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Expanding the chemical diversity of natural esters by engineering a polyketide-derived pathway into *Escherichia coli*

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ABSTRACT

Microbial fatty acid (FA)-derived molecules have emerged as promising alternatives to petroleum-based chemicals for reducing dependence on fossil hydrocarbons. However, native FA biosynthetic pathways often yield limited structural diversity, and therefore restricted physicochemical properties, of the end products by providing only a limited variety of usually linear hydrocarbons. Here we have engineered into *Escherichia coli* a mycocerosic polyketide synthase-based biosynthetic pathway from *Mycobacterium tuberculosis* and redefined its biological role towards the production of multi-methyl-branched-esters (MBEs) with novel chemical structures. Expression of FadD28, Mas and PapA5 enzymes enabled the biosynthesis of multi-methyl-branched-FA and their further esterification to an alcohol. The high substrate tolerance of these enzymes towards different FA and alcohol moieties resulted in the biosynthesis of a broad range of MBE. Further metabolic engineering of the MBE producer strain coupled this system to long-chain-alcohol biosynthetic pathways resulting in *de novo* production of branched wax esters following addition of only propionate.

1. Introduction

Strong environmental concerns, as well as those related to cost and finite supply of crude oil, have expanded global interest in the development of environmentally friendly fluids and bio-based chemicals from renewable and sustainable sources (Curran and Alper, 2012; Kamm and Kamm, 2004). Fatty esters, for example, have numerous and highly relevant industrial applications. Thus, fatty acid methyl esters, the products of the transesterification of vegetable or animal oils with methanol, are the main constituents of biodiesel used in trucks, buses and trains. On the other hand, wax esters–oxoesters of long-chain fatty alcohols with long-chain fatty acids, present in jojoba and carnauba oil, have extraordinary lubrication properties (Bisht et al., 1993; El Kinawy, 2004) and are used in specialty lubricants as well as in pharmaceutical, personal care and cosmetics applications (Kalscheuer et al., 2006b).

The physical properties, and hence the performance, of these bio-based chemicals are largely defined by the structural features of the fatty acid and alcohol moieties (Knothe, 2005). Therefore, the fatty acid composition of the source organism has a strong influence on the characteristics and end uses of the derived oleochemicals. Fatty esters containing unsaturated fatty acids, especially those containing multiple double bonds, prevent molecular packing at low temperatures, improving compound fluidity. However, the presence of bis-allylic protons in these molecules significantly increases their oxidation rate (Knothe, 2005; Salih et al., 2013). On the other hand, highly saturated esters possess better thermo-stability properties, but as a drawback they have higher melting points and poor performance at low-temperatures (Salih et al., 2013). These limitations of natural products have been partially overcome by the use of synthetic and semi-synthetic esters, which allow the introduction of functional groups by multi-step chemical synthesis (Salih et al., 2013). In this regard, branched-chain fatty acids cause important changes in the physicochemical properties of their esters by lowering the melting points of the fluids while maintaining a high thermo-oxidative stability (Ngo et al., 2011).

As an alternative to plant-derived esters and as a result of the progress made in metabolic engineering and in synthetic and systems biology, microbes have been successfully engineered to produce wax esters and advanced biofuels (Dellomonaco et al., 2010; Guo et al., 2014; Ishige et al., 2003; Nielsen et al., 2013; Peralta-Yahya et al., 2012; Zhang et al., 2011). Using microorganisms

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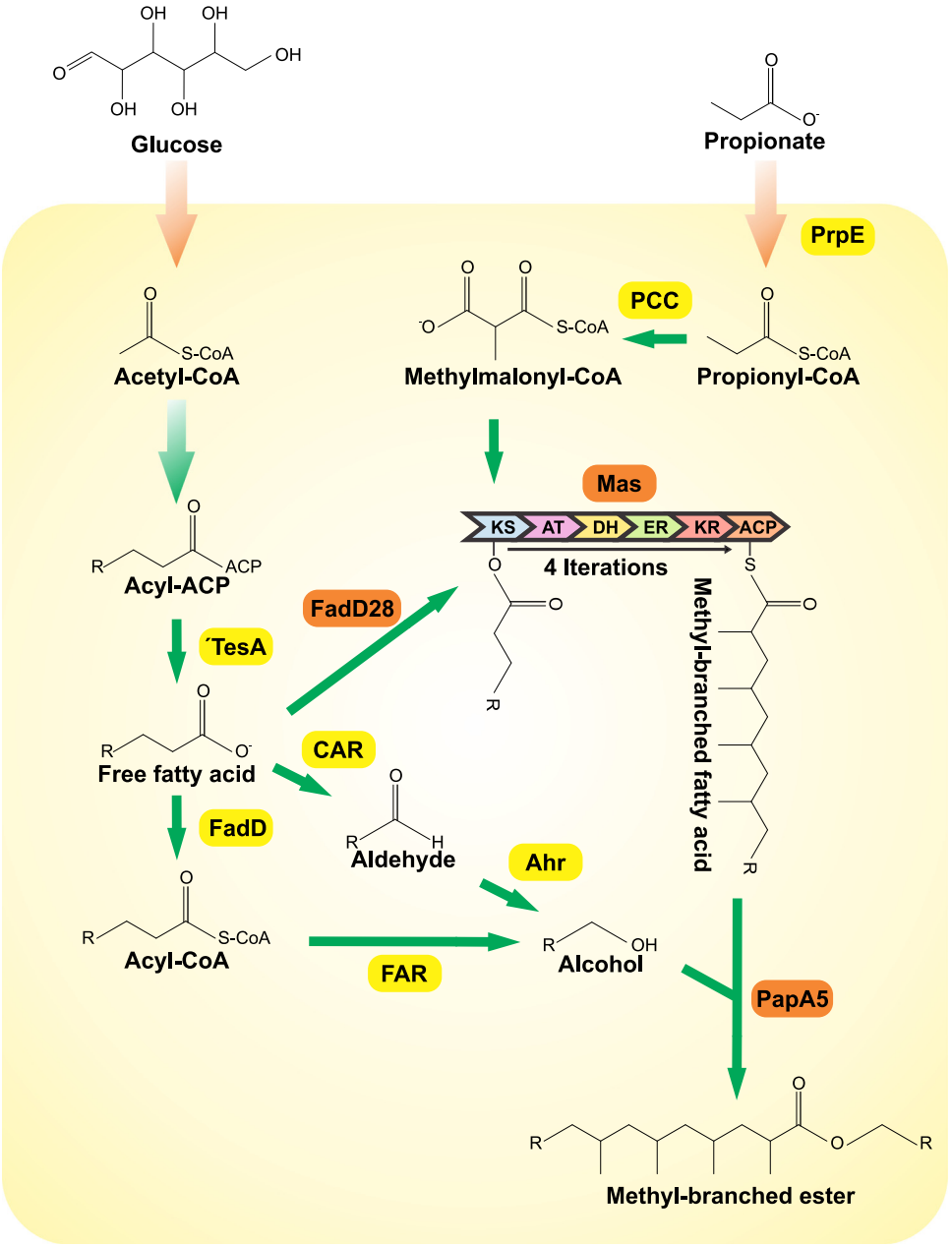

Fig. 1. Microbial biorefinery: Pathways for MBFA-derived lipid production in genetically engineered *E. coli*. Overexpression of native PrpE and heterologous PCC complex from *S. coelicolor* leads to the production of extender unit methylmalonyl-CoA from exogenous propionate. Carbon flux towards accumulation of free fatty acids (FFA) was improved by overexpression of ´TesA. These FFA serve as substrates for FadD28-Mas and CAR enzymes for the production of enzyme-linked MBFA and fatty aldehydes, respectively. Fatty aldehydes are further reduced to fatty alcohols by overexpression of native Ahr. Alternatively, long-chain alcohol molecules can be produced from FFA by conversion to acyl-CoAs, catalyzed by native FadD, and subsequent reduction to fatty alcohols catalyzed by FAR. Finally, PapA5 catalyzes a transesterification reaction between the enzyme-linked MBFA and the alcohol molecule, giving rise to MBE and MBW molecules.

as cell factories not only provides the opportunity to convert renewable feedstocks into high-value chemical commodities (Steen et al., 2010), but also provides an in-depth genetic and biochemical understanding of fatty acid synthesis in model organisms, such as *E. coli* and *Saccharomyces cerevisiae*, to broaden the repertoire of lipid-derived molecules with industrial applications that can be made by these organisms (Akhtar et al., 2013; Choi and Lee, 2013; Liu et al., 2010; Peralta-Yahya et al., 2012; Zhang et al., 2008).

With these considerations in mind, we reasoned that the biosynthesis of multi-methyl-branched fatty acids could generate molecules whose properties would fulfill several of the physicochemical requirements needed for the development of new and improved bio-based fluids. Our objective, therefore, was to seek metabolic pathways that could expand the native fatty acid and lipid biosynthetic capacity of *E. coli* and generate recombinant strains that could produce multi-methyl-branched fatty acids (MFBA) and esters (MBEs).

MBFA and MBE are extremely rare in nature; they are constituents of the waxy material secreted by the uropygial gland of some bird species, and are also part of the complex lipids of the cell wall of certain mycobacteria (Jacob and Poltz, 1974; Minnikin et al., 2002). In particular, we highlight the phthiocerol dimycocerosate (PDIM) biosynthetic pathway of *Mycobacterium tuberculosis*. This lipid arises by esterification of phthiocerol with mycocerosic acid, an MBFA synthesized by the iterative type I polyketide synthase (PKS) mycocerosic acid synthase (Mas) (Fig. 1) (Trivedi et al., 2005). Polyketide biosynthesis is one of the most versatile pathways for producing hydrocarbons with diverse structures, and PKSs are one of the most flexible enzymatic

Table 1
Plasmids and strains.

Plasmid	Description	Reference
pET28a	Vector for expression of N-terminal His-tagged proteins under the T7 promoter; Km^R	Novagen
pBluescript II SK	Phagemid vector; Ap^R	Agilent
pET22b	Vector for protein expression under T7 promoter; Ap^R	Novagen
pBAD33	Vector for protein expression under P_{BAD} promoter; Cm^R	(Guzman et al., 1995)
pRT23	pET21 derivative vector containing *mas* gene; Ap^R	(Trivedi et al., 2005)
pKO3Blue	Vector for gene replacement; Cm^R	(Solano et al., 2009)
pAH162	Plasmid containing φ80 phage *attP* sequence; Tc^R	(Haldimann and Wanner, 2001)
pAH123	Helper plasmid expressing φ80 phage integrase; Ap^R	(Haldimann and Wanner, 2001)
pCC01	pET28a derivative vector containing *accA2* gene; Km^R	This work
pCC02	pET28a derivative vector containing *pccB* gene; Km^R	This work
pCC03	pET28a derivative vector containing *pccE* gene; Km^R	This work
pCC04	pET28a derivative vector containing *pccE and pccB* genes; Km^R	This work
pCC05	pET28a derivative vector containing *accA2*, *pccE* and *pccB* genes; Km^R	This work
pCC06	pBluescript derivative vector containing *GHdel* synthetic DNA fragment; Ap^R	This work
pCC07	pCC06 derivative vector containing *accA2*, *pccE* and *pccB* genes under the control of T7 promoter; Ap^R	This work
pCC08	pKO3Blue derivative vector containing *accA2*, *pccE* and *pccB* genes under the control of T7 promoter, flanked by homology regions to *E. coli* genome; Ap^R	This work
pMB01	pET28a derivative vector containing *papA5* gene; Km^R	This work
pMB02	pET28a derivative vector containing *fadD28* gene; Km^R	This work
pMB03	pET28a derivative vector containing *papA5* and *fadD28* genes; Km^R	This work
pMB04	pBAD derivative vector containing *papA5* gene; Cm^R	This work
pMB05	pBAD derivative vector containing *fadD28* gene; Cm^R	This work
pMB06	pBAD derivative vector containing *papA5* and *fadD28* genes; Cm^R	This work
pMB07	pET28 derivative vector containing *papA5*, *fadD28* and *mas*; Km^R	This work
pMB08	pET28a derivative vector containing *ahr* gene; Km^R	This work
pMB09	pET28a derivative vector containing *acr1* gene; Km^R	This work
pMB10	pET22b derivative vector containing *ahr* gene; Ap^R	This work
pMB11	pET22b derivative vector containing *acr1* gene; Ap^R	This work
pMB12	pET22b derivative vector containing *car* gene; Ap^R	This work
pMB13	pET22b derivative vector containing *far* gene; Ap^R	This work
pMB14	pET22b derivative vector containing *ahr* and *acr1* genes; Ap^R	This work
pMB15	pET22b derivative vector containing *ahr* and *car* genes; Ap^R	This work
pMB16	pET28a derivative vector containing '*tesA* gene; Km^R	This work
pMB17	pAH162 derivative vector containing the T7 promoter sequence; Tc^R	This work
pMB18	pAH162 derivative vector containing '*tesA* gene under the T7 promoter; Tc^R	This work
Strain	**Description**	**Reference**
DH5α	*E. coli* K12 F^-*lacU169* (*Φ80lacZΔM15*) *endA1 recA1 hsdR17 deoR supE44 thi-1 l2 gyrA96 relA1*	(Hanahan, 1983)
BW25142	*lacIq rrnB3 _lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 _phoBR580 rph-1 galU95 _endA9 uidA*(*_Mlu*I)::*pir-116 recA1*	(Haldimann and Wanner, 2001)
BAP1	*E. coli* F^-*ompT hsdSB* (*rB-mB-*) *gal dcm* (*DE3*) *prpRBCD*:: *T7prom-sfp-T7prom-prpE*	(Pfeifer et al., 2001)
K207-3	BAP1 *panD*::*panDS25A ygfG*::*T7prom-accA1–T7prom-pccB-T7term*	(Murli et al., 2003)
RQ1	BAP1 *ygfGH*::*T7prom-accA2-pccE-pccB-T7term*	This work
RQ2	RQ1 $attB_{\phi 80}$:pMB18	This work

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Cm^R, cloramphenicol resistance; Tc^R, tetracicline resistance.

systems known in nature, producing a large family of structurally diverse natural products, including very complex lipids (Minnikin et al., 2002; Staunton and Weissman, 2001); however, they have never been exploited for the production of fuels or biolubricants (Peralta-Yahya et al., 2012). Thus, to expand the natural structural diversity of fatty acids and their use for MBE synthesis on a scalable platform, we aimed to develop a recombinant bacterium with the ability to synthesize MBFA and MBE by taking advantage of the Mas-based metabolic pathway. This enzyme carries out the iterative condensation of long-chain fatty acid starters with methylmalonyl-CoA extender units to yield long tetramethyl-branched fatty acids (Mathur and Kolattukudy, 1992; Onwueme et al., 2004; Trivedi et al., 2005). The long-chain fatty acids are loaded on to the Mas by the acyl-AMP ligase FadD28 and, after four elongation cycles, are released from Mas by the polyketide-associated protein A5 (PapA5). PapA5 interacts with Mas and catalyzes the transesterification of the ACP-bound MBFA with the diol component of phthiocerol (Onwueme et al., 2004; Trivedi et al., 2005). *In vitro*, PapA5 accepts several structurally diverse alcohols as substrates to generate a variety of oxoesters (Onwueme et al., 2004). Therefore, based on the enzymatic properties of these proteins, we set out to reconstitute the basic mycoserosic acid (MA) biosynthesis system -FadD28-Mas-PapA5- in the heterologous host *E. coli*.

In this work we demonstrate that by coupling the fatty acid biosynthetic machinery of *E. coli* with the heterologous mycocerosic biosynthetic pathway of *M. tuberculosis*, we were able to produce a wide range of multi-methyl-branched FA derived esters, thereby expanding the chemical characteristics and properties of these natural molecules. These results provide for the first time a proof of principle for the microbiological production of industrially relevant MBFA-derived compounds.

2. Materials and methods

2.1. Media and growth conditions

E. coli strains were grown either on solid or in liquid Luria–Bertani medium (LB; 10 g Bacto Tryptone, 5 g yeast extract

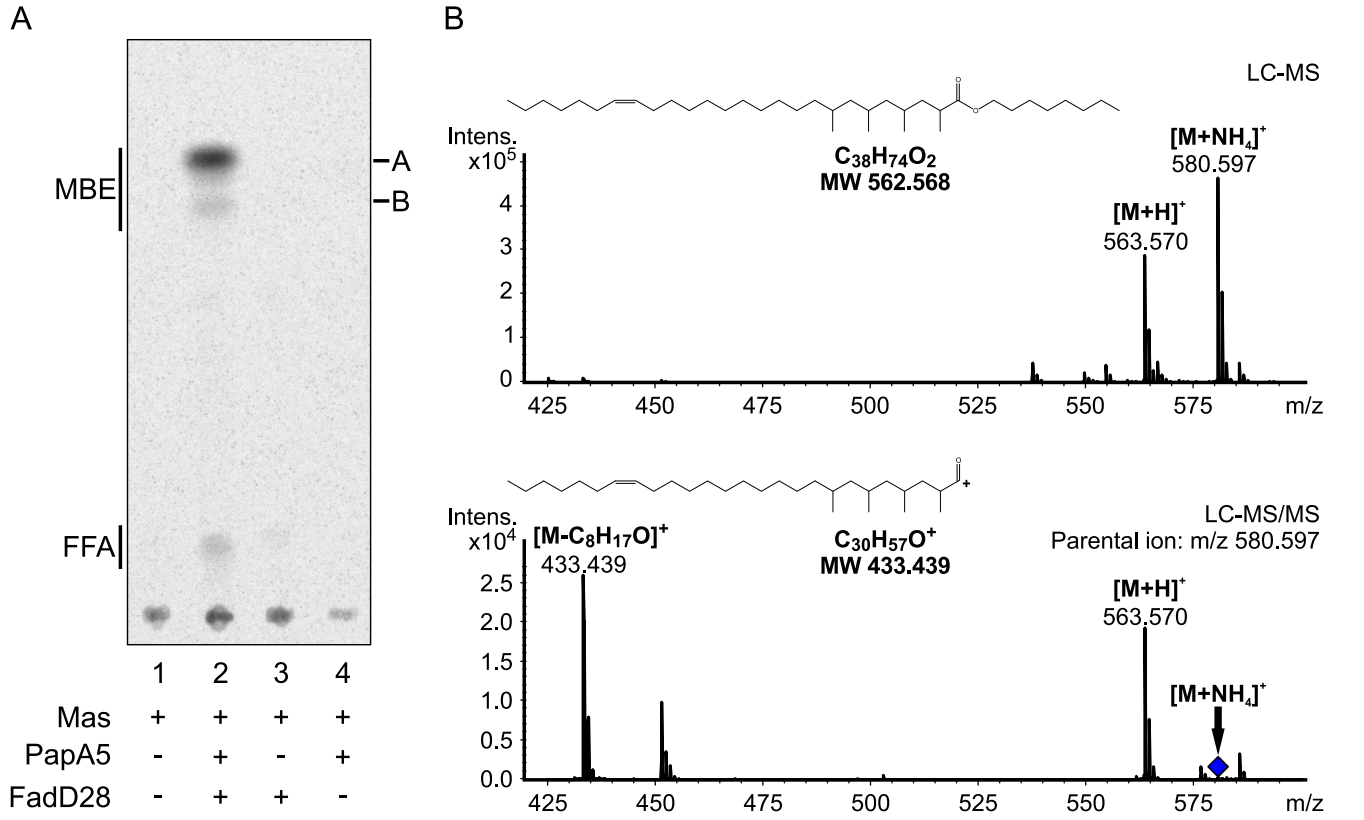

Fig. 2. MBE biosynthesis by recombinant *E. coli*. (A) Radio-TLC analysis of cells cultivated for 20 h at 22 °C in LB medium after induction with IPTG and L-arabinose and supplemented with n-octanol and [^{14}C]-propionate. Lanes: 1, *E. coli* K207-3/pRT23; 2, *E. coli* K207-3/pRT23/pMB06; 3, *E. coli* K207-3/pRT23/pMB05; 4, *E. coli* K207-3/pRT23/pMB04. (B) Identification by LC-MS/MS of major MBE contained in spot A. Transition *m/z* 580.597 to *m/z* 433.439 indicates that chemical formula $C_{38}H_{74}O_2$ corresponds to (Z)-octyl 2,4,6,8-tetramethylhexacos-19-enoate. Spot A also contains the MBE formed by condensation of the MBFA derived from four iterative MAS-catalyzed extension steps of palmitic and palmitoleic acids with n-octanol (Supplementary Fig. 1). In the same way, spot B contains the MBE formed by condensation of cis-vaccenic, palmitic and palmitoleic acid derived MBFA with ethanol (Supplementary Fig. 1).

and 10 g NaCl per liter) at 37 °C and supplemented when needed with the following antibiotics: 100 μg ml^{-1} ampicillin (Ap), 50 μg ml^{-1} kanamycin (Km), 20 μg ml^{-1} chloramphenicol (Cm) or 12.5 μg ml^{-1} tetracycline (Tc).

2.2. Plasmids and strains construction

All the strains and plasmids used in this work are listed in Table 1. Oligonucleotide primers and synthetic DNA fragments are shown in Supplementary Table 1. Gene names and the respective accession numbers are listed in Supplementary Table 2. See Supplementary material for detailed plasmid and strain construction.

2.3. Bioconversion assay

Overnight cultures of the corresponding strains were used to inoculate fresh LB medium supplemented with 20 μM biotin and the appropriate antibiotics. At mid-exponential phase cultures were induced with 0.1 mM IPTG (and 0.2% L-arabinose, if required), labeled with 2 μCi [^{14}C]-propionate (56 mCi mmol^{-1}, Moravek Biochemicals), and supplemented with 1 mM n-octanol. The induced cultures were further incubated for 20 h at 22 °C. Cells were harvested and total lipids were extracted from supernatant and cell material according to the Blight and Dyer method. Radioactivity incorporated into each lipid fraction was quantified using a scintillation counter and total lipid extracts were analyzed by radio-TLC on silica gel 60 F254 plates (0 ± 2 mm, Merck) using the solvent system hexane/diethylether/acetic acid (90:7.5:1, v/v/v). The radioactivity incorporated in each lipid fraction was visualized using a Storm 860 PhosphorImager (Molecular Dynamics) and spots corresponding to methyl-branch esters (MBEs) were identified by comparison with cetyl-palmitate as standard.

To test substrate tolerance of PapA5 towards the acceptor alcohol, a culture of *E. coli* K207-3/pMB07 was induced with IPTG and labelled as already described. In order to avoid the ethanol that carries the commercial solution of [^{14}C]-propionate, the appropriate volume of this reagent was dried in a SpeedVac centrifuge (Savant®) and solubilised in the same volume of water. Aliquots of culture were then supplemented individually with 10 mM methanol, 10 mM propanol, 10 mM isopropanol, 2.5 mM butanol, 2.5 mM isobutanol, 2.5 mM pentanol, 2.5 mM isopentanol, 1 mM 2-octanol, 10 mM ethylenglycol and 10 mM propylenglycol. Cultures were incubated for 20 h at 22 °C and total lipids were extracted and resolved by TLC as previously described.

The same protocols were used to identify the MBE produced by the respective *E. coli* strains by LC–MS/MS. In these cases, cultures were supplemented with 20 mM sodium propionate instead of the ^{14}C labelled substrate.

To evaluate the range of fatty acids accepted by the FadD28-Mas enzymes, a culture of *E. coli* K207-3/pMB07 was induced as described and supplemented with 0.4% Brij-58 and 20 mM sodium propionate. After that, the culture was aliquoted and each aliquot supplemented with 20 mM hexanoic acid, 10 mM octanoic acid, 5 mM decanoic acid, 10 mM dodecanoic acid, 10 mM tetradecanoic acid, 2.5 mM heptadecanoic acid, 2 mM oleic acid or 1 mM docosanoic acid. Cultures were incubated for 20 h at 22 °C and total lipids were extracted as previously described. Cultures supplemented with short-chain fatty acids ($< C_{12}$) were also supplemented with 2 mM of oleic acid in order to induce the fatty acid transport system (Klein et al., 1971). Finally, the organic extracts were subjected to LC–MS/MS analysis.

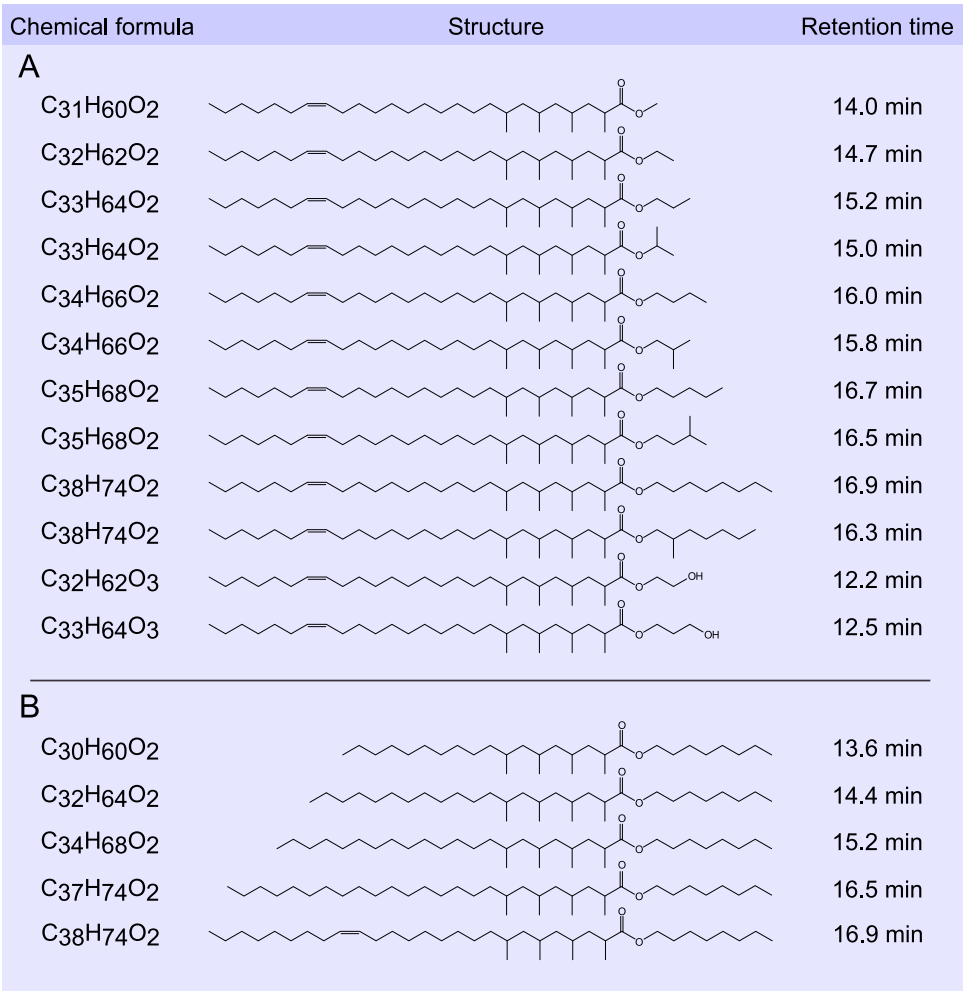

Fig. 3. Diversity of MBE produced by the engineered *E. coli* strain K207-3. (A) MBE synthesized by *E. coli* K207-3/pMB07 supplemented with different alcohols. The table exhibits the chemical formula, structure and retention time of MBE synthesized by condensation of the MBFA, derived from four iterative MAS-catalyzed extension steps of cis-vaccenic acid, with different alcohol molecules. MBE formed by condensation of the different alcohols with palmitic and palmitoleic acid derived MBFA were also identified as minor products. Radio-TLC and LC/MS/MS analysis of each MBE product is detailed in Supplementary Fig. 2. (B) MBE synthesized by *E. coli* K207-3/pMB07 supplemented with different FA. The table exhibits the chemical formula, structure and retention time of the major MBE synthesized by condensation of the corresponding starter FA and four iterative MAS-catalyzed extension steps (See Supplementary Fig. 3 for analytical data).

All the recombinant strains constructed and used in this study displayed the same growth kinetics and comparable final OD.

2.4. Metabolite analysis

Total lipid extracts from cultures or scraped TLC spots were dried under nitrogen, solubilised in methanol:chloroform (4:1 v/v) and separated on a ZORBAX Eclipse XDB-C8 column (3.0×50 mm^2, particle size$=1.8$ μm; Agilent, USA) using a binary solvent system of water (Solvent A) and methanol (Solvent B). A linear gradient from 80 to 100% B was applied between 0 and 25 min. Both solvents were supplemented with 5 mM ammonium acetate. The outlet of the liquid chromatograph was connected to a micrOTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) operating in the positive-ion mode and the data was acquired online in the mass range m/z 35–1000. MBE products were detected as ammonium, sodium and proton adducts in the range of 12–18 min of the chromatography run. Chemical structures of the MBE synthesized were confirmed by MS/MS analysis applying a collision energy of 15 eV and monitoring transitions of the corresponding molecular ions. For quantification of MBW, a calibration curve was done using purified (Z)-octyl 2,4,6, 8-tetramethylhexacos-19-enoate as standard and MBE concentration in the samples was calculated by linear regression equation obtained from the calibration curve.

2.5. Quantitation of PCC activity in cell-free extracts

PCC activity was determined by the method described by Hunaiti and Kolattukudy (1982). The reaction mixture contained 100 mM potassium phosphate, pH 8.0, 0.3 mg BSA, 3 mM ATP, 5 mM MgCl, 50 mM $NaH^{14}CO_3$, 0.5 mM propionyl-CoA and 100 mg of cell-free protein extract in a total volume of 100 ml. The reactions were incubated at 30 °C and stopped by acidification with HCl. Activity was measured by following the incorporation of $H^{14}CO_3^-$ into acid non-volatile material. One unit of enzyme activity catalyzes the incorporation of 1 μmol ^{14}C into acid-stable products per min.

2.6. Analysis of FadD28, Mas and PapA5 expression in E. coli

E. coli K207-3 harboring the corresponding plasmids for protein expression was grown in LB medium supplemented with the respective antibiotics at 37 °C. At mid exponential-phase the

culture was induced with 0.1 mM IPTG (and 0.2% L-arabinose, if required) and incubated for 20 h at 22 °C. Cells were harvested by centrifugation at 5000 $\times g$ for 10 min at 4 °C, washed twice and resuspended in ice-cold disruption buffer (Tris–HCl 50 mM pH 8.0, NaCl 100 mM, EDTA 1 mM, β-mercaptoethanol 10 mM). Cell disruption was carried out by sonication (Bioruptor®, Diagenode s.a., Belgium) in the presence of 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich) and the lysate was cleared by centrifugation at 20,000 $\times g$ for 30 min at 4 °C. Mas expression was analyzed by sodium dodecyl sulfate-polyacrylamide 8% gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. To analyse FadD28 and PapA5 expressions, total soluble protein samples were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane and the proteins identified by Western blotting with monoclonal mouse anti-His antibodies (QIAGEN®) using standard protocols.

3. Results

3.1. Engineering the M. tuberculosis mycocerosic biosynthetic pathway for the production of MBE in E. coli

Besides the general advantages of using *E. coli* as a platform for the production of different fatty acid-derived molecules (Liu and Khosla, 2010; Schirmer et al., 2010; Steen et al., 2010), this organism has also been genetically adapted to produce the methylmalonyl-CoA derived polyketide 6-deoxyerythronolide B (6-dEB) (Murli et al., 2003; Pfeifer et al., 2001). For this purpose, the recombinant *E. coli* strain K207-3 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) production of the PKS substrate (2*S*)-methylmalonyl-CoA from propionate, by expressing the *Streptomyces coelicolor* propionyl-CoA carboxylase (PCC) complex subunits AccA1 and PccB (Murli et al., 2003; Pfeifer et al., 2001) (Table 1). Since this genetic background is well adapted to reconstitute a functional MA system, we engineered into K207-3 the *papA5*, *fadD28* and *mas* genes and tested for the ability of the recombinant strain to produce MBE in bioconversion experiments. IPTG-induced cultures were supplemented with 1 mM n-octanol [one of the preferred *in vitro* substrate of PapA5 (Onwueme et al., 2004)] and ^{14}C-propionate. The *in vivo* synthesis of MBE was analyzed by radio-TLC and confirmed by HPLC coupled to electrospray tandem mass spectrometry (LC–MS/MS) (Fig. 2A and B). Successful production of MBE was observed only in the presence of the complete MA system, confirming the need of the three enzymes for the synthesis of these novel molecules. Analytical characterization of the new esters (Fig. 2A, spots A and B) by LC–MS indicated that each spot contained at least three related species with different chemical formulae. Examination of Spot A on HPLC yielded peaks at 15.9, 16.4 and 16.9 min, producing molecular ion peaks of *m/z* 535.534, 537.549, and 563.570, respectively. These *m/z* values and the associated isotope patterns agree with chemical formulae $C_{36}H_{72}O_2$, $C_{36}H_{70}O_2$ and $C_{38}H_{74}O_2$, respectively (Supplementary Fig. 1). Further analysis by LC–MS/MS generated fragmentation products of *m/z* 405.409, 407.424, and 433.438, respectively. These ions arise from cleavage of the ester bonds, resulting in loss of the alkoxy groups and formation of positively charged species. The identity of the products contained in spot A was thus confirmed as (Z)-octyl 2,4,6,8-tetramethyltetracos-17-enoate, octyl 2,4,6,8-tetramethyltetracosanoate, and (Z)-octyl 2,4,6,8-tetramethylhexacos-19-enoate, respectively. These MBEs are therefore synthesized by the condensation of n-octanol with the MBFA derived from four iterative Mas-catalyzed extension steps of palmitoleic, palmitic and cis-vaccenic acid (the most abundant FA found in *E. coli*) as primer units, respectively. Likewise, we identified the products of spot B as (Z)-ethyl 2,4,6,8-tetramethyltetracos-17-enoate, ethyl 2,4,6,8-tetramethyltetracosanoate and (Z)-ethyl 2,4,6,8-tetramethylhexacos-19-enoate (Supplementary Fig. 1). These compounds arise by the condensation of the corresponding MBFA with ethanol present in the commercial ^{14}C-propionate solution. Together, these results demonstrate that we were able to couple the *E. coli* fatty acid biosynthetic pathway to the *M. tuberculosis* mycocerosic acid biosynthetic enzymes FadD28 and Mas to expand the profile of fatty acids made by this organism. Furthermore, the presence of the phthiocerol dimycocerosyl transferase PapA5 allowed the *in vivo* transesterification of the acyl moiety linked to the ACP domain of Mas to two different chain-length alcohols, generating a diversity of MBE.

3.2. in vivo production of novel MBE through a flexible MA enzyme system

The possibility of combining different MBFA to different alcohol moieties would provide a tractable approach to fine tuning the physicochemical properties of the final ester products. Therefore, after the novel wax ester biosynthetic pathway had been established in recombinant *E. coli*, feeding experiments were conducted to determine the variety of structurally diverse MBE that could be produced by this system.

The apparently high substrate tolerance of PapA5 towards the acceptor alcohols was confirmed by feeding strain K207-3 harboring the MA system with a set of ten different substrates. Subsequent transesterification of Mas-bound MBFA with each of these acceptor alcohols successfully gave rise to a broad variety of new MBE (Fig. 3A). The identity of these new MBE was confirmed by LC–MS/MS (Supplementary Fig. 2). These results demonstrated that PapA5 is able to utilize *in vivo* linear and branched alcohols ranging from C1 to C8, as well as two short-chain carbon diols, such as ethylene glycol and trimethylene glycol. According to the relative yields of each of the MBE produced, we concluded that n-octanol is the preferred substrate of PapA5, consistent with the *in vitro* substrate specificity previously reported for this enzyme (Onwueme et al., 2004). Moreover, *in vivo*, PapA5 is able to catalyze the transesterification of the MBFA to methanol and isopropanol, an activity that had not been previously detected *in vitro* (Onwueme et al., 2004). Experiments carried out with alcohols $>$C10 were difficult to interpret because of the low solubility of these substrates.

To further evaluate the flexibility of the system towards the starter unit used by the FadD28-Mas enzymes, we fed strain K207-3 with n-octanol and with different chain-length fatty acids ranging from C_6 to C_{22}. Under these conditions we observed that fatty acids between C_{10} and C_{18} were successfully accepted as Mas primer units (Fig. 3B and Supplementary Fig. 3). Furthermore, the MS/MS analysis of the products obtained for each fatty acid fed allowed us to determine that the C18:1 derivative MBE was always the most abundant specie.

Overall, these results not only demonstrate the high tolerance of the MA system towards its different substrates –fatty acids and alcohols–but it also allows, within certain limits, the rational manipulation of precursors that could be fed to the MA system to expand the chemical and structural diversity of MBE produced *in vivo*.

3.3. Metabolic engineering to improve MBE production

Although the genetic background of K207-3 is well adapted to reconstitute a functional MA system, previous experiments demonstrated that the ε subunit, encoded by *pccE*, was essential to reach maximal PCC activity *in vitro* (Diacovich et al., 2002).

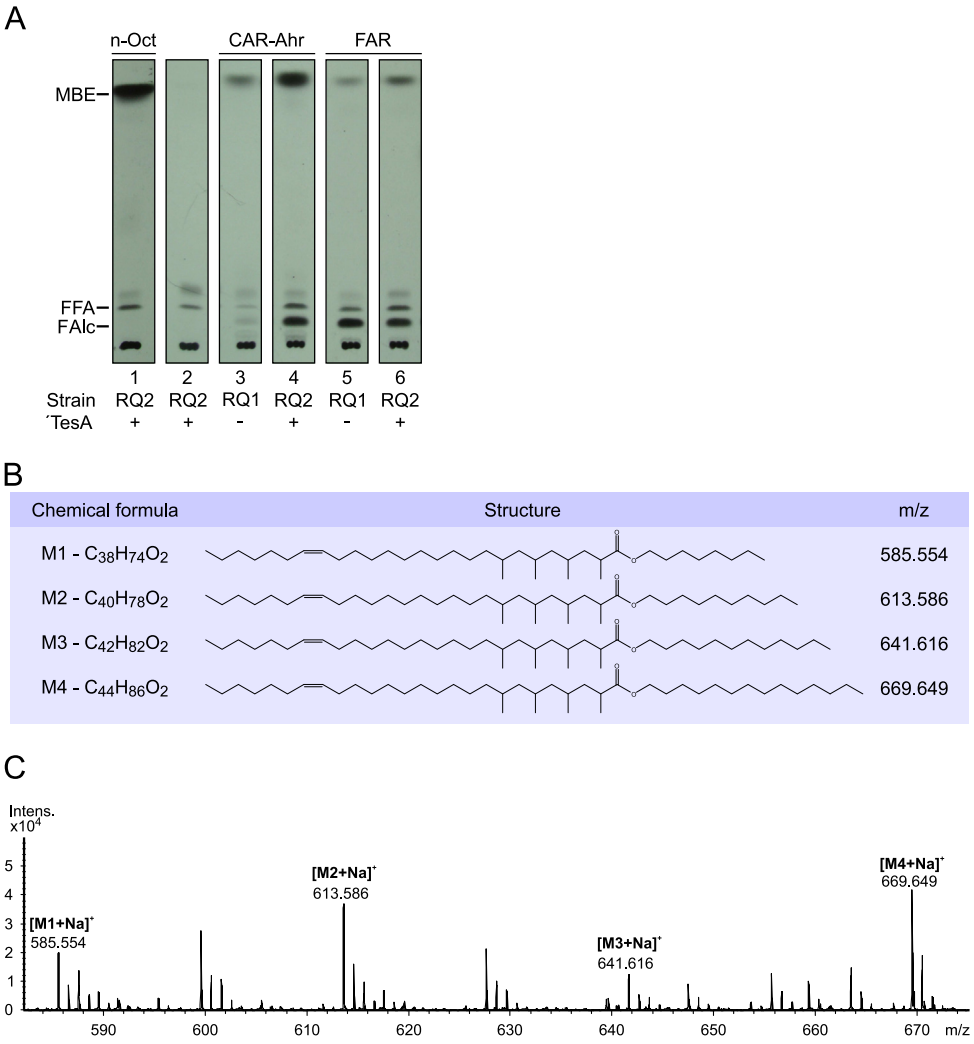

Fig. 4. *De novo* biosynthesis of multimethyl-branched wax esters. (A) Radio-TLC analysis of total lipid extracts from *E. coli* strains RQ1 and RQ2 expressing the MA system together with two different fatty-alcohol biosynthetic pathways. Lanes: 1, *E. coli* RQ2/pMB07 supplemented with 1 mM n-octanol (positive control); 2, RQ2/pMB07 with no alcohol supply (negative control); 3, RQ1/pMB07/pMB15; 4, RQ2/pMB07/pMB15; 5, RQ1/pMB07/pMB13; 6 RQ2/pMB07/pMB13. (B) Diversity of MBW synthesized *de novo* by *E. coli* RQ2/pMB07/pMB15. m/z values correspond to the $[M+Na]^+$ ionized forms of the different MBW. (C) MS identification of major MBW produced *de novo* by *E. coli* RQ2/pMB07/pMB15.

Accordingly, and in order to further improve the availability of (2*S*)-methylmalonyl-CoA, we introduced the *accA2-pccE-pccB* genes into *E. coli* BAP1 (Pfeifer et al., 2001), the parent of K207-3 (Supplementary information, Table S1). For this, we constructed a new artificial operon containing the *accA2-pccE-pccB*- genes under the T7 promoter and inserted it into the *ygfG-ygfH* locus of BAP1 to yield strain RQ1 (Supplementary information, Table S1). PCC activity measured in crude extracts of *E. coli* RQ1 was 8.81 ± 0.06 mU mg^{-1}, 7.8-fold higher compared with that found in the absence of PccE (isogenic strain K207-3). Furthermore, bioconversion experiments showed that K207-3 expressing the MA system produced 57.9 ± 3.1 mg/L of MBE while RQ1 generates 76.1 ± 4.3 mg L^{-1}, a 31.3% increase in the product yield (Supplementary Fig. 4).

As another rational step in platform optimization, we decided to manipulate the endogenous FA pools of *E. coli*. Free fatty acids are one of the substrates of the MA system; thus, to improve their availability we engineered into strain RQ1 the gene encoding the *E. coli* ´TesA (a leaderless version of a periplasmic thioesterase) under the control of the T7 promoter. This enzyme hydrolyzes acyl-ACP pools, deregulating fatty acid biosynthesis and leading to an increase of free fatty acids pools (Cho and Cronan, 1995). The RQ1 derivative strain harboring a unique copy of *´tesA* integrated into the φ80 *attB* sequence was named RQ2 (Supplementary information, Table S1). Bioconversion experiments carried out with this strain produced 97.9 ± 5.2 mg L^{-1} of MBE, a 68.8% increase in the product yield compared with the K207-3 derivative (Supplementary Fig. 4). Attempts to increase FFA concentrations, and therefore MBE production, were carried out by expressing *´tesA* using different strategies. The highest concentration of FFA was obtained when *´tesA* was cloned in a high copy number vector under the control of the T7 promoter. However, under these conditions, the final OD and the MBE productivity were lower than the one obtained by the recombinant RQ2 strain where *´tesA* was integrated as a unique copy in the chromosome.

3.4. *De novo* biosynthesis of multi-methyl-branched wax esters

A central aim in microbial production of advanced chemicals is the generation of consolidated bioprocesses in which product synthesis occurs directly through conversion of a simple carbon source (Keasling, 2010; Steen et al., 2010). In this context, metabolic engineering offers the possibility to genetically compile several complex biosynthetic pathways into a single cell,

simplifying process and raw material requirements (Steen et al., 2010). Therefore, to circumvent the need of an exogenous alcohol supply, we engineered into our MBE producer strain RQ2 three different pathways for the *de novo* synthesis of fatty alcohol acceptor molecules: 1) FAR from *Marinobacter aquaeolei VT8*, which catalyzes the two-step reduction of a fatty acyl-CoA to a fatty alcohol (Hofvander et al., 2011; Willis et al., 2011); 2) CAR from *M. tuberculosis*, an orthologue of *Mycobacterium marinum* carboxylic acid reductase (Akhtar et al., 2013); and 3) Acr1 from *Acinetobacter sp.* strain ADP1, reported as a medium-chain acyl-CoA reductase (Reiser and Somerville, 1997). Given that CAR and Acr1 generate a fatty aldehyde as product, each of these enzymes was overexpressed together with Ahr, a previously described *E. coli* aldehyde reductase (Akhtar et al., 2013). Successful *de novo* production of multi-methyl-branched wax esters (MBW) was achieved by expression of either the FAR or CAR–Ahr pathways in our MBE producer strains, RQ1 and RQ2 (Fig. 4A). The identity of the most abundant species present in the new MBW synthesized by RQ2/pMB07/pMB15 cultures was analyzed by MS/MS (Fig. 4B and C), where transitions of *m*/*z* 585.554, 613.586, 641.616 and 669.649 to *m*/*z* 433.439 (Supplementary Fig. 5) indicate that chemical formulas $C_{38}H_{74}O_2$, $C_{40}H_{78}O_2$, $C_{42}H_{82}O_2$ and $C_{44}H_{86}O_2$ correspond to (Z)-octyl 2,4,6,8-tetramethylhexacos-19-enoate, (Z)-decyl 2,4,6,8-tetramethylhexacos-19-enoate, (Z)-dodecyl 2,4,6,8-tetramethylhexacos-19-enoate and (Z)-tetradecyl 2,4,6,8-tetramethylhexacos-19-enoate, respectively (present in a relative composition of 26.4%, 35.6%, 10.8% and 27.2%, respectively). These results demonstrate that by expressing different fatty alcohol biosynthetic pathways, the MBE producer strain is able to synthesize *de novo* a structurally diverse set of new MBW directly from a simple carbon source. In contrast, expression of Acr1–Ahr did not lead to detectable MBW synthesis, although the long-chain alcohol intermediate product could be detected at low levels in the RQ2/pMB07/pMB14 strain (Supplementary Fig. 6).

The MBW titers for batch cultures of *E. coli* RQ2 expressing the MA system and CAR–Ahr were 37.1 ± 1.8 mg/L of MBW, while the RQ2 strain expressing the MA system and FAR exhibited a titer of 14.3 ± 2.1 mg/L for *de novo* MBW production (Fig. 4A, lanes 4 and 6, respectively). The fact that the CAR–Ahr pathway uses free fatty acid as preferred substrate, and that this substrate is more readily available in the ΔTesA background of RQ2, could be responsible for the higher yields observed when comparing RQ2/pMB07/pMB15 with RQ1/pMB07/pMB15 (Fig. 4A, lanes 4 and 3, respectively). Thus, considering the exciting achievements reported in the optimization of free fatty acid production in *E. coli* (Janssen and Steinbuchel, 2014; Lin et al., 2013; Lu et al., 2008; Torella et al., 2013), future engineering of the MBW-producing strain will certainly enable further yield improvements.

4. Discussion and conclusions

Fatty acid metabolism has received significant attention as a route for producing high-energy density liquid fuels and high-value oleochemicals from renewable feedstocks. For instance, *E. coli* has been extensively engineered to produce many attractive fatty acid-derived oleochemicals, including fatty acid ethyl esters, bioplastics, oils, fatty alcohols, fatty aldehydes, alkanes and alkenes (Kalscheuer et al., 2006a; Liu and Khosla, 2010; Suriyamongkol et al., 2007). However, a major constraint on the chemical and structural diversity of these compounds occurs because they all derive from native lipid metabolism. In this work we sought to broaden the endogenous fatty acid diversity of *E. coli* and for that we took advantage of an iterative PKS system constituted by FadD28 and Mas enzymes to develop a bacterial platform with the capacity to synthesize novel fatty acids containing multiple methyl branches on their carboxy termini. Furthermore, by introducing the acyl–ACP transferase PapA5 together with FadD28 and Mas, we retuned the *M. tuberculosis* MA system towards the synthesis of a wide variety of structurally diverse MBE.

The limited diversity of natural esters has restricted the industrial applications of these compounds. Such limitation, however, has been overcome by the use of synthetic and semi-synthetic esters, which allows the introduction of a variety of chemical modifications, although at the expense of multi-step chemical synthesis. In this regard, the biolubricant industry has developed synthetic and semi-synthetic esters containing branched-chain fatty acids adding a new dimension to the physicochemical properties of the resulting compounds, demonstrating that carbon chain length, location and number of branches all impact on the performance of the final compound (Ngo et al., 2011). Branched esters derived from straight-chain FA and isopropanol or isobutanol also had improved properties as biodiesel, as indicated by their lower cloud and pour point values, confirming the relevance of the methyl-branches, either in the FA or in the alcohol moiety, for the properties and performance of the fluids (Knothe, 2005). Thus, the MBE producing platform developed in this work has expanded the diversity of natural esters, and opened up the possibility of producing new compounds with potentially improved physicochemical properties and promising industrial applications.

Previous reports demonstrated the ability of *E. coli* to produce esters derived from straight-chain fatty acids, by diverting the fatty acid metabolism to fatty acyl-CoA, a general substrate for the production of esters (Kalscheuer et al., 2006a; Kalscheuer et al., 2006b). The key enzyme used in all these approaches was the wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) AtfA from *Acinetobacter baylyi* strain ADP1. This enzyme has a relaxed substrate specificity since it can synthesize wax esters and triacylglycerol utilizing different chain-length acyl–CoAs as acyl donors and different chain-length fatty alcohols or diacylglycerol as acyl acceptors, respectively (Waltermann et al., 2007). Our system, which uses a completely novel metabolic pathway for the biosynthesis of MBE has also benefited from the relaxed substrate specificity of the acyltransferase PapA5 towards the alcohol moiety (Onwueme et al., 2004). As a result of this, the MA system of *M. tuberculosis* was shown to efficiently utilize linear and branched alcohols as well as different chain-length FA to produce a broad range of unnatural MBE.

By engineering the ΔTesA and the FAR and CAR-Ahr reductases into the recombinant strain containing the MA system, *de novo* synthesis of MBE was obtained with only the addition of propionate to the medium. ΔTesA releases the acyl moieties from the acyl-ACP pool to yield free FA, which serve as substrates for FadD28 to synthesize the acyl-AMP derivatives and load the Mas for MBFA synthesis, as well as for the CAR-Ahr reductases to produce the alcohol moiety. High levels of free FA accumulate with time after IPTG induction, most probably as a consequence of the high levels of ΔTesA expression or of a limited use of the FA by the MA system or by the FA reductases. Therefore, a rational approach towards understanding the key bottleneck(s) of the system will be required in order to improve the yields of these compounds.

The use of different thioesterases (Lennen et al., 2010; Voelker et al., 1992; Zheng et al., 2012) or of *E. coli* strains containing specific mutants in the *fabF* gene (Torella et al., 2013) allow the endogenous production of different chain-length FA. On the other hand different pathways have been engineered into *E. coli* to produce short-, long- or branched-chain alcohols (Akhtar et al., 2013; Zhang et al., 2008). Thus, taking into account the relaxed substrate specificity shown by the components of the MA system it may be highly plausible to customize our products by 'plugging-in'

into our production platform the correct combination of the MBFA and alcohol biosynthetic genes.

Finally, modular PKSs have been extensively engineered via catalytic domain swapping to produce a vast array of novel molecules (Staunton and Weissman, 2001). Iterative PKSs, such as Mas, are also organized into well-defined catalytic domains that could be replaced by comparable domains from other PKS in order to attempt to change the starter precursor, the structure of the extension unit or even the chain length of the molecule, diversifying even further the structural features of the produced lipids. In summary, the flexibility of our system towards using a wide range of substrates and the reliable potential to modify the Mas enzyme via the redefinition of PKS catalytic activity lay the foundations for the biosynthesis of structurally diverse lipid compounds and offers the unprecedented possibility to design microbial lipids in a customizable manner.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.05.002.

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