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 abbreviations used are: ACP, acyl carrier protein; RB, rich broth.

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 bacterium *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* FabB, a protein that plays no specific role in unsaturated fatty acid synthesis. Therefore, *E. faecalis* FabZ1 is a bifunctional dehydratase/isomerase, an enzyme activity hitherto confined to a group of Gram-negative bacteria. The FabZ2 protein is unable to replace the function of *E. coli* FabZ, 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* FabF, 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 fabZ genes encode β-hydroxyacyl-ACP 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 each fatty acid elongation cycle (5). FabA and FabZ differ in that FabZ catalyzes only the dehydratase 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 dehydrate β-hydroxymyristoyl-ACP, a lipid A precursor. This enzyme activity was demonstrated, and thus the FabZ was called β-hydroxymyristoyl-ACP dehydratase (4). However, FabZ was later shown to dehydrate β-hydroxyacyl-ACPs of all chain lengths tested (15), and thus, the designation as β-hydroxymyristoyl-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 appeared that 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). Oulet neutralized with KOH was added to RB medium at final concentration of 0.1% and solubilized by the addition of Brij-58 detergent to final concentration of 0.1–0.2%. Antibiotics were used at the following concentration 100 mg/liter sodium ampicillin, 30 mg/liter chloramphenicol, 30 mg/liter kanamycin sulfate, and 200 mg/liter rifampicin. L-Arabinose and fucose were used at final concentrations of 0.01%. Isopropyl- β-D-thiogalactoside was used at final concentration of 1 mM.

**Recombinant DNA Techniques and Construction of Plasmids**—To clone the *E. faecalis* V583 *fabF* and *fabZ* homologues from genomic DNA, the PCR primers for *fabF1*, *fabF2*, *fabZ1*, and *fabZ2* were AAA-CTCGGAGGTACAtcATGAAAAGAGTGGTC plus CATAATTTCTGCT- GCAGTCAATT, CGAAGGGAGTCAAAAaacATGAAATCGAGTA plus TCTAACTGGCAGTCAATATCTC, GAGAAAAAGGGAGAAAACATcA- TGAAAAAAD plus CTCTACTGcAgatatcAAG, and CCGAGTTGC- TCTAACTGCAgGTTAATCCTC, GAAGAAATGGGAGGAAAACTAtcA-

**TABLE I**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>lacZΔM15ΔlacZYA-argF/U169 recA1 endA1 hsdR17</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>CY244</td>
<td>fabB15(Ts) fabF, Ref. 39</td>
<td></td>
</tr>
<tr>
<td>JWC275</td>
<td>fabB15(Ts) fabF::kan, Ref. 40</td>
<td></td>
</tr>
<tr>
<td>CY242</td>
<td>fabB15(Ts), Ref. 19</td>
<td></td>
</tr>
<tr>
<td>K1606</td>
<td>fabB5, Ref. 19</td>
<td></td>
</tr>
<tr>
<td>MR52</td>
<td>fabB::kan, Ref. 41</td>
<td></td>
</tr>
<tr>
<td>CY57</td>
<td>fabA(Ts)</td>
<td>Ref. 42</td>
</tr>
<tr>
<td>MI121</td>
<td>Cm, fabA::lacZ-cat, Ref. 43</td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td><em>E. coli</em> wild type</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>E. coli B F ompT rpsL mcr A (DE3)</td>
<td></td>
</tr>
<tr>
<td>HW1</td>
<td>Transductant of K1606 (pHW1) with phase P1 grown on MR52</td>
<td>This work</td>
</tr>
<tr>
<td>HW5</td>
<td>K1606 (pHW13) Km, Amp, fabB5 fabF::kan</td>
<td>This work</td>
</tr>
<tr>
<td>HW7</td>
<td><em>E. coli</em> DY330 fabF::kan carrying pHW22</td>
<td>This work</td>
</tr>
<tr>
<td>HW8</td>
<td>Transductant of MH121 with phase P1 grown on the cfa::kan strain YCY1257 (44)</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Plasmids**

| pBAD24             | Amp, expression vector, Ref. 45 |        |
| pCR2.1TOPO         | Amp, Km, TA cloning vector, Invitrogen |        |
| pET28(b)           | Km, expression vector, Novagen |        |
| pSU21              | Cm, cloning vector, Ref. 46 |        |
| pHW11              | Amp, Km, fabF1 of *E. faecalis* in pCR2.1TOPO | This work |
| pHW12              | Amp, Km, fabF2 of *E. faecalis* in pCR2.1TOPO | This work |
| pHW13              | Amp, fabF1 from pHW11 cut with BspHI and PestI and ligated between the NcoI and PestI sites of pBAD24 | This work |
| pHW14              | Amp, fabF2 from pHW12 constructed as pHW13 | This work |
| pHW17              | Amp, Km, fabZ1 of *E. faecalis* in pCR2.1TOPO | This work |
| pHW18              | Amp, Km, fabZ2 of *E. faecalis* in pCR2.1TOPO | This work |
| pHW19              | Amp, fabZ1 from pHW17 constructed as pHW13 | This work |
| pHW20              | Amp, fabZ2 from pHW16 constructed as pHW13 | This work |
| pHW22              | Amp, pBAD24 carrying *C. acetobutylicum* fabZ | This work |
| pHW26              | Km, fabF1 from pHW11 cut with BspHI and EcoRI ligated between the NcoI and EcoRI sites of pET28(b) | This work |
| pHW27              | Km, fabF2 from pHW12 constructed as pHW26 | This work |
| pHW29              | Km, fabZ1 from pHW17 constructed as pHW26 | This work |
| pHW30              | Km, fabZ2 from pHW18 constructed as pHW26 | This work |
| pHW71              | Km, fabZ1 from pHW19 digested with BamHI and PestI ligated between the same sites of pSU21 | This work |
| pHW72              | Cm, fabZ2 from pHW20 constructed as pHW26 | This work |
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. FabZ1 was expressed as a recombinant protein and was detected by autoradiography as described previously (10). Thin layer chromatography and detected by autoradiography as described previously (10). Thin layer chromatography and detected by autoradiography as described previously (10).

**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* FabF 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* fabI, a gene expected to encode an enoyl-ACP reductase. The fabB2 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 several different *E. coli* vectors. These included the phage T7 RNA polymerase-dependent vector pET28, 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...
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 cfa (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 (FabI) for the trans-2-decenoyl-ACP (8). FabI 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 (FabI) 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 and retain 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 strain 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, respectively, of triclosan, the extracts of a cell expressing FabZ2. In the absence of oleate supplementation, 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).

The Role of *E. faecalis* FabZ2—These results implied that fabZ2 should be the enzyme responsible for the general dehydrogenation 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 chromophenicol-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

![Fig. 2.](image-url)
resistance cassette. The deletion replacement event was done in the presence of an ampicillin-resistant pBR322-derived plasmid expressing the fabZ gene of Clostridium acetobutylicum to allow survival of the fabZ deletion strain. Growth of strain HW7 was dependent on arabinose addition because transcription of the C. acetobutylicum fabZ gene was from a vector araBAD promoter (Fig. 3). However, following introduction of the E. faecalis fabZ2 plasmid (which is compatible with the C. acetobutylicum fabZ plasmid), the E. coli fabZ deletion strain grew in the absence of arabinose or in the presence of fucose, an anti-inducer of the araBAD promoter (Fig. 3). Moreover, derivatives of this strain that had lost the C. acetobutylicum fabZ plasmid were readily isolated by screening for ampicillin-sensitive colonies. Therefore, E. faecalis fabZ2 can functionally replace E. coli fabZ. Note that growth of strain HW7 in the absence of arabinose (or in the presence of fucose) was specific to fabZ2. Introduction of the fabZ1 plasmid failed to allow arabinose-independent growth (Fig. 3). Hence, although the sequences of FabZ1 and FabZ2 argue that both are homologues of E. coli FabZ, only FabZ2 could functionally replace the E. coli protein.

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 mutations, 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 permits 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

### 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 pHW13 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 FabZ 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.

### Fig. 4. Growth of transformants of E. coli strains CY242, K1060, and JWC275 with plasmids carrying E. faecalis fabF1 or fabF2. Transformants of strain K1060 (an unconditional fabB mutant) were grown at 37 °C, whereas the transformants of strain CY242 (a temperature-sensitive fabB mutant) were grown at the nonpermissive temperature of 42 °C. Strain JWC275 (fabB (Ts) fabF::kan) was also grown at 42 °C. The strains carried the pBAD24-derived fabF1 and fabF2 plasmids pHW13 and pHW14, respectively. The oleate-supplemented RB medium is the permissive medium for the fabF strains that fail to grow on unsupplemented RB medium. Strains carrying a lesion in fabF in addition to the fabB (Ts) mutation are unable to grow at the nonpermissive temperature on oleate-containing medium because of an inability to synthesize long chain saturated fatty acids.
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 encoding plasmid gave only traces of unsaturated acids (Fig. 5).

**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 used 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 FabB 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 residues are conserved in E. coli FabZ, which lacks isomerase activity. The single nonconserved residue, Asp-84 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 extant β-hydroxyacyl-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 aligns significantly more closely with S. pneumoniae FabM than with E. faecalis FabF1 and FabF2. Cell-free extracts of strain MG1655 (wild type) and JWC275 (fabB/Ts fabF::kan) carrying plasmids encoding E. faecalis FabF1 and FabF2 were prepared, and fatty acid synthesis reactions were run and analyzed as for Fig. 2. Lane 1, MG1655; lanes 2–4, derivatives of JWC275 carrying pHW13 (fabF1), pHW14 (fabF2), or pBAD24 (vector), respectively. The spot between cis-vaccenate and the saturated acids in lane 1 has the migration rates of cis-13-eicosenoic acid.

**FIG. 5. In vitro synthesis of acyl-ACP and fatty acid species by E. faecalis FabF1 and FabF2.** Cell-free extracts of strain MG1655 (wild type) and JWC275 (fabB/Ts fabF::kan) carrying plasmids encoding E. faecalis FabF1 and FabF2 were prepared, and fatty acid synthesis reactions were run and analyzed as for Fig. 2. Lane 1, MG1655; lanes 2–4, derivatives of JWC275 carrying pHW13 (fabF1), pHW14 (fabF2), or pBAD24 (vector), respectively. The spot between cis-vaccenate and the saturated acids in lane 1 has the migration rates of cis-13-eicosenoic acid.
ACP reductase is unaffected by the inhibitor. Unfortunately, we could not adopt this approach because the enoyl-ACP reductase of *E. faecalis* is of the FabI (triclosan-sensitive) type. 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.

REFERENCES


*E. faecalis Fatty Acids*