Functional Replacement of the FabA and FabB Proteins of *Escherichia coli* Fatty Acid Synthesis by *Enterococcus faecalis* FabZ and FabF Homologues*

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The anaerobic unsaturated fatty acid synthetic pathway of *Escherichia coli* requires two specialized proteins, FabA and FabB. However, the fabA and fabB genes are found only in the Gram-negative α- and γ-proteobacteria, and thus other anaerobic bacteria must synthesize these acids using different enzymes. We report that the Gram-positive bacteria *Enterococcus faecalis* encodes a protein, annotated as FabZ1, that functionally replaces the *E. coli* FabA protein, although the sequence of this protein aligns much more closely with *E. coli* FabZ, a protein that plays no specific role in unsaturated fatty acid synthesis. Therefore *E. faecalis* FabZ1 is a bifunctional dehydratase/isomerase, an enzyme activity here-fore confined to a group of Gram-negative bacteria. The FabZ2 protein is unable to replace the function of *E. coli* FabZ2, although FabZ2, a second *E. faecalis* FabZ homologue, has this ability. Moreover, an *E. faecalis* FabF homologue (FabF1) was found to replace the function of *E. coli* FabB, whereas a second FabF homologue was inactive. From these data it is clear that bacterial fatty acid biosynthetic pathways cannot be deduced solely by sequence comparisons.

*Escherichia coli* provides the paradigm for the dissociated (or type II) fatty acid biosynthetic systems (1, 2). In type II systems, which are found in most bacteria and plants, the individual synthetic steps are catalyzed by a series of discrete proteins encoded by unique genes (1, 2). Four reactions are required to complete each round of fatty acid elongation. In some cases, multiple enzymes are available to catalyze a given step, suggesting that these proteins have different substrate specificities and/or physiological functions. The *E. coli* fabA and fabB genes encode β-hydroxyacyl-ACP3 dehydratases, enzymes that convert β-hydroxyacyl-ACPs to trans-2 unsaturated acyl-ACPs (3–6). The trans-2 unsaturated acyl-ACPs produced are the substrates of enoyl-ACP reductases that catalyze the last step of fatty acid elongation cycle (5). FabA and FabZ differ in that FabZ catalyzes the dehydration reaction (4), whereas FabA is a bifunctional enzyme that also catalyzes isomerization of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP (3, 6–8), the key step of the classical anaerobic unsaturated fatty acid biosynthetic pathway (3). Unlike the trans-2 double bond, the cis-3 double bond cannot be removed by enoyl-ACP reductase but instead is retained to form the double bonds of the unsaturated fatty acid moieties of the membrane lipids. The fabA gene is essential for growth of *E. coli* and *Pseudomonas aeruginosa* as shown by both mutational studies (9–11) and by inhibition with a substrate analogue (3, 12). It is clear that unsaturated fatty acid synthesis is the essential physiological role of FabA because loss of FabA activity in *vivo* specifically blocks the synthesis of unsaturated fatty acids (10, 12). Moreover, fabA mutant strains grow when supplemented with appropriate unsaturated fatty acids, whereas saturated fatty acids fail to support growth (9, 10). It was thought that all bacteria that synthesize unsaturated fatty acids during anaerobic growth utilize a FabA protein. However, recent bacterial genome sequences show that many organisms lack a recognizable FabA homologue, although anaerobically grown cells of these organisms are known to contain unsaturated fatty acids (for review see Refs. 13 and 14). Indeed in the extant genome sequences FabA homologues are encoded only in the genomes of α- and γ-proteobacteria. Therefore, there seem to be two possibilities to explain anaerobic unsaturated fatty acid synthesis in those bacteria that lack FabA. The first possibility is that chemistry of the pathway is similar to that of *E. coli*, but the amino acid sequences of the required proteins are sufficiently different from FabA such that they are not recognized as FabA homologues. The second possibility is that different chemistry is used that involves markedly different proteins. It seems that several anaerobic unsaturated fatty acid biosynthetic pathways may exist because *Streptococcus pneumoniae*, which lacks a FabA homologue, has an enzyme called FabM that performs the key trans-2 to cis-3 isomerization reaction in vitro (the pathway has not yet been confirmed by mutant studies) (8). However, FabM seems specific for streptococci and hence irrelevant to other FabA-lacking organisms that synthesize unsaturates during anaerobic growth. In the latter bacteria it seems possible that a FabA homologue is present, but the gene has been annotated as encoding a different enzyme. For example several bacterial genomes contain two copies of genes annotated as encoding FabZ proteins. *E. coli* FabZ is a protein having weak homology (28% identical residues) to FabA. This sequence homology plus the location of the fabZ gene in a cluster of genes involved in lipid A biosynthesis was sufficient for Raetz and co-workers (4) to test whether *E. coli* FabZ could dehydrogenate β-hydroxyacyl-ACP, a lipid A precursor. This enzyme activity was demonstrated, and thus the FabZ was called β-hydroxyacyl-ACP dehydratase (4). However, FabZ was later shown to dehydrogenate β-hydroxyacyl-ACPs of all chain lengths tested (15), and thus, the designation as β-hydroxyacyl-ACP dehydratase is a misnomer. If the FabZs...
of other organisms have some broad chain length specificity as *E. coli* FabZ (15), then the presence of a second fabZ gene seems redundant unless the encoded protein performs another function such as introduction of a cis double bond. Therefore, it seemed possible that a class of proteins having much stronger sequence similarity to *E. coli* FabZ than to *E. coli* FabA might possess the enzymatic capability of FabA, i.e. the ability to introduce a cis double bond. We report that this is the case in the Gram-positive pathogenic bacterium, *Enterococcus faecalis* V583, an organism having a fatty acid composition very similar to that of *E. coli* (16, 17).

FabA is not the sole *E. coli* enzyme specifically required for unsaturated fatty acid synthesis, FabB is also essential (18). A similar result has been reported for *P. aeruginosa* (11). FabB is β-ketoacyl-ACP synthase I (19, 20), an enzyme thought to channel the biosynthetic intermediate made by FabA into the mainstream fatty acid synthetic pathway. Consistent with this view FabA and FabB show covariance within bacterial genomes (14). Indeed, in the α-proteobacteria and pseudomonads (unlike other γ-proteobacteria such as *E. coli*), the fabA and fabB genes are found in two-gene operons (11, 14). Therefore, given a protein with FabA function, we expected that although *E. faecalis* has no recognizable FabB homologue, the genome should encode a protein having FabB function. We report that one of the FabF homologues of *E. faecalis* has FabB function. Therefore, the synthesis of unsaturated fatty acids in *E. faecalis* appears to be catalyzed by two proteins that have been consistently annotated as having no specific roles in the introduction of the cis double bond.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains, Plasmids, and Growth Media

The *E. coli* strains and plasmids used in this study are listed in Table I. Luria-Bertani medium (17) was used as the rich medium for *E. coli* growth. The phenotypes of fab strains were assessed on rich broth (RB) medium (24).

| Strains | Relevant characteristics | Source |
|---------|--------------------------|--------|
| DH5α | lacZΔM15(ΔlacZYA-argF)U169 recA1 endA1 hsdR17 | Laboratory collection |
| CY244 | fabB15(Ts) fabF | Ref. 39 |
| JWC275 | fabB15(Ts) fabF::kan | Ref. 40 |
| CY242 | fabB15(Ts) | Ref. 19 |
| K1060 | fabB5 | Ref. 19 |
| MR52 | fabF::kan | Ref. 41 |
| CY57 | fabA::kan | Ref. 42 |
| MI121 | Cm, fabA::lacZ-cat | Ref. 43 |
| MG1655 | E. coli wild type | |
| BL21(DE3) | E. coli B F ompT rpsL mcrA (DE3) | |
| HW1 | Transductant of K1060 (pHW1) with phage P1 grown on MR52 | This work |
| HW5 | K1060 (pHW13) Km, Amp, fabB5 fabF::kan | This work |
| HW7 | *E. coli* DY330 fabZ::kan carrying pHW22 | This work |
| HW8 | Transductant of MH121 with phage P1 grown on the cfa::kan strain YYY1257 (44) | This work |

### Plasmids

| Plasmids | Relevant characteristics | Source |
|----------|--------------------------|--------|
| pBAD24 | Amp+, expression vector | Ref. 45 |
| pCR2.1TOPO | Amp+, Km<sup>+</sup>, TA cloning vector | Invitrogen |
| pET28(b) | Km<sup>+</sup>, expression vector | Novagen |
| pSU12 | Cm<sup>+</sup>, cloning vector | Ref. 46 |
| pH11 | Cm<sup>+</sup>, pSU19 carrying H. influenzae fabB | Ref. 27 |
| pH12 | Amp<sup>+</sup>, Km, fabF1 of *E. faecalis* in pCR2.1TOPO | This work |
| pH13 | Amp<sup>+</sup>, fabF1 from pH11 cut with BspHI and PstI and ligated between the NcoI and PstI sites of pBAD24 | This work |
| pH14 | Amp<sup>+</sup>, fabF2 from pH12 constructed as pH13 | This work |
| pH17 | Amp<sup>+</sup>, Km, fabZ1 of *E. faecalis* in pCR2.1TOPO | This work |
| pH18 | Amp<sup>+</sup>, Km, fabZ2 of *E. faecalis* in pCR2.1TOPO | This work |
| pH19 | Amp<sup>+</sup>, fabZ1 from pH17 constructed as pH18 | This work |
| pH20 | Amp<sup>+</sup>, fabZ2 from pH16 constructed as pH13 | This work |
| pH22 | Amp<sup>+</sup>, pBAD24 carrying C. acetobutylicum fabZ | This work |
| pH26 | Km<sup>+</sup>, fabF1 from pH11 cut with BspHI and EcoRI ligated between the NcoI and EcoRI sites of pET28(b) | This work |
| pH27 | Km<sup>+</sup>, fabF2 from pH12 constructed as pH26 | This work |
| pH29 | Km<sup>+</sup>, fabZ1 from pH17 constructed as pH28 | This work |
| pH30 | Km<sup>+</sup>, fabZ2 from pH18 constructed as was pH26 | This work |
| pH71 | Cm<sup>+</sup>, fabZ1 from pH19 digested with BamHI and PstI ligated between the same sites of pSU21 | This work |
| pH72 | Cm<sup>+</sup>, fabZ2 from pH20 constructed as was pH26 | This work |
E. faecalis Fatty Acids

Expression and activity of the E. faecalis FabZ1, FabZ2, and FabF1 proteins in E. coli. A, derivatives of E. coli strain BL21(DE3) carrying plasmids encoding the E. faecalis proteins under control of a phage T7 promoter were induced, and then rifampicin was added to inhibit the host RNA polymerase. The proteins were then labeled with [35S]methionine and analyzed by SDS gel electrophoresis followed by autoradiography. B, argenation thin layer chromatographic analysis of [1-14C]acetate-labeled E. coli strain H8S carrying either pWH13 (fabZ1) or pWH14 (fabZ2). Cultures of plasmid-containing strains were induced with arabinose (ARA) or left uninduced. Following overnight growth, the phospholipids were extracted, and their fatty acid moieties were converted to their methyl esters by transesterification with sodium methoxide. The methyl esters were then separated by argentation thin layer chromatography followed by autoradiography. The migration positions of the fatty acid species are shown. The designations are: Sat, saturated fatty acids; 39:1C16:1, palmitoleic (cis-9-hexadecenoic) acid; 31:1C18:1, cis-vaccenic (cis-11-octadecenoic) acid. Triclosan (TCL) was added at 0.1 μg/ml. S denotes the E. coli fatty acid standard (strain MG1655 labeled with [1-14C]acetate), whereas V denotes the culture carrying the vector plasmid pBAD24.

a French pressure cell at 18,000 lb/in². The lysate was centrifuged in a JA-20 rotor at 35,000 × g at 4 °C for 1 h to remove cell debris. Ammonium sulfate was added to the supernatant to 45% of saturation, and the precipitated protein was removed by centrifugation. Additional ammonium sulfate was added to bring the supernatant to 80% of saturation, and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 2 ml of lysis buffer and dialyzed for 5 h at 4 °C against 2 liters of the same buffer. Protein concentrations were determined by the Bradford assay with bovine serum albumin as the standard.

In Vitro Fatty Acid Synthesis Assay—The fatty acid synthesis assay mixtures contained 0.1 mM L-leucine, 0.1 mM sodium phosphate, pH 7.0, 1 mM β-mercaptoethanol, 100 μM octanoyl-ACP, 0.17 mM NADH, 0.149 mM NADPH, 54 μM ACP, and 30 μg of protein extracts of strain MG1655 or of MH121 carrying various plasmids in a final volume of 40 μl. Triclosan, when present, was added to final concentration of 1 mM. The reaction mixtures were incubated for 5 min at room temperature to allow triclosan to inactivate FabI followed by the addition of 45 μM [2,14C]malonyl-CoA (specific activity, 55 mCi/mmol) to initiate the reaction. After incubation at 37 °C for 10 min, the reactions were stopped by placing the tubes in ice slush. Samples of the mixtures were mixed with gel loading buffer and analyzed by conformationally sensitive gel electrophoresis on 15% polyacrylamide gels containing 2.5 mM urea at 4 °C (22). The gels were fixed, soaked in Enlightning (DuPont), dried, and exposed to x-ray film.

Phospholipid Fatty Acid Compositions—The cultures were grown aerobically at different temperature in RB medium overnight, the phospholipids were extracted, and the fatty acid compositions were analyzed by mass spectroscopy as described previously (14). Fatty acid residues that comprise the active site and central helices share 58.7% identical residues. In each case all FabA active site residues (excepting Asp-84) are conserved. Indeed, if we adopt the focus of Leesong et al. (24) and consider the amino acid residues that comprise the active site and central helices (residues 60–90 of FabA), then FabZ1 shares 46.4 and 73.3% identical residues with E. coli FabA and FabZ, respectively, whereas the respective values for FabZ2 are 47.1 and 71.0%. Hence, within this key region the two E. faecalis proteins have higher sequence identities to E. coli FabZ than to E. coli FabA, and thus it was very reasonable to annotate these proteins as FabZ homologues rather than FabA homologues. In the case of the E. faecalis FabF proteins both sequences align more closely with E. coli FabF than with E. coli FabB. Both FabF1 and FabF2 are about 36% identical to E. coli FabB and 47–50% identical to E. coli FabF (E. coli FabB and FabF are 37.8% identical, whereas the two E. faecalis FabF proteins are 58.0% identical). The fabZ1 and fabF1 genes are adjacent and transcribed in the same direction with fabF1 being located upstream of fabZ1 (25). Located upstream of fabF1, but transcribed from the other DNA strand, is a homologue of E. coli fabL, a gene expected to encode an enoyl-ACP reductase. The fabZ2 and fabF2 genes are located within a large cluster of genes that encode homologues of all the proteins known to be required for saturated fatty acid biosynthesis in E. coli.

To test the functions of these proteins we cloned the fabZ and fabF genes of E. faecalis into different E. coli vectors. These included the phage T7 RNA polymerase-dependent vector pET28a, which allowed assay of expression of the encoded proteins by labeling cultures with [35S]methionine following the addition of rifampicin to block the synthesis of chromosomally encoded proteins (Fig. 1A). Plasmids that carried the

RESULTS

The E. faecalis genome encodes two homologues each of FabZ and FabF. The FabZ1 protein shares 25.3 and 41.4% identical residues with E. coli FabA and FabZ, respectively, whereas the respective values for FabZ2 are 22.0 and 50.0 (FabZ1 and FabZ2 share 58.7% identical residues). In each case all FabA active site residues (excepting Asp-84) are conserved. Indeed, if we adopt the focus of Leesong et al. (24) and consider the amino acid residues that comprise the active site and central helices (residues 60–90 of FabA), then FabZ1 shares 46.4 and 73.3% identical residues with E. coli FabA and FabZ, respectively, whereas the respective values for FabZ2 are 47.1 and 71.0%. Hence, within this key region the two E. faecalis proteins have higher sequence identities to E. coli FabZ than to E. coli FabA, and thus it was very reasonable to annotate these proteins as FabZ homologues rather than FabA homologues. In the case of the E. faecalis FabF proteins both sequences align more closely with E. coli FabF than with E. coli FabB. Both FabF1 and FabF2 are about 36% identical to E. coli FabB and 47–50% identical to E. coli FabF (E. coli FabB and FabF are 37.8% identical, whereas the two E. faecalis FabF proteins are 58.0% identical). The fabZ1 and fabF1 genes are adjacent and transcribed in the same direction with fabF1 being located upstream of fabZ1 (25). Located upstream of fabF1, but transcribed from the other DNA strand, is a homologue of E. coli fabL, a gene expected to encode an enoyl-ACP reductase. The fabZ2 and fabF2 genes are located within a large cluster of genes that encode homologues of all the proteins known to be required for saturated fatty acid biosynthesis in E. coli.

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FabZ1, fabZ2, and fabF1 genes expressed proteins of 17, 15, and 45 kDa that correspond to the values expected from the deduced protein sequences of FabZ1 (16.2 kDa), FabZ2 (15.2 kDa), and FabF1 (43.2 kDa). (Note that the expression levels of FabZ1 and FabZ2 are essentially identical when corrected for the differing methionine contents of the two proteins.) Comparable results were obtained in similar experiments with fabF2 (data not shown). Given that these genes could be satisfactorily expressed in the heterologous host (the ribosome-binding sites used were those of vectors pET28 and pBAD24, which are identical), we tested the function of the genes by introduction of various plasmids into several E. coli fab mutant strains.

E. faecalis FabZ1 Has FabA Activity Both in Vivo and in Vitro—The fabZ1 and fabZ2 genes were cloned into the arabinose-inducible vector pBAD24 to give pHW19 (fabZ1) and pHW20 (fabZ2), respectively. These plasmids were introduced into two E. coli fabA mutant strains, MH121 and CY57, and the resulting transformants were tested for growth in absence of oleate supplementation. No growth of transformants of either strain was found in absence of oleate (data not shown). Although E. faecalis fabZ1 and fabZ2 failed to complement the E. coli fabA mutants, it remained possible that one or both of these plasmids supported unsaturated fatty acid synthesis, but that the levels of unsaturated fatty acids synthesized were insufficient for growth of the host strain. To test this possibility we introduced the pBAD24-derived plasmids into a derivative of the fabA null mutant strain MH121 that also carried a null mutation in cfa (44). These strains were grown in a medium supplemented with a cyclopropane fatty acid, induced with arabinose, and then labeled with [14C]acetate. The cellular phospholipids were extracted, and their fatty acid moieties were converted to their methyl esters that were analyzed by argentation thin layer chromatography, which resolves each of the unsaturated species from the saturated species and from one another. The cfa null mutation was introduced to increase the sensitivity of the assay by preventing conversion of any radioactive unsaturated fatty acids synthesized to their cyclopropane derivatives (which migrate with the saturated species). We found that the strain carrying the fabZ1 plasmid synthesized unsaturated fatty acids, whereas the strain carrying the fabZ2 plasmid did not (Fig. 1B). Similar results were seen in CY57, a strain that carries a temperature-sensitive point mutation in fabA (data not shown). The levels of unsaturated fatty acid synthesized were very low, presumably because of competition between FabZ1 and the host enoyl-ACP reductase (FabB) for trans-2-decenoyl-ACP (8). FabB would convert trans-2-decenoyl-ACP to decanoyl-ACP and thereby deprive FabZ1 of the isomerization reaction substrate. We tested the competition hypothesis by the addition of low concentrations of triclosan, a specific inhibitor of the E. coli enoyl-ACP reductase (FabB) to the strain carrying the fabZ1 plasmid. Upon the addition of 0.1 μg/ml triclosan the ratio of unsaturated fatty acids to saturated fatty acids synthesized increased by >10-fold (Fig. 1B). In contrast triclosan addition to the strain carrying the fabZ2 plasmid failed to result in unsaturated fatty acid synthesis. We attempted to find a concentration of triclosan that would increase the trans-2-decenoyl-ACP to FabZ1 while retaining sufficient enoyl-ACP reductase activity for growth. We were unsuccessful in these attempts, although we did find conditions that blocked the growth-dependent lysis that occurs upon starvation of fabA strains for unsaturated fatty acids (26) (data not shown).

We also tested FabZ1 for its ability to replace FabA in a cell-free fatty acid synthesis system prepared from an E. coli fabA null mutant strain (Fig. 2A). Incubation of a cell-free extract of a wild type strain of E. coli with [2-14C]malonyl-CoA, octanoyl-ACP, NADPH, NADH, and ACP results in formation of saturated and unsaturated fatty acids with the latter species being the dominant products. Upon the addition of triclosan the loss of enoyl-ACP reductase activity results in accumulation of saturated fatty acids. The ACP-bound products were analyzed by conformationally sensitive gel electrophoresis (47) using 15% polyacrylamide gels containing 2.5 M urea (8, 48) followed by fluorography. The migration positions of trans-2, cis-5-dodecadienoyl-ACP (C12:2 (Δ9,15c) and decanoyl-ACP are shown. It is clear from several other gels that C12:2 (Δ9,15c) is the middle band of the three major bands of lanes 2 and 5. The fastest moving band is a mixture of long chain acyl-ACPs. The abbreviations pEZ1, pEZ2, and VECTOR denote strain MH121 carrying plasmids pHW19 (fabZ1), pHW20 (fabZ2), and pBAD24 (vector), respectively. B, fatty acid products formed in vitro. The fatty acids synthesized in a reaction run as in A, expecting a 30-min incubation time were recovered after base hydrolysis and converted to their methyl esters, which were separated by argentation thin layer chromatography followed by autoradiography. Lane 1 was the extract of the wild type strain MG1655, whereas lanes 2–4 were, respectively, extracts of strain MH121 carrying plasmids pHW19 (fabZ1), pHW20 (fabZ2), or pBAD24 (vector). Sat, saturated fatty acids.

**FIG. 2.** *In vitro* synthesis of acyl-ACP and fatty acid species by a fabA strain expressing E. faecalis FabZ1 or FabZ2. A, cell-free extracts of arabinose-induced cultures of wild type (WT) strain MG1655 or of plasmid-containing derivatives of strain MH121 (fabA-cat) were supplemented with NADPH, NADH, octanoyl-ACP, and [2-14C]malonyl-CoA. Fatty acid synthesis was allowed to proceed for 10 min in the presence or absence of triclosan (added to 1 mM to block enoyl-ACP reductase activity) as shown. The ACP-bound products were analyzed by conformationally sensitive gel electrophoresis (47) using 15% polyacrylamide gels containing 2.5 M urea (8, 48) followed by fluorography. The migration positions of trans-2, cis-5-dodecadienoyl-ACP (C12:2 (Δ9,15c) and decanoyl-ACP are shown. It is clear from several other gels that C12:2 (Δ9,15c) is the middle band of the three major bands of lanes 2 and 5. The fastest moving band is a mixture of long chain acyl-ACPs. The abbreviations pEZ1, pEZ2, and VECTOR denote strain MH121 carrying plasmids pHW19 (fabZ1), pHW20 (fabZ2), and pBAD24 (vector), respectively. B, fatty acid products formed in vitro. The fatty acids synthesized in a reaction run as in A, expecting a 30-min incubation time were recovered after base hydrolysis and converted to their methyl esters, which were separated by argentation thin layer chromatography followed by autoradiography. Lane 1 was the extract of the wild type strain MG1655, whereas lanes 2–4 were, respectively, extracts of strain MH121 carrying plasmids pHW19 (fabZ1), pHW20 (fabZ2), or pBAD24 (vector). Sat, saturated fatty acids.

The Role of E. faecalis FabZ2—These results implied that fabZ2 should be the enzyme responsible for the general dehydration step of the fatty acid synthetic cycle and that FabZ1 might lack FabZ activity. To test these predictions we cloned fabZ1 and fabZ2 into the chloramphenicol-resistant vector pSU21 such that the E. faecalis genes were transcribed from the vector lac promoter. The plasmids were then transformed into E. coli strain HW7, a strain in which the chromosomal fabZ gene had been deleted and replaced with a kanamycin.
E. faecalis Fatty Acids

Fig. 3. Complementation of arabinose-dependent growth of strain HW7 by plasmids expressing E. faecalis fabZ1 or fabZ2. The E. faecalis genes together with the ribosome-binding site was moved from the pBAD24-derived plasmids into plasmid pSU19 resulting in pHW1 and pHW72, which, respectively, express fabZ1 and fabZ2 from the vector lac promoter. These plasmids were then transformed into E. coli HW7, a strain in which the chromosomal fabZ gene had been deleted, and FabB function was provided by a compatible plasmid carrying C. acetobutylicum fabZ under control of the vector arabinose (pBAD) promoter. The Petri plates shown contain RB medium supplemented as shown with arabinose and fucose as the inducer or anti-inducer, respectively, of C. acetobutylicum fabZ expression or isopropyl-β-D-thiogalactopyranoside (IPTG) to induce fabZ1 or fabZ2 expression (although induction was not required for growth because of the leakiness of the lac promoter). The plates were incubated overnight at 30 °C. The plasmids carried by the strains are shown on the schematic. The strain was grown at 30 °C because of the temperature-sensitive λ prophage carried by the host strain.

An E. faecalis FabF Homologue That Complements E. coli FabB Mutants—Our finding that FabZ1 had FabA activity suggested that E. faecalis might also encode a protein functionally analogous to E. coli FabB. The obvious candidate was FabF1 because it is encoded by the gene immediately upstream of fabZ1. Although E. faecalis is only a very distant relative of pseudomonads and α-proteobacteria, this situation was reminiscent (albeit with the opposite gene orientation) of the fabA fabB gene arrangement found in those bacteria. We therefore cloned E. faecalis fabF1 into an arabinose-inducible vector and tested for complementation of two E. coli fabB mutant strains. Both fabB mutations are point mutants, one of which results in temperature-sensitive growth. Transformants of both strains carrying the fabF1 plasmid grew well in the absence of unsaturated fatty acid supplementation, indicating complementation of the fabB mutations (Fig. 4). The fatty acid composition of the E. coli fabB strain K1060 carrying the fabF1 plasmid was determined by mass spectroscopy (27) and was found to contain levels of unsaturated fatty acids comparable with those given by introduction of a plasmid encoding E. coli fabB (data not shown). Similar fatty acid compositions were seen in the fabB(Ts) fabF mutant strain JWC275 (data not shown). To further test the specificity of E. faecalis FabF1 function, we introduced pHW13 into E. coli fabB fabF strain HW1 (27). This strain contains an unconditional fabB mutation plus a fabF null mutation. This strain would be unviable but for the presence of a p15 origin chloramphenicol-resistant plasmid that encodes the fabB of Haemophilus influenzae and thereby per-

mits growth (27). Because the replication origin of pHW13 is compatible with that of the H. influenzae fabB plasmid, we introduced pHW13 into strain HW1 and tested whether the resident fabB plasmid could be lost from the transformed strain. We screened for chloramphenicol-sensitive strains on LB medium containing ampicillin and kanamycin and obtained strain HW5. Despite loss of the H. influenzae fabB plasmid strain HW5 grew well over a wide temperature range in the absence of oleate, indicating that E. faecalis FabF1 could fully replace FabB function. Note that expression of FabF2 in strain K1060 gave very weak growth in the absence of oleate, although no growth was seen in a parallel experiment with strain CY242. Thus, FabF2 might have traces of FabB activity.

The Physiological Role of E. faecalis FabF2—We also tested the fabF1 and fabF2 genes for FabB function. The plasmids were transformed into two fabB(Ts) fabF strains, CY244 and JWC275, and subsequently tested on media supplemented with
or lacking oleate at 42 °C (Fig. 4). At this temperature these strains lack the β-ketoacyl-ACP synthase activities needed to elongate long chain (>C4) substrates and therefore are unable to grow even when the medium is supplemented with oleate (19, 20, 28). Restoration of FabB function allows growth without oleate supplementation, whereas restoration of FabF function allows growth only in presence of oleate (19, 20, 27). As expected from the data of Fig. 4, transformants carrying the fabF1 plasmid grew at 42 °C in either the presence or absence of oleate, thereby demonstrating complementation of the fabB mutation. In contrast the transformants carrying the fabF2 plasmid grew at 42 °C in the presence of oleate but failed to grow in the absence of oleate, demonstrating complementation of the fabF mutation (Fig. 4). Therefore E. faecalis FabF2, like E. coli fabF, catalyzed all of the elongation reactions required for the synthesis of saturated fatty acids but, unlike E. faecalis FabF1, could not replace the function of E. coli FabB in the synthesis of unsaturated fatty acids. Consistent with these data cell-free extracts of the fabB strain K1060 carrying the fabF1-encoding plasmid synthesized high levels of unsaturated fatty acids, whereas a parallel experiment with the fabF2-encoding plasmid gave only traces of unsaturated acids (Fig. 5). Reverse phase chromatography showed that the chain lengths of the saturated acids synthesized in vitro were found to be 12, 14, 16, and 18 carbon atoms when either fabF1 or fabF2 provided 3-ketoacyl-ACP synthase function (data not shown). The fact that fabZ1 complemented the fabB mutant precluded testing this gene for complementation of fabF.

**DISCUSSION**

We report the first evidence that a Gram-positive bacterium encodes a bifunctional dehydratase/isomerase. The two other Gram-positive bacteria thus far examined use either a desaturase (Bacillus subtilis) or an isomerase (S. pneumoniae) (8, 29, 30). The E. faecalis open reading frame annotated as FabZ1 functions like E. coli FabA and lacks FabZ function, although it aligns significantly more closely with E. coli FabZ than with E. coli FabA. Therefore, in this organism and perhaps numerous others the gene encoding a functional homologue of FabA masquerades as a FabZ-encoding gene. A parallel example is E. faecalis FabF1, a protein that appears to be a homologue of E. coli FabF but that has the function of E. coli FabB. It might be supposed that high resolution crystallographic structures of the known proteins might allow functional assignments to be made by the presence or absence of key amino acid residues without recourse to in vivo analyses such as those we report. However, in both of the present instances, this is not the case.

High resolution (2 Å) crystal structures of E. coli FabA and of the protein bound to a covalently bound model substrate are available (24). These structures show that nine residues face the active site. However, all but one of these resides are conserved in E. coli FabZ, which lacks isomerase activity. The single nonconserved residue, Asp84 of FabA, which is Glu in E. coli FabZ, was proposed to be responsible for the different products synthesized by these enzymes (24). However, both FabZ1 and FabZ2 of E. faecalis have Glu at this position, and hence this proposal cannot explain the presence or absence of isomerase activity. The three gaps in the E. coli FabZ sequence relative to E. coli FabA (consisting of FabA surface loops) are conserved in both E. faecalis FabZ1 and FabZ2, which also rules out a function in isomerization for these regions. It seems interesting that the extent β-hydroxacyl-ACP dehydratases cleanly group into two classes: those that are FabA-like and those that resemble FabZ. The lack of proteins having sequences intermediate between FabA and FabZ seems curious given that E. faecalis FabZ1 has isomerase activity and that FabA can replace FabZ in a defined in vitro fatty acid synthesis system (15).

In the case of the 3-ketoacyl-ACP synthases, crystal structures of very high resolution (1.3–1.5 Å) of both FabB and FabF are available as well as structures of each protein bound to substrate analogues and inhibitors (31–36). However, despite this richness of structural information, the different in vivo substrate specificities of the two enzymes are not understood (37, 38). Therefore, particularly when the annotations cannot account for the ability of an organism to make molecules known to be essential for growth (e.g. unsaturated fatty acids), the current state of the art requires functional analysis such as those we report. Note that simple genetic complementation data might not be sufficient to identify the function of a gene; biochemical analysis might also be needed. This was the case with E. faecalis FabZ1, where based on the lack of genetic complementation of an E. coli fabA mutant, we would have concluded that the enzyme was unable to introduce cis double bonds into growing fatty chains. However, analysis of the products formed upon expression of the protein in E. coli showed that FabZ1 was proficient in the introduction of cis double bonds, but that the low levels of unsaturates produced were unable to support growth.

Consistent with the work of Marrakchi et al. (8), it seems clear that the production of unsaturates in cells expressing E. faecalis FabZ1 was limited by competition for trans-2-decenoyl-ACP between FabZ1 and the host enoyl-ACP reductase. This is based on the finding that the addition of triclosan, a specific inhibitor of the E. coli enoyl-ACP reductase (FabI), to an E. coli fabA strain expressing E. faecalis FabZ1 resulted in a markedly increased synthesis of unsaturated chains relative to the saturated species, although triclosan also inhibited the overall rate of fatty acid synthesis (and hence in growth inhibition; data not shown). Marrakchi et al. (8) found that expression of the cognate enoyl-ACP reductase allowed growth of an E. coli fabA strain expressing S. pneumoniae FabM in the presence of triclosan because the S. pneumoniae FabK enoyl-
ACP reductase is unaffected by the inhibitor. Unfortunately, we could not adopt this approach because the enoyl-ACP reductase is unaffected by the inhibitor. From the competition with FabI it seems clear that FabZ1, like FabA (3, 6, 7), must release at least a portion of the trans-2-decenoyl-ACP formed by the dehydratase activity. Based on our analyses the *E. faecalis* fabZ1 and fabF1 genes must be renamed. We propose the names fabN and fabO for fabZ1 and fabF1, respectively, whereas the number designations should be dropped from fabZ2 and fabF2.

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