Biology of Triacylglycerol Accumulation by *Rhodococcus*

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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

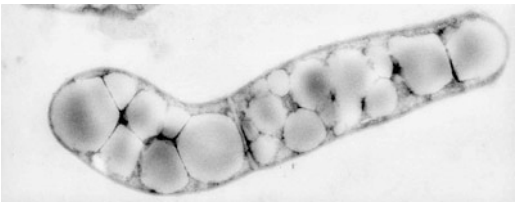

Fig. 1 Micrograph showing a cell of *Rhodococcus opacus* PD630 containing several TAG granules during growth on gluconate as sole carbon and energy source. Picture: F. Mayer from the Georg-August University of Göttingen, Germany

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

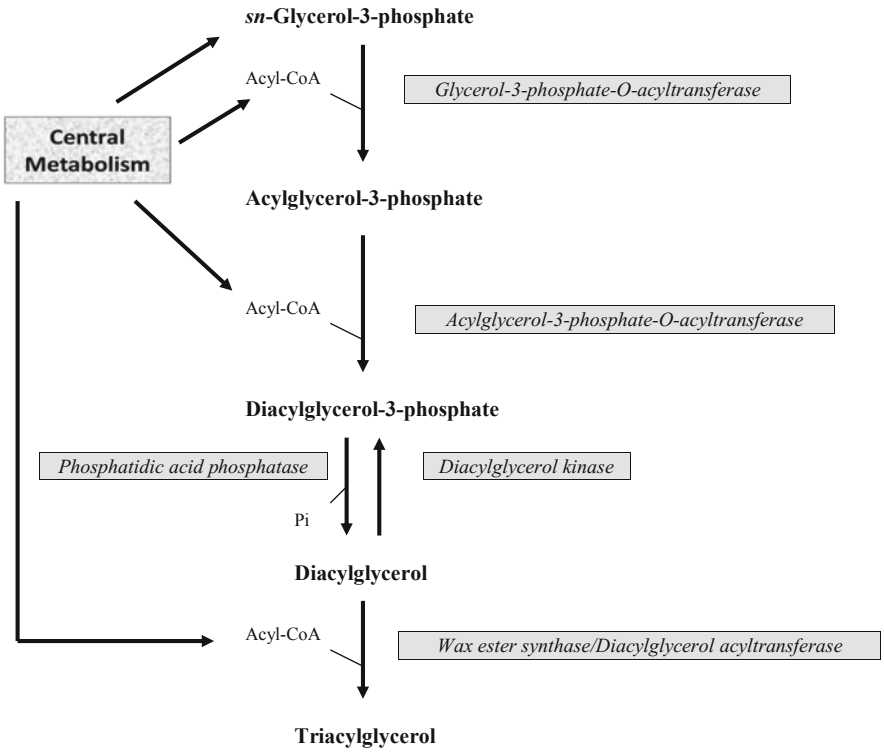

Fig. 2 Pathway for TAG biosynthesis (Kennedy pathway) in rhodococci

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.

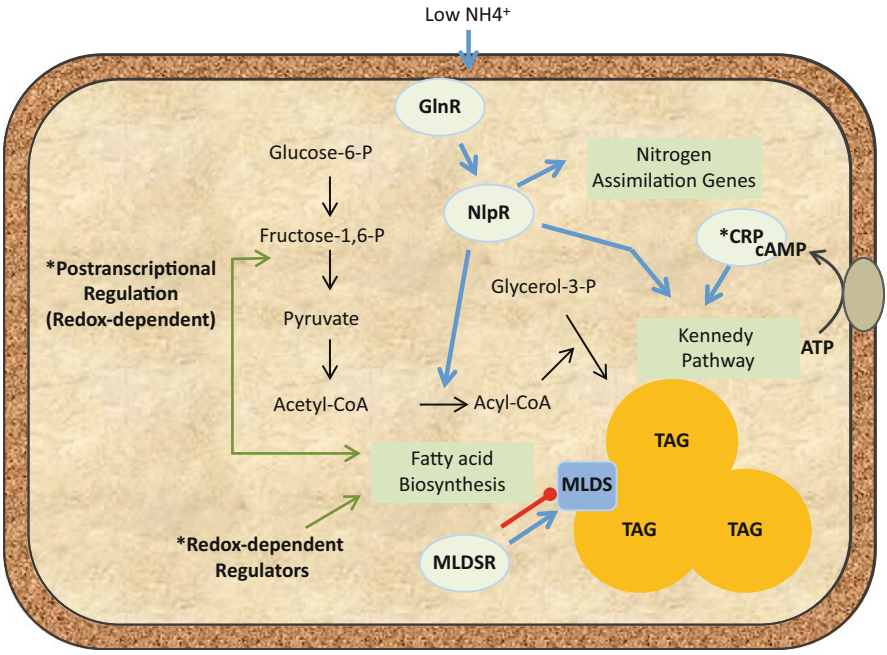

Fig. 3 Some components of the regulatory network controlling TAG biosynthesis and accumulation in oleaginous rhodococci. Blue arrows indicate activation, red bars indicate repression, and green arrows indicate control of gene expression or enzyme activities by unknown mechanisms (activation or repression). 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).

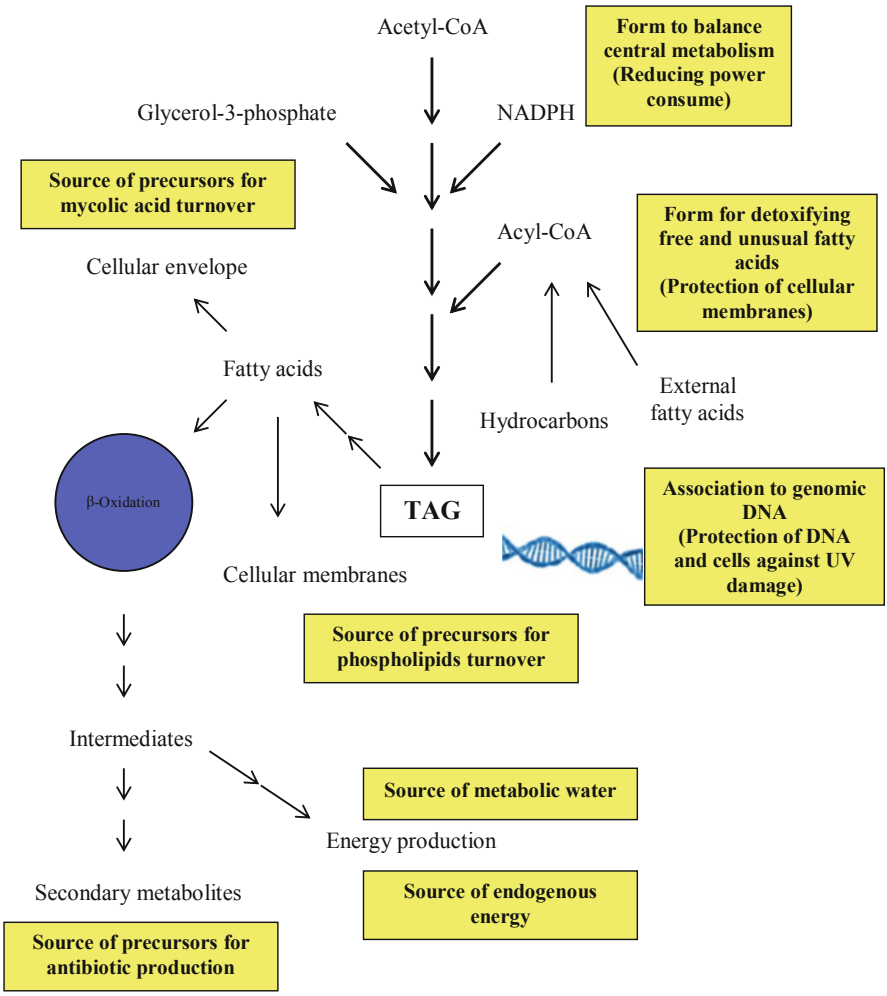

Fig. 4 Physiological functions of TAG proposed for TAG-accumulating rhodococci

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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