Wax Ester and Triacylglycerol Biosynthesis in Bacteria

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Abstract

Bacteria are an extremely diverse group of organisms, some of which possess the ability to synthesize and accumulate neutral lipids, such as triacylglycerols (TAG) and wax esters (WE). Among these microorganisms, Actinobacteria are specialized in the accumulation of TAG, whereas Gram-negative Proteobacteria, such as *Acinetobacter* and *Marinobacter*, produce predominantly WE. The capability for accumulating large amounts of TAG seems to be restricted to some members of Actinobacteria, such as those belonging to *Rhodococcus*, *Gordonia*, and *Streptomyces* genera, and to the Gram-negative *Alcanivorax borkumensis*. The biosynthesis

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and accumulation of TAG and/or WE require the occurrence of a set of genes/proteins working in a coordinated metabolic and regulatory context in the cell. Some components of the lipid-accumulating machinery in native producers have been identified and characterized. They include genes coding for: (1) enzymes catalyzing the last reactions of TAG and/or WE synthesis; (2) enzymes involved in the reduction of fatty acids to the respective fatty alcohols for the synthesis of WE; (3) enzymes of central metabolism which generate NADPH for fatty acid synthesis; (4) a structural protein involved in the assembly and stabilization of lipid inclusion bodies; and (5) a lipid transporter protein involved in the balance and homeostasis of cellular lipids. Some of these genes identified in native producers have been used for engineering bacterial hosts, which are naturally unable to produce these lipids, in order to produce TAG/WE with bacterial strains of biotechnological relevance.

1 Introduction

Bacteria have developed diverse strategies for surviving under the fluctuating conditions of the environment. The synthesis and accumulation of storage lipids, such as polyhydroxyalkanoates (PHA), wax esters (WE), and triacylglycerols (TAG), is one of the mechanisms evolved by some prokaryotes to colonize and thrive in natural environments. Interestingly, the production of TAG or WE might represent an ancestral biosynthetic innovation and a conservative strategy that facilitated bacterial colonization of harsh, early terrestrial environments (Finkelstein et al. 2010). The ability to produce TAG (and WE to minor extent) allowed cells to adapting and tolerating the main stresses found in soil environments, such as desiccation, oxidative stress, and osmotic stress, among others. Neutral lipids seem to play multiple roles in the physiology of lipid-accumulating bacteria. TAG are excellent reserve materials, since their oxidation produces higher yields of energy compared to other storage compounds, such as carbohydrates or PHA. The energy obtained by the mobilization of stored lipids may provide bacterial cells of energetic autonomy and a temporal independence from the environment and contributes to cell survival when they do not have access to energy resources in the environment. In addition, their extremely hydrophobic properties allow their accumulation in large amounts in cells without changing the osmolarity of the cytoplasm. On the other hand, TAG may also provide a source of metabolic water from their oxidation under desiccation conditions. Bacterial TAG or WE may play additional roles in cells, such as: (1) a source of lipid precursors for membranes and cell envelopes; (2) a source of metabolic precursors for the synthesis of secondary metabolites, such as antibiotics; (3) a form to balance central metabolism and the redox state of the cell, and (4) a form to detoxify unusual fatty acids and the excess of free fatty acids, among others.

The biosynthesis and accumulation of neutral lipids by bacteria is a process related to the stationary growth phase or as a response to stress. The limitation of the nitrogen source in the presence of an excess of the carbon source usually

stimulates lipid accumulation in diverse bacteria; thus, the nutritional stress seems to be the main condition that influences lipid accumulation in prokaryotes. The total amount of TAG or WE accumulated by bacteria depends on both the strain (taxonomic affiliation) and the carbon source used for growth. The stored lipids are usually mobilized by bacterial cells in the absence of any carbon source.

Beyond the physiological relevance of the stored TAG and WE in prokaryotes, these lipids have recently gained the interest of the industry because of its potential biotechnological applications. Neutral lipids produced by bacteria may serve as a source of precursors and components for the production of a variety of biofuels, biolubricants, and oleochemicals, among other commercial products. This situation driven recent advances in basic research in this field and the identification of different genes coding for proteins involved in TAG and WE metabolism in Gram-positive as well as in Gram-negative bacteria.

2 Synthesis of TAG and WE by Bacteria

The ability to synthesize TAG or WE is found in a diversity of bacteria, although there are some qualitative and quantitative differences in their accumulation profiles. In general, Gram-positive Actinobacteria are specialized in the accumulation of TAG, whereas WE are synthesized and accumulated predominantly by Gram-negative bacteria, as is shown in Fig. 1. Interestingly, some members of Proteobacteria, such as *Alcanivorax borkumensis* and *Alcanivorax jadensis*, produce TAG as main storage lipids.

On the other hand, some of the lipid-accumulating bacteria are able to produce high amounts of TAG (higher than 20% of cellular dry weight, CDW); thus, they can be considered as oleaginous microorganisms. Among them, Actinobacteria belonging to *Rhodococcus*, *Gordonia*, and *Streptomyces*, and Proteobacteria belonging to *Alcanivorax*, exhibit "oleaginous phenotypes" during growth on different carbon sources (Fig. 1). Diverse substrates support growth and lipid accumulation in bacteria, including sugars, organic acids, hydrocarbons, as well as agro-industrial wastes, such as whey, sugar cane molasses, carob, and orange wastes, among others.

In summary, the combination of the carbon source used for cultivation of cells and the metabolic characteristics of the bacterial strain influence the type and the amount of neutral lipid accumulated by microorganisms.

3 Triacylglycerol Biosynthesis in Bacteria

TAG are composed of three fatty acyl groups esterified to a glycerol backbone at the *sn*-1, *sn*-2, and *sn*-3 positions. Bacterial TAG are predominantly synthesized via the acyl-CoA-dependent acylation of the glycerol backbone derived from *sn*-glycerol-3-phosphate (G3P), referred to as the "Kennedy pathway" (Fig. 2). G3P is generated from dihydroxyacetone phosphate derived from the glycolysis through the catalytic action of *sn*-glycerol-3-phosphate dehydrogenase. The first acylation step of G3P is

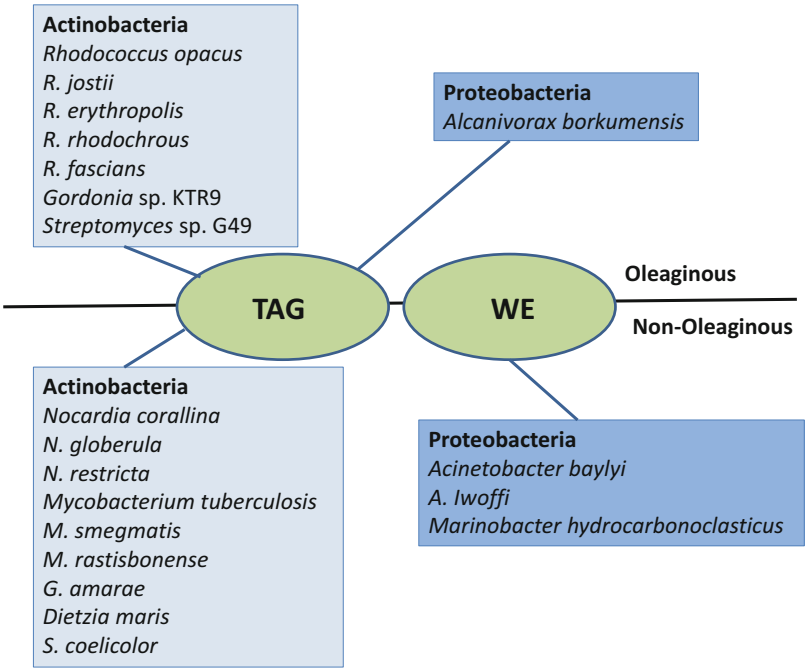

Fig. 1 Bacteria able to synthesize TAG and/or WE. The figure shows the relation between microorganisms and the predominant lipid species which they produce. It is noteworthy that some TAG-accumulating Actinobacteria are able to produce traces of WE and some Gram-negative Proteobacteria that produce WE are also able to synthesize minor amounts of TAG

catalyzed by an *sn*-glycerol-3-phosphate-*O*-acyltransferase (GPAT), whereas the second acylation by an acylglycerol-3-phosphate-*O*-acyltransferase (AGPAT). A subsequent removal of the phosphate group by a phosphatidic acid phosphatase enzyme to generate diacylglycerol (DAG) occurs prior to the final acylation. The final acyl-CoA-dependent acylation of the resulting DAG is catalyzed by a diacylglycerol-acyltransferase enzyme (DGAT) (Fig. 2). Genes and enzymes involved in Kennedy pathway have been intensively investigated during the last years in diverse TAG-synthesizing bacteria. Several DGAT enzymes have been characterized in Actinobacteria as well as in Proteobacteria, since the presence of these enzymes seems to be a key feature that differentiates bacteria capable for synthesizing TAG or WE to those unable to produce these lipids. Kalscheuer and Steinbüchel (2003) reported the first WS/DGAT in *Acinetobacter baylyi* ADP1, which is able to produce WE as main storage lipids plus minor amounts of TAG. This enzyme exhibited simultaneously both acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) and diacylglycerol acyltransferase (DGAT) activities. Later, other DGATs were identified and characterized in several TAG-synthesizing bacteria, such as those belonging to *Alcanivorax*, *Streptomyces*, *Mycobacterium*, *Gordonia, Marinobacter*, and *Rhodococcus* genera (Daniel et al. 2004; Holtzapple

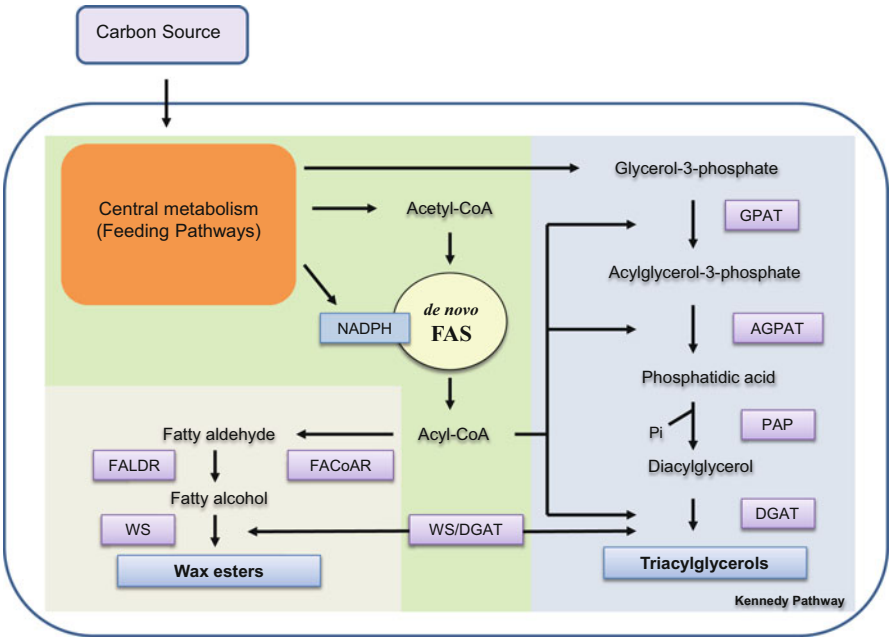

Fig. 2 Schematic of metabolic pathways involved in the synthesis of TAG and WE in bacteria. Major metabolic pathways are shown with different colored backgrounds according to their occurrence in Gram-positive Actinobacteria (*light blue*), Gram-negative Proteobacteria (*violet*), or in both bacteria (*green*). *Abbreviations*: *FAS* fatty acid synthesis, *WS* wax ester synthase, *DGAT* diacylglycerol acyltransferase, *GPAT* glycerol-3-phosphate *O*-acyltransferase, *AGPAT* acyl-glycerol-3-phosphate acyltransferase, *PAP* phosphatidic acid phosphatase, *FALDR* fatty aldehyde reductase, *FACoAR* fatty acyl-CoA reductase

and Schmidt-Dannert 2007; Kalscheuer et al. 2007; Alvarez et al. 2008; Arabolaza et al. 2008; Indest et al. 2015). Interestingly, bacterial DGATs belong to a new class of TAG-synthesizing enzymes, exhibiting no extended sequence similarities to any known eukaryotic acyltransferase. Genes coding for DGAT enzymes are particularly abundant in TAG-accumulating Actinobacteria, although the number of isoenzymes occurring in cells seems to be a strain-/species-dependent feature. Some members of *Mycobacterium* and *Rhodococcus* genera possess up to 15–17 DGAT isoenzymes in their genomes. This feature may represent an ecological advantage, providing more physiological and regulatory flexibility to lipid metabolism in these microorganisms. The abundance of DGAT enzymes and the high promiscuity of their activities may confer to Actinobacteria the ability to produce TAG and/or WE, using a diversity of intermediates from the cellular metabolism, and under a wide range of environmental conditions.

Other studies led to the identification and characterization of genes encoding additional enzymes of the Kennedy pathway, such as AGPAT in *Mycobacterium bovis* BCG and phosphatidic acid phosphatase (PAP2) in *Streptomyces coelicolor*

(Low et al. 2010; Comba et al. 2013) and *Rhodococcus jostii* RHA1 (Hernández et al. 2015; Fig. 2). In addition to the Kennedy pathway, other routes may be involved in the synthesis of TAG in prokaryotes, as occur in yeasts and plants. The existence of phospholipid:diacylglycerol acyltransferase-like enzymes (PDAT) in TAG-producing bacteria is expected. PDAT catalyzes the transfer of fatty acids at the *sn*-2 position of phospholipid species to DAG to generate TAG. The occurrence of PDAT-like enzymes in prokaryotes remains to be investigated. On the other hand, the key intermediate for TAG synthesis (DAG) can be produced by different metabolic reactions in bacteria. Phospholipase C produces DAG by cleavage of phospholipids, whereas the headgroup transfer from phospholipids to membrane-derived oligosaccharides or periplasmic cyclic glucans is also a source of DAG (Sohlenkamp and Geiger 2015).

Holder et al. (2011) proposed that 261 genes are implicated in TAG metabolism in the oleaginous *R. opacus* strain PD630. Thus, the ability to synthesize and accumulate TAG (principally in oleaginous bacteria) depends not only on the presence of DGAT enzymes but also on the existence of a special metabolic and regulatory network, which includes several genes/enzymes involved in the central metabolism, the fatty acid synthesis, and the production of reducing equivalents, as well as several genes/enzymes involved in the assembly, stabilization, and structure of lipid inclusion bodies, lipid transport required for maintaining a balanced distribution of lipids between lipid fractions in the cell, and regulation of the lipid accumulation process. Some of the components of the TAG-accumulating machinery have been identified and characterized in detail, such as TadA protein that plays a structural role in the formation of lipid bodies by *R. opacus* PD630 (MacEachran et al. 2010), Ltp1 protein which is an ATP-binding cassette transporter that functions as an importer of long-chain fatty acids in *R. jostii* RHA1 (Villalba and Alvarez 2014), and an acyl-CoA synthetase which participates in the activation of fatty acids for TAG synthesis in *Mycobacterium tuberculosis* H37Rv (Daniel et al. 2014).

The de novo fatty acid biosynthesis, which is a key pathway for the subsequent TAG formation, demands the generation of high levels of NADPH and ATP by cells, since the synthesis of one molecule of palmitic acid ($C_{16:0}$) requires eight molecules of acetyl-CoA, 14 molecules of NADPH, and seven molecules of ATP. In this context, previous studies identified and characterized $NADP^+$-dependent enzymes in TAG-producing Actinobacteria, which provide reducing equivalents and participate in the carbon flux homeostasis during fatty acid and TAG synthesis. Rodriguez et al. (2012) reported the occurrence of two genes coding for malic enzymes in *Streptomyces coelicolor* A3(2), whereas MacEachran and Sinskey (2013) identified and characterized a gene encoding a non-phosphorylative glyceraldehyde dehydrogenase enzyme in *R. opacus* PD630, which is specifically activated during TAG accumulation.

According to the current knowledge on TAG-producing prokaryotes, the ability of bacterial cells to synthesize and accumulate those lipids is not only supported by the presence of key specific enzymes involved in their synthesis, such as WS/DGAT's; but also by the occurrence of an integrated metabolic and regulatory network in lipid-accumulating bacteria.

4 Wax Ester Biosynthesis in Bacteria

Waxes are esters of a long-chain alcohol and a long-chain fatty acid (WE). The key step for WE synthesis is catalyzed by a WS, or by the bifunctional WS/DGAT enzyme, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol (Fig. 2). In general, WS/DGATs accept acyl groups with carbon chain length of C_{16} to C_{18} and *n*-alcohols with carbon chain length between C_{12} and C_{20} (Shi et al. 2012). The WS/DGAT from *Acinetobacter baylyi* ADP1 (AtfA) exhibits a preference for C_{14} and C_{16} acyl-CoA substrates with C_{14} to C_{18} fatty alcohols (Kalscheuer and Steinbüchel 2003). Interestingly, *Marinobacter hydrocarbonoclasticus* DSM 8798 was able to synthesize isoprenoid WE after growth on phytol as sole carbon source (Rontani et al. 1999). Two WS/DGAT enzymes (WS1 and WS2) were involved in the biosynthesis of isoprenoid WE by strain DSM 8798 (Holtzapple and Schmidt-Dannert 2007).

The ability to produce WE from unrelated carbon sources, such as acetate or glucose, depends not only on the occurrence of a WS/DGAT enzyme but also on the capability of the microorganism for reducing fatty acids to the respective fatty alcohols. Fatty alcohols can be produced by a two-step reduction of either acyl-ACP or acyl-CoA to the respective fatty alcohol via an intermediate fatty aldehyde (Fig. 2). *A. baylyi* ADP1 possesses this route for the sequential reduction of fatty acids to fatty alcohols for WE synthesis (Reiser and Somerville 1997). On the other hand, *Marinobacter aquaeolei* VT8 possesses two fatty acyl-CoA reductases (FarA and AcrB) that able to reduce not only fatty acids to fatty aldehydes but also fatty acids to fatty alcohols directly with consumption of NADPH (Hofvander et al. 2011; Lenneman et al. 2013). Fatty acyl-CoA reductases also occur in Actinobacteria, as has been reported for *M. tuberculosis* (Sirakova et al. 2012). These enzymes produce WE that are accumulated in the cell wall of mycobacteria as a permeability barrier limiting replication by inhibition of nutrient uptake under dormancy state.

In summary, the ability to synthesize WE in bacteria depends on the occurrence of, at least, two types of enzymes: the fatty acid reductases for the de novo production of key alcohol precursors and WS (or WS/DGAT enzymes) for the esterification of fatty acids and fatty alcohols to produce WE. The accumulation of WE in intracellular inclusion bodies is a property only restricted to a few prokaryotes, predominantly Gram-negative Proteobacteria. Other bacteria, such as mycobacteria, produce WE for their accumulation in the cell wall in order to modify its permeability for controlling the uptake of nutrients. Thus, only few bacteria can accumulate large amounts of WE, and most of them produce just trace of these lipids.

5 Metabolic Engineering in TAG Biosynthesis

During the last years, the increasing availability of genomic databases, omic studies, and molecular tools led to a diversity of interesting approaches to enhance lipid production in native oil-accumulating bacteria and enlarge their application

spectrum, or to establish lipid production in the canonical bacterial hosts, which are naturally not able to produce TAG and/or WE, through genetic engineering. In the first line of study, bacteria belonging to *Rhodococcus* and *Acinetobacter* genera have been predominantly used as lipid production platforms via genetic engineering. The overexpression of native genes has been the preferred strategy to improve TAG production in rhodococci. A variable increase of TAG accumulation has been obtained after overexpression of the following genes in rhodococci: (1) *atf1* and *atf2* genes coding for DGAT enzymes in *R. opacus* PD630 (Alvarez et al. 2008; Hernández et al. 2013), and *atf8* in *R. jostii* RHA1 (Amara et al. 2016); (2) a gene coding for a phosphatidic acid phosphatase (PAP2) enzyme in *R. jostii* RHA1 (Hernández et al. 2015); (3) a gene encoding a non-phosphorylative glyceraldehyde dehydrogenase enzyme in *R. opacus* PD630 for enhancing NADPH formation (MacEachran and Sinskey 2013); (4) a gene encoding a thioesterase enzyme in *R. opacus* PD630 (Huang et al. 2016); (5) a gene coding for an ATP-binding cassette transporter protein for the uptake of long chain fatty acids (Villalba and Alvarez 2014); and (6) genes encoding enzymes involved in the catabolism of glycerol from *R. fascians* transferred to *R. opacus* during growth on glycerol as sole carbon source (Herrero et al. 2016). On the other hand, the overexpression of genes encoding $NADP^+$-dependent malic enzymes and PAP2 enzymes also improved TAG accumulation by *Streptomyces coelicolor* (Rodríguez et al. 2012; Comba et al. 2013).

Rhodococci have been also engineered to utilize lignocellulosic raw materials, which are important agro-industrial wastes, as substrates for oil production. In this context, some genes/enzymes from *Streptomyces* involved in the xylose metabolism pathway were used to construct *R. jostii* RHA1 and *R. opacus* PD630 xylose-fermenting strains (Xiong et al. 2012; Kurosawa and Sinskey 2013). The successful engineering of xylose (Xiong et al. 2012; Kurosawa and Sinskey 2013) and cellobiose (Hetzler and Steinbüchel 2013) catabolic pathways in *Rhodococcus* strains suggested that lignocellulosic biomass can be utilized by rhodococci to produce TAG.

Other studies considered the Gram-negative *Acinetobacter baylyi* as a convenient model organism for studying the potentiality and modifiability of WE in a natural host by means of synthetic biology. Santala et al. (2014) successfully expressed a fatty acid reductase complex LuxCDE with an inducible arabinose promoter in *A. baylyi* ADP1 for the production of WE. The introduction of the synthetic pathway also led to a modification of fatty acid and alcohol species in the produced WE, demonstrating the potentiality of recircuiting a biosynthesis pathway in a natural producer, for a regulated production of a customized bioproduct (Santala et al. 2014).

The second line of study involves the possibility to transfer the capability for producing TAG and/or WE by host strains that are naturally not able to synthesize those lipids, such as *Escherichia coli*. Different molecular approaches have been successfully used to produce TAG and/or WE by recombinant *E. coli* strains. Rucker et al. (2013) engineered an *E. coli* strain to synthesize and accumulate TAG from cell wall phospholipid precursors through heterologous expression of two enzymes, phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT) (Fig. 2). Other study investigated the production of TAG in cells of the wild type of

E. coli and of a strain with a deleted diacylglycerol kinase gene (*dgkA*). The overexpression of *atfA* from *A. baylyi* ADP1 and *fadD* from *E. coli* in the *dgkA* deletion mutant led to a production of approximately 4.9% (w/w) of TAG in batch cultivation (Janβen and Steinbüchel 2014). On the other hand, Comba et al. (2014) used an *E. coli* BL21 derivative strain containing a deletion in the *dgkA* gene as platform for TAG production and the heterologous expression of some TAG biosynthesis genes from *Streptomyces coelicolor*, such as the acyl-CoA: diacylglycerol acyltransferase (DGAT) Sco0958 and the phosphatidic acid phosphatase (PAP) Lppβ. Moreover, the authors performed a series of stepwise optimizations of the chassis: (1) fine-tuning the expression of the heterologous *Sco0958* and *lppβ* genes, (2) overexpression of the *S. coelicolor* acetyl-CoA carboxylase complex, and (3) mutation of *fadE*, the gene encoding for the acyl-CoA dehydrogenase that catalyzes the first step of the β-oxidation cycle in *E. coli*. They obtained a recombinant strain able to produce up to 4.85% cell dry weight (CDW) TAG in batch cultivation (Comba et al. 2014). In other study, Röttig et al. (2015) engineered an *E. coli* strain by expressing *fadD* (acyl-CoA synthetase), *fadR* (transcriptional activator of fatty acid biosynthesis genes), *pgpB* (phosphatidic acid phosphatase), *plsB* (glycerol-3-phosphate acyltransferase), and *tesA* (thioesterase A) in combination with *atfA* (WS/DGAT) from *Acinetobacter baylyi*, which led to a total fatty acid contents of up to 16% (w/w) on complex media, corresponding to approximately 9% (w/w) of TAG. *E. coli* has been also used for the biosynthesis of WE upon metabolic engineering. Kalscheuer et al. (2006) co-expressed in a recombinant *E. coli* strain a fatty alcohol-producing bifunctional acyl-coenzyme A reductase from the jojoba plant and the bacterial WS from *A. baylyi* strain ADP1 for the synthesis of WE. In the presence of oleate, jojoba oil-like WEs such as palmityl oleate, palmityl palmitoleate, and oleyl oleate were produced, amounting to up to ca. 1% of CDW.

Recently, Plassmeier et al. (2016) reported the use of *Corynebacterium glutamicum* as platform for TAG biosynthesis through metabolic engineering. Firstly, they performed the heterologous expression of four genes involved in TAG biosynthesis and accumulation in rhodococci, such as *atf1* and *atf2*, encoding DGAT enzymes; *pgpB*, encoding the phosphatidic acid phosphatase enzyme to complete the TAG biosynthesis pathway; and *tadA* coding for a structural protein for lipid body assembly. On the other hand, the authors applied four metabolic strategies to increase TAG accumulation: (i) boosting precursor supply by heterologous expression of *tesA* (encoding a thioesterase to form free fatty acid to reduce the feedback inhibition by acyl-ACP) and *fadD* (encoding acyl-CoA synthetase to enhance acyl-CoA supply), (ii) reduction of TAG degradation and precursor consumption by deleting four cellular lipases (*cg0109*, *cg0110*, *cg1676* and *cg1320*) and the diacylglycerol kinase (*cg2849*), (iii) enhancement of fatty acid biosynthesis by deletion of *fasR* (*cg2737*, TetR-type transcriptional regulator of genes for the fatty acid biosynthesis), and (iv) elimination of the observed by-product formation of organic acids by blocking the acetic acid (*pqo*) and lactic acid production (*ldh*) pathways. The recombinant *C. glutamicum* strain was able to produce TAG, and the intracellular fatty acid content amounted approximately 17% of the CDW.

All these studies demonstrated that the synthesis and accumulation of TAG and/or WE can be engineered using the native oil producers or other nonlipid-accumulating bacterial hosts as cellular production platforms.

6 Research Needs

Oleagenicity demands not only the occurrence of key enzymes for the esterification of alcohols (glycerol or long-chain fatty alcohols) with fatty acids, such as WS and DGAT enzymes, but also the integration of these reactions with the metabolic and regulatory network of cell. Oleaginous microorganisms must be able to generate a high carbon flux through metabolic pathways that provide key intermediates for lipogenesis; they need a set of reactions which generate the necessary NADPH pool for supporting high levels of fatty acid biosynthesis, an efficient mechanism that coordinate and balance phospholipid and TAG pathways which share common reactions: a set of proteins involved in the assembly, stabilization and structure of lipid inclusion bodies, and efficient regulatory circuits for the fine regulation of the lipid accumulation process. This could be one of the main reasons why bacterial host such as *E. coli*, which is naturally unable to produce TAG and/or WE, is not able to accumulate large amounts of lipids after expression of heterologous genes from lipid-accumulating bacteria. Although promising advances on basic aspects of lipid accumulation in native bacterial producers and the metabolic engineering of diverse bacterial hosts were done, further efforts are necessary for understanding the ecological role of these lipids and for their application in the industry. It is necessary to focus research on the identification and characterization of new components of the lipid-accumulating machinery in native bacteria. The combination of these results obtained in model microorganisms with the increasing availability of a wide variety of new genetic tools and genome sequences and the application of high-throughput techniques and synthetic biology strategies will led to promising advances in the basic knowledge of lipid-accumulating process and in the metabolic engineering of diverse bacterial hosts for the design of new oil-derived bioproducts.

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