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

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Contents

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

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H.M. Alvarez (ed.), *Biology of Rhodococcus*, Microbiology Monographs 16,
DOI 10.1007/978-3-642-12937-7_10,

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field, including the most recent knowledge that has emerged from studies on TAG accumulation by rhodococci and some unpublished results.

1 Introduction

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

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

2 Triacylglycerol Accumulation by *Rhodococcus*

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

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

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

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

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

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

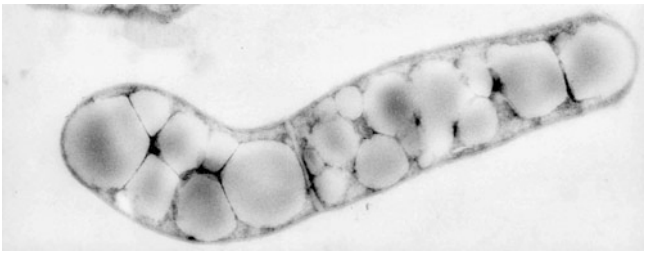

Fig. 1 Micrograph showing a cell of *Rhodococcus opacus* PD630 containing several TAG granules during growth on gluconate as sole carbon and energy source (Alvarez 2006). Picture: F. Mayer from the Georg-August University of Göttingen, Germany

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

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

3 Composition and Structure of Rhodococcal Triacylglycerols

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

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

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

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

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

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

4 Conditions for Triacylglycerol Accumulation and Mobilization

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

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

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

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

5 Triacylglycerol Biosynthesis by *Rhodococcus*

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

5.1 Production of Key Metabolic Precursors for Fatty Acid Biosynthesis

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

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

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

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

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

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

5.2 Biosynthesis of Fatty Acids

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

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

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

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

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

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

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

5.3 Biosynthesis of Triacylglycerols

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

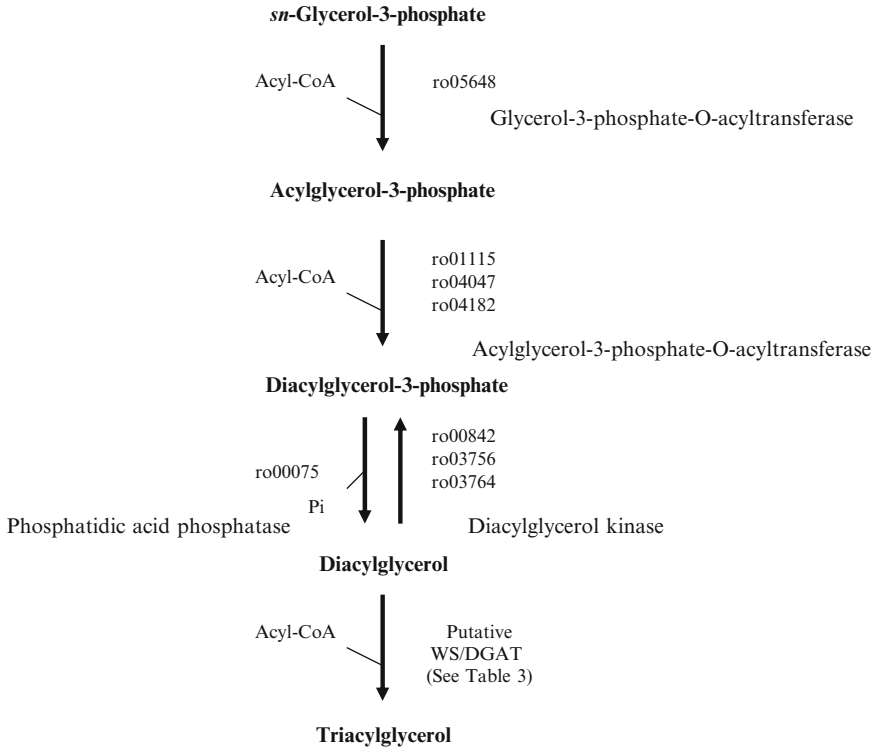

Fig. 2 Pathway for TAG biosynthesis in rhodococci. Genes encoding for putative enzymes for the Kennedy pathway occurring in *R. jostii* RHA1 genome are shown

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

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

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

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

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

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

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

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

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

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

[a]Length aa

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

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

6 Biogenesis of TAG Inclusion Bodies

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

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

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

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

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

7 Physiological Functions of TAGs in *Rhodococcus*

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

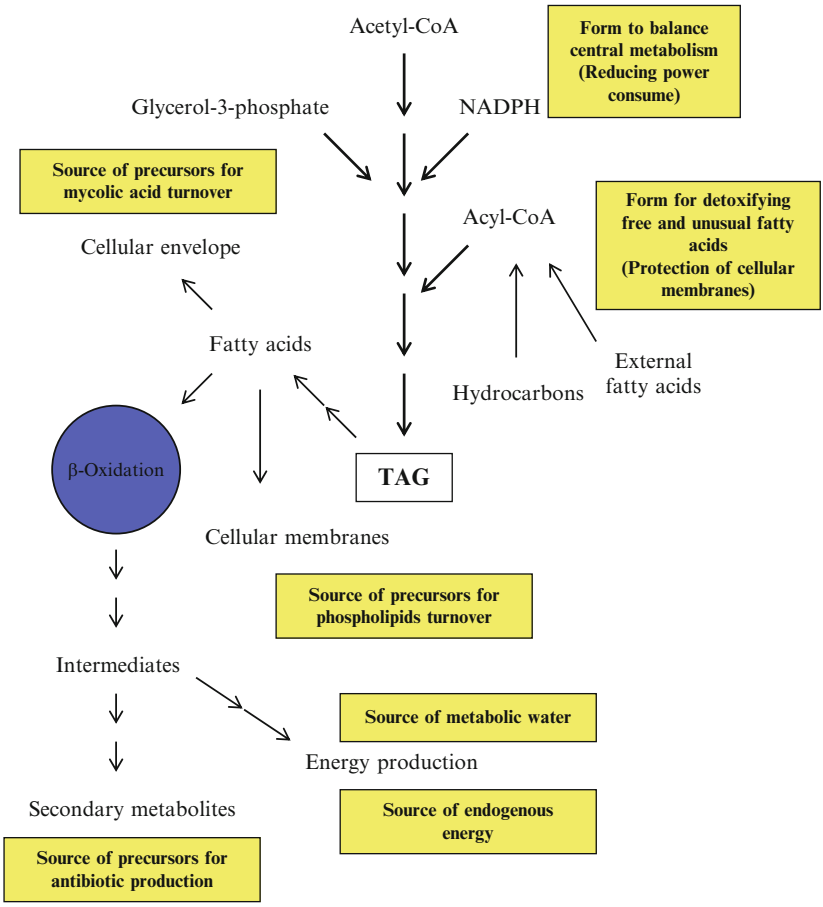

Fig. 3 Physiological functions of TAG proposed for TAG-accumulating rhodococci

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

7.1 TAGs as Endogenous Carbon and Energy Sources

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

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

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

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

7.2 TAGs as Source of Precursors for Membranes and Cell Envelope

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

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

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

7.3 TAGs as a Form to Detoxify Free Fatty Acids

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

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

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

7.4 TAGs as a Form to Balance Central Metabolism

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

7.5 TAGs as Source of Intermediates for Secondary Metabolism

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

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

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

8 Biotechnological Significance of Rhodococcal TAGs

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

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

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

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

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

9 Concluding Remarks

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

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

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