

Expression of *Vibrio harveyi* Acyl-ACP Synthetase Allows Efficient Entry of Exogenous Fatty Acids into the *Escherichia coli* Fatty Acid and Lipid A Synthetic Pathways[†]

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ABSTRACT: Although the *Escherichia coli* fatty acid synthesis (FAS) pathway is the best studied type II fatty acid synthesis system, a major experimental limitation has been the inability to feed intermediates into the pathway in vivo because exogenously supplied free fatty acids are not efficiently converted to the acyl–acyl carrier protein (ACP) thioesters required by the pathway. We report that expression of *Vibrio harveyi* acyl-ACP synthetase (AasS), a soluble cytosolic enzyme that ligates free fatty acids to ACP to form acyl-ACPs, allows exogenous fatty acids to enter the *E. coli* fatty acid synthesis pathway. The free fatty acids are incorporated intact and can be elongated or directly incorporated into complex lipids by acyltransferases specific for acyl-ACPs. Moreover, expression of AasS strains and supplementation with the appropriate fatty acid restored growth to *E. coli* mutant strains that lack essential fatty acid synthesis enzymes. Thus, this strategy provides a new tool for circumventing the loss of enzymes essential for FAS function.

The *Escherichia coli* fatty acid biosynthetic pathway (Figure 1) is the archetypical type II fatty acid synthesis (FAS) system (1). Although genetic, enzymological, and structural analyses have rendered *E. coli* FAS by far the best understood type II system, it has one serious disadvantage, the inability of exogenous fatty acids to efficiently enter the fatty acid synthetic pathway. Fatty acids added to cultures of *E. coli* are readily transported into the cell where they are converted to CoA thioesters and degraded to acetyl-CoA by the β -oxidation system (2). However, if the acyl chains are sufficiently long (> 12 carbons), the acyl-CoAs can be used directly as acyl donors by the acyltransferases of glycerophospholipid synthesis (1). However, there is little or no elongation of the fatty acids, indicating they cannot enter the fatty acid synthetic pathway (3). For example, labeled 14-carbon fatty acids are incorporated into glycerophospholipids only as 14-carbon fatty acids, not as a 16- or 18-carbon fatty acid (4). This indicates that *E. coli* lacks the means to efficiently convert either free fatty acids (FFAs)¹ or acyl-CoA thioesters to the acyl–acyl carrier protein (ACP) thioester substrates required by the fatty acid synthesis system. This inability to form acyl-ACPs from exogenous fatty acids has precluded feeding fatty acid synthetic intermediates (or analogues thereof) to address mechanistic and genetic questions in vivo. We report that expression of *Vibrio harveyi* acyl-ACP synthetase (AasS), a soluble cytosolic enzyme

that ligates FFAs to ACP to form acyl-ACPs (5–7), allows exogenously supplemented FFAs to enter the *E. coli* fatty acid synthesis pathway and become elongated. Moreover, AasS expression allowed growth of *fabA*, *fabB*, and *fabH* null mutant strains on fatty acids that do not support growth in the absence of the synthetase. Finally, AasS expression allowed labeling of the primary and secondary acyl chains of the lipid A component of the outer membrane with exogenous dodecanoic and tetradecanoic acids. We also report that the very low level of background incorporation seen in the absence of AasS is dependent on conversion of exogenous acids to their acyl-CoA thioesters.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids are listed in Table 1. All *E. coli* strains are K-12 derivatives. The *aasS* coding sequence was PCR amplified from pYFJ64 (7) with primers AAS NcoI (5'-CCATG-GACCAGTATGTAAATGAT-3') and AAS HindIII (5'-AAG-CTTTTACAGATGAAGTTTACGCAGT-3'), followed by cloning of the PCR product into pCR2.1-TOPO, resulting in pYFJ82. Plasmid pYFJ82 was digested with NcoI and HindIII, and the *aasS*-containing fragment was then ligated into the same sites of pBAD24 (8) to give pYFJ85. Plasmids pYFJ85 and pBAD24 were transformed into strains K19 (*fadE62*), K27 (*fadD88*), and JWC255 (*cfa::kan*, *fadE62*). The last strain was constructed by transduction of strain K19 with a phage P1 lysate grown on strain YYC1273 (9). Strains K19 and K27 were obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT).

The Δ *fabH* strains were derived by elimination of the functional copy of *fabH* from strain CL111 by phage P1 transduction with lysates grown on strains CAG12094 and CAG18466 as previously described (10), except that the strain carried the AasS

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¹Abbreviations: AasS, soluble acyl-ACP synthetase; ACP, acyl carrier protein; FFA, free fatty acid; FabA, 3-hydroxydecanoyl-ACP dehydratase; *trans*-2-decenoyl-ACP isomerase; FabB, 3-ketoacyl-ACP synthase I; FabF, 3-ketoacyl-ACP synthase II; FabH, 3-ketoacyl-ACP synthase III; *cfa*, gene encoding cyclopropane fatty acid synthase.

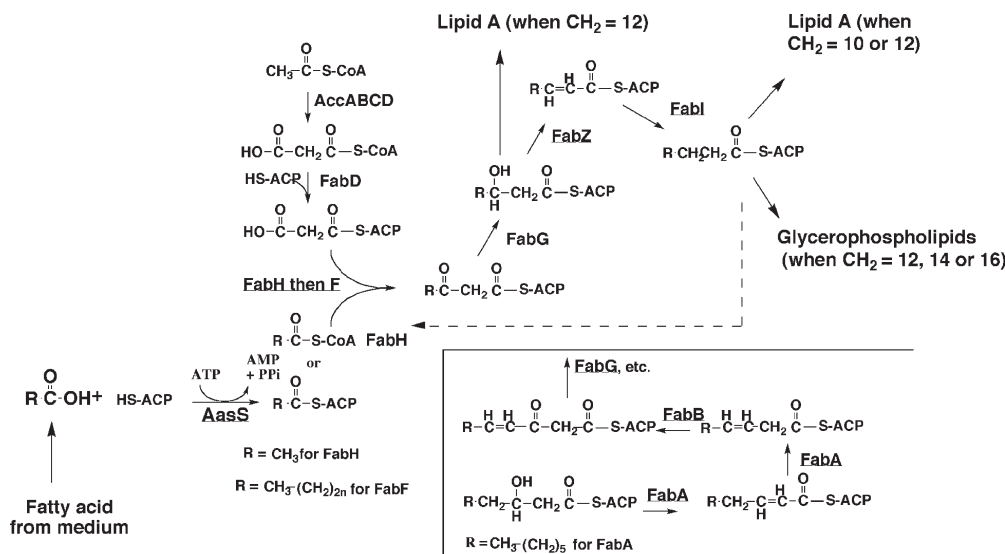


FIGURE 1: Fatty acid synthesis pathway of *E. coli*, its intersection with *V. harveyi* AasS, and incorporation of acyl chains into complex lipids. Unsaturated fatty acid synthesis is shown below the bracket at the bottom right. Unsaturated fatty acids are not found in lipid A unless the cells are grown at low temperatures (30, 31).

encoding plasmid pYFJ85 or the vector plasmid, pBAD24, to give strain YFJ249 or YFJ248, respectively. The transduction mixtures were plated on LB supplemented with 1 mM octanoate, 0.2% oleate, 0.02% arabinose, 15 $\mu\text{g/mL}$ tetracycline, 50 $\mu\text{g/mL}$ kanamycin, and 100 $\mu\text{g/mL}$ ampicillin. The plates were incubated at 37 °C for 48 h to allow growth of small colonies. Both the large and small colonies were screened for sensitivity to a mixture of spectinomycin and streptomycin. Colonies sensitive to the mixture (17.5 $\mu\text{g/mL}$ each) of spectinomycin and streptomycin (the *fabH* bypass mutants) were only seen among the transductants of strain YFJ249, and all were small colonies. The bypass strains were designated as YFJ260 (P1 grown on CAG12094) and YFJ262 (P1 grown on CAG18466).

To construct strains YFJ303, YFJ304, and YFJ305, a P1 lysate grown on CL111 was used to transduce strains YFJ294 (UB1005/pYFJ85), YFJ293 (UB1005/pBAD24), and UB1005, respectively. The transductants were plated on LB plates supplemented with 1 mM octanoate, 0.2% oleate, 0.02% arabinose, 50 $\mu\text{g/mL}$ kanamycin, 50 $\mu\text{g/mL}$ neomycin, and 100 $\mu\text{g/mL}$ ampicillin (for recipient strains YFJ294 and YFJ293 only). Although addition of octanoate, oleate, and arabinose was not expected to aid growth of YFJ293 and UB1005 transductants, these supplements were routinely added to the plating medium. The transduction plates were again incubated at 37 °C for 2–3 days to allow growth of small colonies. Strain YFJ303 was essentially a *fabH* bypass strain similar to YFJ262 except for the absence of a *Tn10* element. Strains YFJ304 and YFJ305 were expected to exhibit the *fabH* deletion mutant phenotype in the absence of AasS expression. The *cfa* derivatives of YFJ303, YFJ304, and YFJ305 were obtained by P1 transduction of a *cfa::Cm* locus from strain GRT26 into the corresponding recipient strains to give strains YFJ315, YFJ316, and YFJ317, respectively. To confirm that the *fabH* locus of YFJ305 had the expected insertion mutation (that of donor strain CL111), PCR analysis was performed using primers 5'-GAGTCACTCGCAAAGCGAACG-3' and 5'-CGTTACTGAGTACGCAGCC-3' (which flank the chromosomal *fabH* region) followed by restriction mapping of the PCR products in wild-type (UB1005), YFJ305, and CL111 strains (Figure 1S of the Supporting Information). The *fabH* bypass and deletion mutant strains were routinely grown on LB supplemented

with 1 mM octanoate, 0.2% oleate, 0.02% arabinose, and the appropriate antibiotics. For testing the growth phenotypes of these strains on various fatty acids, each fatty acid at 1 mM (final concentration) was added to the growth medium.

Radioactive Labeling, Lipid Extraction, and Fatty Acid Analysis. For the *fabH* deletion strains, the *cfa* derivatives of YFJ315 (carrying *aasS* plasmid pYFJ85), YFJ316 (carrying vector pBAD24), and YFJ317 (no plasmid) together with the corresponding wild-type strain CHC41 (UB1005 *cfa::Kan*) were grown at 37 °C either in LB or in LB supplemented with 0.5 mM octanoic acid and 0.2% arabinose. Derivatives of strain K19, K27, or JWC255 carrying either pYFJ85 or pBAD24 were grown overnight in LB containing 15 μM 1- ^{14}C -labeled fatty acid (50–55 mCi/mmol), ampicillin (100 $\mu\text{g/mL}$), and various concentrations of arabinose. Phospholipids were extracted by the method of Bligh and Dyer (11), whereas lipid A was obtained by the following procedure. Labeled cells were recovered by filtration using solvent-resistant 0.2 μm syringe filters (Chromafil Xtra PET-20-25 from Macherey-Nagel) and washed several times with LB to remove unincorporated fatty acids. The filters were then washed three times with 10 mL of a chloroform/methanol mixture (1/2, v/v) to remove phospholipids. Forcing air through them dried the filters. Each filter was then connected to a fresh syringe that was used to draw 1 M KOH into the filter, and the syringe plunger was partially withdrawn to seal the top of the filter and impede drainage of the KOH by creation of a vacuum. The filters were then incubated at 42 °C for 3 days to hydrolyze the ester-linked fatty acids. The KOH was then expelled from the filter and acidified, and the fatty acids were recovered by Bligh–Dyer extraction. The fatty acids were analyzed by reverse phase chromatography on Partisil KC18 thin layer plates of octadecyl-modified silica gel 60 (Whatman) using an acetonitrile/acetic acid/acetone solvent system (7:1:1, by volume) followed by autoradiography. Note that only the ester-linked lipid A acyl groups were analyzed since amide-linked 3-hydroxytetradecanoate moieties are not released by base treatment. The phospholipid acyl chains were analyzed as their methyl esters which were obtained by base-catalyzed transesterification (12).

The methyl esters were analyzed by reverse phase chromatography as described above, except that the solvent consisted of

Table 1: *E. coli* K-12 Strains and Plasmids Used in This Work

relevant characteristics		source or reference
strains		
UB1005	<i>metB1 relA1 spoT1 gyrA216^{f1}</i>	lab collection
CL81	UB1005, <i>att_{HK022}::(plsX⁺fabH, Spc^r Str^r)</i> 10	
CL111	UB1005, <i>att_{HK022}::(plsX⁺fabH, Spc^r Str^r)</i> , 10 <i>fabH::Kan</i>	
YFJ248	CL111/pBAD24	this work
YFJ249	CL111/pYFJ85	this work
CAG12094	MG1655, <i>zcb-3059::Tn10</i>	28
CAG18466	MG1655, <i>zcb-282::Tn10</i>	28
YFJ260	<i>fabH::Kan, zcb-3059::Tn10, Spc^s Str^s/pYFJ85</i>	this work
YFJ262	<i>fabH::Kan, zcb-282::Tn10, Spc^s Str^s/pYFJ85</i>	this work
YFJ293	UB1005/pBAD24	this work
YFJ294	UB1005/pYFJ85	this work
YFJ303	YFJ294 <i>fabH::Kan</i>	this work
YFJ304	YFJ293 <i>fabH::Kan</i>	this work
YFJ305	UB1005 <i>fabH::Kan</i>	this work
GRT26	MC1061 <i>cfa::Cm</i>	lab collection
YFJ315	YFJ303 <i>cfa::Cm</i>	this work
YFJ316	YFJ304 <i>cfa::Cm</i>	this work
YFJ317	YFJ305 <i>cfa::Cm</i>	this work
CHC41	UB1005, <i>cfa::kan</i>	this work
K19	<i>fadE62</i>	23
K27	<i>fad88</i>	23
CAG18483	<i>fadL771::Tn10</i>	28
CAG18496	<i>fadA 101::Tn10</i>	28
JWC255	<i>fadE62 cfa::kan</i>	this work
YYC1273	<i>cfa::kan</i>	9
plasmids		
pBAD24	expression vector with the <i>pBAD</i> promoter	8
pCR2.1-TOPO	TOPO TA cloning vector	Invitrogen
pYFJ82	<i>aasS</i> PCR amplified and inserted into pCR2.1-TOPO	this work
pYFJ85	<i>aasS</i> fragment of pYFJ82 inserted into the <i>NcoI</i> and <i>HindIII</i> sites of pBAD24	this work
pMON5839	expression vector	29
pBB277	<i>Saccharomyces cerevisiae</i> FAA1 gene in pMON5839	29

acetonitrile, methanol, and water (65:35:0.5 by volume) or by argentation thin layer chromatography on 20% AgNO₃ (Analtech Silica Gel GHL) plates developed twice in toluene at -20 °C followed by autoradiography. The chromatograms were dried and exposed to Kodak BioMax XAR film. Mass spectral analyses of the fatty acid compositions of the membrane phospholipids were conducted as described previously (13) on phospholipid extracts from cultures grown overnight with the fatty acid to be tested at a final concentration of 0.1 mM.

RESULTS

AasS Expression Allows Efficient Incorporation and Elongation of Exogenous Short and Medium Chain Fatty Acids into Phospholipids and Lipid A. We first tested the ability of strains expressing AasS to elongate exogenous short chain fatty acids to chain lengths of sufficient length to be accepted by the glycerophospholipid acyltransferases for incorporation into membrane phospholipids. In our first experiments, the exogenous acids were labeled in the carboxyl group with ¹⁴C and both even and odd chain length acids were tested in the

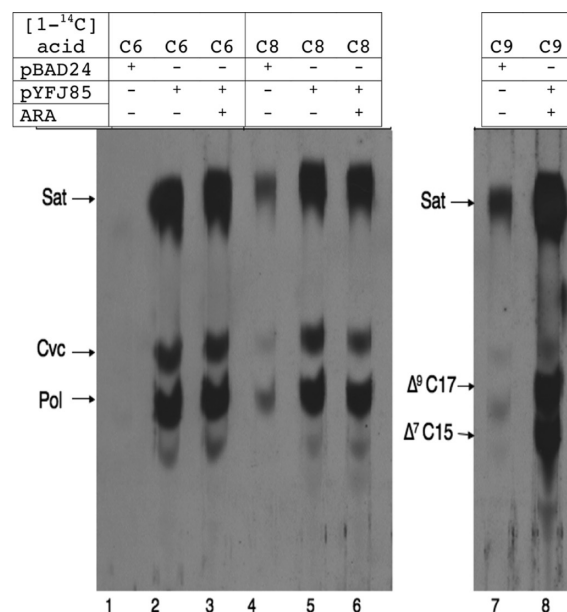


FIGURE 2: Strain YYC1273 (*cfa::kan*) carrying either vector plasmid pBAD24 or AasS encoding plasmid pYFJ85 was grown overnight at 37 °C on each of the ¹⁴C-labeled short chain fatty acids shown at 15 μM. The acyl chains were analyzed as their methyl esters by argentation chromatography. When added, arabinose was present at a concentration of 0.02%. Sat, saturated fatty acid esters; Cvc, *cis*-vaccenic acid (*cis*-11-octadecenoic acid); Pol, palmitoleic acid (*cis*-9-hexadecenoic acid, Δ⁹-C17, *cis*-9-heptadecenoic acid, and Δ⁷-C15, *cis*-9-pentadecenoic acid). Identification of the fatty acids was accomplished using authentic standards as described previously (19), except the odd chain unsaturated acids that were identified by reference to the prior literature (32, 33).

presence or absence of AasS expression (Figure 2). The concentration used was 15 μM since this concentration is more than twice the Michaelis constants of the C8, C12, and C14 acids (5). The most striking result was observed with [1-¹⁴C]hexanoate. In the absence of AasS expression (the empty vector control), no detectable label was incorporated into phospholipid acyl chains whereas either induced or basal expression of AasS resulted in high levels of incorporation into both saturated and unsaturated acyl chains (the strains used with the short chain acids were unable to convert unsaturated phospholipid acyl chains to their cyclopropane derivatives due to inactivation of the *cfa* gene). The lack of labeling of the empty vector control culture indicated that labeling was not due to β-oxidation of the labeled fatty acid followed by incorporation of labeled acetyl-CoA. Moreover, identical labeling was seen in wild-type and β-oxidation negative (*fadE*) strains (data not shown). When the supplements were ¹⁴C-labeled with C8 or C9 fatty acids, some incorporation was seen in the vector control cultures in both wild-type and β-oxidation negative (*fadE*) strains. As expected, the C6 and C8 acids were incorporated into both saturated and unsaturated acyl chains (due to inactivation of the *cfa* gene, the strains used were unable to convert unsaturated phospholipid acyl chains to their cyclopropane derivatives which chromatograph as saturated fatty acids). Unsaturated fatty acids were also synthesized from the C9 acid, indicating that the unsaturated fatty acid synthetic enzymes, FabA and FabB, can function (albeit poorly) with a C11 substrate in place of the usual C10 substrate. This is consistent with early enzymatic studies of FabA (14). The C9 acid would be elongated to 3-ketoundecanoyl-ACP which would be reduced to 3-hydroxyundecanoyl-ACP and then dehydrated

and isomerized by FabA. The products expected would be *cis*-7-pentadecenoic and *cis*-9-heptadecenoic acids. Analysis of both the argentation chromatography of the radioactive products (Figure 2) and mass spectra of the unlabeled products (see below) provides further evidence of the synthesis of these fatty acids.

Encouraged by these results, we asked if the dodecanoate and tetradecanoate secondary acyl groups of lipid A could be labeled when these fatty acids were provided exogenously in the presence of AasS. The lipid A acyltransferases are considered specific for ACP thioesters (15), although it was recently reported that LpxL has some activity with dodecanoyl-CoA (16). In this case, the exogenous acids are sufficiently long to induce the β -oxidation pathway which could result in randomization of the label; hence, we used a *fadE62* strain (lacking acyl-CoA dehydrogenase). We first examined incorporation into glycerophospholipids. Supplementation of an AasS expressing strain with [1-¹⁴C]dodecanoic acid resulted in incorporation of the label into C12, C14, and C16 glycerophospholipid acyl chains, indicating efficient elongation of the exogenous acid (Figure 3A). As expected, no label was found in unsaturated acids (data not shown). In a similar manner, the [1-¹⁴C]tetradecanoic acid was efficiently elongated to the C16 species. In both cases, some incorporation of the labeled acids was seen in cultures lacking AasS (Figure 3A). This seemed primarily due to their incorporation as acyl-CoA thioesters since the chains were incorporated without elongation in the case of the C14 acid and with little elongation in the case of the C12 acid. When the lipid A fractions of these cultures were examined, [1-¹⁴C]dodecanoic acid labeled both the C12:0 and C14:0 secondary acyl groups as well as the primary 3-hydroxymyristate acyl groups, indicating that the labeled fatty acid had entered the elongation cycle. In contrast, supplementation with [1-¹⁴C]tetradecanoic acid labeled only the C14:0 secondary acyl group as expected (Figure 3B). The lack of longer acyl chains showed that the lipid A preparations were largely free of glycerophospholipids. In the case of the C12 acid, the strain lacking AasS exhibited some labeling consistent with the recent report that the LpxL acyltransferase has low activity with dodecanoyl-CoA (16). It should be noted that labeling with the C12 acid was significantly more efficient than labeling with the C14 acid. This can probably be attributed to the 8-fold lower Michaelis constant of AasS for the shorter substrate (5).

The Defective Growth of *E. coli fabH* Null Mutant Strains Is Rescued by Expression of *V. harveyi* Acyl-ACP Synthetase with Exogenous Fatty Acids. Most of the fatty acid synthetic genes are involved in the elongation cycle and therefore are needed throughout the synthetic process. An exception is FabH, the short chain 3-ketoacyl-ACP synthetase (KAS III) thought to be required for only the first elongation, that of an acetate unit to acetoacetyl-ACP, but not for the subsequent elongations required to make long chain length acyl-ACPs. Hence, FabH is thought to provide the "primer" of fatty acid synthesis and to be used only once per acyl chain synthesized (1). *E. coli fabH* null mutants grow very slowly, indicating that the enzyme plays an essential role in the normal fatty acid synthetic pathway (10). However, the slow growth of *E. coli fabH* null mutants indicates that another inefficient mechanism for generating the primer is present. Indeed, *fabH* null mutants of *Lactococcus lactis* were found to synthesize fatty acids at ~10% of the normal rate (10). If the role of FabH is to make the primer, it seemed possible that loss of the enzyme should be compensated by the combination of AasS expression and supplementation with an exogenous short chain fatty acid such as octanoate.

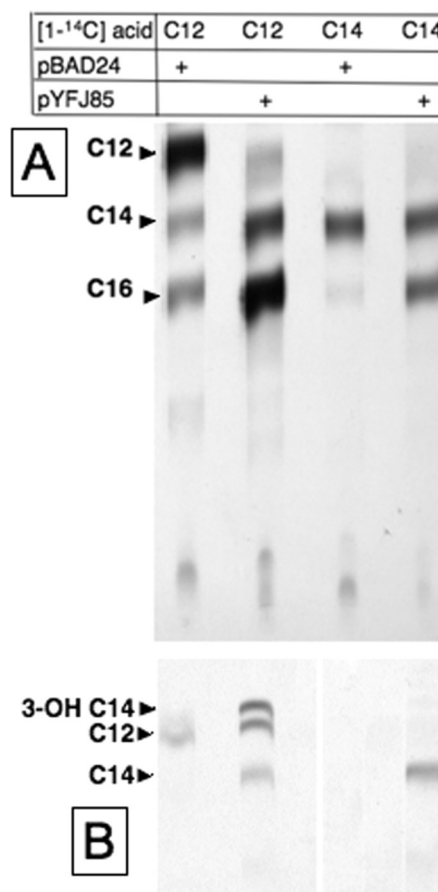


FIGURE 3: Incorporation of [1-¹⁴C]dodecanoic acid or [1-¹⁴C]tetradecanoic acid into the phospholipid (A) or lipid A (B) acyl chains of strain K19 (*fadE62*) carrying either vector plasmid pBAD24 or AasS encoding plasmid pYFJ85 assayed by reverse phase chromatography. The phospholipid acyl chains were analyzed as their methyl esters, whereas the lipid A acyl chains were analyzed by reverse phase chromatography as free fatty acids. Note that the two right-hand lanes of panel B were exposed 3 times as long as the two left-hand lanes (two exposures of the same plate were conducted). Arabinose was present at a concentration of 0.1%. C12, C14, and C16 denote dodecanoic, tetradecanoic, and hexadecanoic acids, respectively, whereas 3-OH C14 denotes 3-hydroxytetradecanoic acid. The fatty acids were identified using authentic standards as described previously (19).

To test this hypothesis, we used strain CL111 previously constructed in this laboratory (10). This strain was constructed by insertion of a second copy of *fabH* (that of *Salmonella enterica*) into the *attHK022* attachment site of strain UB1005 followed by the deletion of the chromosomal *fabH* copy. A control strain, CL81, that contained both the *S. enterica* and *E. coli fabH* genes was also constructed. It was then shown that the copy of *S. enterica fabH* could be deleted in strain CL81 (where a second *fabH* copy was present), but not in CL111 (which lacked the second copy). These data indicated that a functional FabH is essential for normal cell growth of *E. coli* (10).

We first introduced AasS expression plasmid pYFJ85 into strain CL111. The *aasS* gene was under the control of the arabinose inducible *pBAD* promoter in the high-copy number vector, pBAD24. To remove the functional (*S. enterica*) copy of *fabH*, strain YFJ249 was then transduced with phage P1 stocks grown on two strains that carried different transposon *Tn10* insertions closely linked to the *attHK022* site into which the *S. enterica fabH* gene (marked with a spectinomycin/streptomycin resistance element) had been inserted (10). The transductants

were selected on LB supplemented with octanoate with oleate and arabinose (the inducer of AasS expression) with selection for tetracycline resistance. The tetracycline resistant colonies were then screened for sensitivity to a mixture of spectinomycin and streptomycin. The colonies sensitive to spectinomycin and streptomycin were *fabH* null mutant strains formed by homologous recombination with the transduced DNA segment that removed the *S. enterica fabH* gene together with the spectinomycin and streptomycin resistant cassette. Indeed, after incubation for 48 h at 37 °C, both large and small colonies formed. All of the small colonies (~86% of the total tetracycline resistant colonies) were sensitive to spectinomycin and streptomycin, whereas all of the large colonies were spectinomycin and streptomycin resistant. In contrast in a parallel experiment, a strain carrying the empty vector plasmid (YFJ248) in place of the *aasS* plasmid gave no spectinomycin and streptomycin sensitive colonies when exposed to the same protocol.

Given these results, we asked if a *fabH* null strain could be constructed by direct transduction of the *fabH::kan* null allele from strain CL111 into strains carrying a single functional copy of *fabH*. We used wild-type strain UB1005 and two different derivatives of this strain as transduction recipients. Strain YFJ294 carried pYFJ85 and expressed AasS, whereas strain YFJ293 carried vector plasmid pBAD24. The transduction mixtures were plated on LB medium supplemented with octanoate, oleate, and arabinose and incubated at 37 °C for 2 days to permit the strains lacking AasS activity to form colonies.

All three strains gave kanamycin resistant colonies, and a colony from each strain background was chosen for further analysis. The strains were designated as strains YFJ303 (derived from YFJ294, the pAasS strain), YFJ304 (derived from YFJ293, the vector plasmid strain), and YFJ305 (derived from the wild-type strain, UB1005). The latter two strains, YFJ304 and YFJ305, formed very small colonies irrespective of fatty acid supplementation (data not shown). In contrast, the growth of the AasS-containing construct, YFJ303, was essentially identical to that of strain YFJ262 (Figure 4) (strains YFJ303 and YFJ262 were interchangeable except when the *cfa* mutation of the former strain was required). In addition, we confirmed by PCR amplification of the chromosomal *fabH* region followed by restriction mapping of the PCR products (Figure 1S of the Supporting Information) that the insertion of strain YFJ305 (10) was as expected. However, strains YFJ304 and YFJ305 could not be used for physiological experiments because significantly larger colonies often appeared on plates after prolonged incubation in the presence or absence of a fatty acid supplement (Figure 2S of the Supporting Information). When these larger colonies were picked and purified, they gave uniformly large colonies comparable in size to those of the wild-type strain after overnight incubation at 37 °C (Figure 2S of the Supporting Information). Since the strains retained the $\Delta fabH::kan$ marker in which the kanamycin resistance determinant replaced the FabH active site region, they must be extragenic suppressors. Since such fast growing cells would readily take over liquid cultures of the authentic $\Delta fabH::kan$ strains, we used strains in which the $\Delta fabH$ phenotype was bypassed by the presence of AasS with octanoate supplementation and engendered the $\Delta fabH$ phenotype by removal of the fatty acid supplement or by shutting down AasS expression (or both). However, this bypass approach did not result in cells that grew as rapidly as the extragenic suppressor strains, and thus, the possibility of takeover of liquid cultures by suppressor strains remained. We are currently in the process of

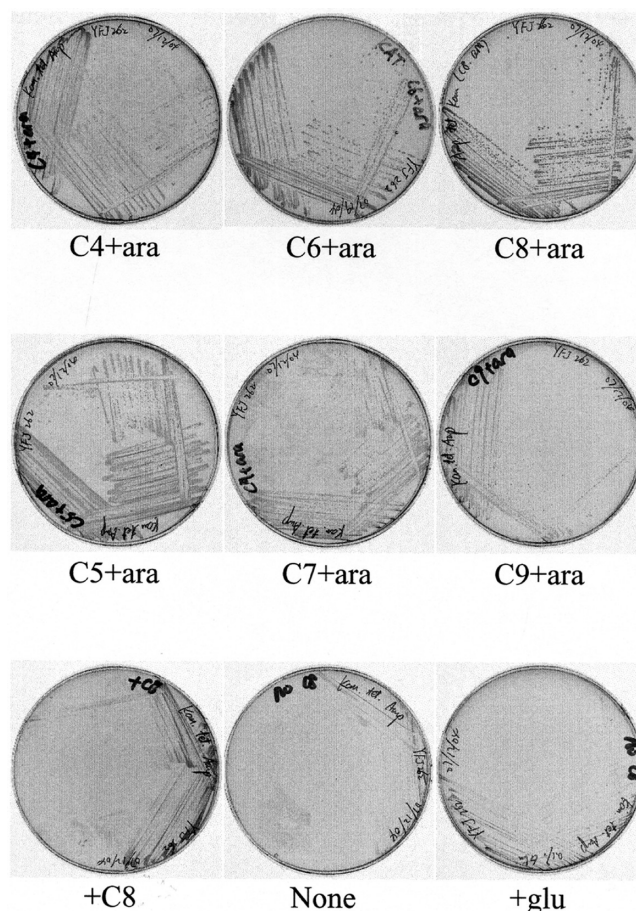


FIGURE 4: Growth of the $\Delta fabH$ pYFJ85 (AasS)-containing strain YFJ262 streaked on LB plates containing ampicillin and a fatty acid (1 mM) and/or arabinose (0.02%) as shown. The control plates (bottom row) were as follows: +C8, plates containing octanoate but no arabinose (the residual growth can be attributed to basal expression from the pBAD promoter); None, plates with no fatty acid or arabinose supplements; and +glu, plates containing 0.1% glucose (which represses basal expression of the pBAD promoter). The plates were incubated at 37 °C for 24 h.

identifying the extragenic suppressor mutation(s) that allows growth of $\Delta fabH$ strains. It should be noted that in their systematic deletion analysis of the *E. coli* chromosome (the Keio collection), Baba and co-workers (17) reported that *fabH* is not essential for normal growth. However, we find (A. Smith and J. E. Cronan, unpublished data) that the *fabH721::kan* strain, JW1077-1, of the Keio collection obtained from the Coli Genetic Stock Center (Yale University) carries a suppressor mutation as well as the *fabH* deletion allele. Phage P1 transduction of the JW1077-1 *fabH* allele into wild-type strain MG1655 with selection for kanamycin resistance resulted in the same syndrome (very small colonies that give rise to large colonies) that we observed in our constructions. (The selection for the suppressor mutation in the Keio strain is probably due to the natural tendency to select large colonies and discount small colonies as abortive transformants.)

Our original hypothesis was that growth of *fabH* null mutant strains would require both AasS induction and exogenous fatty acid supplementation. This was only partially the case (Figure 4). First, the strains grew, albeit very slowly, on medium lacking fatty acid supplementation. This very slow growth was also seen when chemically defined medium was used in place of LB medium and when basal level expression of AasS from the pBAD

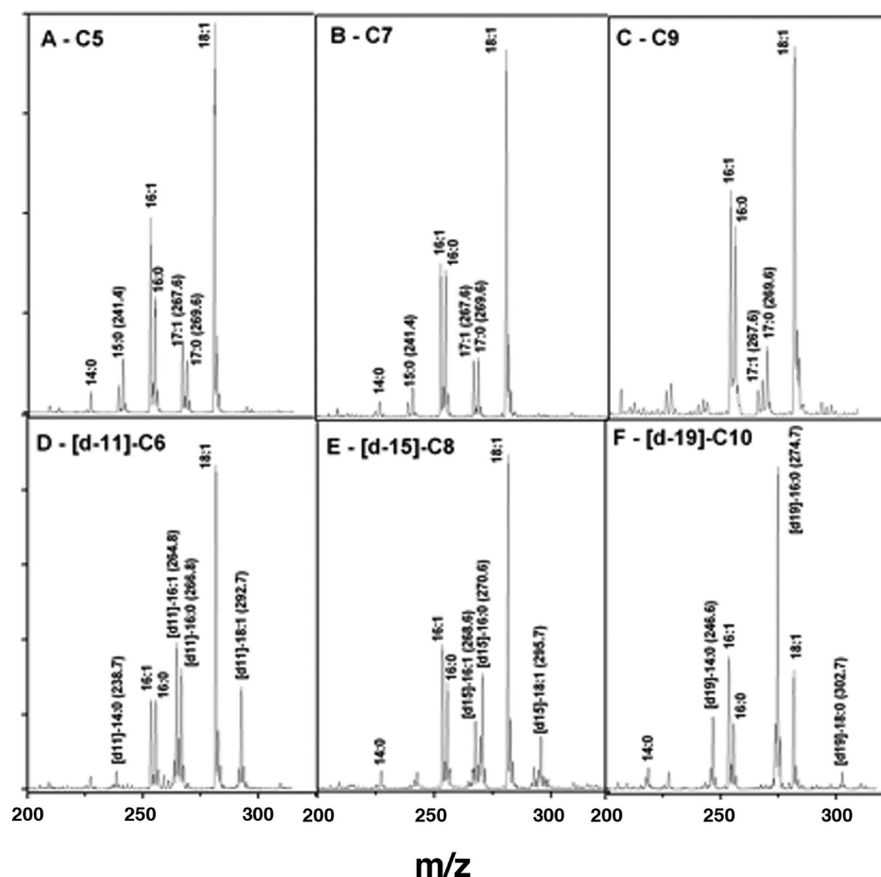


FIGURE 5: Mass spectra (CID ES-MS) of the phospholipid acyl chains of strain YFJ305 $\Delta fabH$ *cfa::kan* carrying the AasS encoding plasmid, pYFJ85. The cultures were grown overnight in the presence of 100 μ M fatty acid and 0.02% arabinose. The spectra shown are those of experiment 2 of Table 1S of the Supporting Information.

promoter was suppressed by addition of glucose (Figure 4). These findings suggested that the *fabH* null mutant retained some fatty acid synthetic activity. [Prior work (10) did not mention the very slow growing colonies formed by the $\Delta fabH$ strains presumably because the plates were scored before these colonies appeared.] Upon supplementation with a fatty acid with induction of AasS expression by addition of arabinose, much more rapid growth was realized (Figure 4). Note that during our construction of the original mutant strains two fatty acid supplements were present, oleate (to alleviate the need for unsaturated fatty acid synthesis) and octanoate. Octanoate was chosen because it is a good AasS substrate (5, 7, 18) and because octanoyl-ACP enters fatty acid synthesis immediately before the branch point where fatty acid synthesis splits into saturated and unsaturated pathways. Hence, octanoate should provide a primer for synthesis of both saturated and unsaturated fatty acids. This was the case; rapid growth did not require oleate. Further studies showed that butyrate and hexanoate were less effective supplements whereas decanoate (not shown) was inhibitory to growth (Figure 4). The odd chain length fatty acids, pentanoic, heptanoic, and nonanoic acids, also supported growth, with pentanoic acid being the best of these supplements, although the growth on these acids was less robust than seen on the even chain fatty acids (Figure 4) perhaps due to a decreased level of synthesis of unsaturated fatty acids.

To test if the *fabH* strain used exogenous short chain fatty acids as primers, we grew cultures of a $\Delta fabH$ Δcfa strain supplemented with either a deuterium-labeled fatty acid of even chain length ([*d*-11]-C6:0, [*d*-15]-C8:0, or [*d*-19]-C10:0) or an unlabeled odd chain length fatty acid (C5:0, C7:0, or C9:0).

Incorporation was then assayed by electrospray collision-induced mass spectrometry (Figure 5). Incorporation of these acids would test if significant levels of exogenous fatty acids were elongated and incorporated into glycerophospholipid acyl chains. Moreover, if the labeled acids retained their deuterium contents, this would indicate that they had been incorporated intact. Elongation of each of the fatty acid supplements was observed in AasS-expressing strain YFJ315 (Figure 5A), whereas as expected from the ^{14}C -labeled fatty acid incorporation studies, no incorporation was detected in vector control strain YFJ316 (data not shown). The deuterated C6 and C8 acids were incorporated with full retention of deuterium into long chain saturated fatty acids (C14:0 and C16:0) and unsaturated fatty acids (C16:1 and C18:1). The exception was the third supplement, [*d*-19]-C10:0. As expected, this precursor gave no labeled unsaturated fatty acids ([*d*-19]-C16:1 or [*d*-19]-C18:1) because the chain would enter fatty acid synthesis beyond the point where unsaturated fatty acid synthesis branches from the common (saturated) pathway. A large accumulation of labeled [*d*-19]-C16:0 and a dramatically reduced peak of de novo synthesized C18:1 were observed. A straightforward explanation for the decreased level of de novo synthesis of C18:1 would be that the exogenously supplied C10:0 chains would titrate the capacity of the FabF 3-ketoacyl-ACP synthase, leading to a deficiency of the longer unsaturated acid (which is the reaction unique to FabF) (7). The growth defect could be due to the decreased fluidity of the cell membrane due to the increased saturated:unsaturated ratio in the glycerophospholipids. Indeed, the defective growth of this strain on decanoate was largely rescued by addition of the unsaturated acid, oleate, to the

medium (data not shown). It should be noted that although the strain used was β -oxidation proficient, this was of no consequence since fatty acids shorter than C12 are not degraded by *E. coli* due to the mechanism that regulates expression of the genes of the pathway (2).

The *cfa* mutation was essential for analysis of products of the odd chain length fatty acid supplements because the cyclopropane fatty acids of *E. coli* have the same masses as the unsaturated elongation products (C15:1 and C17:1) that would result from elongation of the odd chain length fatty acids. Although *E. coli* does not normally synthesize odd chain length fatty acids, both C15:0 and C17:0 saturated fatty acids and a C17:1 unsaturated fatty acid were detected in the phospholipids of the cells grown with any of the three odd chain length fatty acid supplements (C5, C7, and C9) (Figure 5). These data indicated that *E. coli* fatty acid biosynthetic machinery tolerated the odd chain length acyl-ACPs. As expected from the [^{14}C]nonanoic acid labeling studies (Figure 2), the C9 supplement like the C5 and C7 supplements resulted in a C17:1 unsaturated fatty acid, indicating that FabA can use 3-hydroxyundecanoyl-ACP as a substrate. It was also noteworthy that no C19:1 acid was formed in any of the odd chain fatty acid supplementation experiments, indicating that FabF (3-ketoacyl-ACP synthase II), the enzyme responsible for elongation of the C16:1 acid in *E. coli*, was unable to elongate C17:1-ACP.

AasS Expression Allows Supplementation of *fabA* and *fabB* Strains with *cis*-5-Dodecenoic Acid. Mutants of *E. coli* with lesions in either the *fabA* or *fabB* genes are defective in unsaturated fatty acid biosynthesis and require supplementation with an appropriate unsaturated fatty acid (e.g., oleate) for growth (1). FabA is a bifunctional enzyme that introduces the double bond of these acids by first dehydrating 3-hydroxydecanoyl-ACP to *trans*-2-decenoyl-ACP, the double bond of which is then isomerized to *cis*-3-decenoyl-ACP (Figure 1). Several cycles of elongation then result in the unsaturated acyl chains of the membrane lipids (1). Recent *in vivo* labeling studies have shown that FabB is required for the first of these elongations, that of *cis*-3-decenoyl-ACP to *cis*-5-dodecenoyl-ACP (19). These data argued that if *cis*-5-dodecenoic acid was a suitable AasS substrate, then growth of a *fabB* mutant strain should result in the presence of *cis*-5-dodecenoic acid upon expression of AasS. This was the case (Figure 6). The combination of *cis*-5-dodecenoic acid supplementation and AasS expression allowed growth of strains carrying null mutations in either *fabB* or *fabA*, thereby confirming the *in vivo* labeling results. However, growth was slow, and high levels of AasS expression and high concentrations of the supplement were required. This was expected since unsaturated fatty acids are known to be poor substrates for AasS (5). Indeed, we expected to be able to supplement growth of a *fabA* strain with *cis*-3-decenoic acid, but this failed. Subsequent *in vitro* assays showed that AasS was unable to couple this acid to ACP (data not shown). It should be noted that 0.2% arabinose was needed for growth of the *fabA* and *fabB* strains whereas lower concentrations (or no arabinose) sufficed in other experiments. This is presumably due to the relative activities of AasS with the various fatty acid substrates. Note also that arabinose at 0.2% results in modest growth inhibition of wild-type strains carrying plasmid pYFJ85, perhaps due to competition between AasS and malonyl-CoA:ACP transacylase (FabD) for ACP.

DISCUSSION

Expression of AasS in *E. coli* clearly allows exogenous fatty acids to access both the fatty acid and lipid A synthetic pathways

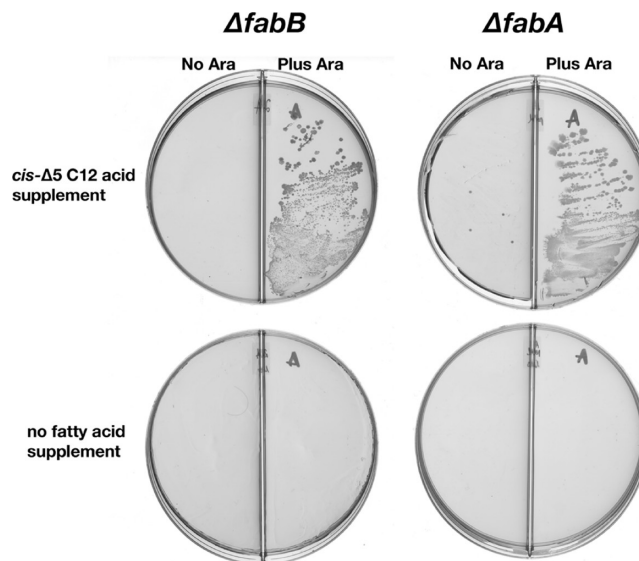


FIGURE 6: Growth of strains JWC280 ($\Delta fabA \Delta fadA::cml \Delta fadII::kan$) and GRT23 ($\Delta fabB \Delta cfa::kan$) carrying AasS encoding plasmid pYFJ85 in the presence or absence of *cis*-5-dodecenoic acid (0.5 mg/mL from Aldrich) and in the presence or absence of arabinose (0.2%). The LB plates were incubated for 3 days at 37 °C. The plates are divided into halves by a wall. Both strains are derivatives of strain BW25111 (34) and were constructed by phage λ Red-mediated recombination and transduction (34). None of these media supported detectable growth of the same strains carrying vector plasmid pBAD24 in place of pYFJ85 (data not shown).

of *E. coli*. The acids are efficiently activated to their ACP thioesters and are elongated or incorporated directly into phospholipids or lipid A. The fact that the radioactive short chain fatty acids labeled in the carboxyl group were converted to normal length glycerophospholipid acyl chains argues that at least a portion of the acids had been incorporated intact since the first cycle of β -oxidation would have removed the label from the chain. Subsequent mass spectral studies with deuterated and odd chain length acids showed that the acids were incorporated intact. These findings indicate that mechanistic questions of fatty acid synthesis and subsequent distribution of the acyl chains can now be approached *in vivo* as well as *in vitro*. It should be noted that prior work with *V. harveyi*, the source of AasS, had shown that exogenous fatty acids were efficiently taken up, elongated, and incorporated into the complex lipids of this bacterium (20). However, the routes of incorporation remain unclear (21). Recent work showed that strains of *V. harveyi* with an inactive *aasS* gene retained the ability to incorporate exogenous fatty acids, although the $\Delta aasS$ strains were somewhat defective in incorporation at low exogenous acid concentrations (7).

The AasS-engendered uptake and elongation of short and medium chain fatty acids also allowed growth of unsaturated fatty acid auxotrophs and of strains lacking *fabH*-encoded 3-ketoacyl-ACP synthase III, the enzyme responsible for priming fatty acid synthesis. However, the mass spectral data of Figure 5 show that an appreciable level of fatty acid synthesis occurred in the $\Delta fabH$ strain without AasS-facilitated incorporation of the exogenous acid (the unlabeled acids in the deuterated acid experiments and the even numbered chains in the odd chain supplementation experiments). Moreover, the extent of incorporation of the exogenous acid varied markedly in the two experiments (Table 1S of the Supporting Information). We believe this is due to extragenic suppressor mutations that allow growth of

$\Delta fabH$ strains through bypass of the enzyme defect (Figure 2S of the Supporting Information); the inactivated *fabH* allele remained intact. These suppressor mutations arose spontaneously, and the suppressed strains grew much more rapidly than $\Delta fabH$ strains and more rapidly than $\Delta fabH$ strains expressing AasS in the presence of fatty acid supplementation (Figure 2S of the Supporting Information). The faster growth rates allowed the suppressor strains to rapidly take over liquid cultures which almost certainly accounts for the variability seen in Table 1S of the Supporting Information. Hence, the mass spectral data should not be taken as an indicator of the residual fatty acid synthetic capacity of $\Delta fabH$ strains, but only as an indication that exogenous fatty acids are incorporated intact.

It should be noted that in the absence of AasS expression (in the empty vector control cultures) some incorporation and elongation of short chain fatty acids occurred (Figure 2). This incorporation was chain length-dependent and favored octanoate and nonanoate over hexanoate (Figure 2). Elongation of these acids indicated that they somehow had been converted to acyl-ACPs. This was consistent with prior work from this lab showing that low levels of octanoate were attached to the pyruvate and 2-oxoglutarate dehydrogenases as an intermediate in lipoic acid synthesis in a strain in which the only known pathway of octanoate attachment to these proteins was ACP-dependent (22). In both cases, only traces of fatty acids are incorporated. For example, the amounts of deuterated or odd chain length fatty acids incorporated are insufficient to alter the fatty acid compositions of the phospholipids as assayed by mass spectrometry (Figure 3S of the Supporting Information). What is the source of this trace incorporation? Incorporation is almost entirely dependent on the FadD acyl-CoA synthetase (Figure 4S of the Supporting Information), indicating that the incorporation proceeds through an acyl-CoA intermediate. The FadD dependence is also consistent with the chain length dependence because octanoate is an appreciably better FadD substrate than hexanoate (23, 24). Moreover, FadD was not unique in this regard; its function could be replaced by the yeast FAA1 acyl-CoA synthetase (Figure 5S of the Supporting Information). The only known route for transfer of an acyl chain from CoA to ACP involves the 3-ketoacyl-ACP synthases (25). The acyl chain would be transferred from CoA to the active site thiol of a 3-ketoacyl-ACP synthase and would subsequently be transferred from the active site thiol to ACP to form an acyl-ACP. Although we were unable to demonstrate such an acyl-CoA:ACP transacylase activity with any of the purified *E. coli* 3-ketoacyl-ACP synthases (data not shown), recent work with the 3-ketoacyl-ACP synthases of Gram-positive bacteria shows that these enzymes can be efficiently primed with long chain acyl-CoA substrates (26, 27). In any case, the activity seen in the absence of AasS is not sufficient to support either lipoic acid synthesis or growth of strains lacking the fatty acid synthetic enzyme FabH, FabA, or FabB. Direct incorporation of the longer fatty acids, dodecanoate and tetradecanoate, into phospholipids was expected since in vitro assays with the relevant acyltransferases showed that acyl-CoA esters were competent acyl donors. Indeed, for dodecanoate and tetradecanoate, direct incorporation into complex lipids was dominant over elongation, whereas the opposite results were seen upon AasS expression (Figure 3).

SUPPORTING INFORMATION AVAILABLE

Analyses of the genetic constructs, mass spectra, a table of mass spectroscopic values, and figures of data characterizing the

endogenous elongation activity. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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