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MINI-REVIEW

Metabolic engineering of microorganisms for the production of structurally diverse esters

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Abstract Conventional petroleum-based chemical industry, although economically still thriving, is now facing great socio-political challenges due to the increasing concerns on climate change and limited availability of fossil resources. In this context, microbial production of fuels and commodity oleochemicals from renewable biomass is being considered a promising sustainable alternative. The increasing understanding of cellular systems has enabled the redesign of microbial metabolism for the production of compounds present in many daily consumer products such as esters, waxes, fatty acids (FA) and fatty alcohols. Small aliphatic esters are important flavour and fragrance elements while long-chain esters, composed of FA esterified to fatty alcohols, are widely used in lubricant formulas, paints, coatings and cosmetics. Here, we review recent advances in the biosynthesis of these types of mono alkyl esters in vivo. We focus on the critical ester bond-forming enzymes and the latest metabolic engineering strategies employed for the biosynthesis of a wide range of products ranging from low-molecular-weight esters to waxy compounds.

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Introduction

Esters are organic compounds formed by condensation between a carboxylic acid and an alcohol molecule. They have the general chemical formula RCOOR' and can be roughly classified according to their chain length in low-molecular-weight (LMW) esters and high-molecular-weight (HMW) esters.

LMW esters have chain lengths shorter than 10 carbon atoms and are usually colorless and volatile (Beekwilder et al. 2004; El Hadi et al. 2013). Many of them are important components of essential plant oils with pleasant aromas creating the fragrances and flavours of many flowers and fruits. The cosmetic and fragrance industries, with a current estimated value of $27 billion (Carroll et al. 2016), use small esters to reproduce particular floral or fruity odours (Barney 2014).

HMW esters are formed from long-chain carboxylic acids and fatty alcohols and have been found in a variety of organisms including bacteria, mammals, birds, insects and plants (Iven et al. 2013). HMW esters have a wide range of biological functions which strongly depends on both their chemical structure and their cellular localization. Intracellular waxes (WE) as well as triglycerides (TAG), the major component of natural oils, serve as important carbon storage compounds; whereas cuticular waxes protect cells against water loss and pathogens (Barney 2014). Various types of long chain wax esters are widely used in the manufacture of commercial products such as lubricants, printing inks, rubbers, adhesives, cosmetics, candles and coating stuffs. On this regard, fossil material constitutes the principal source of industrially used waxes, although their chemical structure does not exclusively

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correspond to an ester. That is, the vast majority of these industrial waxes are based either on alkane and/or ester structures: fossil waxes, are extracted from crude oil and coal deposits, yielding alkanes and alkyl ester mixtures (together with the corresponding free acids and alcohols), respectively (Jetter and Kunst 2008). On the other hand, synthetic waxes are mainly generated by the Fischer–Tropsch process ($CO + H_2$) and olefin (ethylene, propylene) polymerization, giving rise to mixtures of straight- and branched-chain alkanes (Bekker et al. 2013; Jia et al. 2016). As an alternative to synthetic production, lipase-catalysed methods provide an attractive approach for the production of ester-based waxes that are difficult to be synthesized by conventional chemical-catalysed methods (Adachi and Kobayashi 2005; Stergiou et al. 2013). Lipases possess several characteristics that make them useful biocatalysts for industry: they exhibit broad substrate spectrum, excellent chemo-, regio- and stereoselectivity, high stability towards harsh reaction conditions and independence of cofactors (Zorn et al. 2016). Indeed, lipase-based wax ester production usually requires mild reaction conditions, which makes them environmentally friendly. Lipases from *Candida antarctica*, *Candida parapsilosis* and *Pseudozyma antarctica* have been extensively studied regarding their determinant structural characteristics (Jan et al. 2016; Subileau et al. 2015; Zorn et al. 2016). These studies provided the molecular bases for further improvement of their activities by protein engineering. However, besides all their advantages, lipase-based methods for ester production still depend on fatty alcohols as substrates, which at present are chemically synthesized.

In this context, bio-esters represent a sustainable alternative to fossil and synthetic waxy materials due to the possibility of obtaining specific products with attractive physicochemical properties through an environmentally friendly biosynthetic process. Currently, the demand of these compounds is growing rapidly and steadily. For instance, beeswax constitutes a representative commodity obtained from an animal source, which major component is myricyl palmitate, an ester formed from triacontanol and palmitic acid (Fratini et al. 2016). Regarding plants, the most important sources for commercial production of wax esters are carnauba (*Copernicia cerifera*), candelilla (*Euphorbia cerifera* and *Euphorbia antisyphilitica*), ouricouri (*Syagros coronata*), sugar cane (*Saccharum* sp.) and jojoba (*Simmondsia chinensis*) (Kunst and Samuels 2009). Jojoba plant, for example, has the distinguishing characteristic of producing oil constituted mainly of wax esters (97%), rather than TAG. These waxes contain C_{38} to C_{44} linear unsaturated esters and, due to their physicochemical characteristics, jojoba oil has become a significant additive used in the cosmetic and personal care industries (Bhatia 1993; Miwa 1971). As another example, leaves from the carnauba plant contain wax esters companied by small amounts of free acids, alcohols, hydrocarbons and resins in its leaves. The waxes represent about 85% of the total lipid content of the leaves cuticle and are comprised by linear C_{46} to C_{54} molecular species (Freitas et al. 2016). However, the high price of these types of plant oils has limited their use and commercialization, restricting them to medical and cosmetics applications.

In the recent years, remarkable progresses have been made in the understanding of microbial global biosynthesis pathways and their regulation. In the context of searching for new sustainable ester-production processes, the knowledge achieved have led to the designing and successful application of a variety of metabolic engineering strategies (Janßen and Steinbüchel 2014; Lennen and Pfleger 2013; Sorger and Daum 2003). The many published works on this regard showed that designed microbial cell factories represent a potential source of bio-esters that could mitigate plants seasonal dependent growth, intensive farming and expensive extraction methods (Lynch 2016). Here, we review the latest and most original microbial metabolic engineering approaches for the production of industrially relevant bio-esters. There are several means by which esters are formed in vivo; here, we focus on proteins members of three different acyltransferase families: (1) the wax synthase/diacylglycerol acyltransferase (WS/DGAT, AtfA-type) family, (2) the alcohol acetyltransferase family, and (3) the condensation family. This subdivision is based on the enzyme that catalyses the ester bond formation and dictates the type of ester compound that will be produced. We make emphasis in particular examples of acyltransferases that have been successfully utilized in different metabolic engineering approaches oriented to obtain microbial esters. The main strategies developed to overproduce diverse alkyl esters in microorganisms as well as recent discoveries and current challenges to optimize their yield are chronologically described. Finally, we highlight the "versatility-uses" of these ester forming enzymes as well as their biotechnological applications in two model microorganisms: *Escherichia coli* and *Saccharomyces cerevisiae*.

The WS/DGAT (AtfA-type) family

One kind of reaction for ester formation comprises acyltransferase enzymes involved in neutral lipid storage compounds. Annika Röttig and Alexander Steinbüchel described six phylogenetically different families of acyltransferases involved in TAG and/or WE biosynthesis: (i) the DGAT1 family, (ii) the jojoba WS family, (iii) the DGAT2 family, (iv) an avian WS type, (v) a protozoan DGAT2-related type from *Tetrahymena* and (vi) the WS/DGAT (AtfA-type) family (Röttig and Steinbüchel 2013). In prokaryotes, only enzymes belonging to the WS/DGAT group have been identified so far.

Prokaryotic WS/DGATs: substrate specificity and product diversity

AtfA of *Acinetobacter baylyi* ADP1 was the first characterized member of this acyltransferase family (Kalscheuer and Steinbüchel 2003; Stöveken et al. 2005). This enzyme has relaxed substrate specificity; it can synthesize WE and TAG by utilizing different chain-length acyl-CoAs and different chain-length fatty alcohols or diacylglicerol (DAG), respectively (Janßen and Steinbüchel 2014; Stöveken et al. 2005). Regarding the acyl donor, AtfA accepts saturated or unsaturated acyl-CoA thioesters ranging from C_2 to C_{20}. However, biochemical characterization revealed that palmitoyl-CoA was accepted with highest specificity (Stöveken et al. 2005). On the other hand, linear alcohols from C_2 to C_{30}, branched alcohols, cyclic and aromatic alcohols and mono- and DAG are recognized as acyl acceptor substrates; although the highest activity in vitro was obtained for medium-chain-length alcohols (Stöveken et al. 2005). The catalytic active-site motif HHxxxDG is highly conserved in members of this class of acyltransferases (Wältermann et al. 2007). This motif is part of an HxxxxD-like pattern present in the catalytic active-site of a conserved condensation domain (Pfam 00668) of several nonhomologous enzyme classes (Wältermann et al. 2007). In general, they catalyse the transfer of thioester-activated acyl substrates to hydroxyl or amine acceptor to form ester or amide bonds. Examples of such nonhomologous enzymes are acyltransferases that synthesize glycerolipids, nonribosomal peptide synthetases, acyltransferases involved in lipid A biosynthesis and polyketide-associated acyltransferases (see below) (Röttig and Steinbüchel 2013).

Many members of the WS/DGAT family have been identified and characterized from several lipid-accumulating bacteria such as *Marinobacter* (Holtzapple and Schmidt-Dannert 2007), *Streptomyces* (Arabolaza et al. 2008; Kaddor et al. 2009), *Mycobacterium* (Elamin et al. 2011) and *Rhodococcus* (Alvarez et al. 2008). *Marinobacter hydrocarbonoclasticus* DSM 8798, for example, is capable of producing an isoprenoid wax from phytanoyl-CoA and phytol. Two of the three WS/DGAT cloned from this microorganism (i.e., WS1 and WS2) were capable of synthesizing isoprenoid WE in vitro, showing activity with palmitoyl-CoA (C16) and phytol as tested substrates (Holtzapple and Schmidt-Dannert 2007). Whereas the recombinant expression of AtfA was extensively used for heterologous biosynthesis of lipid-derived relevant compounds (Janßen and Steinbüchel 2014; Kalscheuer et al. 2006a, b), other WS/DGAT enzymes have also been studied for biotechnological purposes. As an example, the expression of the WS/DGAT SCO0958 from *Streptomyces coelicolor* led to TAG biosynthesis in an *E. coli* diacylglycerol kinase (*dgkA*) mutant strain (Comba et al. 2014). More recently, ten promising bacterial WS/DGAT enzymes were tested and compared for their TAG and FA ethyl ester (FAEE) production in engineered *E. coli* strains (Röttig et al. 2015). This work showed that the expression of WS1 from *M. hydrocarbonoclasticus* DSM 8798, AtfA1 from *Alcanivorax borkumensis* SK2 and the model AtfA from *A. baylyi* ADP1 resulted in efficient TAG and FAEE production in *E. coli* derivative strains (for FAEE biosynthesis, exogenous ethanol addition was needed). Interestingly, all the WS/DGAT tested displayed the capacity of synthesizing TAG and FAEE, although a marked variation in the yields of these two compounds was observed (Röttig et al. 2015).

Metabolic engineering involving the promiscuous WS/DGAT pathway

The heterologous production of esters derived from FA and diverse chain-length alcohols has been the focus of numerous studies in recent years. As we mentioned above, most of these studies were carried out with the *A. baylyi* ADP1 AtfA enzyme. Particularly, heterologous overproduction of FAEE (biodiesel) has been extensively investigated in both prokaryotic and eukaryotic models. *De novo* production of FAEE requires FA and ethanol precursors to be engineered in the recombinant microorganism.

In *E. coli*, ethanol production can be achieved by the heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* (Pdc and AdhB, respectively) (Fig. 1) (Lawford and Rousseau 1991). In 2006, Kalscheuer et al. expressed *atfA, pdc*, and *adhB* genes from a plasmid in *E. coli* and obtained up to 1.28 g/L of FAEE in a fed-batch fermentation supplemented with exogenous oleic acid (Kalscheuer et al. 2006a). Later, this group achieved a final titer of 14.8 g/L of FAEE with a volumetric productivity of 0.32 g/L h after 47 h in pilot scale 20 L-fermentation of this engineered *E. coli* strain using glycerol as sole carbon source followed by glucose and oleic acid feeding (Elbahloul and Steinbüchel 2010) (Table 1). In 2011, Duan et al. combined the *atfA-pdc-adhB* route with overexpression of *'tesA* (leaderless version of a periplasmic thioesterase), *fadD* (acyl-CoA synthase) and the *E. coli* ACC complex (acetyl-CoA carboxylase, *accABCD*) in order to enhance precursors supply (Duan et al. 2011). In this work, they obtained a FAEE production of 0.9 g/L in fed-batch cultures of *E. coli*. In 2012, Zhang et al. designed an elegant dynamic sensor-regulator system (DSRS) to control the expression of *atfA, pdc, adhB* and *fadD* genes (Zhang et al. 2012). The DSRS is based on the ligand-responsive transcription factor FadR which coordinately regulates the expression of the enzymatic steps that provide ethanol and its condensation with fatty acyl-CoAs in response to the availability of FA. By using this approach, they were able to obtain 1.5 g/L of FAEE in a recombinant *E. coli* strain (Zhang et al. 2012).

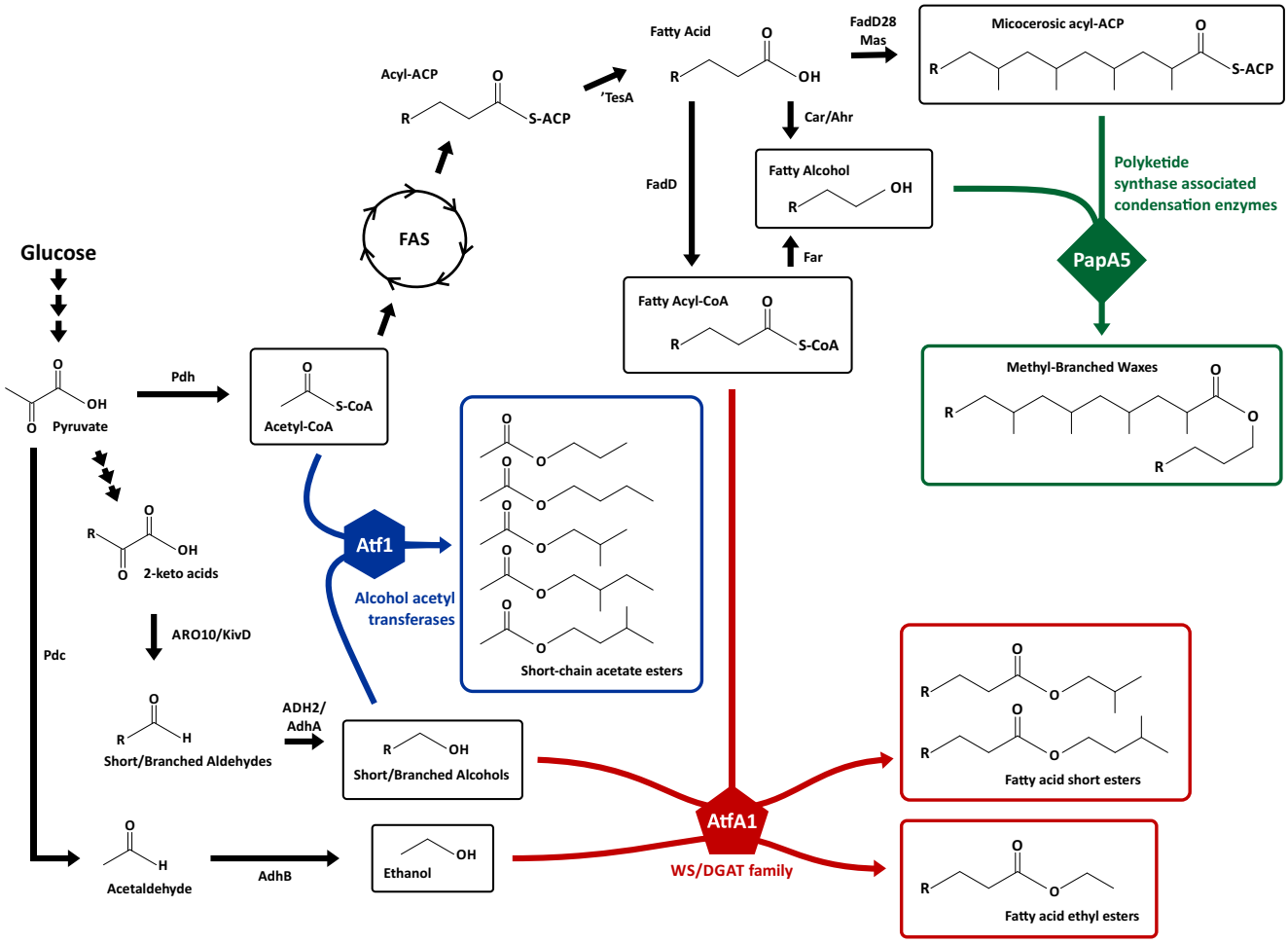

Fig. 1 Scheme of the metabolic pathways involved in the bioproduction of esters. *Black arrows* correspond to native/heterologous enzymatic reactions that provide the precursor moieties for ester biosynthesis. *Colored arrows* indicate the three ester forming enzyme groups described in the text, with the respective products highlighted in *colored boxes*. *Pdh* pyruvate dehydrogenase complex, *FAS* fatty acid synthase, *'TesA* cytoplasmic acyl-ACP thioesterase, *FabD* fatty acyl-CoA synthetase, *FAR* fatty acyl-CoA reductase, *CAR/Ahr* carboxylic acid reductase/aldehyde reductase, *FadD28* fatty acyl:AMP ligase, *Mas* mycocerosic acid synthase, *Pdc* pyruvate decarboxylase, *ARO10/KivD* 2-keto acid decarboxylases, *AdhA* ADH2 and *AdhB* alcohol dehydrogenases, *PapA5* polyketide synthase associated protein 5, *Atf1* alcohol:acetyl transferase 1, *AtfA1* wax synthase-diacylglycerol:acyl transferase

In 2014, Guo et al. utilized AtfA for FA-derived short chain esters production (FASE) combining FA metabolism with 2-keto acid pathways (Guo et al. 2014). The authors overexpressed ARO10 (2-ketoacid decarboxylase) and ADH2 (alcohol dehydrogenase) from *S. cerevisiae* YPH499 in a *fadE* (acyl-CoA dehydrogenase involved in FA degradation) knockout strain of *E. coli* that also overexpressed *tesA*' and *fadD*. This engineered strain led to the production of short-chain alcohol molecules, mainly isobutanol and isopentanol, and allowed the synthesis of up to 1 g/L of FASE in fed-batch cultures (Guo et al. 2014) (Table 1). In 2015, as we mentioned, Röttig et al. tested ten different bacterial WS/DGAT in a genetically engineered *E. coli* strain towards the production of FAEE and/or TAG, respectively (Röttig et al. 2015). While the highest TAG storage was achieved with *A. baylyi* ADP1 AtfA overexpression, the mutated variant AtfA-G355I turned out to be most suitable for FAEE biosynthesis and enabled an accumulation of approximately 0.5 g/L without external ethanol supplementation.

In yeasts, when the *A. baylyi atfA* gene was expressed in an engineered strain of *S. cerevisiae*, 0.24–0.52 g/L of FAEE were produced when cells were fed with FA (Yu et al. 2012) (Table 1). In 2012, Shi et al. carried out a comparison of five different WS/DGAT towards FAEE production in *S. cerevisiae* (Shi et al. 2012). WS2 from *M. hydrocarbonoclasticus* had the best performance and led to the production of 6.3 mg/L of FAEE. A titer improvement to 8.3 mg/L of FAEE was achieved when the *S. cerevisiae* acetyl coenzyme A carboxylase (ACC1) was up-regulated (Shi et al. 2012). Later, Shi et al. re-designed the pathway in order to overcome plasmid instability and metabolic burden. Also, they enhanced precursors supply by the overexpression of genes encoding endogenous acyl-CoA binding protein (*ACB1*) and a bacterial NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (*gapN*) (Shi et al.

Table 1 Higher titers achieved by expression of AtfA, ATF1 or PapA5 condensing enzymes

Condensing Enzyme	Microbial Host	Product	Maximum Titer (g/L)	Ref.
AtfA	*E. coli*	FASE	1.00	(Guo et al. 2014)
		FAEE	14.80	(Elbahloul and Steinbüchel 2010)
	S. cerevisiae	FAEE	0.52	(Yu et al. 2012)
ATF1	*E. coli*	EA, PA, IBA, 2MBA, 3MBA, 2PEA	0.10	
		TA	0.14	(Rodriguez et al. 2014)
		IBA	17.20	(Rodriguez et al. 2014)
			19.70	(Tashiro et al. 2015)
			36.00	(Tai et al. 2015)
PapA5	*E. coli*	MBE	0.79	(Menendez-Bravo et al. 2016)

FASE fatty acid short ester, *FAEE* fatty acid ethyl ester, *EA* ethyl acetate, *PA* propyl acetate, *IBA* isobutyl alcohol, *2MBA* 2-methyl-butyl acetate, *3MBA* 3-methyl-butyl acetate, *2PEA* 2-phenylethyl acetate, *TA* tetradecyl acetate, *IAA* isoamyl alcohol, *MBE* multi-methyl-branched ester

2014). Although effective, these strategies led to a FAEE production of 48 mg/L; a titer significantly lower than those obtained when *E. coli* is used a chassis.

The alcohol acetyltransferase family

The alcohol acetyltransferase family (AATase, PF07247) is a member of the CoA-dependent acyltransferase superfamily (clan CL0149). AATase enzymes convert alcohols and acetyl-CoA (mainly) to their corresponding esters (EC 2.3.1.84). AATase genes (also denoted as ATFs) from several yeasts and fungi have been cloned and sequenced and it was observed that there are considerable differences among the encoded enzymes regarding substrate specificity and activity (Park et al. 2009).

Eukaryotic AATases: substrate specificity and product diversity

In *S. cerevisiae*, most acetate-derived esters are formed by two alcohol acetyltransferases: ATF1 and ATF2. ATF1, the most relevant enzyme for the production of acetate esters, is responsible for 80% of isoamyl acetate formation, 75% of phenyl ethyl acetate production and about 40% of ethyl acetate synthesis; as it was shown by deletion analysis (Verstrepen et al. 2003, 2004). In addition, overexpression of the *ATF1* gene results in a more than 100-fold increase in isoamyl acetate production, as well as a 10–200-fold increase in the production of other esters, such as ethyl acetate, phenyl ethyl acetate and C_3-C_8 acetate esters (Verstrepen et al. 2003, 2004). On the other hand, ATF2 was characterized to have a lesser role than ATF1 in ester formation and it was observed that a double *ATF1*$^{-}$*ATF2*$^{-}$ mutant did not produce isoamyl acetate but still produced some ethyl acetate (Verstrepen et al. 2003). Although *S. cerevisiae* enzymes are the most studied eukaryotic AATases, it seems likely that this activity is widespread among protozoa. In 2008, Van Laere et al. identified sequences coding for putative homologous to ATF1 and ATF2 in several fungi such as *Saccharomyces bayanus*, *Saccharomyces kudriavzevii*, *Saccharomyces mikitase*, *Saccharomyces paradoxus*, *S. pastorianus*, *Torulopsis castelii*, *Candida glabrata*, *Kluyveromyces waltii* and *Kluyveromyces lactis* (Van Laere et al. 2008). These authors validated their in silico analysis for one of the identified candidates: the AATase orthologue from *K. lactis*. This coding sequence was heterologous expressed in *S. cerevisiae* and the produced metabolites were analysed by GC-MS. The obtained metabolite profiles suggested a functional similarity between *K. lactis* ATF and the *S. cerevisiae* ATF2 that reinforces the idea that the other identified orthologues could be involved in aroma-active acetate ester formation (Van Laere et al. 2008).

Neurospora sp. ATCC46892, a strong producer of ethyl hexanoate, expressed an AATase which was purified to homogeneity and biochemically characterized (Yamauchi et al. 2014). Analysis of substrate specificity showed that *Neurospora* AATase is highly active on various acyl-CoAs longer than n-hexanoyl-CoA but did not react with acetyl-CoA. For the alcohol substrate, *Neurospora* AATase showed higher activities with n-butyl alcohol and isobutyl alcohol compared with ethanol. However, to date, the *Neurospora* AATase coding gene has not been assigned. In contrast, AATase coding genes from other sources like fruit of wild strawberry (*Fragaria vesca*), cultivated strawberry (*Fragaria x ananassa*) and banana (*Musa sapientum*) have been isolated and partially characterized in 2004 by Beekwilder and

collaborators (Beekwilder et al. 2004). To determine the substrate specificity profiles of these enzymes, the authors carried out activity assays with recombinant enzymes expressed in *E. coli* using a variety of alcohols and acyl-CoAs and observed that the in vitro substrate preferences of the recombinant enzymes was not necessarily reflected in the volatile esters profile of the corresponding fruit. When the results were compared with a phylogenetic analysis of the various members of the AATase family, it was suggested that substrate preference could not be predicted on the basis of sequence similarity (Beekwilder et al. 2004).

Metabolic engineering involving the AATase pathway

In the recent years, *E. coli* has been successfully engineered to produce a variety of short-chain esters through the action of recombinant AATase enzymes in combination with an alcohol biosynthetic route.

In 2014, Rodriguez et al. were able to synthesize diverse alcohol-acetate esters by co-expression of a chimeric 2-ketoacid-derived alcohol biosynthetic pathway and ATF1 from *S. cerevisiae*. Short-chains alcohols can be generated by a promiscuous 2-keto acid decarboxylase (Kdc), that produces the corresponding aldehydes, followed by the action of endogenous or heterologous NAD(P)H-dependent aldehyde reductases/alcohol dehydrogenases (Adhs) (Fig. 1). In this work, the 2-keto acid decarboxylase gene *kivD* from *Lactococcus lactis* was expressed in *E. coli* to convert pyruvate, 2-ketobutyrate, 2-ketoisovalerate, 2-keto-3-methylvalerate, 2-keto-4-methylvalerate and phenylpyruvate into ethanol, 1-propanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol, respectively (Rodriguez et al. 2014). In combination with ATF1, this pathway led to the production of ethyl acetate, propyl acetate, isobutyl acetate (IBA), 2-methyl- butyl acetate, 3-methylbutyl acetate and 2-phenylethyl acetate giving a total ester concentration of 100 mg/L (Table 1). Similar experiments using the Lux-CDE system to synthesize tetradecanol enabled the production of 137 mg/L of tetradecyl acetate (Rodriguez et al. 2014) (Table 1). Since isobutanol production demonstrated to approach maximal theoretical yields (with titers up to 50 g/L), Rodriguez et al. performed a further optimization of the IBA biosynthesis pathway. To do this, they combined the isobutanol route reconstructed from AlsS (acetolactate synthase from *Bacillus subtilis*), IlvC (ketoacid reductoisomerase *from E. coli*), IlvD (dihydroxy-acid dehydratase from *E. coli*), KivD (*L. lactis*) and AdhA (*L. lactis*) with the ester-forming enzyme ATF1 in the *E. coli* strain JCL260 (Δ*adhE* Δ*frd* Δ*ldhA* Δ*pta* Δ*pflB* Δ*fnr*, gene deletions introduced to increase precursors levels). IBA production was maximized in a fed-batch fermentation process by using a hexadecane layer to avoid product toxicity. As a result, 17.2 g/L of IBA was produced after 72 h, this value represents 80% of the theoretical maximum yield (Rodriguez et al. 2014) (Table 1).

In 2015, Yi-Shu Tai et al. (Tai et al. 2015) studied and compared different alcohol acyltransferases from various organisms for their capability to catalyze esterification reactions to produce IBA and isoamylacetate (IAA) in *E. coli*. Among the benzylalcohol O-benzoyl transferase encoded by *luxE* from *Clarkia breweri* (D'Auria et al. 2002), *ATF1* (Fujii et al. 1994, 1996, 1997), *ATF2* (Nagasawa et al. 1998), benzylalcohol O-benzoyl transferase encoded by BPBT from *Petunia hybrida* (Orlova et al. 2006) and strawberry alcohol acyltransferase encoded by *SAAT* (Aharoni et al. 2000), the highest titer of IBA was achieved at 2.14 g/L with ATF1 and the co-expression of the corresponding alcohol pathway enzymes (*ilvD, alsS, kivd and yqhD* alcohol dehydrogenase from *E. coli*). The strain containing ATF2 produced 1.69 g/L of IBA. For IAA, 0.39 g/L and 0.25 g/L were obtained after the overexpression of ATF1 and ATF2, respectively (Table 1). LuxE, BPBT and SAAT showed very low titers for both esters. Finally, by deletion of native competitive pathways (pyruvate oxidase *poxB*, lactate dehydrogenase *ldh* and phophotransacetylase *pta*) and an adjusted fed-batch fermentation process with in situ product removal, these authors obtained a production of 36.0 ± 1.5 g/L of IBA and a yield of 42% of theoretical maximum after 72 h (Table 1).

Interestingly, Tashiro et al. carried out the construction of a novel pathway for IBA production where pyruvate and acetyl-CoA biosynthesis was dissociated (Tashiro et al. 2015). For this, the IBA synthesis pathway was split into two modules in which acetate was used for acetyl-CoA generation and glucose was derived for its conversion into isobutanol. Acetate assimilation route (acetate kinase AckA and phosphotransacetylase Pta from *E. coli*) was engineered to produce acetyl-CoA without carbon loss or the need for cofactors such as NADPH. Through this method, isobutanol is solely produced from pyruvate and is condensed with acetyl-CoA by ATF1; IBA production reached a titer of 19.7 g/L after 120 h of batch culture in a medium supplemented with glucose and acetate (Tashiro et al. 2015) (Table 1).

ATF1 and ATF2 overexpression was also carried out in order to analyse and compare the ester concentrations and aroma profiles of wines and distillates obtained by fermentation with genetically modified yeast strains. The *S. cerevisiae* industrial strain VIN13 carrying an integrated extra copy of *ATF1* under the control of the constitutive *PGK1* promoter increased the concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl caproate. Meanwhile, the overexpression of ATF2 affected the concentrations of ethyl acetate and isoamyl acetate to a lesser degree (Lilly et al. 2000). Additionally, the overexpression of ATF1 in brewing yeast strains resulted in beer with increased levels of ethyl acetate and isoamyl acetate, and decreased concentrations of the corresponding alcohols (Lee et al. 1995). Although these

genetically modified prototype yeasts cannot be used for commercial purposes, these data could be useful for the screening of ester producing natural yeasts through the analysis of ATF1 and ATF2 expression.

Despite the successful production of acetate-derived esters, medium-chain esters, such as isobutyl isobutyrate, presented very low titers after designing a short chain-acyl-CoA biosynthesis route. This is mainly due to the unusually high preference for acetyl-CoA, and very low specificity for higher-chain acyl-CoA (C_4-C_6), exhibited by Atf1 (Verstrepen et al. 2003, 2004). Given that only a few ATF have been studied and characterized, the low ATF1 specificity towards higher-chain-CoA (C4-C6) remained as a limiting factor.

The condensation family

The condensation family of enzymes (PF00668) constitutes another member of the CoA-dependent acyltransferase superfamily (clan CL0149). Among this family lies the "polyketide-associated proteins" (Pap) subfamily, which is not strictly CoA-dependent. This general name refers to a unique set of proteins encoded within polyketide synthase (PKS) coding regions and their function remained unclear over a long period of time.

Polyketide-associated proteins: substrate specificity and product diversity

In 2004, Onwueme and coworkers characterized one of these Pap from *M. tuberculosis*, designated PapA5, as the first member of this new subfamily and demonstrated that it is essential for the acylation of complex mycobacterial PKS-derived compounds (Onwueme et al. 2004). The *M. tuberculosis* genome comprises five such genes, *papA1* to *papA5*, and homologous genes could be identified in others mycobacteria. All of these sequences share the highly conserved motif HxxxD(x14Y) where PapA5 exhibits the extended motif HHxxxDG. PapA1 and PapA2 are responsible for the sequential acylation of trehalose-2-sulfate required for the synthesis of Sulfolipid-1 (SL-1) (Kumar et al. 2007). PapA3 is essential for the biosynthesis of PAT, a penta-acylated trehalose-based glycolipid (Hatzios et al. 2009). The *papA4* gene from *M. tuberculosis* is predicted to encode a truncated protein of only 165 amino acids, instead of the 465 amino acid Pap4 (MMAR_2343) involved in lipooligosaccharides biosynthesis of *M. marinum* (Rombouts et al. 2011). Finally, *papA5* of *M. tuberculosis* is crucial for the diesterification of phthiocerol with two residues of mycocerosate to produce the cell wall phthiocerol dimycocerosate (PDIM). In vitro, PapA5 showed broad substrate specificity for both the acyl donors and the alcohol moiety, being the long-chain acyl-CoA thioesters and 1-octanol, the preferred ones (Onwueme et al. 2004). The flexible substrate tolerance showed by PapA5 in vitro set the system formed by this protein and the mycocerosic acid biosynthesis enzyme as an interesting platform for the production of non-natural esters with novel structures.

Metabolic engineering involving the PKS-acyltransferase pathway

A novel strategy was used by Menendez-Bravo et al. in order to produce non-natural multi-methyl-branched wax esters in *E. coli* (Menendez-Bravo et al. 2014). The authors engineered a PKS-based biosynthetic pathway from *M. tuberculosis* and redefined its biological role towards the production of long-chain branched carbon ester with novel chemical structures. This pathway is constituted by the mycocerosic acid synthase Mas, which is an iterative type I PKS, the fatty-acyl-AMP ligase FadD28, and PapA5 (Fig. 1) (Menendez-Bravo et al. 2014). FadD28 is required for the activation of FA into their acyl-AMP derivatives and their loading on to the KS domain of Mas (Arora et al. 2009; Trivedi et al. 2005). This PKS elongates a C_{16}-C_{18} fatty acid derivative using methylmalonyl-CoA as a substrate, generally by four iterative catalytic cycles. Finally, the tetramethyl-branched fatty acid (MBFA) is transferred to a phthiocerol molecule by the PapA5 acyltransferase (Arora et al. 2009; Trivedi et al. 2005). Taking into account that in vitro PapA5 could utilize a variety of alcohols as acyl-acceptors (Onwueme et al. 2004), the authors were able to couple these three enzymes to exogenous supplied alcohols to generate a variety of new multi-methyl-branched esters in vivo. For this, the recombinant *E. coli* background strain was made proficient for (1) the postranslational modification of heterologous PKS ACP domains by expressing the promiscuous Sfp phosphopantetheinyl transferase from *Bacillus subtilis* and (2) the production of the PKS substrate (2*S*)-methylmalonyl-CoA from propionate by expressing the *Streptomyces coelicolor* propionyl-CoA carboxylase (PCC) complex subunits AccA1, PccB and PccE (Diacovich et al. 2002; Murli et al. 2003; Pfeifer et al., 2001).

Based in the high flexibility of this heterologous biosynthetic pathway, this *E. coli* platform allowed the synthesis of a wide set of related molecules by feeding different alcohols and different long-chain FA to the culture medium. In vivo, C_{10} to C_{18} FA were successfully accepted as Mas primer units (Menendez-Bravo et al. 2014); while PapA5 was able to utilize linear and branched alcohols ranging from C_1 to C_8, as well as two short-chain diols: ethylene glycol and trimethylene glycol. Moreover, PapA5 was also capable to catalyse the transesterification of the MBFA to methanol and isopropanol in vivo, an activity that had not been previously detected in vitro (Onwueme et al. 2004). Batch cultures bioconversion assays demonstrated that, by feeding propionate and octanol to the growth medium, multi-methyl-branched

esters of octanol (MBE) were produced with a titer of 97.9 mg/L (Menendez-Bravo et al. 2014). Fed-batch microbial fermentation process was also optimized achieving a maximum yield of 790 ± 6.9 mg/L of MBE with a volumetric productivity of 15.8 ± 1.1 mg/L h (Menendez-Bravo et al. 2014) (Table 1). The presence of several methyl branches in these esters should offer favourable physicochemical properties to the molecules, making them more stable to oxidation and suitable for the formulation of new biolubricant (Knothe 2005; Salimon et al. 2012a, b). Indeed, the bacterial MBE, analyzed by DSC, showed improved cold flow properties while exhibiting high oxidation stability at elevated temperatures (Menendez-Bravo et al. 2016).

Conclusion and perspectives

The major advantage of microbial production of esters over other biological processes is that product composition can be controlled by the choice of the starting materials, growth-conditions and, mainly, strain design (Ishige et al. 2003). In this review, we described three main strategies by which esters can be synthesized in native or heterologous microbial host according to the enzyme that performs the ester-bond formation. At the same time, each of these pathways gives rise to a different kind of ester molecule. Among the WS/DGATs, AtfA from *A. bayli* ADP1 is the most utilized enzyme in metabolic engineering for FAEEs production (Choi and Lee 2013; Janßen and Steinbüchel 2014; Kaddor et al. 2009; Kalscheuer et al. 2006a; Stöveken et al. 2005). On the other hand, expression of an AATase, almost exclusively ATF1 from S. *cerevisiae*, in combination with an alcohol biosynthetic route (commonly derived from 2-ketoacids metabolism) was widely utilized for the production of short branched-chain acetate-derived esters (Atsumi et al. 2008; Carroll et al. 2016; Nozzi et al. 2014; Rodriguez et al. 2014; Tashiro et al. 2015). Finally, Pap enzymes derived from PKS-based pathways, particularly PapA5 from *M. tuberculosis*, show great potential as condensing enzymes for the production of a wide variety of structurally diverse esters (Menendez-Bravo et al. 2014, 2016).

It is worth noting the great difference in titers achieved in the production of esters by prokaryotic and eukaryotic model microorganisms; basically, *E. coli* and *S. cerevisiae*, respectively. For example, overexpression of AtfA leads to 14.8 g/L of FAEE in a metabolically engineered *E. coli* (Elbahloul and Steinbüchel 2010) while the same enzyme produces 48 mg/L of FAEE when expressed in *S. cerevisiae* (Shi et al. 2014). However, the lower yields observed in *S. cerevisiae* are not specific for the production of esters since low efficiency has also been observed for the production of free FA and TAG (Lian and Zhao 2015). Although the specific metabolic bottlenecks have not been determined yet, the low titers obtained in the production of FA-derived compounds in *S. cerevisiae* may have multiple origins. The limited supply of precursors (acetyl-CoA and malonyl-CoA), the tight regulation of FA biosynthesis (Lian and Zhao 2015), the incorrect folding and/or the mislocation of the heterologously expressed proteins (the main acyl-CoA metabolism occurs at the membrane of the lipid droplets whereas heterologous proteins remain cytoplasmic (Liu et al. 2013)), are only some of the multiple reasons to explain the poor performance of *S. cereviseae* in the production of FA derived metabolites. However, the compatibility with high-density and large-scale fermentation, the resistance to phage infection and the high tolerance against toxic inhibitors and products makes *S. cerevisiae* an ideal host for bio-esters and other FA-derived compounds production and supports increasing efforts towards improving lipid production in this organism.

Although the three types of ester bond-forming enzymes discussed here have shown to be effective for the in vivo production of their corresponding esters, the full potential of microbial ester biosynthesis has not been reached so far. Improving production titers and yields are challenging when producing carbon molecules with increasing carbon length and chemical complexity. Even, the achievement of broader product diversity remains as an uncompleted task.

AtfA is proficient in synthesizing FAEE; however, the highest activity of this enzyme is obtained with medium to long-chain-length alcohols (C_{14} to C_{18}) (Stöveken et al. 2005). Short-chain-length alcohols such as ethanol, butanol, hexanol and octanol can also be accepted as substrates by AtfA although with much lower catalytic efficiency. Interestingly, even the mutant enzyme AtfA-G355I which acylates ethanol does it at lower rates compared with longer carbon chain alcohols (Stöveken et al. 2005). On the other hand, *S. cerevisiae* ATF1 is highly efficient for producing acetate-derived esters but its activity towards acyl-CoA others than acetyl-CoA showed to be negligible (Verstrepen et al. 2003, 2004). In the same way, PapA5 is the only enzyme of the Pap family reported to acylate an alcohol molecule (Menendez-Bravo et al. 2014; Onwueme et al. 2004). Other mycobacterial Pap-like enzymes utilize sugars or complex glycolipids as substrates, which makes them not suitable for the production of bio-esters with the characteristics of the ones reviewed here. Also, the limited understanding of the basis of the protein-protein interaction between Pap enzymes and the corresponding PKS domains restricts their use to its cognate PKS and substrate(s). Therefore, narrow substrate specificity constitutes one of the main restricting factors for the production of structurally diverse Pap-derived esters. On this regard, the detailed knowledge about the residues involved in the active site of PapA5 will enable the designing of variants capable of using specific alcohols, such as short-chain alcohols, with higher affinity than the wild type enzyme.

Overall, state of the art in microbial production of bio-esters shows that strategies of protein engineering as well as identification, characterization and optimization of new ester-forming enzymes represents a demand but also an opportunity towards expanding microbial ester structural diversity.

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