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MINIREVIEW

Insights into the Metabolism of Oleaginous *Rhodococcus* spp.

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ABSTRACT Some species belonging to the *Rhodococcus* genus, such as *Rhodococcus opacus*, *R. jostii*, and *R. wratislaviensis*, are known to be oleaginous microorganisms, since they are able to accumulate triacylglycerols (TAG) at more than 20% of their weight (dry weight). Oleaginous rhodococci are promising microbial cell factories for the production of lipids to be used as fuels and chemicals. Cells could be engineered to create strains capable of producing high quantities of oils from industrial wastes and a variety of high-value lipids. The comprehensive understanding of carbon metabolism and its regulation will contribute to the design of a reliable process for bacterial oil production. Bacterial oleagenicity requires an integral configuration of metabolism and regulatory processes rather than the sole existence of an efficient lipid biosynthesis pathway. In recent years, several studies have been focused on basic aspects of TAG biosynthesis and accumulation using *R. opacus* PD630 and *R. jostii* RHA1 strains as models of oleaginous bacteria. The combination of results obtained in these studies allows us to propose a metabolic landscape for oleaginous rhodococci. In this context, this article provides a comprehensive and integrative view of different metabolic and regulatory attributes and innovations that explain the extraordinary ability of these bacteria to synthesize and accumulate TAG. We hope that the accessibility to such information in an integrated way will help researchers to rationally select new targets for further studies in the field.

KEYWORDS metabolism, oleagenicity, regulation, *Rhodococcus*, triacylglycerols

Oleagenicity could be defined as the capacity of an organism to synthesize and accumulate significant amounts of lipids. Oleaginous microorganisms usually accumulate lipids at more than 20% of their weight (dry weight) as intracellular inclusion bodies. Different bacteria are able to synthesize triacylglycerols (TAG), such as those belonging to the genera *Acinetobacter*, *Alkanivorax*, *Streptomyces*, *Rhodococcus*, *Mycobacterium*, and *Nocardia* (1–5). Among them, some members of *Rhodococcus* belonging to the species group C, represented by *Rhodococcus opacus* (6), can be considered oleaginous bacteria for their ability to accumulate large amounts of lipids. This rhodococcal group proposed by Sangal et al. (7) comprises *R. opacus*, *R. jostii*, *R. wratislaviensis* and *R. imtechensis*. These rhodococci usually possess large genomes (between 7.4 and 10.4 Mbp) containing several genes involved in catabolic reactions and the biosynthesis of diverse lipids. *R. opacus* PD630 and *R. jostii* RHA1 are the oleaginous models most frequently used for basic research in the field (8). For this reason, the basic knowledge included in this review article is based almost exclusively on results obtained from these oleaginous strains.

The main trigger for TAG accumulation by rhodococci is the limitation of the nitrogen source and an excess of the available carbon source, or a high C/N ratio in the culture media (9). The imbalance between C and N in cells promotes profound changes in the metabolism with a redirection of the carbon flux to lipogenesis (10–12). Thus, oleagenicity requires a special metabolic network involving several reactions, pathways, and regulatory circuits for the appropriate distribution of precursors, energy, and

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reducing equivalents in the cell. A schematic view of the metabolic circuits that support oleagenicity in rhodococci is shown in Fig. 1.

Oleaginous rhodococci are promising microbial cell factories for the production of lipids with application in the industry (13–14). Special interest in these microorganisms centers on their ability to convert agroindustrial wastes into high-value lipids (15–18). The study of metabolism in oleaginous rhodococci is of interest since its deregulation allows the opportunity of cell engineering to increase the production of lipids. For this reason, it is necessary to improve our understanding of cell metabolism and its regulation in these oleaginous bacteria. In this context, our aim is to provide a broad overview of metabolic and regulatory mechanisms occurring in oleaginous rhodococci and to identify knowledge gaps to guide future studies in the field.

THE ED AND PP PATHWAYS RATHER THAN THE EMP PATHWAY ARE SPECIFICALLY ACTIVATED IN OLEAGINOUS SPECIES

Oleaginous *Rhodococcus* bacteria possess multiple biologically feasible routes for breaking down glucose into incomplete oxidized intermediates like pyruvate. All genes/enzymes of the glycolytic pathways, such as the Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED), and pentose-phosphate (PP) pathways, are present in the genomes of *R. opacus* and *R. jostii* (10–12). This broad metabolic repertoire allows the cell to modify the carbon fluxes and the production of ATP and reducing equivalents according to its physiological needs. Figure 2 provides an integrative view of the main changes in carbon flow in rhodococcal metabolism under conditions of nitrogen limitation and nitrogen excess. Transcriptome and proteome studies demonstrated that enzymes involved in the ED pathway are significantly induced during TAG accumulation in *R. jostii* RHA1 (11, 12). Similarly, *R. opacus* PD630 has also a strong preference for the ED pathway (19). Three genes related to the ED pathway, which are arranged in a gene cluster on RHA1 genome, were highly upregulated under TAG accumulation conditions, as revealed by transcriptome sequencing (RNA-Seq) and reverse transcription-quantitative PCR (RT-qPCR) analyses (RHA1_RS11565 for KHG/KDPG aldolase, *eda*/RHA1_RS11570 for phosphogluconate dehydratase, and *edd*/RHA1_RS11575 [*zwf*] for glucose 6-phosphate 1-dehydrogenase) (11, 12). Although Zwf catalyzes the first step of the PP route producing NADPH, this enzyme is not unique to this pathway, since its product 6-phosphogluconate (6 P-gluconate) can also be further metabolized in the ED pathway (20). Interestingly, *R. jostii* RHA1 possesses at least 5 putative Zwf enzymes distributed in its genome. The physiological role of each Zwf enzyme in this oleaginous bacterium remains to be investigated. On the other hand, several enzymes from the PP pathway, such as transaldolase and transketolase, were highly expressed in RHA1 under TAG accumulation conditions (11). This pathway generates NADPH, which is required as a cofactor for fatty acid biosynthesis, in contrast to the EMP pathway, which produces NADH. Thus, under nitrogen-limiting conditions which promote lipid accumulation, sugar-P intermediaries are channeled to the ED and PP pathways rather than to the EMP route in oleaginous rhodococci. The ED pathway is less ATP efficient than the EMP route but has lower protein costs and avoids the loss of carbon (21), which can be conserved for lipogenesis. Further studies are necessary to better understand how changes in the sugar-P metabolism are coupled with the synthesis of lipids in these bacteria.

SEVERAL METABOLIC REACTIONS PROVIDE REDUCING EQUIVALENTS FOR LIPOGENESIS

Fatty acid synthesis utilizes two molecules of NADPH for each molecule of acetate; thus, the biosynthesis of palmitic acid requires the input of 8 molecules of NADPH and 7 molecules of ATP. For this reason, it is clear that oleagenicity is a process demanding a high level of NADPH. Thus, oleaginous rhodococci must possess robust NADPH-generating systems to support the massive fatty acid biosynthesis for TAG accumulation. The supply of NADPH needed for lipid synthesis in oleaginous rhodococci might be provided by different sources as are found in TAG-accumulating yeasts and fungi

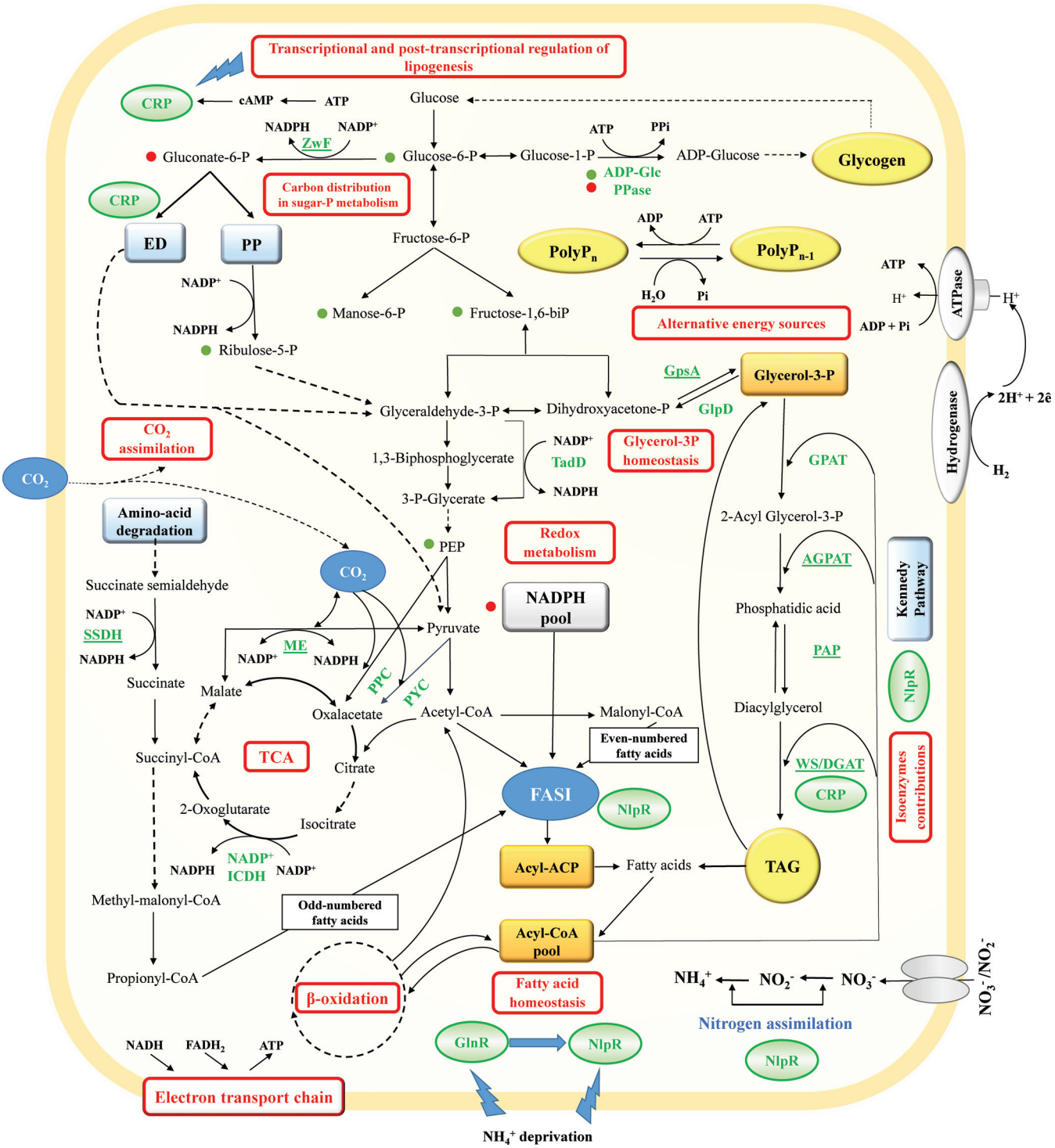

FIG 1 Schematic representation of different metabolic reactions and pathways contributing to oleagenicity in *Rhodococcus*. The enzymes and regulators (circles) mentioned in this article are in green. The enzymes that exhibit redundancy are underlined. The orange boxes indicate the precursors needed for the synthesis of triglycerides, while the yellow circles represent reserve compounds. The activator and inhibitory metabolites for the ADP-Glc PPase activity are marked with green and red dots, respectively. Lightning bolts indicate the existence of an external input that activates a regulatory protein. The red boxes indicate the main knowledge gaps that need to be investigated to get a better integral understanding of oleagenicity in rhodococci. Abbreviations: ED, Entner-Doudoroff pathway; PP, pentose phosphate pathway; FAS, fatty acid biosynthesis; TCA, tricarboxylic acid cycle; TAG, triacylglycerols; ADP-Glc PPase, ADP-glucose pyrophosphorylase; Zwf, NADP$^+$-dependent glucose-6-phosphate dehydrogenase; NADP$^+$-ICDH, NADP$^+$-dependent isocitrate dehydrogenase; ME, malic enzyme; TadD, nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase; SSDH, NADP$^+$-dependent succinate-semialdehyde dehydrogenases; PPC, phosphoenolpyruvate carboxylase; PYC, pyruvate carboxylase; GlpD, flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase; GpsA, NADP$^+$-dependent glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerophosphate acyltransferase; PAP, phosphatidic acid phosphatase enzyme; DGAT, diacylglycerol acyltransferase; GlnR, nitrogen-sensing regulatory protein; NlpR, nitrogen lipid regulator.

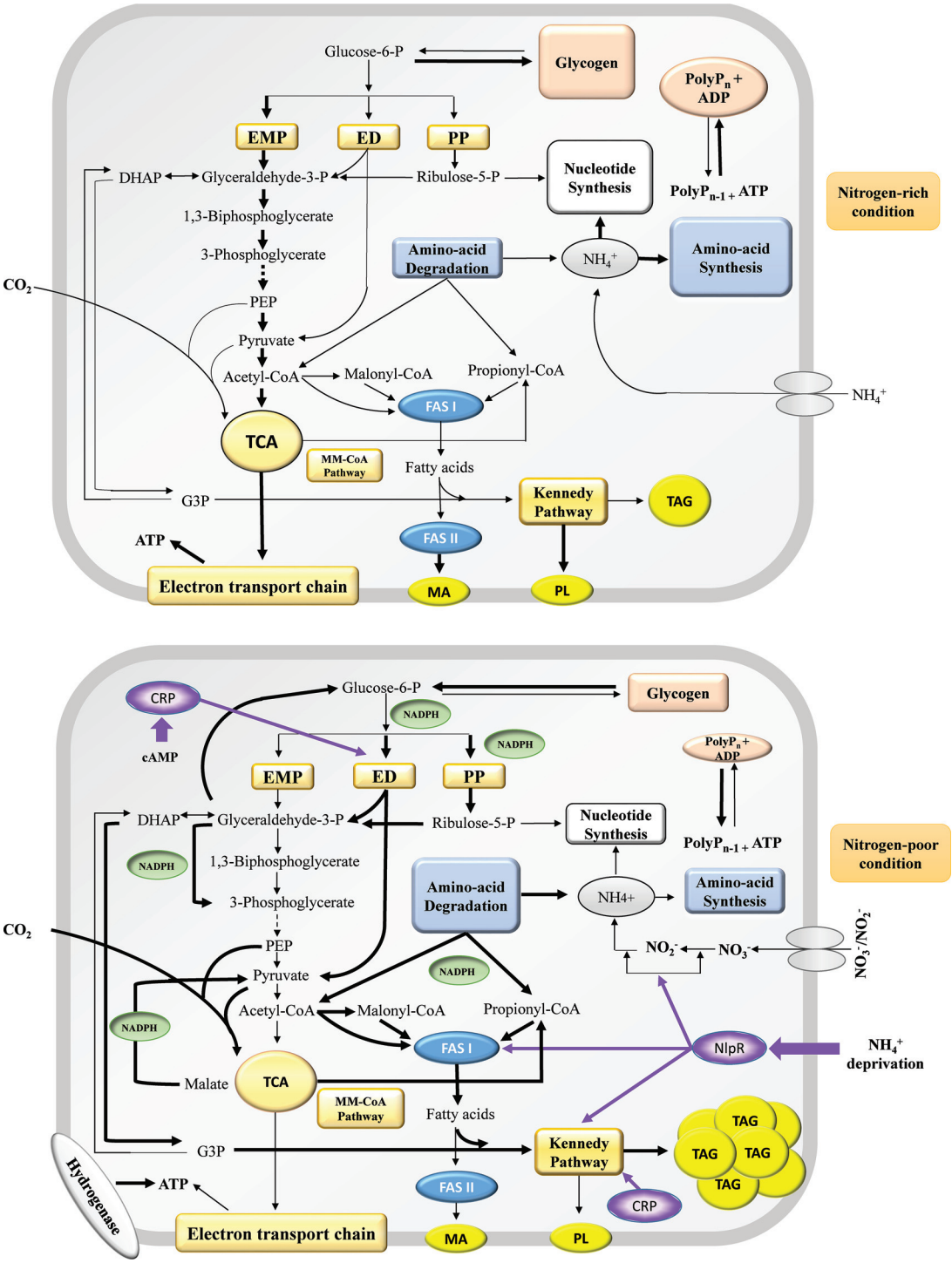

FIG 2 Differences in the carbon flux distribution in oleaginous rhodococci under nitrogen-rich and nitrogen-poor (TAG accumulation) conditions. The network representation integrates changes of carbon flux predicted from results of diverse studies (transcriptomes, proteomes, gene mutagenesis, and gene overexpression experiments, enzymatic activity measurements, and ^{13}C isotope fractionation approaches) in *R. jostii* RHA1 and *R. opacus* PD630 as models of oleaginous rhodococci. Arrows indicate the physiological directions of reactions. The intensity of the metabolic flow is represented by the thickness of the black arrows. Metabolic pathways are shown in boxes, and the occurrence of a higher or lower predominance is represented by combining the box and the font sizes. Thick violet arrows represent signal inputs or effectors of regulatory proteins. Thin violet arrows represent activation of reactions and/or pathways by regulatory proteins. Abbreviations: EMP, Embden-Meyerhof-Parnas pathway; MM-CoA, methyl malonyl-CoA; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; G3P, glycerol 3-phosphate; TAG, triacylglycerols; PolyP, polyphosphate; MA, mycolic acids; PL, phospholipids; CRP, cAMP receptor protein.

(22, 23). $NADP^+$-dependent isocitrate dehydrogenase, the oxidative PP pathway, and the malic enzyme seem to be the major NADPH sources for many oleaginous fungi, whereas the PP pathway rather than malic enzyme is the primary source of NADPH required for fatty acid synthesis in *Yarrowia lipolytica* (24). On the other hand, proteome analysis of *Rhodotorula toruloides* during conversion of xylose to lipids revealed that NADPH is generated primarily through the PP pathway, with the contribution of malic enzyme as well as alcohol and aldehyde dehydrogenases (25). In oleaginous rhodococci the intracellular cofactor pools might be influenced by the changes in the metabolic scenario in cells during the transition between different physiological states (Fig. 2). The induction of the PP pathway during TAG accumulation in *R. jostii* RHA1 might contribute to the increase of NADPH pools in cells (12), together with the activation of other $NADP^+$-dependent reactions. Recently, we demonstrated that the $NADP^+$-malic enzyme activity is linked with fatty acid and TAG biosynthesis in *R. jostii* RHA1 and *R. opacus* PD630 (26). The $NADP^+$-malic enzyme activities in cell-free crude extracts of RHA1 and PD630 from nitrogen-poor medium were higher than those from nitrogen-rich medium. Moreover, the addition of sesamol (3,4-methylenedioxyphenol), which is a natural inhibitor of malic enzyme activity, negatively affected TAG accumulation in RHA1 and PD630 (26). These effects were not observed in *Rhodococcus fascians* F7 (a nonoleaginous strain), suggesting that $NADP^+$-malic enzyme activity did not play a significant role in F7 metabolism independently of the nitrogen concentration present in the culture media (26). On the other hand, the overexpression of RHA1_RS44255, a nonredundant $NADP^+$-malic enzyme from *R. jostii* RHA1, in strains RHA1 and PD630 promoted an increase in total $NADP^+$-malic enzyme activity and an up to 1.9-fold increase in total fatty acid production in both strains when grown with glucose (26). These results confirmed the functional contribution of malic enzymes to lipogenesis in oleaginous rhodococci. However, additional studies are required to elucidate the mechanism (generation of NADPH or acetate) by which these enzymes contribute to lipid accumulation in rhodococci.

The role of $NADP^+$-dependent isocitrate dehydrogenase in lipogenesis in rhodococci has not yet been elucidated, although previous results of proteomic studies have shown a decrease in the abundance of these enzymes in RHA1 during the accumulation of TAG (11). These results suggest a minor or no contribution of $NADP^+$-dependent isocitrate dehydrogenase in lipid biosynthesis in rhodococci, in contrast to oleaginous fungi (24). The switch in rhodococci from vegetative to TAG-accumulating cells might promote alterations in the tricarboxylic acid (TCA) cycle organization and operation according to the metabolic and physiological demands. Since the TCA cycle and the reactions involved in the phosphoenolpyruvate-pyruvate-oxalacetate node control the distribution of carbon between anabolism, catabolism, and energy supply to the cell (27), further studies must be performed to understand how oleaginous rhodococci adapt their metabolic network to support lipogenesis under nitrogen-limiting conditions.

On the other hand, another interesting mechanism for generation of NADPH during lipid accumulation was reported for *R. opacus*. MacEachran and Sinskey (28) reported a gene encoding a nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GapN) in PD630, called TadD protein, as a source of NADPH for TAG biosynthesis under nitrogen-limiting conditions. The authors demonstrated that this gene is specifically induced in PD630 cells during TAG accumulation. GapN catalyzes the oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate while simultaneously reducing $NADP^+$ to NADPH. This enzyme is used in a variant of glycolysis for producing NADPH rather than ATP (Fig. 2). In this way, NADPH and 3-phosphoglycerate can be then used for lipid synthesis. Interestingly, *tadD* overexpression in *R. opacus* PD630 resulted in an increase of TAG accumulation (28).

Proteomic and transcriptomic studies revealed the significant induction of several enzymes involved in the degradation of proteins and amino acids during cultivation of *R. jostii* RHA1 under nitrogen-limiting conditions (11, 29). The degradation of some amino acids, such as L-glutamate, involves $NADP^+$-dependent enzymes, which might

contribute to the cofactor pools for fatty acid biosynthesis and consequently for the synthesis and accumulation of TAG. The high activation of genes encoding $NADP^+$-dependent succinate-semialdehyde dehydrogenases for the conversion of succinate-semialdehyde to succinate in strain RHA1 during cultivation under TAG-accumulating conditions suggests a contribution of these enzymes to oleagenicity in rhodococci. Succinate can be directed to the methyl-malonyl coenzyme A (methyl malonyl-CoA) pathway, which is also induced in RHA1, for the production of propionyl-CoA as a precursor for synthesis of odd-numbered fatty acids (5, 11). The role of $NADP^+$-dependent succinate-semialdehyde dehydrogenases and the methyl-malonyl-CoA pathway in the TAG-accumulating process deserves further studies with oleaginous rhodococci.

The present evidence suggests that oleaginous rhodococci possess a robust NADPH-generating system, which includes multiple $NADP^+$-dependent enzymes, reactions, and pathways that contribute to the necessary cofactor pools for supporting massive fatty acid and TAG biosynthesis (Fig. 1 and 2). The development of powerful NADPH-generating mechanisms during the evolution of rhodococci might represent one of the physiological innovations that differentiate oleaginous rhodococci from nonoleaginous actinobacteria. Moreover, the supply of reducing equivalents to support lipid metabolism is one of the key aspects for the development of engineering processes.

BIOSYNTHETIC PATHWAYS IN COMPETITION WITH LIPOGENESIS

The reorganization of cell metabolism in oleaginous rhodococci might also involve the downregulation of reactions and pathways that consume reducing equivalents or precursors useful for lipogenesis.

Oleaginous rhodococci produce glycogen at small amounts (between 0.3% and 5% of their weight [dry weight]) mostly during the exponential growth phase, independently of the carbon source used (30, 31). In *R. jostii* RHA1, glycogen biosynthesis is allosterically regulated through the activity of the ADP-glucose pyrophosphorylase (ADP-Glc PPase) enzyme (32). Different intermediates from the sugar-P metabolism serve as effectors of its activity, including glucose-6P, mannose-6P, fructose-6P, ribose-5P, and phosphoenolpyruvate as major activators and NADPH and 6 P-gluconate as main inhibitors of the enzyme. The combined availability of these effectors (activators and inhibitors) orchestrates the fine regulation of the ADP-Glc PPase and glycogen synthesis in RHA1 (32). Interestingly, one of the key ED pathway products, such as 6 P-gluconate, downregulates glycogen synthesis in RHA1. At the same time, NADPH, which is required for fatty acid biosynthesis, also inhibits glycogen synthesis, increasing intracellular availability of carbon for lipogenesis. In this context, MacEachran and Sinskey (28) reported that the ratio of NADPH to $NADP^+$ is elevated in PD630 cultures grown under lipid production conditions compared to that in cultures grown under vegetative growth conditions. Finally, enzymes for glycogen degradation, such as glycogen phosphorylase, glycogen-debranching enzyme, and phosphoglucomutase, are induced under nitrogen-limiting conditions according to results of proteomic analysis (11). Thus, the glycogen-recycling system might help to modulate the carbon fluxes into glycolytic pathways in oleaginous rhodococci according to the physiological and energetic status of the cell (32). Glycogen metabolism seems to be part of a complex assemblage of factors that are interconnected with lipid and nitrogen metabolisms (Fig. 2).

On the other hand, proteomic studies revealed that the abundance of the L-ectoine synthase enzyme involved in the biosynthesis of the compatible solute ectoine decreased 41.9-fold in *R. jostii* RHA1 during TAG accumulation (11). The ectoine biosynthesis pathway may compete with TAG synthesis for common intermediates, such as acetyl-CoA and NADPH (33). These results suggest that oleagenicity in rhodococci involves not only the activation of metabolic reactions and pathways that generate NADPH and precursors but also a more profound reorganization of cell metabolism inhibiting biosynthetic pathways in competition with lipogenesis.

CARBON DIOXIDE ASSIMILATION AND ALTERNATIVE ENERGY SOURCES MIGHT CONTRIBUTE TO LIPOGENESIS

R. opacus 1CP highly depends on carboxylation reactions with external CO_2 during heterotrophic growth (34). The authors reported that this strain is not able to grow with glucose as carbon source in the absence of externally provided CO_2 (34). Experiments applying isotopically labeled compounds demonstrated that in this strain CO_2 is fixed by anaplerotic carboxylation reactions (by malic enzyme, and phosphoenolpyruvate and pyruvate carboxylases), mostly for the synthesis of odd-numbered fatty acids and amino acids (34). In this context, the label incorporation into proteins or amino acids of *Rhodococcus* was about 30 to 40% higher than in *Pseudomonas* (34). In addition, about 6- to 8-fold-higher enrichments in ^{13}C, on average, were found in odd-numbered relative to the even-numbered fatty acids in *R. opacus*. TAG produced by oleaginous rhodococci are usually enriched with odd-numbered fatty acids (20 to 30% of the total fatty acids from gluconate). Propionyl-CoA is the precursor for the synthesis of odd-chain-length fatty acids, which are mainly formed from succinyl-CoA via the methyl malonyl-CoA pathway in *R. opacus* (5). These results suggested that the heterotrophic CO_2 assimilation by anaplerotic reactions might play a role in TAG biosynthesis and accumulation in *R. opacus* and also probably in *R. jostii* (Fig. 1 and 2). Moreover, CO_2 assimilation during heterotrophic growth might contribute to the enrichment of odd-numbered fatty acids in the accumulated TAG in these bacteria.

Oleaginous rhodococci possess additional genetic and physiological properties that might contribute to their ability to synthesize and accumulate large amounts of TAG. *R. opacus* and *R. jostii* have the genetic inventory to utilize hydrogen as an alternative energy source (35, 36) (Fig. 1 and 2). On the other hand, both oleaginous rhodococci are able to produce polyphosphates under diverse culture conditions (30). Polyphosphate is a flexible molecule that can serve as a source of short-term energy released during its hydrolysis (37). The operation of these alternative routes could provide greater robustness to the oleaginous metabolism in these bacteria, since they might serve as auxiliary mechanisms for the production of energy and precursors during the accumulation of lipids. The possible association of these processes to lipid accumulation should be investigated in detail to improve our understanding on the biochemistry of oleaginous rhodococci.

A ROBUST GENETIC REPERTOIRE SUPPORTS OLEAGENICITY

One of the special features of the oleaginous rhodococci is the larger size of their genomes (approximately 8 to 10 Mbp) in comparison to those of nonoleaginous rhodococci (approximately 5 to 7 Mbp) (38). Moreover, oleaginous rhodococci exhibit higher redundancy of several genes/enzymes related to the synthesis of lipids and their precursors compared to nonoleaginous rhodococci. In this context, *R. jostii* RHA1 possesses five genes encoding putative glucose-6-phosphate dehydrogenase enzymes (Zwf), which participate in the PP pathway and supplies reducing equivalents (NADPH) for the cell. On the other hand, $NADP^+$-dependent malic enzymes, which are involved in the NADPH and/or acetate generation for supporting biomass and lipid accumulation, are more redundant in *R. jostii* (3 isoenzymes), *R. opacus* (2 isoenzymes), or *R. wratislaviensis* (2 isoenzymes) than in other rhodococci, such as *R. fascians* (1 enzyme), *R. erythropolis* (1 enzyme), *R. rhodochrous* (1 enzyme), *R. equi* (1 enzyme), and *R. pyridinivorans* (1 enzyme) (26).

The genes encoding putative succinate semialdehyde dehydrogenase (SSDH) are highly redundant in *R. jostii* and *R. opacus*, which catalyzes the conversion of succinate semialdehyde in succinate with generation of NADPH. Some of these genes were highly induced by *R. jostii* RHA1 during TAG accumulation, as revealed by a previous proteome study (11). Thus, the high redundancy of genes encoding NADPH-generating enzymes in oleaginous rhodococci (Fig. 1 and 2) might be part of a mechanism for maintaining cell redox homeostasis through the differential expression of isoenzymes with different cofactor specificities.

A previous study suggested the occurrence of the glyceroneogenesis pathway in *R.*

jostii RHA1, which catalyzes the conversion of pyruvate into glycerol-3-phosphate, a key precursor for TAG biosynthesis, under nitrogen-limiting conditions (11). The activation of the glyceroneogenesis pathway together with the activity of the flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase (GlpD) and the reaction catalyzed by $NADP^+$-dependent glycerol-3-phosphate dehydrogenase (GpsA) might regulate the availability of glycerol-3-phosphate during TAG accumulation. In this context, *R. jostii* and *R. opacus* possess two putative GpsA proteins in their genomes, whereas nonoleaginous rhodococci contain only one gene encoding this enzyme (39). The occurrence of GpsA isoenzymes in the oleaginous cells might provide them the possibility to control more finely the homeostasis of the key intermediate, glycerol-3-phosphate, for TAG biosynthesis. The role of both GpsA isoenzymes during lipogenesis in oleaginous rhodococci is other interesting research gap to fill in this field.

In addition to some genes involved in central metabolism, the oleaginous rhodococcal species, such as *R. opacus*, *R. jostii*, and *R. wratislaviensis*, are highly enriched in genes for biosynthesis and degradation of lipids. In contrast, rhodococcal species with a lower ability to accumulate TAG possess a complete but a more simplified set of genes/proteins for supporting TAG accumulation (38). Interestingly, oleaginous rhodococci contain multiple isoforms of enzymes involved in the Kennedy pathway, the main TAG biosynthesis pathway occurring in these microorganisms (Fig. 1). Briefly, the Kennedy pathway consists of the following reactions: (i) the precursor *sn*-glycerol-3-phosphate is esterified by an acyl-CoA residue in a reaction catalyzed by a glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid, and (ii) this, is in turn, is acylated by an acylglycerophosphate acyltransferase (AGPAT) to form a phosphatidic acid molecule. (iii) The phosphate group is removed by a phosphatidic acid phosphatase enzyme (PAP), and finally, (iv) the resultant diacyl-*sn*-glycerol (DAG) is acylated by a diacylglycerol acyltransferase (DGAT), which can utilize a wide range of fatty acyl-CoA residues to form the triacyl-*sn*-glycerol. Numerous isoforms of these enzymes are found in *R. jostii* and *R. opacus* genomes (10, 29, 38). For instance, *R. jostii* RHA1 possesses 8 genes encoding putative AGPATs, 7 genes encoding putative PAP enzymes (including 3 haloacid dehalogenase [HAD] hydrolases proposed by Amara et al. [29]), and 16 putative DGAT isoenzymes (29, 40). It is difficult to determine the exact role of each of these genes/proteins since any attempt to study a specific gene might require deletion of multiple versions of that gene due to redundancy. However, the contribution of some DGAT isoenzymes to TAG biosynthesis has been determined for *R. opacus* PD630 and *R. jostii* RHA1. When the *atf1* gene was disrupted in PD630, cells of the mutant exhibited a significant decrease of total DGAT activity and accumulated up to 50% less fatty acid than the wild type during cultivation on gluconate under nitrogen-limiting conditions (41). On the other hand, the disruption of the *atf2* gene in PD630 resulted in a decrease of approximately 30% of TAG content in comparison to the wild type (42), whereas the disruption of *atf8* in *R. jostii* RHA1, the most abundant DGAT isoenzyme during TAG accumulation, promoted a 70% decrease in TAG content compared to that in the wild-type strain (29). These results suggest that TAG accumulation in oleaginous rhodococci is the result of the contributions of different sets of genes/enzymes probably regulated by different regulatory circuits that work in a highly coordinated manner (Fig. 1). The existence of several isoenzymes for the synthesis of TAG could have boosted the ability of these oleaginous bacteria to accumulate lipids.

TRANSCRIPTIONAL REGULATORS INDUCED IN CONDITIONS OF NITROGEN LIMITATION AND THEIR ROLE IN LIPOGENESIS

Nitrogen limitation seems to be the main trigger of TAG biosynthesis and accumulation by oleaginous rhodococci (9). Thus, the existence of a regulatory circuit linking the nitrogen and lipid metabolisms in these bacteria is expected. Kerkhoven et al. (43) reported that lipid accumulation in the oleaginous yeast *Yarrowia lipolytica* does not involve transcriptional regulation of lipid metabolism but is associated with regulation of amino acid biosynthesis. Under nitrogen limitation *Y. lipolytica* downregulates amino acid synthesis, resulting in a strong redirection of carbon flux from amino acids to lipids.

Simultaneously, this oleaginous yeast activates mechanisms that provide alternative sources of nitrogen, such as protein turnover and autophagy (43). Transcriptome and proteome analyses of *R. jostii* RHA1 revealed a similar association between lipid accumulation and amino acid metabolism under nitrogen starvation conditions (11, 29). Amino acids are degraded under TAG accumulation conditions to provide nitrogen, NADPH, and precursors for lipogenesis. However, in *R. jostii* RHA1 and *R. opacus* PD630 there is an additional, interesting regulatory mechanism that couples the expression of several genes of lipogenesis to the activation of a GlnR-mediated system that provides alternative sources of nitrogen (Fig. 1 and 2). GlnR is a well-known nitrogen-sensing regulatory protein which transcriptionally regulates several genes involved in nitrogen metabolism in diverse actinobacteria, such as *Streptomyces* (44–47), *Mycobacterium* (48, 49), *Saccharopolyspora* (50), and *Amicolatopsis* (51–53). GlnR activates the expression of a cluster of genes involved in the nitrite/nitrate reduction, which also contains a gene encoding a transcriptional regulator known as *nnaR* in *Streptomyces coelicolor* (54). NnaR works as a coactivator together with GlnR of its own gene cluster during ammonium limitation, as has been reported by Amin et al. (54). NnaR and GlnR cooperate in the regulation of gene expression associated with nitrate/nitrite assimilation also in *Mycobacterium smegmatis* (55). The orthologous of NnaR (best hit protein) is also found in the genome of oleaginous rhodococci located in the same gene cluster as nitrite/nitrate reduction. This regulatory protein is significantly induced during cultivation of *R. opacus* and *R. jostii* cells under ammonium limitation (56). As has been reported for *Streptomyces* and *Mycobacterium*, this regulatory protein also controls the expression of nitrate/nitrite assimilation genes in *R. jostii* RHA1. However, this protein additionally contributes to the modulation of lipogenesis and TAG accumulation in *R. jostii* and *R. opacus* (56), in contrast to *Mycobacterium smegmatis* (55). Since this transcriptional regulator modulates simultaneously nitrogen and lipid metabolisms in response to ammonium limitation, the protein was named NlpR (nitrogen lipid regulator) in oleaginous *Rhodococcus* (56). ^{13}C-labeling studies demonstrated that NlpR regulator contributes in oleaginous phenotype of *R. jostii* RHA1 to the allocation of carbon into different lipid fractions, such as TAG, DAG, MAG, fatty acids, phospholipids, and glycolipids, in response to nitrogen levels (57). NlpR is not essential for lipogenesis and TAG accumulation but provides a stronger redirection of carbon flux toward lipid metabolism, enhancing lipid biosynthesis specifically under nitrogen starvation conditions (56) (Fig. 2). Molecular studies demonstrated that NlpR positively modulates the expression of some genes involved in the fatty acid biosynthesis (FASI) and the Kennedy pathway for TAG synthesis in *R. jostii* RHA1, such as those encoding an AGPAT (PlsC), a phosphatidic acid phosphatase (Pap2), and a DGAT (Atf3), among others possible genes/proteins (56). Moreover, the binding of NlpR to the putative binding site located upstream of *nark*, *nirD* from nitrogen metabolism and *fasI*, *plsC*, and *atf3* from lipid metabolism, has also been demonstrated (56). Thus, NlpR regulon of oleaginous rhodococci (at least in RHA1 and PD630) seems to be more extensive than that of *Streptomyces* and *Mycobacterium* strains, including not only genes involved in nitrogen metabolism but also genes involved in lipogenesis and TAG accumulation. In this context, transcriptomic studies recently revealed that this regulatory protein modulates the expression of a small set of genes critical for nitrate/nitrite assimilation in *M. smegmatis* (42). After comparison of an *nnaR*-disrupted mutant of *M. smegmatis* to the wild-type strain during cultivation under nitrogen-limiting conditions, only seven transcripts, all involved in nitrogen utilization pathways, were downregulated in the mutant strain (55). The possible amplification of NlpR/NnaR regulon in oleaginous rhodococci in comparison to other actinobacteria with a lower ability to accumulate TAG could be an interesting innovative feature acquired during the evolution of these microorganisms. This metabolic and regulatory innovation might allow rhodococcal cells to coordinate their lipogenesis more finely according to the levels of available nitrogen source and, on the other hand, to maximize the capacity to accumulate TAG under these conditions.

Interestingly, Maarsingh and Haydel (58) reported that the PrrAB two-component

system influences TAG accumulation in *M. smegmatis* during ammonium stress by repressing some genes associated with TAG and lipid biosynthesis. Whether the homologous PrrAB system from *R. jostii* RHA1 and *R. opacus* PD630 is also associated with lipogenesis under nitrogen-limiting conditions is still unknown and remains to be investigated.

On the other hand, the regulatory network of oleaginous rhodococci also seems to include an energy-dependent regulatory circuit involving CRP (cAMP receptor protein). CRPs are transcription factors widely present in prokaryotes that respond to cAMP by binding at target promoters when the cAMP concentration increases. Juarez et al. (12) reported the occurrence of at least one CRP binding site in genes involved in the ED pathway (cluster RHA1_RS11565/RHA1_RS11570/RHA1_RS11575) and in that coding for a DGAT (Atf8) in *R. jostii* RHA1. Atf8 is the main TAG-biosynthetic enzyme induced by RHA1 during TAG accumulation, as has been reported by Amara et al. (29). These results suggested that oleagenicity in rhodococci is the result of different sets of genes, enzymes, and pathways regulated by different regulatory circuits in response to diverse environmental inputs, such as the changes in the energetic status and the nitrogen availability, among other still unknown factors (Fig. 1 and 2). The knowledge and understanding of the regulatory network components working in oleaginous species of *Rhodococcus* are still in their infancy and deserve more research.

CONCLUSION

Undoubtedly, the metabolism and the regulatory mechanisms of oleaginous rhodococci have been shaped during evolution to become specialists for TAG biosynthesis and accumulation. The fundamental knowledge of rhodococcal metabolism and its regulation will contribute to the economical feasibility of bacterial oil production on an industrial scale. However, the available knowledge of the biochemistry and genetic regulation of lipogenesis in these bacteria is still fragmentary. For this reason, it is necessary to focus future efforts on the following directions: (i) to quantify the relative contributions of the EMP, PP, and ED pathways to the degradation of carbohydrates during TAG accumulation; (ii) to clarify the mechanisms involved in the glycerol-3P homeostasis; (iii) to identify the processes that influence the energy fluxes and redox fluctuations during lipogenesis; (iv) to understand the organization of the phosphoenolpyruvate-pyruvate-oxalacetate node and TCA cycle, which control the distribution of the carbon flux between several pathways during lipogenesis; (v) to determine the contribution of CO_2 assimilation to lipogenesis during heterotrophic cell growth; (vi) to identify the mechanisms that control fatty acid homeostasis during lipid accumulation; (vii) to determine the specific contribution to TAG biosynthesis of the multiple isoenzymes involved in the Kennedy pathway; and (viii) to identify the regulatory circuits involved in the control of lipogenesis and their regulatory components (Fig. 1). A better understanding of the carbon, redox, and energy metabolisms will be indispensable to obtain a clearer picture of TAG accumulation in oleaginous rhodococci.

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