and 1 from permafrost, and 2 endophytes isolated from *Arabidopsis* indicated that these bacteria grouped into two clades representing up to seven different species (Creason et al. 2014b). Both clades contained pathogenic as well as nonpathogenic isolates. Even more, *Next Generation Systematics* based on the genome analysis of 100 rhodococcal strains and 15 representatives of related genera has provided a genomic framework that revealed seven distinct species-groups and three singletons (Sangal et al. 2016). Species-group E consisting of 28 genomes represents the *R. fascians* cluster which can be further subdivided in two subgroups corresponding to the two clades identified by Creason et al. (2014b). Subgroup E1 corresponds to clade II and would comprise two species, whereas subgroup E2, including the type strain and the model strain D188, corresponds to clade I and encompasses six predicted species (Sangal et al. 2016). Based on this information and as discussed by Sutcliffe and colleagues in this issue, a formal proposal of a new and hopefully final genus name for *R. fascians* is to be expected soon.

3 Pistachio Bushy Top Syndrome, a Novel *Rhodococcus*-Associated Disease

In 2011, a novel disease emerged on pistachio "UCB-1" rootstock trees, termed Pistachio Bushy Top Syndrome (PBTS) which significantly affected the pistachio industry in California, Arizona, and New Mexico, causing massive economic losses (Stamler et al. 2015a, 2015b). PBTS is characterized by stunted growth of the rootstock trees, the formation of additional shoots, the strongly reduced capacity of the rootstock to accept a *Pistacia vera* graft, the development of cracked tissue at graft junctions weakening the graft union, the emergence of ectopic shoots from gall-like tissue formed at lateral buds, and, most typically, a complete disturbance of regular root development (Stamler et al. 2015a, 2015b) (Fig. 2). The causative agents of PBTS were found to be two *Rhodococcus* species: *Rhodococcus* sp. strain 1 (PBTS1) genetically related to *R. corynebacterioides* and *Rhodococcus* sp. strain 2 (PBTS2) with high genetic similarity to *R. fascians* (Stamler et al. 2015b, 2016). Little information is available on the former bacterium, but it has been found as an endophyte in xylem of eggplant and leaves of *Arabidopsis* (Traw et al. 2007; Achari and Ramesh 2014). Concerning the *R. fascians* isolate, few woody plants were

Fig. 2 Typical symptoms associated with Pistachio Bushy Top Syndrome on "UCB-1" pistachio rootstock. (**a**) Loss of apical dominance leads to bushy top appearance; (**b**) deformed leaves; (**c**) deformed root system; (**d**) unsuccessful grafting attempts; (**e**) bark cracking at the graft union

shown to be sensitive to leafy gall-inducing *R. fascians* strains (Putnam and Miller 2007), but the Anacardiaceae or cashew family to which pistachio belongs is a new host of this bacterium. While the strategy utilized by the model strain *R. fascians*

D188 to cause leafy galls on the model plant *Arabidopsis* is largely understood and the major players involved in the initiation and maintenance of the symptoms have been identified, as summarized above, to date, virtually nothing is known on the pathogenic strategies deployed by the PBTS strains. Nevertheless, both isolates generated an amplicon with *fasD*-specific primers, and PBTS2 also generated an amplicon with *fasA*-specific primers, suggesting that cytokinins might play a role. Interestingly, while performing Koch's postulates, it appeared that both PBTS *Rhodococcus* strains work in synergism to alter plant development on clonal "UCB-1" pistachio (Stamler et al. 2015b).

Whereas the current knowledge on the mode of action of the PBTS *Rhodococcus* strains is still in its infancy (Stamler et al. 2015a, 2015b, 2016), Koch's postulates were clearly fulfilled. Still the diagnosis of PBTS1 and PBTS2 as outbreak strains of PBTS on pistachio has been challenged because the presence of the virulence genes could not be confirmed, and consequently, the PBTS isolates analyzed were not pathogenic. Hence, the hypothesis has been put forward that the PBTS *Rhodococcus* strains would actually be beneficial bacteria naturally associated with pistachio (Savory et al. 2017). In our opinion, it is premature to assume that leafy gall and PBTS inducers necessarily use the same virulence mechanism and that all aspects of the biology of both syndromes are shared. Additionally, concerning the epidemiology of PBTS, very strong conclusions were drawn based on the analysis of two single PBTS isolates, whereas the epidemiology of leafy gall inducers in ornamental orchards could only be revealed by studying 60 different isolates (Savory et al. 2017). One can only hope that good and integer science will resolve this issue in the future (Randall et al. 2018; Vereecke 2018).

4 Beneficial and Undefined Associations of *Rhodococcus* Species with Plants

The concept of plants serving as a habitat for complex microbial communities interacting with each other and with the plant host is now widely accepted and gaining more research interest as to how we can use these organismal interactions in plant growth and health management. The composition of the microbial communities interacting with different plant parts can vary greatly and reflects niche adaptation. Within these microbiomes, several *Rhodococcus* species, apart from the well-known plant pathogen *R. fascians* (discussed above), have recently been found living in close association with various plant parts. For example, *Rhodococcus* isolates were found as part of the rhizosphere of *Origanum vulgare* in a sub-Himalayan region (Bafana 2013), the phyllosphere of *Arabidopsis thaliana* plants (Bai et al. 2015; Ritpitakphong et al. 2016), and as an endophyte of leaves of *Cercidiphyllum japonicum* (Li et al. 2008) and stem tissues of *Artemisia annua* and *Sorghum bicolor* (Zhao et al. 2012; Maropola et al. 2015). Although the ecological role of most of these plant-associated *Rhodococcus* species remains to

be discovered, a number of isolates have been shown to display plant growth-promoting properties and can confer resistance to plant pathogens.

4.1 Rhodococcus fascians *Beyond Leafy Gall Formation*

Recent work has shown that upon loss of its linear plasmid, the resulting nonpathogenic derivative D188-5 of *R. fascians* strain D188 can stimulate plant growth (Francis et al. 2016). This bacterium is very well equipped for an epiphytic lifestyle and likely acts as a phytostimulator mainly through the production of the plant hormones cytokinin and auxin (IAA) and the degradation of ethylene via 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Francis et al. 2016). The delicate interplay between cytokinin and auxin regulates meristem formation and development in both shoots and roots from early embryogenesis (Su et al. 2011), while ethylene inhibits shoot and root proliferation at high concentrations which generally occurs when the plant is under environmental stress (Sing et al. 2015). The fact that this plasmid-free, nonpathogenic derivative of D188 promotes plant growth suggests that pathogenic isolates may actually be plant growth-promoting bacteria gone astray. This reasoning is supported by the conservation of the plant growth-promoting determinants, especially the chromosomal genes involved in cytokinin and auxin metabolism, in strains covering both clades of the *R. fascians* species group discussed above (Francis et al. 2016). Additionally, plant growth-promoting activity has been attributed to other leafy gall-associated *R. fascians* strains (Savory et al. 2017), but the experimental data to support this conclusion were thought to be insufficient (Randall et al. 2018; Vereecke 2018).

The plant pathogen *R. fascians* was also identified as one of the dominant bacterial species on different cultivars of spring barley in combination with various other pathogenic and nonpathogenic bacteria, yeasts, and fungi (Newton et al. 2010) and as one of the *Rhodococcus* isolates in the rhizosphere of the nickel hyperaccumulator *Thlaspi goesingense* (Idris et al. 2004). The latter isolates displayed moderate to high siderophore production, and some also produced ACC deaminase (Idris et al. 2004). Although the later subjective synonym *R. luteus* (Klatte et al. 1994) was used, *R. fascians* was also isolated as a xylem-inhabiting bacterium from grape cultivars in Nova Scotia (Bell et al. 1995). Importantly, none of these hosts displayed disease symptoms suggesting that the pathogenic lifestyle of *R. fascians* might be strongly condition dependent.

4.2 *Plant-Associated* Rhodococcus *Species Have Plant Growth- Promoting Activities and Confer Plant Resistance to Pathogens*

Apart from the plant growth-promoting ability of the plasmid-free derivative of *R. fascians* D188 (Francis et al. 2016), several other *Rhodococcus* species were shown to stimulate plant growth and development. An isolate from postglacial clay and positive for ACC deaminase significantly increased the root biomass of pea plants in a pot experiment by 25% (Belimov et al. 2001). An endophytic species of tomato roots also tested positive for IAA production, synthesis of ACC deaminase, and had high siderophore activity (Abbamondi et al. 2016). Although not tested on plants, *Rhodococcus* aff. *qingshengii*, isolated from a polluted river in India, produced high levels of IAA (Hasuty et al. 2018). As part of its plant growth-promoting properties, *R. globerulus*, an endophyte of the medicinal plant *Plectranthus amboinicus*, was capable of phosphate solubilization making insoluble organic phosphorus available to plants. This trait opens up the possibility of using the isolate as a biofertilizer (Murugappan et al. 2017).

In a diversity study of the nodule microbiomes of *Lotus japonicus* and *Anthyllis vulneraria* in Sweden, two *Rhodococcus* isolates have been found with the ability to nodulate these two plants. These bacteria probably obtained this exceptional ability through horizontal gene transfer from other nodule-inhabiting bacteria, as their *nodA* gene was closely related to that of *Mesorhizobium loti* (Ampomah and Huss-Danell 2011). Moreover, it is speculated that the combined activity of the nitric oxide synthase of *Rhodococcus* isolate APG1, an endophyte of the aquatic fern *Azolla pinnata*, together with symbiotic *Arthrobacter* species could be beneficial to plants by providing nitrogen oxides for regulatory functions (Cohen and Yamasaki 2003; Cohen et al. 2004). *Rhodococcus* was also found as one of the main genera colonizing the shoot endosphere of a willow cultivar that is particularly well-suited for phytoremediation of heavy metal-contaminated soils. This property was attributed to the very diverse endophytic bacterial population among which a high number of metal-resistant plant growth-promoting bacteria, although for the identified *Rhodococcus* species no specific plant-stimulating properties were reported (Weyens et al. 2013).

Rhodococcus species themselves are commonly used for bioremediation due to their unique biochemical properties, such as biodegradation of hydrophobic natural compounds and xenobiotics, including polychlorinated biphenyls (PCB) (van der Geize and Dijkhuizen 2004; Larkin et al. 2005; Ceniceros et al. 2017; and further discussed in this volume). These bacteria are often found to live in close association with plants grown at such contaminated sites, and it is this unique plant—*Rhodococcus* combination that improves soil bioremediation (Leigh et al. 2006). *R. erythropolis* U23A, isolated from the rhizosphere of plants grown in PCB-contaminated soils, showed increased PCB degradation due to chemotaxis toward plant secondary metabolites (Toussaint et al. 2012; Pham et al. 2015). *R. erythropolis* strain CD 106 not only significantly increased the effectiveness of

the phytoremediation of petroleum hydrocarbon-contaminated soil by 31.2% in combination with ryegrass but also increased the biomass of roots and shoots by 30–49%, respectively (Płociniczak et al. 2017). Also, plant growth inhibition by chromium, more specifically Cr^{6+}, can be alleviated by the psychrotroph *R. erythropolis* MTCC 7905 due to its ability to reduce Cr^{6+} to the nontoxic Cr^{3+} and several plant growth-promoting activities, such as ACC deaminase production, indole and siderophore production, although these latter activities decreased with increasing Cr^{6+} concentrations (Trivedi et al. 2007).

Finally, microbiome studies show that the natural inhabitants of plants such as leaf epi- and endophytic bacteria can confer resistance to plant pathogens. In that respect, *R. kyotonensis* and *R. corynebacterioides*, endophytes isolated from *Arabidopsis*, were shown to confer mild disease suppression against *Pseudomonas syringae* pv. tomato DC3000 (Hong et al. 2015), and another endophyte, *Rhodococcus* sp. KB6, significantly repressed black rot disease in sweet potato caused by *Ceratocystis fimbriata* (Hong et al. 2016). *R. erythropolis* can intercept and degrade the quorum-sensing signals produced by *Pectobacterium atrosepticum* and *P. carotovorum*, the causative agents of blackleg and soft rot disease in potato plants and tubers. These signals are important in the onset of virulence gene expression, and hence, degradation of the quorum-sensing N-acyl homoserine lactone signals limits disease development (Jafra et al. 2006; Cirou et al. 2007; Latour et al. 2013; Kwasiborski et al. 2015).

5 Concluding Remarks

Although the leafy gall-inducing model *R. fascians* strain D188 has been studied for over a quarter of a century, detailed information on the genes implicated in the pathogenicity is only available for the *fas* and the *att* operon. However, the linear plasmid, around 200 kb in size, is highly conserved in all other leafy gall inducers analyzed to date, suggesting that many other determinants encoded by the linear plasmid must be essential for a successful interaction with the plant host. Future experimentation will hopefully give more insight into the role of these other genes in the pathogenicity of *R. fascians*, and time will tell if the *fas* operon and the trick-with-the-cytokinin-mix are indeed determinative for leafy gall formation.

With the outbreak of PBTS on pistachio, the genus *Rhodococcus* acquired an additional pathogenic member, and with the expanding research on plant-associated microbiomes, it is clear that the capacity of members of this genus to interact with plants has been largely underestimated. Given that the genus *Rhodococcus* is renowned for its highly specialized metabolic capacities, the comparison of the genomes of all plant-interacting *Rhodococcus* species, beneficial and adverse, might reveal novel functions specifically utilized by this group of bacteria to interact with a plant host.

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H. M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
https://doi.org/10.1007/978-3-030-11461-9