LETTERS

Microbial production of fatty-acid-derived fuels and chemicals from plant biomass

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Increasing energy costs and environmental concerns have emphasized the need to produce sustainable renewable fuels and chemicals[1]. Major efforts to this end are focused on the microbial production of high-energy fuels by cost-effective 'consolidated bioprocesses'[2]. Fatty acids are composed of long alkyl chains and represent nature's 'petroleum', being a primary metabolite used by cells for both chemical and energy storage functions. These energy-rich molecules are today isolated from plant and animal oils for a diverse set of products ranging from fuels to oleochemicals. A more scalable, controllable and economic route to this important class of chemicals would be through the microbial conversion of renewable feedstocks, such as biomass-derived carbohydrates. Here we demonstrate the engineering of *Escherichia coli* to produce structurally tailored fatty esters (biodiesel), fatty alcohols, and waxes directly from simple sugars. Furthermore, we show engineering of the biodiesel-producing cells to express hemicellulases, a step towards producing these compounds directly from hemicellulose, a major component of plant-derived biomass.

Fuels and chemicals have been produced from the fatty acids of plant and animal oils for more than a century. Today these oils are the raw materials for a growing diversity of products including biodiesel, 'renewable diesel', surfactants, solvents and lubricants. The increased demand and limited supply of these oils has resulted in competition with food, higher prices, questionable land-use practices and environmental concerns associated with their production[3]. A sustainable alternative is to produce these products directly from abundant and cost-effective renewable resources by fermentation. The well-studied industrial microorganism *E. coli* is ideally suited for this purpose. *E. coli* is approximately 9.7% lipid, produces fatty acid metabolites at the commercial productivity of $0.2\,g\,l^{-1}\,h^{-1}$ per gram of cell mass just to grow, can achieve product-dependent mass yields of 30–35%[4], and is exceptionally amenable to genetic manipulation. Combining this natural fatty acid synthetic ability with new biochemical reactions realized through synthetic biology has provided a means to divert fatty acid metabolism directly towards fuel and chemical products of interest (Fig. 1).

The product of microbial fatty acid biosynthesis is fatty acyl-ACP (acyl carrier protein), which can then be directed to cellular components such as structural or storage lipids[5,6]. The accumulation of fatty acyl-ACP feedback inhibits fatty acid biosynthesis. The expression of a cytoplasmic thioesterase was previously shown to result in hydrolysis of these acyl-ACPs, deregulation of fatty acid biosynthesis, and overproduction and secretion of significant levels of free fatty acids[6,7]. Here, by cytosolic expression of a native *E. coli* thioesterase ('*tesA*—a 'leaderless' version of TesA that is targeted to the cytosol), normally localized to the periplasm, we demonstrate free fatty acid production of $\sim 0.32\,g\,l^{-1}$, similar to previous findings (Fig. 2)[6,8]. 'TesA exhibits a preference for C14 fatty acyl-ACPs, although a range of free fatty acids (C8–C18) is observed when 'TesA is produced (Supplementary Fig. 1). The length of the fatty acid chain produced can be controlled by expressing alternative thioesterases from plants[9]. To improve free fatty acid production further, we eliminated the first two competing enzymes associated with β-oxidation, FadD and FadE, resulting in an extra three- to fourfold increase in titre to $\sim 1.2\,g\,l^{-1}$. 'TesA-Δ*fadE* affected a 6% yield of fatty acids from 2% glucose in shake flasks, 14% of the theoretical limit.

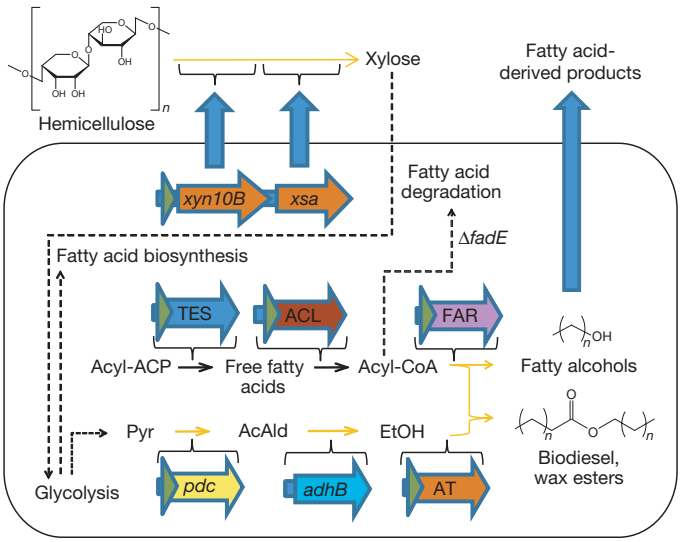

Figure 1 | Engineered pathways for production of fatty acid-derived molecules from hemicelluloses or glucose and depiction of the synthetic operons used in this study. Flux through the *E. coli* fatty acid pathway (black lines) was increased to improve production of free fatty acids and acyl-CoAs by eliminating β-oxidation (knockouts are *fadE*), by overexpressing thioesterases (TES) and acyl-CoA ligases (ACL). Various products were produced from non-native pathways (orange lines) including biodiesel, alcohols and wax esters. Alcohols were produced directly from fatty acyl-CoAs by overexpressing fatty acyl-CoA reductases (FAR); the esters were produced by expressing an acyltransferase (AT) in conjunction with an alcohol-forming pathway; biodiesel was produced by introduction of an ethanol pathway (*pdc* and *adhB*) and wax esters were produced from the fatty alcohol pathway (FAR). Finally, expressing and secreting xylanases (*xyn10B* and *xsa*) allowed for the utilization of hemicellulose. Overexpressed genes or operons are indicated; green triangles represent the lacUV5 promoter. AcAld, acetaldehyde; EtOH, ethanol; pyr, pyruvate.

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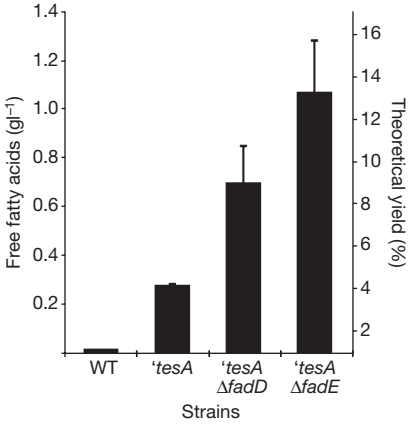

Figure 2 | Total free fatty acid production and respective theoretical yield by engineered *E. coli* strains. Overexpressed and knocked out genes are indicated. WT, wild-type DH1. Values and error bars represent the mean and s.d. of triplicate experiments.

Although free fatty acids are valuable, directly compatible renewable fuels and chemicals are in greater demand. Microbial production of biofuels has focused on gasoline supplements[10,11]; however, diesel is globally in greatest demand, and has a growth rate three times that of the gasoline market[12]. Biodiesel, the primary renewable alternative to diesel, is consumed at greater than 2 billion gallons per year, and is composed of fatty acid methyl and ethyl esters (FAMEs and FAEEs, respectively) derived from the chemical transesterification of plant and animal oils[3]. Previously, *E. coli* was engineered to produce FAEEs by esterifying exogenously added fatty acids with endogenously produced ethanol[13]; however, fatty acids are not a commercially viable feedstock. Thus, we engineered *E. coli* to produce FAEEs directly from glucose and ethanol. Expression of the gene encoding a wax-ester synthase (*atfA*) with expression of *fadD* and '*tesA* and the addition of ethanol (to 2%) resulted in the production of approximately 400 mg l^{-1} of FAEEs in 48 h, with the FAEE composition ranging from C12 to C18. Overexpression of *fadD*, the first step for fatty acid degradation, and deletion of *fadE*, the second step in fatty acid degradation, markedly increases FAEE production as well as other fatty acid products, as described later (Fig. 3a).

In addition to esters, there is a large market for fatty alcohols, aldehydes and wax esters, which are used predominantly in soaps, detergents, cosmetic additives, pheromones and flavouring compounds, and potentially as biofuels; their value was approximately $1,500 per ton (2004 ICIS pricing), and they represent a ~$3 billion market[14]. Fatty alcohols are produced by hydrogenation of plant oil-derived FAMEs or by synthesis from petrochemical precursors. Previous identification and expression of fatty alcohol-forming fatty acyl-CoA reductases, such as that encoded by *acr1* from *Acinetobacter calcoaceticus* BD413, have been described[15–17]. Expressing *acr1* in place of *atfA* (used in the FAEE-producing strains) resulted in the production of medium chain fatty alcohols up to ~60 mg l^{-1} (Fig. 3b). Although FadD improved the production of all products, the lower level of alcohol as compared to FAEEs (400 mg l^{-1}) suggests that Acr1 may be limiting in this pathway.

Fatty acid chain length and saturation directly affect fuel and chemical properties, such as cetane number and melting point. A previous study demonstrated that the poor performance of plant-derived biodiesel can be improved by tailoring its fatty ester composition[18]. To engineer FAEE and fatty alcohol chain-length composition, '*tesA* in the FAEE and fatty alcohol strains was replaced with plant genes encoding thioesterases previously shown to prefer different chain-length fatty acyl-ACPs[9]. This single genetic manipulation directly affected the composition of the fatty acids shunted into these pathways and resulted in controlled FAEE and fatty alcohol chain-length distributions (Fig. 3c, d). This genetic tool provides a means to

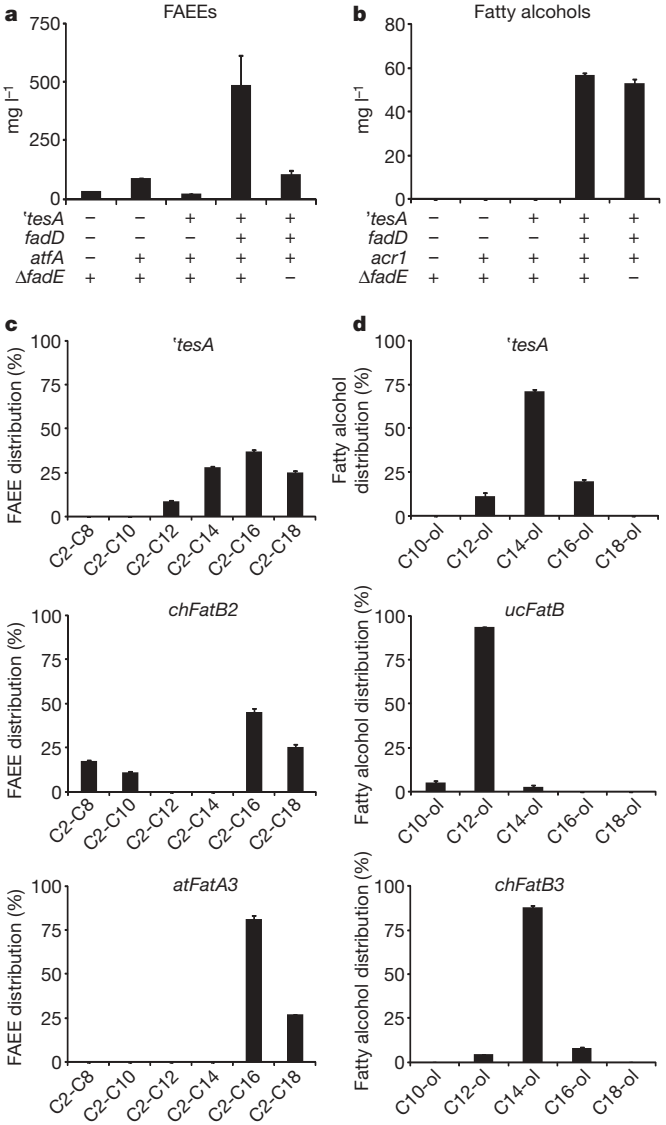

Figure 3 | Engineered production of FAEEs and fatty alcohols with controlled chain length. **a**, **b**, Production of FAEEs (**a**) or fatty alcohols (**b**) by overexpressing '*tesA*, *fadD*, and *atfA* or *acr1* in C41(DE3) Δ*fadE*. **c**, **d**, The chain-length distribution of FAEEs fatty alcohols was varied by expressing thioesterases with different substrate specificities. Shown are the percentage composition of each chain-length FAEE or fatty alcohol resulting from the expression of thioesterase genes '*tesA*, *chFatB2*, *atFatA3*, *ucFatB* and *chFatB3*, together with *fadD* and an ester synthase (*atfA*) or acyl-CoA reductase (*acr1*) in C41(DE3) Δ*fadE*. Values and error bars represent the mean and s.d. of triplicate experiments.

easily tailor the composition, and hence the performance, of the fuel and chemical product being produced.

A benefit of microbial catalysis is the ability to genetically compile several complex, biosynthetic pathways into a single cell, simplifying process and raw material requirements, and decreasing costs. To obviate the need to feed ethanol to produce FAEEs, we engineered our FAEE-producing strain to co-produce ethanol by expressing the *Zymomonas mobilis* genes *pdc* and *adhB*, which encode pyruvate decarboxylase and alcohol dehydrogenase, respectively. *E. coli* expressing *pdc* and *adhB* alone produced ~5 g l^{-1} ethanol after 24 h, as reported previously[19]. The addition of the minimal FAEE biosynthetic pathway ('*tesA* and *atfA*) into the ethanol-producing strain resulted in lowering production of FAEEs to 37 mg l^{-1} (strain LAAP) (Fig. 4) as expected, because the native acyl-CoA ligase (*fadD*) activity was limiting. Overexpression of *FAA2*, an acyl-CoA ligase from *Saccharomyces cerevisiae*, resulted in an approximately 2.5-fold

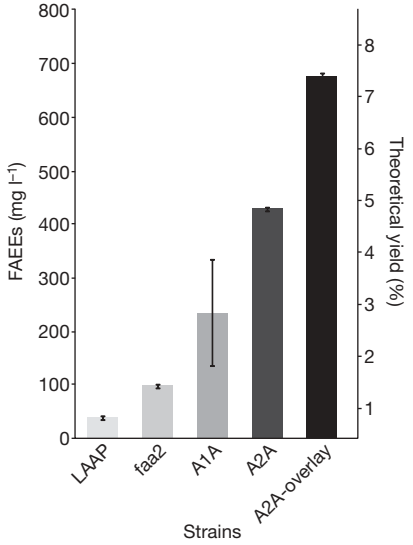

Figure 4 | Towards a single cell catalyst: biodiesel (FAEE) production by various strains without exogenous ethanol supplementation. Strain HE-LAAP: DH1, Δ*fadE*, '*tesA*, *atfA*, *pdc*, *adhB*; strain faa2: HE-LAAP *FAA2*; strain A1A: HE-LAAP *fadD*; strain A2A: A1A containing an extra copy of *atfA*; strain A2A-overlay: A2A grown with a dodecane overlay. Values and error bars represent the mean and s.d. of triplicate experiments.

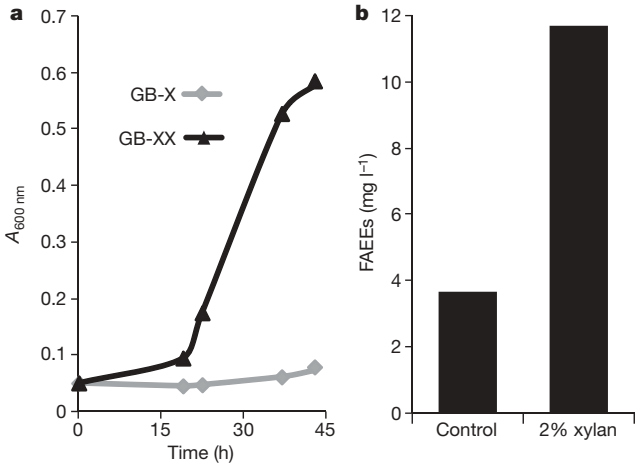

Figure 5 | Consolidated bioprocessing: growth and FAEE production by xylan-using strains. a, Growth of xylan-using strains on 0.2% xylan-containing minimal salts medium. Grey diamonds: GB-X, *E. coli* BL21 *xynB*; black triangles: GB-XX, GB-X expressing *xsa*. **b**, FAEE production from xylan. Control strain HE-GX (*E. coli* DH1 Δ*fadE* expressing *xynB*, *xsa*, '*tesA*, *atfA*, *pdc* and *adhB*) was grown in 0.2% glucose and no xylan; 2% xylan is strain HE-GX grown in 0.2% glucose and 2% xylan.

increase in FAEE production to 96 mg l^{-1} (strain faa2). Another 2.5-fold increase to 233 mg l^{-1} was achieved by overexpression of an *E. coli*-derived *fadD* (strain A1A). Expressing an extra copy of *atfA* resulted in the production of 427 mg l^{-1} FAEE (strain A2A). Finally, by overlaying an organic phase of dodecane to potentially prevent FAEE evaporation, the engineered *E. coli* produced FAEEs at 674 mg l^{-1}, which is 9.4% of the theoretical yield. FAEEs were not growth inhibitory to *E. coli* up to 100 g l^{-1} (Supplementary Fig. 2). By combining the pathways for ethanol and FAEE biosynthesis, FAEE was produced efficiently from glucose as the sole carbon source. In a similar fashion, an *E. coli* strain (strain KS12) engineered to express '*tesA*, *fadD*, *mFar1* and *atfA* produced wax esters including tetradecanoate hexadecyl ester (C14:0-C16), hexadecanoate hexadecyl ester (C16:0-C16), 9-hexadecenoate hexadecyl ester (C16:1-C16), and 9-octadecenoate octadecyl ester (C18:1-C18) from glucose as the sole carbon source (Supplementary Fig. 3).

The production of advanced fuels and chemicals such as FAEEs and fatty alcohols from sugar has a promising advantage over first generation products, such as corn ethanol and plant oil-derived biodiesel. However, a primary goal in renewable energy is the production of these compounds directly from cellulosic plant biomass. At present, biochemical processing of cellulosic biomass requires costly enzymes for sugar liberation. Consolidated bioprocessing, in which the biofuel-producing organism also produces glycosyl hydrolases, eliminates the need to add these enzymes and can improve economics[2]. To further simplify our FAEE process, we engineered into our FAEE-producing *E. coli* the genes encoding an endoxylanase catalytic domain (Xyn10B) from *Clostridium stercorarium*[20] and a xylanase (Xsa) from *Bacteroides ovatus*[21]. To hydrolyse the hemicellulose in the growth medium into xylose, which could then be imported and catabolized using the native *E. coli* metabolism, the hemicellulases were fused on their amino termini to the *E. coli* protein OsmY[22]. Expression of OsmY–xylanase fusions enabled *E. coli* growth on hemicellulose without the need for exogenously added enzymes (Fig. 5a). The addition of the biodiesel genes to the xylan-degrading strain resulted in the production of 3.5 mg l^{-1} FAEE from 0.2% glucose alone (Fig. 5b). Addition of 2% xylan, the hemicellulose substrate, resulted in a threefold increase in FAEE production to 11.6 mg l^{-1}, compared to glucose alone, and demonstrated a step towards microbial biodiesel production from biomass in a consolidated process. Future engineering of the FAEE-producing *E. coli* to secrete various cellulases would enable the production of FAEEs directly from both cellulose and hemicellulose without the addition of expensive enzymes, further reducing the cost of cellulosic biodiesel.

In summary, this work demonstrates the practical use of microbial fatty acid metabolism. By combining the energetically favourable thioesterase-catalysed hydrolysis of fatty acyl-ACP, to overproduce fatty acids and deregulate fatty acid biosynthesis, with fatty acyl-CoA-synthase-catalysed reactivation of the fatty acid carboxylate group, fatty acid metabolism is efficiently diverted to fatty acyl-CoA, an important general substrate for the production of esters, alcohols and other products. This engineering strategy supports yields of these products within an order of magnitude of that required for commercial production. Indeed, strain and process improvements of this magnitude have been achieved during scale-up of other bioprocesses[23], and significant steps to this end have already been achieved for this process. The further production of this biodiesel from hemicellulose demonstrates a necessary and promising achievement towards realizing a consolidated bioprocess. We believe that these data can significantly contribute to the ultimate goal of producing scalable and cost-effective advanced biofuels and renewable chemicals.

METHODS SUMMARY

Strains and plasmids. *E. coli* strains DH1 and C41 (DE3) were used as the wild-type strain for all studies, except where indicated. Knockouts of *fadD* and *fadE* in DH1 were performed as previously described[24]. A list of strains and plasmids constructed is given in Supplementary Table 1. Construction of plasmids was carried out with standard molecular biology methods and is described in detail in Methods.

Growth and production parameters. Strains were cultivated in baffled flasks at 37 °C in M9 minimal medium supplemented with trace elements and the appropriate antibiotics (50 mg l^{-1} ampicillin, 20 mg l^{-1} chloramphenicol, 5 mg l^{-1} tetracycline, 100 mg l^{-1} carbenicillin, 100 mg l^{-1} spectinomycin and 50 mg l^{-1} kanamycin). Pathway induction was achieved by the addition of 1 mM isopropylthiogalactoside (IPTG) at an absorbance of 0.5–1 measured at a wavelength of 600 nm ($A_{600\,nm}$). For the thioesterase expression studies, 2% ethanol was added and the post-induction temperature was 25 °C for production of FAEEs. Xylan media was prepared by addition of 2% beechwood xylan and 0.2% glucose to M9 minimal media. Metabolites were identified and quantified by gas chromatography–mass spectrometry, further described in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions E.J.S., Y.K., G.B., Z.H., A.S., A.M., S.B.d.C. and J.D.K. conceived of the experiments. E.J.S. and Y.K. constructed the strains and metabolic pathways for fatty-acid-derived products and performed the production experiments. LS9 engineered and evaluated FAEE and fatty alcohol producing strains for thioesterase evaluations. G.B. conceived, constructed and performed the xylan-metabolizing pathway growth experiments. E.J.S. and Y.K. constructed the xylan-metabolizing, fatty acid production strain and performed the production experiments. E.J.S., Y.K., A.S., S.B.d.C. and J.D.K. drafted the manuscript. All authors approved the final manuscript.

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METHODS

Reagents. All chemicals were purchased from Sigma-Aldrich and include FAME standards, FAEE standards, fatty aldehyde standards, fatty alcohol standards, and beechwood xylan.

Plasmid and strain construction. For the study in which various thioesterases were used, *E. coli* C41 (DE3) with a deletion of the acyl-CoA dehydrogenase (Δ*fadE)* was used as an expression strain. The *fadE* deletion strain of *E. coli* C41 (DE3) was constructed using the temperature-sensitive plasmid pKO3 (a gift from G. M. Church). DNA sequences down- and upstream of *fadE* were amplified with the downstream primers, LF_NotI (5′-CAACCAGCGGCCGCGCG ACGAAGCTGCCGCTTC-3′) and LR (5′-CCTACAAGTAAGGGGCTTTTCG TTATGAATAACGGAGCCGAAAGGCTCC-3′), and the upstream primers, RF1 (5′-TTTCGGCTCCGTTATTCATAACGAAAAGCCCCTTACTTGTAGGA GG-3′) and RR_Bam (5′-CCAGGATCCAGGTCGGATGCGGCGTGAAC-3′), using genomic DNA from C41 (DE3) as the template. (Underlined bases denote restriction sites).The two PCR products were mixed and joined together by PCR amplification with primers, LF_NotI and RR_Bam described earlier. This PCR product (~1.8 kb) was digested with NotI and BamHI and ligated between the NotI and BamHI sites of pKO3. The pKO3 derivative was used to construct the *fadE* in-frame deletion as described[25].

E. coli DH10B and DH5α were used for bacterial transformation and plasmid amplification in the construction of the expression plasmids used in this study. Native *E. coli* genes were cloned from DH1. Mouse *Far1* (*Mus musculus*, GenBank accession BC007178)[15] was synthesized and codon-optimized for *E. coli* expression (Epoch biolabs). *atfA* (*Acinetobacter* sp. strain ADP1) was synthesized (Epoch biolabs). *pdc* and *adhB* were cloned from *Z. mobilis* genomic DNA (ATCC 31821). *FAA2* was cloned from *Saccharomyces cerevisiae* (BY4742) genomic DNA. DNA encoding the catalytic domain of *xynB* from *C. stercorarium* NCIMB 11754 was synthesized and codon-optimized by DNA 2.0. The *E. coli osmY* gene was synthesized with the native sequence by DNA 2.0. The full-length *xsa* gene was codon-optimized and synthesized in-house[26]. A BglII site followed by a strong ribosome-binding site was appended to the *osmY* gene, which ended with a glycine–serine linker, followed by a BamHI site. *xynB* and *xsa* were cloned with a BglII site on their 5′ end and BamHI-TAA-XhoI sites on their 3′ ends. Gene fusions were constructed by digestion of the OsmY-carrying plasmid with BamHI/XhoI and ligation with the BglII/XhoI-cut inserts (*xynB* and *xsa*). The plasmid bearing the OsmY–Xyn10B fusion was subseqently re-cut with BamHI/XhoI, and the OsmY–Xsa insert cut with BglII/XhoI was ligated. To generate biodiesel from xylan, the OsmY–XynB, OsmY–Xsa bicistronic gene was cut with EcoRI/BamHI and ligated in front of pKS104 cut with EcoRI/BglII. Before this the BglII site within *fadD* was removed with Quickchange mutagenesis. Other plasmids were constructed using the 'sequence and ligation independent cloning' (SLIC) method[27] or standard methods[28]. All genes were overexpressed under the control of the IPTG-inducible lacUV5 or trc promoters as indicated; LS9 vectors use the T7 promoters (pDuet vectors).

For the thioesterase studies, pDuet vectors were used for plasmid-based-expression of wax synthase (AtfA), fatty acyl-CoA reductase (Acr1), acyl-CoA synthetase (FadD) and the thioesterases '*tesA* and FatA. All other thioesterases were expressed from pMAL-c2x (New England Biolabs). The wax synthase gene (*aftA*) from *Acinetobacter baylyi* ADP1 was amplified with primer adp1ws_NdeI (5′-TCATATGGCGCCCATTACATCCG-3′) and adp1ws_AvrII (5′-TCCTAGG AGGGCTAATTTAGCCCTTTAGTT-3′). After amplification, the PCR product was digested with NdeI and AvrII (underlined sites) and ligated with pCOLADuet-1 cut with NdeI and AvrII to produce pLS9-atfA. The *fadD* gene of *E. coli* was amplified with forward primer (5′-CCATGGTGAAGAAGGTTTG GCTTAA-3′) and reverse primer (5′-AAGCTTTCAGGCTTTATTGTCCAC-3′), using genomic DNA of *E. coli* strain XL-Blue (Stratagene) as a template. The PCR product was digested with NcoI and HindIII and ligated with pCDFDuet-1 linearized with NcoI and HindIII. The resulting plasmid, pLS9-fadD, was digested with NotI and AvrII, and the fragment was ligated with the NotI/AvrII fragment from pLS9-atfA to generate plasmid pLS9-atfA-fadD. The NcoI and HindIII fragment from pLS9-fadD was also cloned into NcoI- and HindIII-digested pLS9-acr1 generating plasmid pLS9-acr1-fadD.

Metabolite analysis. Free fatty acids were extracted from 5-ml cultures by addition of 500 μl HCl and 5 ml of ethyl acetate, spiked with 10 mg l^{-1} of methyl nonadecanoate as an internal standard. The culture tubes were vortexed for 15 s followed by shaking at 200 r.p.m. for 20 min. The organic layer was separated and a second extraction was performed by the addition of another 5 ml ethyl acetate to the culture tubes. The free fatty acids were then converted to methyl esters by the addition of 200 μl TMS-diazomethane, 10 μl HCl and 90 μl methanol[29]. This reaction was allowed to proceed for 2 h and then was applied to a Thermo Trace Ultra gas chromatograph (GC) equipped with a Triplus AS autosampler and a TR-WAXMS column (Thermo Scientific). The GC program was as follows: initial temperature of 40 °C for 1.2 min, ramped to 220 °C at 30 °C per min and held for 3 min. Final quantification analysis was performed with Xcalibur software.

FAEEs, fatty alcohols and wax esters were extracted from cultures by the addition of 10% (v/v) ethyl acetate, spiked with 10 mg l^{-1} methyl nonadecanoate, followed by shaking at 200 r.p.m. for 20 min. Analysis of FAEEs was performed on an HP 6890 Series GC with an Agilent 5973 Network MSD equipped with a DB5 column (Thermo). The GC program was the same as for quantifying FAMES. Fatty alcohols and aldehydes were separated with a TR-Wax column (Agilent). The GC program was as follows: initial temperature of 70 °C, held for 1 min, ramped to 240 °C at 25 °C per min and held for 3 min. *N*, *O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization of fatty alcohols aided in their identification and separation. Where described, a 10% (v/v) dodecane overlay was added to cultures at the time of induction. Twenty microlitres of dodecane was sampled and diluted 25 times in 480 μl ethyl acetate.

Ethanol was measured by sampling 1 ml of culture, centrifuging at 18,000*g* for 5 min, and applying the supernatant to an Agilent 1100 series HPLC equipped with an Aminex HPX-87H ion exchange column (Biorad). The flow rate of the solvent, 4 mM H_2SO_4, was 0.6 ml min^{-1} and the column was maintained at 50 °C. All metabolites were detected with an Agilent 1200 series DAD and RID detectors.

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