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Matching protein interfaces for improved medium-chain fatty acid production

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Abstract

Medium-chain fatty acids (MCFAs) are key intermediates in the synthesis of medium-chain chemicals including α -olefins and dicarboxylic acids. In bacteria, microbial production of MCFAs is limited by the activity and product profile of acyl-ACP thioesterases. Here, we engineer a heterologous bacterial acyl-ACP thioesterase for improved MCFA production in *Escherichia coli*. Electrostatically matching the interface between the heterologous medium-chain *Acinetobacter baylyi* acyl-ACP thioesterase (AbTE) and the endogenous *E. coli* fatty acid ACP (*E. coli* AcpP) by replacing small nonpolar amino acids on the AbTE surface for positively charged ones increased secreted MCFA titers more than three-fold. Nuclear magnetic resonance titration of *E. coli* ¹⁵N-octanoyl-AcpP with a single AbTE point mutant and the best double mutant showed a progressive and significant increase in the number of interactions when compared to AbTE wildtype. The best AbTE mutant produced 131 mg/L of MCFAs, with MCFAs being 80% of all secreted fatty acid chain lengths. This work demonstrates that engineering the interface of heterologous enzymes to better couple with endogenous host proteins is a useful strategy to increase the titers of microbially-produced chemicals.

Introduction

Medium-chain fatty acids (MCFAs, C8–C12) are useful as antimicrobials and emulsifying agents, and their derivatization results in an array of chemicals including alkenes, α -olefins, esters, ω -hydroxy-carboxylic acids, α,ω -dicarboxylic acids, alcohols, and ketones¹. Microbially, MCFAs are generated via hydrolysis of medium-chain fatty acyl-ACPs obtained from Type II fatty acid synthase (FAS)², or medium-chain fatty acyl CoAs produced by Type I FAS³ or the reverse β -oxidation pathway⁴. Hydrolysis of fatty acyl intermediates is performed by medium-chain acyl-ACP thioesterases (TEs) from bacteria, higher plants (FatBs), and mammals, or medium-chain acyl-CoA TEs from bacteria. While

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Supplementary information.

Materials, homology model and docking, AbTE mutant generation, SDS-PAGE gel of abTE:WT and AbTE variants.

bacterial acyl-ACP TEs tend to hydrolyze C8–C16 acyl-ACPs⁵, such as *Acinetobacter baylyi* TE⁶, FatBs have a narrower substrate profile, such as *Cuphea palustris* TE (C8:0 acyl-ACPs)⁷, and *Umbellularia californica* TE (C12:0 acyl-ACPs)⁸. The rat acyl-ACP TE has been shown to produce primarily C6–C8 fatty acids⁹. On the acyl-CoA front, *E. coli* ydiI hydrolyzes C6–C10 acyl-CoAs⁵.

Microbes do not naturally synthesize MCFAs extensively, and microbial MCFA production is hindered by the inefficient expression of heterologous plant TEs and the broad substrate profile of bacterial TEs. Long chain acyl-ACP TEs have been engineered for improved MCFA production via active site mutagenesis and computationally-guided approaches. For example, *E. coli* ‘TesA:L109P (signal peptide removed) preferentially hydrolyzes 12:0 and C14:0 acyl-ACPs, unlike the wild type’s preference for C16:0 acyl-ACPs¹⁰. Similarly, *Pseudomonas aeruginosa* TesA:D17S/L462R has improved activity on C12:0 acyl-CoAs¹¹. More recently, using an iterative protein redesign and optimization algorithm ‘TesA sequences that preferentially bind C8–C12 substrates were selected leading to ‘TesA:S122K/Y145K/L146K and ‘TesA:M141L/Y145K/L146K, which had a 1.8-fold improvement in C12 mole fraction and a 10-fold improvement in C8 mole fraction over wild type, respectively¹².

Using a natural medium-chain acyl-ACP TE as the engineering starting point, however, has the potential to result in a TE variant with an almost exclusive MCFA product profile, potentially leading to higher MCFA yields. Engineering medium-chain acyl-ACP TEs has a unique set of challenges. First, medium-chain acyl-ACP-TEs are heterologous to *E. coli* and may have problems interfacing with the host machinery. Second, active site engineering of medium-chain acyl-ACP TEs may not prove to be as fruitful, as mutations may need to be much more subtle, potentially in the second shell, and overall more difficult to identify. We hypothesized that engineering the interface of a heterologous medium chain TE to better complement the surface of *E. coli* fatty acid biosynthesis ACP (*E. coli* AcpP) may improve MCFA titers (Fig. 1). In Type II FAS, ACP is bound to the fatty acyl-chain and interacts with all the proteins in fatty acid biosynthesis. The fatty acyl chain is sequestered in the ACP hydrophobic core, and protein-protein interactions between the ACP and partner enzymes guide the maturing acyl chain from the ACP core and into each partner enzyme’s active site¹³. It is the selective binding of ACP to its enzyme partners, including the *de novo* FAS subunits, that enables efficient fatty acid biosynthesis¹⁴. For instance, crosslinking studies show that *E. coli* Type II FAS ACP binds more selectively to its cognate *E. coli* Type II FAS ketoacid synthase (KS) than *Streptomyces maritimus* Type II polyketide synthase KS¹⁴.

Here, we engineer the medium-chain acyl-ACP TE from *Acinetobacter baylyi* (AbTE) to better interface with *E. coli* AcpP to improve MCFA production. First, we docked *E. coli* AcpP with the endogenous *E. coli* TE ‘TesA to identify potential contact residues involved in stabilizing the AcpP-‘TesA interaction. Next, we mutated the equivalent positions in AbTE to the amino acids found in *E. coli* ‘TesA and measured its fatty acid profile. We find that mutation of just two residues on the AbTE surface, G17 and A165 to arginines, improves MCFA titers more than 3-fold when compared to expression of AbTE wild type in *E. coli*. We proved that the AbTE mutations lead to new selective protein-protein interactions with *E. coli* ACP by performing NMR titrations between ¹⁵N-octanoyl-AcpP

and the AbTEs. This work demonstrates that engineering the interface of heterologous enzymes to better couple with endogenous host enzymes is a useful strategy to improve the microbial production of chemicals that require the expression of heterologous enzymes. Improving the microbial production of MCFAs is significant because MCFAs are key intermediates in the biosynthesis of medium-chain chemicals, including α -olefins, dicarboxylic acids and hydroxyacids, which are important targets in the polymer industry.

Results

Screening medium-chain acyl-ACP TE and *E. coli* hosts for MCFA production.

To identify the acyl-ACP TE that results in the highest secreted MCFA titers, we expressed the bacterial TE from *Acinetobacter baylyi*⁶, and the plant TEs from *Cocos nucifera*⁷, *Cuphea palustris*⁷, and *Umbellularia californica*¹⁵ in *E. coli* MG1655 (Fig. 2A). The percent sequence identity of these TEs to one another ranges from 15–17%; *A. baylyi* TE has the highest percent identity with *E. coli* ‘TesA at 38% (Fig. SI1). We measured secreted fatty acids as they could be continuously extracted from the culture broth, overcoming issues with cell lysis, and potentially reducing the overall cost for MCFA production. AbTE results in the highest secreted MCFA titers at 29 mg/L, consisting of octanoic, decanoic and dodecanoic acid. *C. nucifera* TE produced only dodecanoic acid at 5 mg/L, while *C. palustris* TE produced mostly octanoic acid at 8 mg/L. *U. californica* TE produced a mixture of decanoic and dodecanoic acid at 3 mg/L and 12 mg/L respectively. Given that AbTE resulted in the highest MCFA titers, we decided to engineer this enzyme.

The *E. coli* genomic background has been shown to affect chemical production¹⁶. We expressed AbTE in five different *E. coli* hosts: DH5 α , BL21, DH10B, MG1655, and BW25113 *fadE*, and measured secreted MCFA titers. We included the *fadE* deletion as it has been shown to improve fatty acid production in *E. coli*¹⁷. Surprisingly, *E. coli* hosts BL21 and MG1655 resulted in the highest MCFA productions at 26 mg/L and BW25113 *fadE* produced only 12 mg/L (Fig. 2B). Based on these results, we moved forward with AbTE expressed in *E. coli* MG1655.

Engineering AbTE for improved MCFA titers.

Expression of the non-functional AbTE:S11A in *E. coli* produces only saturated long-chain (C14-C18) fatty acids (LCFA) due to the presence of endogenous long chain TEs. Expression of AbTE wild type (AbTE:WT) in *E. coli* produced ~29 mg/L of secreted MCFAs or ~52% of all secreted fatty acid chain lengths. When total fatty acids were measured, i.e. secreted fatty acids plus intracellular and membrane bound fatty acids, AbTE:WT expressed in *E. coli* produced ~48 mg/L of MCFAs, which is ~22% of total fatty acid chain lengths. While MCFA levels increased by 65% when taking into account intracellular and membrane bound fatty acids, LCFA levels increase more than 6-fold. Specifically, AbTE:WT produced octanoic, decanoic, and dodecanoic acid at 9 mg/L, 6, mg/L and 14 mg/L, respectively (Fig. 2A). In addition to saturated fatty acids, AbTE:WT also produced small levels of unsaturated C12-C16 fatty acids.

To identify the interface between *E. coli* 'TesA and *E. coli* AcpP we used ClusPro¹⁸, which takes into account only the 'TesA-AcpP protein interactions to dock *E. coli* AcpP (PDB ID: 2FAE) and *E. coli* 'TesA bound to octanoic acid (PDB ID: 1U8U) (Fig. 2D). Using the model, we identified eight positions on 'TesA that are potentially part of the AcpP-'TesA interface: Y15, R16, R77, N112, R115, R116, D153, and R160. Structural alignment of 'TesA with a AbTE homology¹⁹ model revealed that all positions except for R16 (AbTE: G17), R115 (AbTE: T120), R116 (AbTE: A121), D153 (AbTE: N158) and R160 (AbTE: A165) had the same amino acids in these two proteins (Fig. 2E, Fig. SI2). Interestingly, four of the five amino acids that are different between 'TesA and AbTE are positively charged arginines, which could help stabilize the 'TesA-AcpP interaction as *E. coli* AcpP has a highly negative surface. We hypothesized that we could replicate these interactions between AbTE and *E. coli* AcpP to improve MCFA production.

We mutated positions 17, 120, 121, and 165 on AbTE to arginines to generate AbTE:G17R, AbTE:T120R, AbTE: A121R and AbTE:A165R, and measured their secreted fatty acid titers (Fig. 3A, Fig. SI3). Expression of AbTE:G17R in *E. coli* resulted in ~76mg/L of secreted MCFAs, more than double the secreted MCFA titers from AbTE:WT. The MCFAs produced by AbTE:G17R accounted for ~74% of secreted fatty acids of all chain lengths. In particular, octanoic, decanoic and dodecanoic acid were produced at 30 mg/L, 18 mg/L and 28 mg/L, respectively. Expression of AbTE:A165R in *E. coli* resulted in slightly lower secreted MCFA titers than AbTE:WT. To determine if the effects of these mutations on MCFA titers were additive, we constructed all double mutants using AbTE:G17R as the scaffold. Expression of AbTE:G17R/A165R in *E. coli* resulted in ~98 mg/L of secreted MCFAs. This is a 29% increase in secreted MCFA titers when compared to expression of AbTE:G17R and a more than 3-fold in secreted MCFA titers when compared to AbTE:WT. Although AbTE:G17R/A165R secreted MCFA titers improved, the MCFA percentage was only 55% of secreted fatty acids of all chain lengths. The major constituent in the AbTE:G17R/A165R secreted MCFA profile was dodecanoic acid at 45 mg/L. The increase in secreted MCFA titer achieved by AbTE:G17R/A165R was unexpected as AbTE:A165R resulted in the lowest MCFA titers from all single mutants. Finally, we generated the triple mutants using AbTE:G17R/A165R as the scaffold. At this stage, we also mutated the fifth position on AbTE that varies from 'TesA, AbTE:N158D. This fifth position does not change the amino acid to a positively charged arginine, but to a negatively charged aspartate. Nevertheless, the aspartate could still form part of a stabilizing interaction. As Figure 3C shows, none of the triple mutants resulted in improved secreted MCFA titers. Of note, positions A121 and T120 are located on the other side of the AbTE binding pocket than G17 and A165. For completion, we generated the remaining double mutants AbTE:T120R/A121R, AbTE:T120R/A121R, and AbTE:A121R/A165R and measured their secreted fatty acid titers (Fig. SI4). While two of these double mutants produced comparable secreted MCFA titers to AbTE:WT, AbTE:T120R/A121R produced ~45 mg/L of secreted MCFAs. Taken together, AbTE:G17R/A165R results in the highest secreted MCFA titers (98 mg/L), yet AbTE:G17R has the highest percentage of secreted MCFAs (74%), a trend that holds true whether analyzing secreted or total fatty acids (Table 1).

Extending cultivation time to increase MCFA titers.

Expecting to accumulate higher MCFA titers during a longer cultivation period, we increased the cultivation time from 24hrs to 72hrs (Fig. 3B). When expressing AbTE:WT in *E. coli*, secreted MCFA titers almost tripled between 24hrs and 72hrs from ~29 mg/L to ~79 mg/L, jumping from being 52% to 69% of all secreted fatty acid chain lengths. We speculate that LCFAs are more likely to be ligated to CoA by endogenous FadD and enter the β -oxidation pathway²⁰, thus leading to a reduction of LCMA levels over time. Interestingly, cultivating *E. coli* expressing AbTE:G17R for 72hrs did not result in any changes in MCFA titers. When expressing AbTE:G17R/A165R in *E. coli*, secreted MCFAs increased from 98 mg/L to 131 mg/L when extending the cultivation from 24hrs to 72 hrs. This increasing in MCFA titers increased the percentage of secreted MCFAs from 55% to 80%. It is possible that the low increase in MCFA titers observed with the AbTE mutants between 24hrs and 72hrs is due to lost in activity after 24hrs. It has been shown that adding solubility tags to heterologous proteins increases their viability inside the cell²¹. Attaching maltose binding protein (MBP) to the N-terminus of the AbTE mutants, the terminus at the opposite end of the AcpP-AbTE interface, results only in LCFAs and no MCFAs were detected (Fig. SI5).

AbTE:G17R and AbTE:G17R/A165R mode of action.

It is possible that the improved MCFA profile of AbTE:G17R and AbTE:G17R/A165R when compared to AbTE:WT is the result of the mutants' superior expression in *E. coli*. Positions 17 and 165 are located on the AbTE surface and mutating small hydrophobic amino acids to positively charged arginines may improve solubility. A SDS-PAGE gel of *E. coli* expressing AbTE:WT, AbTE:G17R or AbTE:G17R/A165R showed comparable soluble expression of the three enzymes over 3 dilutions of cell lysate (Fig. 3C, Fig SI6). In *E. coli*, arginines on the surface of FAS enzymes are essential for interaction with AcpP, which has a highly negatively charged surface²². Mutation of these arginines in FAS enzymes to negatively charged amino acids results in decreased interactions with ACP (FabA), decreased specific activity with ACP substrates (FabH, FabG), and decrease K_{cat} and increased K_M with ACP substrates (FabD)^{23–26}. If the newly introduced arginines on AbTE help stabilize AbTE-AcpP interactions, mutating AbTE positions 17 and 165 to a negatively charged amino acids, such as glutamate, should disrupt *E. coli* AcpP interactions resulting in lower MCFA titers. *E. coli* expression of both the single AbTE:G17E and AbTE:G17E/A165E result in overall lower secreted fatty acid titers than AbTE:WT (Fig. 3A). AbTE:G17E and AbTE:G17E/A165E results in ~21mg/L and 14 mg/L of secreted MCFAs, respectively, which is ~69% of secreted fatty acids of all chain lengths. Interestingly, AbTE:G17R/A165E results in ~57 mg/L of secreted MCFAs, which is ~79% of secreted fatty acids of all chain lengths. This favorable effect in secreted MCFAs or secreted fatty acids of all chain lengths is not seen in AbTE:G17E/A165R, making position 17 key in stabilizing AbTE-AcpP interactions. Taken together, the arginines on the AbTE surface do not affect the protein expression in *E. coli*, and enhance AbTE's interaction with *E. coli* AcpP.

To directly test whether there are increased interactions between *E. coli* AcpP and AbTE:G17R or AbTE:G17R/A165R when compared to AbTE:WT, we performed a two dimensional $^1\text{H}/^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) titration experiment.

Specifically, ^{15}N -octanoyl-AcpP was titrated with an increasing molar ratio of AbTE:WT, AbTE:G17R, or AbTE:G17R/A165R, and movement of AcpP residues on the HSQC spectra were analyzed²⁷. The degree of movement of each residue's peak on the HSQC spectra is quantified as a chemical shift perturbation (CSP), which combines the ^1H and ^{15}N dimension movement with a scaling factor to yield a simple value²⁸. This method yields a residue-by-residue quantification of the rapid binding events that normally result in catalysis. In the presence of AbTE:WT, *E. coli* AcpP CSPs are very low, indicating only a few sites of weak interaction between these two enzymes (Fig. 4B, Fig. SI7). In the presence of AbTE:G17R, *E. coli* AcpP showed significantly more interactions (Fig. SI8). In particular, there are increased perturbations with *E. coli* AcpP surface residues 20E, 36S, 38D, 41E, 49E, 68A, and 71Y. In the presence of AbTE:G17R/A165R, *E. coli* AcpP showed global increases in CSPs across all residues, indicating significant increase in protein-protein interactions (Fig. SI9). Of note, there are large CSPs at *E. coli* AcpP residue 36S and 41E, and many of the CSPs occur at negative residues demonstrating the need to match the positive residues of the partner to AcpP's negative face. Furthermore, 36S, the residue which is functionalized with phosphopantetheine, is greatly perturbed most likely due to AbTEG17R/A165R interacting with the acyl chain. Taken together, the titration experiments show that AbTE:G17R/A165R has significantly increased molecular contacts with *E. coli* AcpP compared to AbTE:WT. Although AbTE:G17R/A165R induced *E. coli* AcpP CSP values that are two orders of magnitude smaller than the ones triggered by *E. coli* native enzymes²⁷, the CSP values triggered by AbTE:G17R/A165R are ten times larger than the ones induced by AbTE:WT. The data herein demonstrates that the previously overlooked protein-protein interactions between the interfaces are critical for the development of novel functional interactions.

Discussion

We engineered the surface of a heterologous enzyme (AbTE) to better couple to an endogenous *E. coli* enzyme (AcpP) to increase chemical titers (MCFAs). Replacement of two small nonpolar residues on the AbTE surface predicted to contact *E. coli* AcpP, which has a highly negatively charged surface, with positively charged arginines, the amino acid found at the equivalent positions in *E. coli* 'TesA, resulted in more than 3-fold improvement in secreted MCFA titers. Titration NMR experiments demonstrate that the mutations indeed affect the AbTE-AcpP binding interface, demonstrating the power of protein interface engineering between heterologous proteins and interacting host proteins to increase the titers of microbially-produced chemicals.

In the future, engineering the interface of heterologous proteins to better match the interface of host enzymes could be applied to more distantly related proteins, such as plant TEs. Such an approach may prove even more beneficial for more distantly related enzymes that may only marginally interact with endogenous host proteins. A key limitation will be the low sequence homology between heterologous and endogenous proteins, making it difficult to identify key residues for mutagenesis (SI Fig. 1). The recently determined structure of *U. californica* TE should assist with the identification of plant TE-AcpP interactions²⁹. In addition to MCFA production, the matching interface strategy could also be applied to

medium-chain methyl ketone production by better coupling *E. coli* AcpPs to heterologous β -ketoacyl-ACP TEs, such one from *Solanum habrochaites* (ShMKS2)³⁰.

Methods

Plasmid construction.

Non-codon optimized *Acinetobacter baylyi* TE (AbTE), *S. cerevisiae* codon-optimized *Cuphea palustris* TE (CpTE), *E. coli* codon-optimized *Umbellularia californica* TE (UcTE) were commercially synthesized and cloned under P_{TRC} in pMB1-P_{TRC}-AgGPPS-(GSG)₂-AgPS (pSS185) between *NcoI/XmaI* to generate pMB1-P_{TRC}-AbTE (pSS192), pMB1-P_{TRC}-CpTE (pSS183), and pMB1-P_{TRC}-UcTE (pSS193). *S. cerevisiae* codon-optimized CnTE was amplified from pESC-LEU2-P_{TEF1}-P_{HXT7}-CnTE (pSS81) with primers SS455/SS456 and cloned under P_{TRC} in pSS185 between *NcoI/XmaI* to generate pMB1-P_{TRC}-CnTE (pSS174). AbTE mutants were generated using QuikChange protocol with some modifications (SI). Details on templates and primers used are in Table SI4. For protein expression, a C-terminal His₆-tag was introduced into AbTE:WT, AbTE:G17R and AbTE:G17R/A165R using primers TB1/TB2 and cloned into pET-28b (amplified using primers TB3/TB4) to generate pET-28b -AbTE:WT, pET-28b -AbTE:G17R, and pET-28b -AbTE:G17R/A165R.

AbTE and ACP expression and purification.

pET-AbTE:WT, pET-AbTE:G17R, and pET-AbTE:G17R/A165R were transformed in *E. coli* BL21 (DE3) and grown in the presence of 50 mg/L of kanamycin. Cells were induced with 1mM IPTG at OD₆₀₀=0.8 and grown for 16 to 18 hours at 16° C. Cells pellets were resuspended in 50 mM Hepes (pH 7.4), 250 mM NaCl, and 10% glycerol before lysis by sonication and clarification at 22,000 rcf. Clarified lysate was allowed to batch bind the Ni-NTA resin for 20 minutes followed by washing with buffer containing 25 mM imidazole. Final elution was performed with buffer containing 250mM imidazole, followed by dialysis into 50 mM Tris buffer (pH 7.4), 150 mM NaCl and 10% glycerol. After concentration to ~2mL, the enzymes were purified on a GE Superdex 200 gel filtration column and the fractions containing the desired protein were checked by UV trace and SDS PAGE before concentration. The same procedure was followed for the ¹⁵N-AcpP, however, AcpP was grown in 1g ¹⁵N ammonium chloride, 4g of ¹²C glucose, 1L of M9 media, and 50mg of kanamycin.

¹⁵N-octanoyl-AcpP synthesis.

¹⁵N-AcpP was incubated overnight with *Pseudomonas aeruginosa* ACP hydrolase to generate pure apo ¹⁵N-AcpP as confirmed by a urea PAGE³¹. C8-pantethenamide³² was loaded onto apo ¹⁵N-AcpP to generate ¹⁵N-octanoyl-AcpP using 12.5mM MgCl₂, 10mM ATP, 0.1μM *E. coli* CoaA, 0.1μM *E. coli* CoaD, 0.1μM *E. coli* CoaE, 0.2μM *Bacillus subtilis* surfactin phosphopantetheinyl transferase, 0.02% Triton X, 0.01% azide, and 0.1% tris(2-carboxyethyl)-phosphine (TCEP).

NMR titration experiments.

AbTE:WT, AbTE:G17R, AbTE:G17R/A165R and ^{15}N -octanoyl-AcpP were purified separately on a Superdex 75 gel filtration column into 50 mM pH 7.4 potassium phosphate with 0.01% azide and 0.5 mM TCEP. Samples were prepared to a volume of 500 μL with 50 μL of D_2O . The AbTE:WT, AbTE:G17R, and AbTE:G17R/A165R experiments were performed with 0.75 mM, 0.101 mM, and 0.74 mM ^{15}N -octanoyl-AcpP. The AcpPs were titrated to a final concentration of 1.5, 2.0, and 1 molar equivalents of AbTE: WT, AbTE :G17R, and AbTE G17R/A165R, respectively. Experiments were performed on a Bruker Avance 800MHz spectrometer, in 50 mM potassium phosphate pH 7.4 with 0.5mM TCEP and 0.1% sodium azide. Chemical shift perturbations were calculated using the equation $\text{CSP} = \sqrt{\left(\frac{1}{2}\right)\left[\delta_H^2 + (\alpha\delta_N)^2\right]}$ where $\alpha=0.2$.

MCFA production, derivatization and quantification.

MCFA Production: Overnight cultures of *E.coli* MG1655 expressing AbTE:WT or AbTE variants were diluted 1:50 in 5 mL of M9 media (0.5% glucose, amp^{100}) and grown at 37°C , 250 r.p.m. until reaching an $\text{OD}_{600} = 0.3\text{--}0.4$. The cells were then induced with 500 μM of IPTG (500 mM stock) and grown at 30°C , 250 r.p.m. for 24 or 72 hrs. *Fatty acid analysis:* For secreted fatty acids, *E. coli* cultures were vortexed for 3 sec, 600 μL of culture removed and centrifuged for 10 min at $7354g$. Next, 400 μL of the supernatant was removed for derivatization. For total fatty acids, 400 μL of culture was used for derivatization. *Fatty acid derivatization:* Fatty acids were derivatized to fatty acid methyl esters and analyzed via GC/MS as described in Torella et. al., 2013³³ with some modifications. To the 400 μL of sample, 50 μL of 10% (wt/vol) NaCl, 50 μL of glacial acetic acid, 20 μL of 90.5 mg/L nonanoic acid (internal standard), and 200 μL of ethyl acetate were added and the mixture was vortexed for 5 sec. The mixture was then centrifuged at $12,098g$ for 10 min. Methyl esters were generated by mixing 100 μL of the ethyl acetate layer with 900 μL of a 30:1 mixture of methanol and 37% (vol/vol) HCl in a 2 mL microcentrifuge tube, vortexed for 5 sec, and incubated at 50°C for 1 hr. After cooling to room temperature, 500 μL of water and 500 μL of hexanes were added. The mixture was vortexed for 5 sec, 100 μL of the hexane layer was taken and mixed with 400 μL of ethyl acetate for analysis via GC-MS. *FAME quantification:* The samples were analyzed using Agilent 7890A/Agilent 5975 MS detector using a DB-5MS column. The inlet temperature was set to 300°C , flow at 1 mL/min, the oven at 70°C for 1 min, ramp at $30^\circ\text{C}/\text{min}$ to 290°C , and held for 1 min at 290°C . Standard curves of C8–C18 fully saturated FAMES (Alfa Aesar/TCI) were used for sample quantification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Medium-chain fatty acid biosynthesis in *Escherichia coli*. **A.** *E. coli* Type II fatty acid synthase (FAS) extends and reduces an acyl chain bound to acyl-carrier protein (ACP). All enzymes in FAS interact with ACP. Thioesterases (TEs) hydrolyze acyl-ACPs to free fatty acids of different chain lengths according to their substrate specificity. **B.** Matching the surface interface between the native *E. coli* AcpP and heterologous medium-chain *Acinetobacter baylyi* TE, improves medium-chain fatty acid production in *E. coli*.

Figure 2.

Medium-chain fatty acid production and calculated *E. coli* AcpP interphase with TEs. **A.** Secreted fatty acid titers of *E. coli* (MG1655) expressing four heterologous TEs. **B.** Secreted fatty acid titers by different *E. coli* strains expressing *A. baylyi* TE (AbTE:WT). **C.** Secreted and total (secreted plus intracellular and membrane bound) fatty acid titers produced by *E. coli* expressing AbTE:WT and inactive AbTE (AbTE:S11A). The experiments were done in triplicate and the error bars represent the standard deviation from the mean. **D.** Docking of *E. coli* AcpP (magenta, PDB ID: 1FAE) and *E. coli* 'TesA (cyan, PDB ID 1U8U) identifies potential residues on the 'TesA surface that are important for interactions between 'TesA and AcpP. **E.** Homology model of AbTE with surface residues equivalent to 'TesA labelled.

Figure 3.

Secreted fatty acid and protein levels of *E. coli* expressing *A. baylyi* TE (AbTE) and AbTE mutants. **A.** Secreted fatty acid levels of *E. coli* expressing AbTE single, double, and triple arginine mutants, as well as AbTE single and double glutamate mutants. All experiments were done in triplicate and the error bars represent the standard deviation from the mean. **B.** Time course of saturated fatty acid titers produced by *E. coli* expressing AbTE, AbTE:G17R, and AbTE:G17R/A165R. **C.** Coomassie stained SDS-PAGE gel of Serial dilution of induced *E. coli* cultures expressing AbTE:WT, AbTE:G17R and AbTE:G17R/A165R.

Figure 4.

Solution NMR analysis of molecular interactions between *A. baylyi* thioesterase (AbTE) and *E. coli* AcpP. **A.** HSQC titration of AbTE:G17R/A165R with *E. coli* ¹⁵N-octanoyl-AcpP. Right: Zoom of two selected cross-peaks 36S and 40V. **B.** CSPs were measured for each ⁵N-octanoyl-AcpP residue in the presence of AbTE:WT (red), AbTE:G17R (blue), or AbTE:G17R/A165R (orange) and plotted by residue number. **C.** CSPs are plotted onto the structure of AcpP (PDB ID = 2FAD) in the presence of AbTE:WT, AbTE:G17R, or AbTE:G17R/A165R. Shifts in ¹H and ¹⁵N dimensions of ¹⁵N-octanoyl-AcpP upon titration with AbTE:WT, AbTE:G17R and AbTE:G17R/A165R used to calculate chemical shift perturbations can be found in Fig.SI 9.

Table 1

: Saturated fatty acid percent composition produced by AbTE:WT and variants

	Thioesterase	<i>E. coli</i> strain	Media	C8:0 (%)	C10:0 (%)	C12:0 (%)	C14:0 (%)	C16:0 (%)	C18:0 (%)
Supernatant	AbTE:WT	MG1655	M9	16.5	10.5	25.2	11.5	17.1	19.2
	AbTE:G17R	MG1655	M9	29.3	17.5	27.3	4.7	9.1	12.1
	AbTE:G17R/A165R	MG1655	M9	18.5	11.7	25.4	21.2	14.2	9
Total	AbTE:WT	MG1655	M9	4.7	3.3	14.4	41.7	30.4	5.5
	AbTE:G17R	MG1655	M9	16.1	9.9	25.0	29.4	13.9	5.7
	AbTE:G17R/A165R	MG1655	M9	9.4	6.0	18.8	38.7	18.6	8.5