The Two Functional Enoyl-Acyl Carrier Protein Reductases of *Enterococcus faecalis* Do Not Mediate Triclosan Resistance

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ABSTRACT **Enoyl-acyl carrier protein (enoyl-ACP) reductase catalyzes the last step of the elongation cycle in the synthesis of bacterial fatty acids. The *Enterococcus faecalis* genome contains two genes annotated as enoyl-ACP reductases, a FabI-type enoyl-ACP reductase and a FabK-type enoyl-ACP reductase. We report that expression of either of the two proteins restores growth of an *Escherichia coli fabI* temperature-sensitive mutant strain under nonpermissive conditions. *In vitro* assays demonstrated that both proteins support fatty acid synthesis and are active with substrates of all fatty acid chain lengths. Although expression of *E. faecalis fabK* confers to *E. coli* high levels of resistance to the antimicrobial triclosan, deletion of *fabK* from the *E. faecalis* genome showed that FabK does not play a detectable role in the inherent triclosan resistance of *E. faecalis*. Indeed, FabK seems to play only a minor role in modulating fatty acid composition. Strains carrying a deletion of *fabK* grow normally without fatty acid supplementation, whereas *fabI* deletion mutants make only traces of fatty acids and are unsaturated fatty acid auxotrophs.**

IMPORTANCE **The finding that exogenous fatty acids support growth of *E. faecalis* strains defective in fatty acid synthesis indicates that inhibitors of fatty acid synthesis are ineffective in countering *E. faecalis* infections because host serum fatty acids support growth of the bacterium.**

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Fatty acid synthesis (FAS) is essential for the formation of cellular membranes and hence for survival in mammals, plants, fungi, and bacteria (1–3). Moreover, in bacteria the fatty acid synthesis pathway allows diversion of intermediates to other end products, such as quorum-sensing molecules (4, 5), lipid A (6, 7), and the vitamins biotin and lipoic acid (8–10). Bacterial fatty acid synthesis, which differs significantly from the mammalian and fungal system (FAS I), is catalyzed by a set of discrete enzymes that are collectively known as the type II, or dissociated, fatty acid synthases (7, 11–13). Enoyl-acyl carrier protein (enoyl-ACP) reductases (ENRs) reduce *trans*-2-enoyl-ACPs to the fully saturated ACP species in the last step of the elongation cycle in the synthesis of bacterial and plant fatty acids (7, 11–14) (Fig. 1A). *Escherichia coli* has a single ENR encoded by the essential *fabI* gene (7, 12, 13, 15) which catalyzes reduction of all the enoyl intermediates of the pathway (15, 16). FabI plays a determinant role in completing rounds of fatty acid elongation and is also feedback inhibited by long-chain ACPs (17).

The growing interest in ENRs is mainly due to the fact that a variety of both synthetic and natural antibacterial compounds specifically target ENR activity (13, 18–23). However, unlike most enzymes of type II fatty acid synthesis, ENRs display extensive sequence and structural diversity among bacteria (14). Investigation of the molecular basis for the differing sensitivities of bacteria to triclosan, a synthetic compound that targets the FabI form of ENR, led to the identification of three additional ENR enzymes, FabL (24, 25), FabK (18, 26), and FabV (27–29). FabI, FabL, and FabV are members of the short-chain dehydrogenase reductase (SDR) superfamily, whereas FabK is a flavoprotein that has no structural similarity to members of the SDR superfamily (14). Although all ENR isozymes catalyze reduction of *trans*-2-enoyl-ACPs to the saturated ACP species, some bacteria encode multiple ENRs. The physiological rationale for multiple ENRs is poorly explored. In *Bacillus subtilis*, the genes encoding either of the two FabI and FabL ENRs can be inactivated without blocking growth (24). Since these ENRs are functionally redundant, it is unclear why both enzymes have been retained. Two ENRs, FabI and FabV, are present in *Pseudomonas aeruginosa* (29, 30). Although either of the encoding genes can be deleted, the strain lacking FabV grew much more slowly than the wild-type strain (29). Deletion of *fabV* resulted in sensitivity to triclosan and a defect in swimming and swarming motility. Moreover, the *fabV* and *fabI* deletion strains produced significantly more unsaturated fatty acids (UFA) than the wild-type strain (29, 31, 32). Although *Streptococcus pneumoniae* has only a single ENR, FabK (26), it competes for substrates with FabM, the isomerase required to introduce *cis* double bonds into its unsaturated fatty acids (33). Although it has been suggested that the relative levels of FabK and FabM must be closely controlled to allow the synthesis of unsaturated fatty acids (34), the detailed mechanism remains unclear.

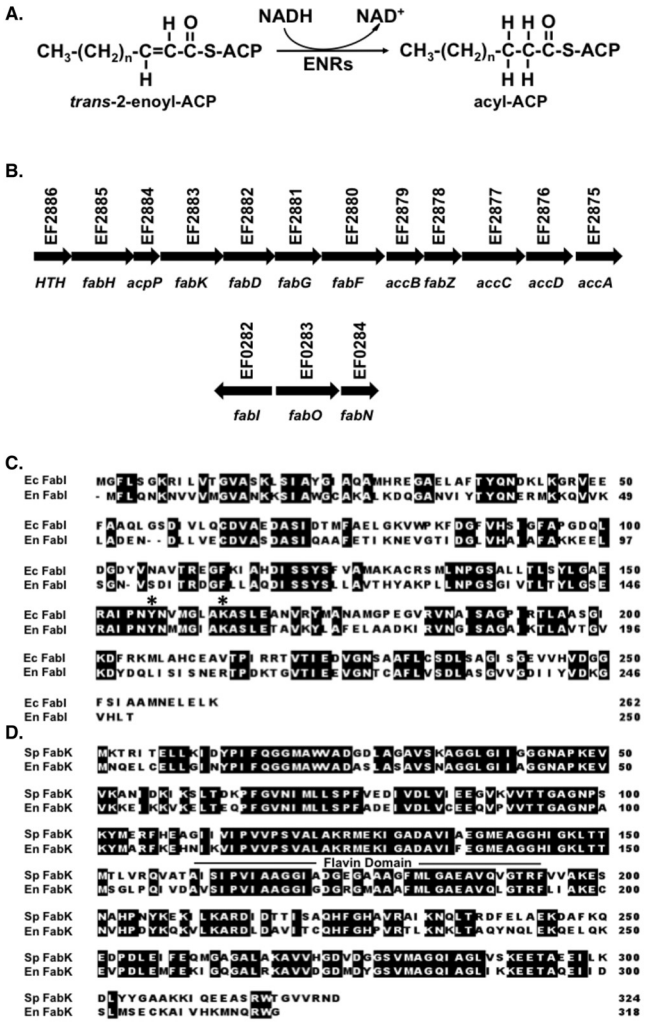

FIG 1 The enoyl-ACP reductase (ENR) reaction, organization of the *E. faecalis* fatty acid biosynthesis gene clusters, and alignment of *E. faecalis* FabI and FabK with ENRs of known structure, *E. coli* FabI and *S. pneumoniae* FabK. (A) The enoyl-ACP reductase reaction. (B) Organization of the *E. faecalis* fatty acid biosynthesis gene clusters. The thick arrows indicate the relative sizes of the genes. The numbers above the arrows indicate the gene designations in the *E. faecalis* CMR database, and the gene names below the arrows indicate the *E. coli* genes that correspond to the open reading frames in the *E. faecalis* cluster. (C) Alignment of *E. faecalis* FabI with *E. coli* FabI. En and Ec denote *E. faecalis* and *E. coli*, respectively. The (Y-(Xaa)$_6$-K) catalytic triad is denoted by asterisks. (D) Alignment of *E. faecalis* FabK with *S. pneumoniae* FabK. En and Sp denote *E. faecalis* and *S. pneumoniae*, respectively. The central regions of the proteins that contain a consensus flavin-binding site are indicated by an underline.

E. faecalis is a Gram-positive bacterium that, although a normal member of the digestive microflora of humans and many other animals, has emerged as a serious nosocomial pathogen responsible for endocarditis and infections of the urinary tract, bloodstream, meninges, wounds, and the biliary tract (35). Moreover, many present-day *E. faecalis* strains are resistant to virtually all clinically useful antibiotics (36). Fatty acid synthesis inhibitors have been proposed as antienterococcal agents (18), but the fatty acid synthetic pathway of this bacterium is little explored. Bioinformatic analyses of the type II fatty acid biosynthetic genes in *E. faecalis* indicate that they cluster at two locations within the genome (Fig. 1B). One gene cluster contains *fabH*, *acpP*, *fabK*, *fabD*, *fabG*, *fabF*, *accB*, *fabZ*, *accC*, *accD*, and *accA* and apparently encodes the entire pathway of saturated fatty acid (SFA) synthesis, whereas the second, smaller cluster contains three genes, *fabI*, *fabN*, and *fabO*, annotated as encoding the enzymes of unsaturated fatty acid synthesis (37) (Fig. 1B). In 2000, Heath and Rock (18) noted that the *E. faecalis* genome encodes two ENR homologues, FabK and FabI, and suggested that *E. faecalis* FabK ENR was responsible for the characteristic triclosan resistance of this bacterium. They also proposed that two different ENR inhibitors would be required to inhibit *E. faecalis* lipid synthesis. To date, however, the physiological functions of FabK and FabI in *E. faecalis* have been unclear. Indeed, it was unknown if the putative FabK protein had ENR activity since other putative homologues of *S. pneumoniae* FabK have been found to lack activity (26). In the present study, we examined the physiological and enzymatic properties of *E. faecalis* FabI and FabK and tested the role of FabK in the relatively high-level intrinsic triclosan resistance of this bacterium.

RESULTS

The two ENRs of *E. faecalis*. The *E. faecalis* genome contains two genes annotated as ENRs, EF0282, encoding a FabI-type ENR, and EF2883, encoding a FabK-type ENR (37) (Fig. 1B). Alignment of the FabI homologue showed that *E. faecalis* FabI is 47% identical to *E. coli* FabI and contains the Tyr-(Xaa)$_6$-Lys motif of *E. coli* FabI (Fig. 1C). The *E. faecalis* FabK homologue is 68% identical to *S. pneumoniae* FabK and contains the expected consensus flavin-binding motif (Fig. 1D). Moreover, both putative ENR genes are adjacent to genes that are likely to encode other fatty acid synthetic proteins (Fig. 1B). Based on these criteria, it seemed reasonable that FabK and FabI could both be functional ENRs that play roles in *E. faecalis* fatty acid synthesis.

E. faecalis fabK and fabI functionally replace E. coli fabI in vivo. FabI is the sole *E. coli* ENR, and strain JP1111 harbors a temperature-sensitive *fabI*(Ts) allele and fails to grow at 42°C (15, 16). To test the function of *E. faecalis fabK* (En*fabK*) and En*fabI*, these genes plus *E. coli fabI* (Ec*fabI*) were inserted into the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible pHSG575 vector (38), which has the low-copy-number (1 to 5 copies/cell) pSC101 replication origin, and the resulting plasmids were introduced into strain JP1111. Strain JP1111 carrying the plasmid with *E. coli fabI* (Ec*fabI*) (pZL32) grew at 42°C in the presence or absence of IPTG.

In contrast, strains carrying En*fabI* or En*fabK* (pZL30 or pZL31) grew at 42°C only in the presence of IPTG, whereas the strain carrying the vector plasmid failed to grow under either condition (Fig. 2A). Similar results were seen in liquid medium (Fig. 2B). Therefore, both En*fabI* and En*fabK* complemented growth of the *E. coli fabI*(Ts) strain, indicating that both proteins catalyze the ENR reaction. It should be noted that, in liquid medium, IPTG induction inhibited growth of strain JP1111 expressing *E. coli* FabI (pZL32) and that growth of the strains expressing En*fabI* or En*fabK* in medium containing IPTG was slower than that of the strain expressing *E. coli* FabI in the absence of inducer. Our data are consistent with the report that FabI overproduction is toxic to growth of *E. coli* and that this toxicity is offset by low doses of the FabI inhibitor, triclosan, indicating that overproduction of enzyme activity rather than of FabI protein causes inhibition (39). Derivatives of strain JP1111 expressing EnFabI (pZL30)

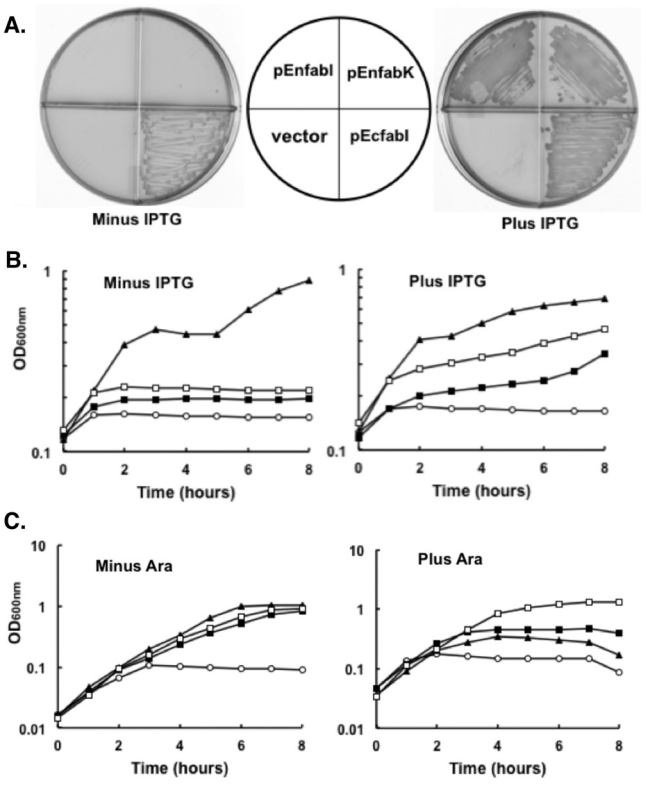

FIG 2 Expression of *E. faecalis fabI* and *fabK* restores growth of *E. coli fabI*(Ts) strain JP1111. (A) Transformants of *fabI*(Ts) *E. coli* strain JP1111 were grown at 42°C on LB medium in either the presence or the absence of IPTG (strain JP1111 grows at 30°C but not at 42°C). The strains carried plasmids encoding En*fabK*, En*fabI*, or Ec*fabI* (pZL30, pZL31, or pZL32, respectively) or the vector plasmid, pHSG575. (B) Growth of *E. coli* strain JP1111 carrying plasmids with En*fabK*, En*fabI*, or Ec*fabI* (pZL31, pZL30, or pZL32, respectively) or the vector plasmid, pHSG575, in LB medium in either the presence or the absence of IPTG. Symbols: ❍, vector plasmid (pHSG575 or pBAD24M); ▲, plasmid carrying Ec*fabI* (pZL32 or pZL18); ❑, plasmid carrying En*fabI* (pZL30 or pZL23); ■, plasmid carrying En*fabK* (pZL31 or pZL24). (C) Growth of *E. coli strain* JP1111 with plasmids carrying En*fabK*, En*fabI*, or Ec*fabI* (pZL24, pZL23, or pZL18, respectively) or the vector plasmid, pBAD24M, in LB medium in either the presence or the absence of arabinose (Ara). Symbols are as described for panel B. OD_{600}, optical density at 600 nm.

or EnFabK (pZL31) grew slowly even in the presence of IPTG, perhaps due to poor expression of EnFabI and EnFabK because of the low copy number of the vector and the quite different codon usages of *E. coli* and *E. faecalis*. To test the possibility of low expression, the three ENRs were expressed from the high-copy-number arabinose-inducible vector pBAD24M. These plasmids were introduced into strain JP1111, and the growth of these derivatives was followed at the nonpermissive temperature in liquid media in the presence or absence of arabinose (Fig. 2C). All three ENR plasmid strains grew in liquid medium in the presence or absence of arabinose. However, in the absence of arabinose, the strain carrying the Ec*fabI* plasmid grew faster than the strains carrying the En*fabI* and En*fabK* plasmids. In contrast, the presence of arabinose gave much-improved growth of the strain expressing En*fabI*, whereas arabinose severely inhibited growth of the strains expressing either Ec*fabI* or En*fabK*.

It has been suggested that high FabI activity competes with the isomerase activity of FabA for the *trans*-2-decenoyl-ACP intermediate, resulting in an unsaturated fatty acid deficiency in *E. coli* which, if severe, would result in cell lysis (14). We assayed fatty acid synthesis in JP1111-derived strains carrying the three ENR plasmids by measuring *de novo* fatty acid synthesis by [1-^{14}C]acetate incorporation into membrane phospholipids. At the nonpermissive temperature, derivatives of strain JP1111 carrying the plasmids with Ec*fabI*, En*fabI*, or En*fabK* incorporated high levels of [1-^{14}C]acetate into membrane phospholipids (see Fig. S1 in the supplemental material), whereas the parental strain showed no incorporation. Strain JP1111 expressing En*fabI* produced almost entirely unsaturated fatty acids, whereas the strains expressing either Ec*fabI* or En*fabK* produced high levels of saturated fatty acids. Hence, it seems that high levels of EnFabK, like those of EcFabI, enable it to compete effectively with FabA for *trans*-2-decenoyl-ACP, thereby causing the *E. coli* strain to increase production of saturated fatty acids. In contrast, upon high-level expression of EnFabI, this ENR competed poorly with FabA, thereby resulting in increased unsaturated fatty acid synthesis.

TABLE 1 Triclosan resistance of *E. coli* and *E. faecalis* strains

Strain	Gene	Triclosan MIC (μg/ml)
E. coli		
W3110/pHSG575	Empty vector	0.2
W3110/pZL32	Ec*fabI*	2
W3110/pZL30	En*fabI*	2
W3110/pZL31	En*fabK*	20
W3110/pBAD24M	Empty vector	0.2
W3110/pZL18	Ec*fabI*	2
W3110/pZL23	En*fabI*	3
W3110/pZL24	En*fabK*	120
E. faecalis		
FA2-2		10
FAZL1(Δ*fabI*)		7.5[a]
FAZL1(Δ*fabI*)/pZL023	En*fabI*	10
FAZL1(Δ*fabI*)/pZL024	En*fabK*	10
FAZL2(Δ*fabK*)		10
FAZL2/pZL024	En*fabK*	10

[a] This value was obtained in cultures grown with oleic acid, which forms micelles in solution which bind the very hydrophobic triclosan and thereby lowers the effective triclosan concentration (44).

The sensitivity to triclosan of the wild-type *E. coli* strain W3110 carrying plasmids with Ec*fabI*, En*fabI*, or En*fabK* was tested (Table 1). As expected, derivatives of strain W3110 carrying either vector plasmid (pHSG575 or pBAD24M) were very sensitive to triclosan. In contrast, expression of the *E. coli* and *E. faecalis* FabI proteins from pHSG575-derived or pBAD24M-derived plasmids increased triclosan resistance; the triclosan MICs were shifted from 0.2 μg/ml to 2 to 3 μg/ml (Table. 1). In contrast, introduction of the plasmid carrying En*fabK* into strain W3110 shifted the triclosan MIC from 0.2 μg/ml to 20 (pHSG575 vector) or 120 (pBAD24M vector) μg/ml (Table 1), indicating that *E. faecalis* FabK, like *S. pneumoniae* FabK (26), is not inhibited by triclosan.

Expression, purification, and characterization of the *E. faecalis* FabI and FabK proteins. The *E. faecalis fabK* and *fabI* genes were expressed in *E. coli* as described in Materials and Methods. The hexahistidine-tagged fusion proteins were purified by nickel chelate chromatography and gel filtration. As measured by denaturing gel electrophoresis, the purified EnFabI and FabK proteins had monomeric molecular masses of 29 kDa and 38 kDa, respectively (see Fig. S2A in the supplemental material), in good agreement with the values calculated from the sequences of the

tagged proteins (28.9 and 36.4 kDa, respectively). *E. coli* FabI and the other FabI enzymes described are homotetramers (40, 41), whereas the solution structure of *S. pneumoniae* FabK has not been reported, although it has been crystallized (42). We therefore examined the solution structures of native FabK and FabI by gel filtration chromatography. The molecular masses of EcFabI, EnFabI, and EnFabK were estimated to be 94.6, 65.9, and 68.9 kDa, respectively, by graphic analysis of a standard curve based on the elution volumes of protein molecular mass markers (see Fig. S2B). Thus, surprisingly, EnFabI seems to exist as a dimer in solution while EnFabK may also be a dimeric protein.

Analysis of EnFabK and EnFabI ENR activities *in vitro*. In order to confirm the ENR activities of *E. faecalis* FabI and FabK, the ability to function in fatty acid synthesis reactions was assayed *in vitro*. We purified the N-terminal hexahistidine-tagged versions of *E. coli* fatty acid biosynthetic proteins FabD, FabA, FabH, FabG, and EcFabI plus *Vibrio harveyi* AasS (ACP synthetase) by nickel-chelate chromatography. *E. coli* holo-ACP was also purified. We first reconstituted the initiation steps of the fatty acid synthesis reaction by the sequential addition of purified components followed by incubation and analysis by conformationally sensitive gel electrophoresis. The addition of malonyl-coenzyme A (CoA):ACP transacylase (FabD) plus malonyl-CoA led to the formation of malonyl-ACP (Fig. 3A, lane 2). Subsequent additions of 3-ketoacyl-ACP synthase III (FabH) and 3-ketoacyl-ACP reductase (FabG) resulted in the accumulation of 3-hydroxybutyryl-ACP (Fig. 3A, lane 3). After the addition of 3-hydroxyacyl-ACP dehydrase (FabA), the reaction mixture should accumulate crotonyl-ACP (*trans*-2 butyryl-ACP). However, because the FabA reaction rapidly reaches equilibrium in favor of the 3-hydroxyacyl-ACP species (Fig. 3A, lane 4) (24), crotonyl-ACP was not seen. The addition of EnFabK, EnFabI, or EcFabI plus NADH to the reaction mixture gave butyryl-ACP (Fig. 3A, lanes 5 to 7). These data showed that, like EcFabI (24), EnFabK and EnFabI functioned with respect to the prior steps of fatty acid synthesis such that 3-hydroxybutyryl-ACP was converted to butyryl-ACP.

To test the abilities of EnFabK and EnFabI to reduce long-chain enoyl-ACPs, 3-hydroxydecanoyl-ACP was synthesized from 3-hydroxydecanoic acid and *E. coli* holo-ACP using AasS (Fig. 3B, lane 2, and Fig. 3C, lane 2) and converted to *trans*-2-decenoyl-ACP by incubation with FabA (Fig. 3B, lane 3, and Fig. 3C, lane 3). NADH and an ENR were then added to the reaction mixtures followed by incubation. The production of decanoyl-ACP was demonstrated by conformationally sensitive gel electrophoresis in the presence of 2.5 M urea (Fig. 3B, lane 4, and Fig. 3C, lane 4). As expected, both EnFabK and EnFabI quantitatively converted *trans*-2-decenoyl-ACP to decanoyl-ACP. However, the activity of EnFabK was lower than that of EnFabI and some *trans*-2-decenoyl-ACP remained in the reaction mixture (Fig. 3C).

It has been demonstrated that *S. pneumoniae* FabK is a triclosan-resistant ENR (18, 26) whereas *E. coli* FabI is the paradigm triclosan-sensitive enzyme (14, 43). To examine the triclosan sensitivities of the *E. faecalis* ENRs *in vitro*, the reaction mixtures containing *trans*-2-decenoyl-ACP were divided into aliquots in tubes, various amounts of triclosan were added, and the synthesis of decanoyl-ACP products was assayed. In agreement with expectations, EnFabK remained active in the conversion of *trans*-2-decenoyl-ACP to decanoyl-ACP in the presence of 1,000 μg/ml triclosan (Fig. 3C), whereas 50 μg/ml triclosan almost completely eliminated the EnFabI activity (Fig. 3B). However, the known nonspecific protein binding of triclosan, a highly hydrophobic molecule (44), complicated the EnFabI experiments. Given the high protein concentration (ca. 1.1 mg/ml) of these reactions at low triclosan concentrations, an appreciable portion of the triclosan could be sequestered by nonspecific binding (45). For this reason, we directly tested triclosan inhibition of the purified enzyme in parallel with the well-characterized *E. coli* FabI and found that EnFabI was only slightly less sensitive to triclosan than was the *E. coli* enzyme (Fig. 4). Our 50% inhibitory concentration (IC_{50}) value for *E. coli* FabI is similar to that reported by others (24), although somewhat lower than the values reported earlier from that laboratory (46, 47). Note that much tighter binding of triclosan to *E. coli* FabI can be obtained if the enzyme is preincubated with triclosan and NAD^+ prior to the assay (41).

The kinetic properties of EnFabK and EnFabI were measured with *trans*-2-decenoyl-ACP as the substrate. The observed maximal velocity of EnFabI (159.0 $\pm$ 68.9 pmol min^{-1} ng^{-1}) with NADH was much higher than the maximal velocity of EnFabK (10.9 $\pm$ 3.2 pmol min^{-1} ng^{-1}) with the same substrate, and EnFabI had lower K_m values (197.0 $\pm$ 95.1 μM) than EnFabK (394.3 $\pm$ 171.0 μM). The K_{cat} values for EnFabI and EnFabK were 76.3 $\pm$ 33.1 s^{-1} and 6.9 $\pm$ 2.0 s^{-1}, respectively. Note that the ACP used was that of *E. coli*.

Construction of *fabI* and *fabK* deletion mutant strains. In order to determine the physiological functions of the two ENRs in *E. faecalis* fatty acid biosynthesis, strains in which either *fabI* and *fabK* genes had been deleted were constructed by allelic replacement (see Fig. S3A in the supplemental material). Suicide vector pBVGh-borne *fabI* or *fabK* deletion cassettes were constructed (see Materials and Methods in the supplemental material), and single-crossover integrants of each plasmid into the strain *E. faecalis* FA2-2 genome were selected by erythromycin resistance and scoring for blue colonies. Cultures from the integrant colonies were grown in AC medium containing oleic acid and plated on medium with oleic acid and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The successful construction of the designed mutations was assayed by PCR analysis using the primers listed in Table S1 in the supplemental material. As expected for the *fabI* mutant, FAZL1, the primers (EnI check UP and EnI check DOWN) amplified a 1.25 kb Δ*fabI*-containing fragment (see Fig. S3B, lane 2), whereas in wild-type FA2-2, this fragment was 1.80 kb (see Fig. S3B, lane 1). The strain was further verified by sequencing of the 1.25-kb fragment, which validated the construction. A Δ*fabK* strain (named FAZL2) was made and verified by exactly the same methods (see Fig. S3C).

Deletion of *fabI* renders *E. faecalis* almost totally deficient in fatty acid synthesis. The growth of the deletion mutants of strain FAZL1 (Δ*fabI*) on AC medium was tested, and although strain FAZL1 (Δ*fabI*) was viable, the growth was less than that of wild-type strain FA2-2 (wild type) and strain FAZL3 (Δ*fabK*) (Fig. 5A). The function of *fabI* in *E. faecalis* fatty acid synthesis was tested by measuring *de novo* fatty acid synthesis by [1-^{14}C]acetate incorporation into membrane phospholipids. The Δ*fabI* strain synthesized only traces of fatty acid (Fig. 5B, lane 4 and 8). The results indicated that, although the wild-type *fabK* gene was present in the Δ*fabI* strain, the level of FabK ENR activity could not support sufficient fatty acid synthesis for growth and thus that the Δ*fabI*

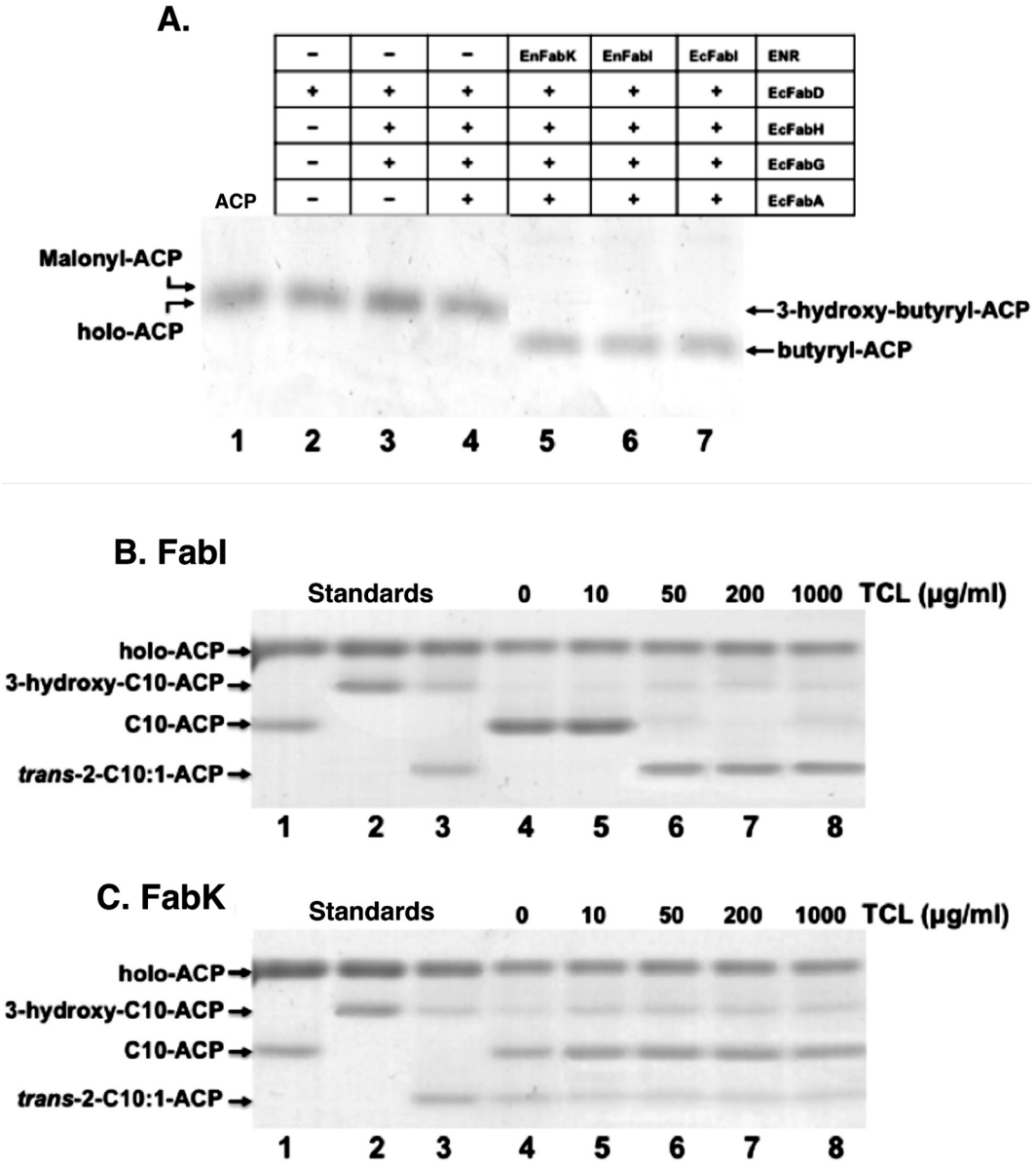

FIG 3 Enzymatic characterization of *E. faecalis* FabI and FabK. (A) The first cycle of fatty acid biosynthesis was reconstructed *in vitro* by the sequential addition of each purified enzyme to a reaction mixture containing NADPH, NADH, and *E. coli* ACP as cofactors and acetyl-CoA and malonyl-CoA as substrates as described in Materials and Methods and indicated in the key above the gel. The first lane represents an ACP standard which comigrates with malonyl-ACP in this gel system. (B and C) The reaction mixture for assays of the reduction of *trans*-2-enoyl-ACPs by EnFabI (B) or EnFabK (C) contained 0.1 M sodium phosphate (pH 7.0), 1 mM 2-mercaptoethanol, 50 μM 3-hydroxydecanoyl-ACP, and EcFabA. The first two lanes of each gel represent standards generated *in situ* by acylation of ACP with the fatty acid catalyzed by AasS. The third lane of each gel contained 3-hydroxydecanoyl-ACP synthesized with AasS that was dehydrated to *trans*-2-decanoyl-ACP by FabZ. To test the effect of triclosan on the activities of EnFabI or EnFabK, the appropriate amounts of triclosan (TCL) solution were added to 1.5 ml tubes and the solvent was evaporated. The reactions were initiated by the addition of EnFabI or EnFabK and the reaction mixtures incubated for an additional 1 h. The reaction products were resolved by conformationally sensitive gel electrophoresis on 17.5% polyacrylamide gels. Note that the apparent resistance of FabI to 10 μg/ml triclosan can be attributed to nonspecific protein binding of the very hydrophobic triclosan (45) due to the high protein concentrations of the reconstituted system. Higher triclosan concentrations saturate the nonspecific binding and are able to inhibit. When assayed in the absence of other proteins, EnFabI was slightly more sensitive to triclosan than EcFabI (Fig. 4).

strain was dependent on acquiring free fatty acids from the medium.

To demonstrate that the lack of growth was due to loss of FabI, we introduced plasmid pZL35 (wild-type En*fabI* carried by vector pBM02) into the Δ*fabI* strain and found that the resulting strain grew as well as wild-type strain FA2-2 (Fig. 5A) and produced fatty acids normally (Fig. 5B, lane 3). We also introduced plasmid pZL36 (wild-type *fabK* carried by pBM02) into the Δ*fabI* strain and found full restoration of growth (Fig. 5A) and fatty acid synthesis (Fig. 5B, lane 6). All these results indicated that the ENR activity encoded by *fabK* was low in strain FAZL1. In order to test if growth of the Δ*fabI* mutant strain was dependent on incorporation of free fatty acids from medium, the incorporation of free fatty acids from AC medium into the phospholipids of Δ*fabI* mu-

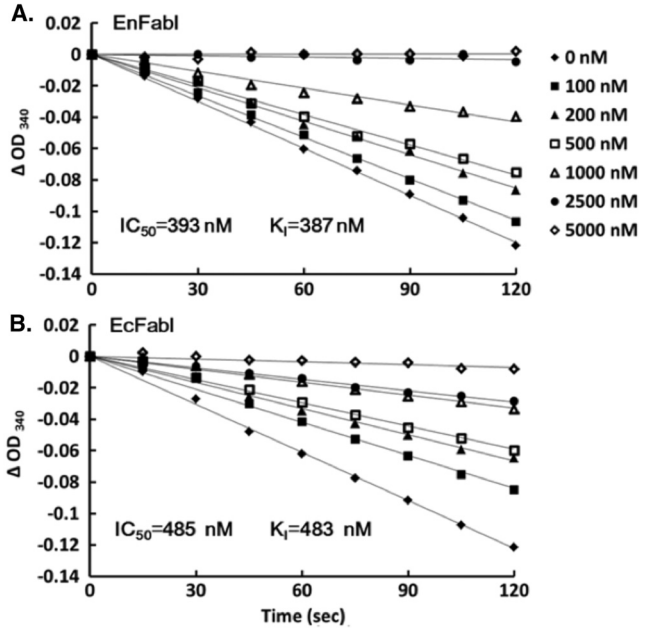

FIG 4 Triclosan inhibition of FabI. The 100-μl reaction mixtures contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 200 μM crotonoyl-CoA, 100 μM NADH, 50 μM NADH, 5% dimethyl sulfoxide (DMSO), and differing concentrations of triclosan as shown (the symbols are the same in both panels). The activity of 100 ng EnFabI (A) or 50 ng EcFabI (B) was determined using a UV-visible light (Vis) spectrophotometer at 25°C. The IC_{50} and K_i values were determined from Dixon plots. It should be noted that much lower K_I values were obtained when FabI was preincubated with triclosan and NAD^+, reflecting its properties as a slow binding inhibitor (41).

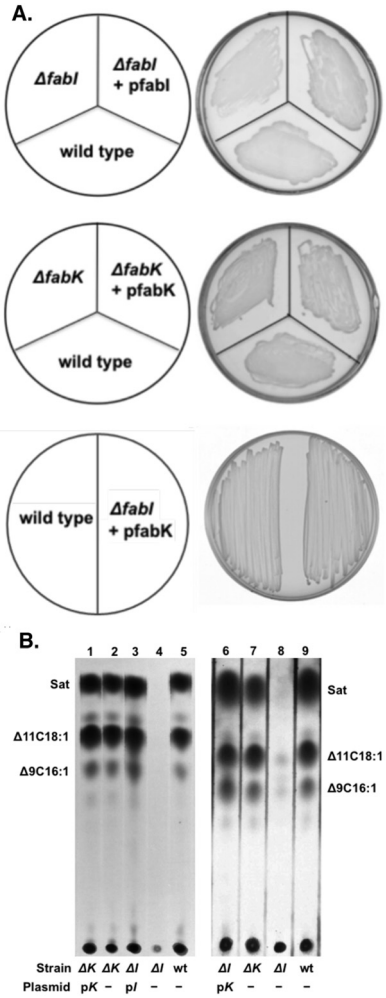

FIG 5 Growth and fatty acid synthesis of *E. faecalis* mutant strains. (A) Growth of *E. faecalis* mutant strains on AC medium. The pfabI designation indicates the strain carrying plasmid pZL35, whereas pfabK denotes the strain carrying plasmid pZL36. The plus and minus signs denote the presence or absence of the plasmid given above. (B) Analysis of fatty acid synthesis in *E. faecalis* mutant strains by argentation thin-layer chromatography of methyl esters derived from the membrane [1-^{14}C]acetate-labeled phospholipids. Lanes 1, 2, and 7 represent the Δ*fabK* strain carrying either an *EnfabK* plasmid (pZL36, lane 1) or no plasmid. Lanes 3, 4, and 8 represent the Δ*fabI* strain carrying an *EnfabI* plasmid (pZL35, lane 3) or no plasmid. Lanes 5 and 9 are from the wild-type strain, whereas lane 6 represents the Δ*fabI* strain carrying an *EnfabK* plasmid (pZL36). Abbreviations: Sat, saturated fatty acids; Δ11C18:1, *cis*-vaccenic acid; Δ9C16:1, palmitoleic acid. The residual growth of the Δ*fabI* strain was due to the low levels of unsaturated fatty acids present in AC medium.

tant was examined by addition of 1-^{14}C-labeled octanoic, decanoic, or dodecanoic acids to AC medium. The cellular phospholipids were extracted and their fatty acid moieties converted to their methyl esters and analyzed by argentation thin-layer chromatography. Dodecanoic acid is a normal component of *E. faecalis* phospholipids (see Table S2 in the supplemental material), and we therefore expected that it could be incorporated without elongation, and this was the case; both the wild-type and Δ*fabI* mutant strains incorporated dodecanoic acid into phospholipids (see Fig. S4A, lanes 1 and 4, in the supplemental material). Since dodecanoyl-ACP is past the origination point of the branch between saturated and unsaturated species, only SFA labeling occurred. Much weaker labeling was seen with decanoic acid (see Fig. S4A, lanes 2 and 5), probably because it must be elongated for efficient incorporation into phospholipids. Still weaker labeling was seen with octanoic acid because at least two elongation cycles would be needed for its incorporation as a saturated fatty acid and four or five additional cycles for its incorporation as a phospholipid UFA. Despite the cycles needed, the wild-type strain showed trace labeling of both saturated and unsaturated species, whereas no labeling of the Δ*fabI* strain was obtained (see Fig. S4A, lanes 3 and 6).

We also tested the abilities of both strains to elongate dodecanoic acid to longer chain fatty acids by C18 reverse-phase thin-layer chromatography (see Fig. S4B in the supplemental material) and found that the wild-type strain and Δ*fabK* strains elongated dodecanoic acid mainly to palmitic acid (C16), whereas the Δ*fabI* strain failed to extend the acid. These data indicated that fatty acid synthesis of the Δ*fabI* strain was severely inhibited such that growth was dependent on acquiring suitable long-chain free fatty acids (e.g., oleic acid) from the medium. We also tested the growth of the Δ*fabI* mutant on fatty acid-depleted AC medium and found that the wild type grew well on this medium, whereas the Δ*fabI* strain mutant failed to grow (Fig. 6A). The growth defect of Δ*fabI* mutant was bypassed by supplementation of the medium with oleic acid, whereas saturated fatty acids such as palmitic or stearic acids were unable to support growth (Fig. 6B). Note that increasing or decreasing the growth temperature (to 42°C or 30°C, respectively) of the Δ*fabI* strain did not give improved growth, in-

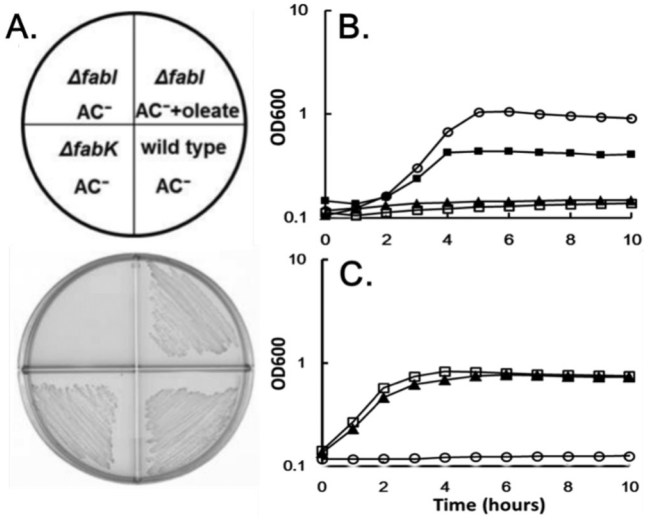

FIG 6 Essentiality and growth phenotype of the *E. faecalis* Δ*fabI* strain on fatty acid-depleted AC medium. (A) *E. faecalis* strains were streaked onto fatty acid-depleted AC medium (AC$^-$) or the same medium supplemented with oleic acid as depicted in the diagram (the plate was sectored by plastic walls). (B) Growth of the *E. faecalis* Δ*fabI* strain in fatty acid-depleted AC medium in the absence or presence of fatty acid supplements. Symbols: ❍, wild-type strain without fatty acid supplement; ■, Δ*fabI* strain supplemented with oleic acid; ▲, Δ*fabI* strain supplemented with the saturated fatty acid (stearic acid); ❑, Δ*fabI* in fatty acid-depleted AC medium without supplement. (C) Growth of the *E. faecalis* Δ*fabI* strain carrying the *EnfabI* plasmid pZL35 in fatty acid-depleted AC medium. ❑, wild-type strain; ▲, Δ*fabI* strain carrying plasmid pZL35; ❍, Δ*fabI* strain.

dicating that FabK remained functionally silent under these conditions. Hence, the Δ*fabI* strain is a UFA auxotroph. We confirmed that the deficiency in the UFA synthesis of Δ*fabI* mutant strain was due to the absence of a functional FabI protein by introducing plasmid pZL35 expressing the wild-type *fabI* gene. The plasmid completely eliminated the growth defect in fatty acid-depleted AC medium and restored fatty acid synthesis (Fig. 5B, lane 3, and Fig. 6C). We conclude that the *fabI*-encoded ENR plays a key role in *E. faecalis* fatty acid synthesis.

FabK moderates the phospholipid fatty acid composition of *E. faecalis*. Like the wild-type strain, the Δ*fabK* strain grew well on fatty acid-depleted AC medium (Fig. 6A) and incorporated [1-^{14}C]acetate into membrane phospholipids. However, this strain synthesized more unsaturated fatty acid than the wild-type strain (Fig. 5B, lanes 2 and 7). To quantitate this effect, the fatty acid compositions of strains grown in fatty acid-depleted AC medium were determined by gas chromatography-mass spectrometry (GC-MS) (see Table S2 in the supplemental material). The ratio of unsaturated to saturated fatty acids (UFA/SFA) of the wild-type strain was 1.38, but for the Δ*fabK* strain this ratio increased to 2.36, indicating that FabK moderates phospholipid fatty acid compositions. We constructed a plasmid (pZL36) that carried *E. faecalis* wild-type *fabK* and introduced this plasmid into the Δ*fabK* and Δ*fabI* strains. The Δ*fabI* strain carrying this plasmid grew well on fatty acid-depleted AC medium (data not shown), indicating that, upon overproduction, *fabK* could complement the Δ*fabI* strain. Hence, the inability of chromosomally encoded FabK to rescue loss of FabI is due to low enzyme activity.

The UFA/SFA ratio of the Δ*fabI* strain carrying the FabK plasmid was 0.99, whereas the ratio of UFA/SFA of the Δ*fabK* strain carrying the same plasmid was 1.99, further indicating that FabK moderates *E. faecalis* fatty acid composition.

To further characterize the functions of FabK and FabI, cell-free extracts of *E. faecalis* wild-type, Δ*fabI*, and Δ*fabK* strains were tested for the ability to synthesize fatty acids *in vitro*. Incubation of a cell-free extract of the wild-type strain with [2-^{14}C]malonyl-CoA, acetyl-CoA, NADPH, NADH, and ACP resulted in formation of saturated and unsaturated fatty acids (see Fig. S5, lane 6, in the supplemental material). As expected, the cell-free extract of the Δ*fabI* strain was unable to synthesize any fatty acids (see Fig. S5, lane 4), whereas upon addition of purified FabI or FabK protein to the cell-free extract of the Δ*fabI* strain, the reaction mixture formed saturated and unsaturated fatty acids (see Fig. S5, lanes 2 and 3). It should be noted that addition of FabK protein to the extract of the Δ*fabI* strain resulted in increased production of saturated fatty acids, whereas addition of FabI to the Δ*fabI* strain extract resulted in essentially the same fatty acid species as in the wild-type strain extract. On the other hand, the amount of unsaturated fatty acids synthesized by Δ*fabK* strain extract was greater than that formed by the wild-type strain extract (see Fig. S5, lane 5). Thus, we conclude that although the ENR activity of FabK is not major, it plays a role in modulation of *E. faecalis* fatty acid composition.

FabK plays no apparent role in *E. faecalis* triclosan resistance. The *E. faecalis fabK* gene was shown to encode a triclosan-resistant ENR by expression of *fabK* in *E. coli* and by the triclosan resistance of FabK ENR activity *in vitro* (Fig. 5). *E. faecalis*, for which the triclosan MIC is 10 μg/ml, displays inherent resistance; given the presence of FabK, we examined the triclosan resistance of the Δ*fabI* and Δ*fabK* strains. The MIC for triclosan for the Δ*fabK* strain was the same (10 μg/ml) as that for the wild-type strain (Table 1), whereas the MIC for the Δ*fabI* mutant was slightly lower (7.5 μg/ml) than that for the wild-type strain (Table 1). Interpretation of the latter result is problematic because the micelles formed by the fatty acid used to support growth can sequester triclosan (44) or conversely can act to solubilize the antimicrobial compound. When plasmid-borne *fabI* or *fabK* genes were introduced into the Δ*fabI* or Δ*fabK* strains, the triclosan MIC for these strains was the same as that for the wild-type strain (Table 1) and the presence of these plasmids had no effect on the triclosan resistance of the wild-type strain (Fig. 7). These data indicate that neither of the *E. faecalis* ENRs plays a role in the inherent triclosan resistance of this bacterium.

DISCUSSION

It is apparent that the plausible prediction (18) that *E. faecalis* FabK is responsible for the triclosan resistance of this bacterium is incorrect. FabK plays only a secondary role in modulating fatty acid composition and is expressed at levels that are too low to support growth, at least under the growth conditions we have tested. The primary *E. faecalis* ENR is FabI, a triclosan-sensitive enzyme, and this seems likely to be a cellular triclosan target, although the triclosan sensitivity is masked in *E. faecalis* by another process(es), for which efflux of the antibacterial compound seems a likely candidate. Moreover, oleic acid supplementation reverses growth inhibition of *E. faecalis* by cerulenin, a specific and well-characterized inhibitor of the enzyme(s) required for the elongation reactions of long-chain fatty acid synthesis (13), but fails to reverse growth inhibition by triclosan, suggesting that triclosan may target a cellular process unrelated to fatty acid synthesis in

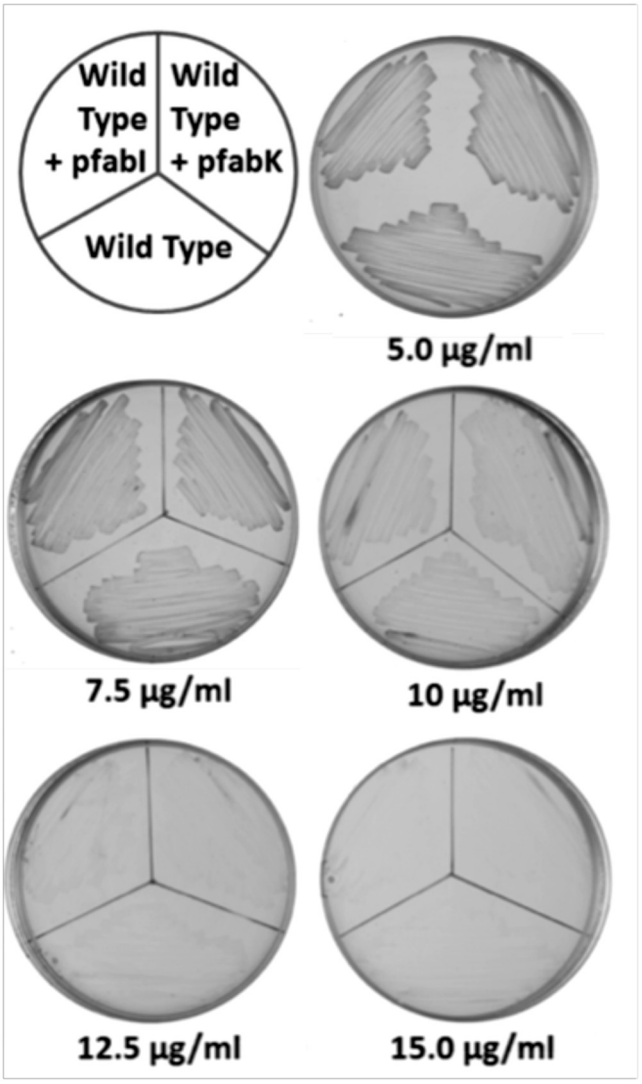

FIG 7 ENR overproduction does not alter the triclosan inhibition profile of *E. faecalis*. The concentration of triclosan is shown below each plate. For simplicity, the plate lacking triclosan was omitted from the figure, but its appearance was essentially identical to that of the plate containing 5 μg/ml triclosan. The pfabI designation indicates the strain carrying plasmid pZL35, whereas pfabK denotes the strain carrying plasmid pZL36.

this bacterium (data not shown). The phenotype of the Δ*fabI* strain shows that antibacterial compounds targeted at the fatty acid synthetic pathway would be of no avail in coping with *E. faecalis* infections. It should be noted that until recently discussions concerning the efficacy of fatty acid synthesis inhibitors in blocking growth of Gram-positive bacteria were highly contentious due to the assumption that the lipid metabolism processes in these diverse bacteria were essentially identical. Brinster and coworkers (48) reported that exogenous fatty acids and human serum were highly effective sources of fatty acids that supported growth of deletion mutants of *Streptococcus agalactiae* blocked in fatty acid synthesis. Soon thereafter, other workers (49) reported that exogenous fatty acids were unable to restore growth to *Staphylococcus aureus* treated with a FabI inhibitor. It now seems clear that both observations are correct and that the mechanisms of feedback inhibition of fatty acid synthesis differ in the two bacteria (44). Exogenous fatty acids can completely replace the *de novo*-synthesized fatty acids of *Streptococcus pneumoniae* but can replace only half of the phospholipid fatty acid moieties of *S. aureus*; the remaining half must come from synthesis (44). Our *E. faecalis* observations strongly resemble those reported for the two *Streptococcus* species in that supplementation with either oleic acid or linoleic acid bypasses the loss of FabI. Since these two unsaturated fatty acids are the major fatty acids of serum (50), we expect that *E. faecalis* would multiply unimpeded in a host treated with a fatty acid synthesis inhibitor.

Although several bacteria are known to encode multiple ENRs, this is the first known case where each enzyme has been shown to have a discrete physiological role (other than triclosan resistance). FabI does the "heavy lifting" in the *E. faecalis* fatty acid synthetic pathway, whereas FabK modulates the composition of the phospholipid acyl chains. However, since *E. faecalis* can make a lipid bilayer from a single unsaturated fatty acid that is fully functional, at least in the laboratory, it is not clear why modulation of fatty acid composition is necessary. A role for fatty acid modulation in the native habitat of this bacterium seems indicated, since *fabK* is conserved in all 24 extant *E. faecalis* complete genome sequences. Moreover, it should be noted that our FA2-2 wild-type strain is representative of *E. faecalis*. The nucleotide sequence of the FA2-2 strain chromosomal segment located between the end of the *acpP* coding sequence and the beginning of the *fabK* sequence (Fig. 1B) is completely conserved in about 230 of the 290 complete and draft *E. faecalis* genome sequences currently available. Those sequences that do not align perfectly with the FA2-2 sequence have only a single base substitution. Finally, it seems noteworthy that a related emerging pathogen, *Enterococcus faecium*, has only *fabI*. Perhaps this reflects differing ecological niches for the two enterococci.

MATERIALS AND METHODS

Materials. For details of the materials used in the study, see Materials and Methods in the supplemental material (Text S1). AC medium contains (in g/liter): tryptone, 10; yeast extract, 10; glucose, 1; K_2HPO_4, 5; pH 7.2).

Bacterial strains, plasmids, and growth conditions. For details of the bacterial strains, plasmids, and growth conditions used in the study, see Materials and Methods in the supplemental material.

Protein expression and purification. For details of the protein expression and purification methods used in the study, see Materials and Methods in the supplemental material.

Construction of *E. faecalis* deletion strains. For details of the construction of the *E. faecalis* deletion strains used in the study, see Materials and Methods in the supplemental material.

Cell-free extract preparation and *in vitro* fatty acid synthesis assay. For details of the cell-free extract preparation and *in vitro* fatty acid synthesis assay used in the study, see Materials and Methods in the supplemental material.

Assay of EnFabK and EnFabI activities *in vitro*. To test ENR function in the first cycle of fatty acid synthesis, EnFabK and EnFabI were assayed in reaction mixtures containing 0.1 M sodium phosphate (pH 7.0); 0.1 μg each of EcFabD, EcFabH, EcFabG, and EcFabA; 50 μM NADH; 50 μM NADPH; 1 mM β-mercaptoethanol; 100 μM acetyl-CoA; 100 μM malonyl-CoA; and 50 μM holo-ACP in a final volume of 40 μl. To investigate the reduction of long-chain intermediates, conversion of *trans*-2-decenoyl-ACP to decanoyl-ACP was measured. The *trans*-2-decenoyl-ACP was synthesized in a preincubation reaction using *Vibrio harveyi* AasS (29). Briefly, the AasS reaction mixtures which contained 10 mM ATP, 20 mM $MgSO_4$, 0.1 M Tris-HCl (pH 7.4), 1 mM dithiothreitol,

0.1 mM *E. coli* holo-ACP, 0.5 mM *trans*-2-decenoic acid, and 40 μg/ml of His-tagged *V. harveyi* ACP synthetase were incubated at 37°C for 2 h. NADH and ENR were then added, the mixture was incubated at 37°C for 1 h, and then the reaction products were resolved by conformationally sensitive gel electrophoresis on 17.5% polyacrylamide gels containing a concentration of urea optimized for the separation (51). The gel was stained with Coomassie brilliant blue R250. A stock solution of triclosan was prepared in 95% ethanol. In order to assess the possible inhibitory effects of triclosan on enzyme activities, the appropriate volumes of triclosan solution were added into the assay tubes, and the solvent was evaporated before the addition of the assay ingredients. The conformationally sensitive gel electrophoresis method takes advantage of the differential partial denaturation of ACP species under alkaline conditions in the presence of urea.

ACP species carrying a hydrophobic acyl chain are more stable than holo-ACP, which, in turn, is more stable than apo-ACP and ACP species acylated with hydrophilic acyl groups (e.g., malonyl-ACP). In the gel systems commonly used to resolve ACP species, partially denaturing conditions are maintained by urea and alkaline pH and the mobility of a protein is inversely related to its hydrodynamic radius (all species of a given ACP have essentially the same net charge). The method was previously discussed in more detail by Cronan and Thomas (51).

ENR activity was directly monitored spectrophotometrically by measurement of the decrease in the absorbance at 340 nm using an NADH extinction coefficient of 6,220 M^{-1} (29). Each reaction was performed in UV light-transparent microcuvettes. The reaction mixtures (100 μl) for activity assays of EnFabI or EnFabK contained 150 μM NADH, 10 ng of the purified native EnFabI or 100 ng of EnFabK, 100 μM enoyl-ACP, 0.1 M LiCl, and 0.1 M sodium phosphate buffer (pH 7.0). For K_m determinations, the concentration of the enoyl-ACP substrate was adjusted. In triclosan inhibition experiments, the enoyl substrate was crotonyl-CoA (Sigma), which was used as a model enoyl-ACP substrate. Since triclosan inhibition is due to hydrophobic interaction (***π***–stacking) of one of the two linked chlorine-substituted aromatic rings with the nicotinamide ring of the NAD^+ product of the ENR reaction, the enoyl-thioester used is irrelevant. Note that, unlike the *S. pneumoniae* FabK (26), EnFabK was unable to reduce crotonyl-CoA. Kinetic constants were determined using GraphPad Prism software, version 4.

Fatty acid biosynthetic analysis of *E. faecalis* strains. *E. faecalis* strains were cultured in AC medium (0.1 mM fatty acid was added, if required) at 37°C to the log phase. The cells were harvested, and their fatty acids were analyzed by gas chromatography-mass spectrometry as described above. Fatty acid biosynthesis was analyzed by [1-^{14}C]acetate incorporation as follows. *E. faecalis* strains were cultured in AC medium (containing 0.01 mM oleic acid, if required) at 37°C overnight. The cells were washed twice using water and resuspended in AC medium. The suspensions were diluted to an optical density at 600 nm of 0.3. After addition of sodium [1-^{14}C]acetate (final concentration, 1 μCi/ml), the cells were grown at 37°C for 4 h. The cells were collected, and labeled fatty acid methyl esters were prepared and analyzed by thin-layer chromatography and autoradiography as described above.

Saturated fatty acid elongation by *E. faecalis* strains was analyzed by labeling with 1-^{14}C-labeled octanoic, decanoic, or dodecanoic acids as follows. *E. faecalis* strains were cultured in AC medium (containing 0.01 mM oleic acid, if required) at 37°C overnight. The cells were washed twice using water and resuspended in AC medium. The suspensions were diluted to an optical density at 600 nm of 0.3. After addition of the 1-^{14}C-labeled acid (final concentration, 0.1 μCi/ml), the cultures were shaken at 37°C for 4 h. The cells were collected, and the labeled fatty acids were prepared and separated by reverse-phase thin-layer chromatography (52).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00613-13/-/DCSupplemental.

Text S1, DOCX file, 0.4 MB.
Figure S1, DOCX file, 0.4 MB.
Figure S2, DOCX file, 0.5 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 3.9 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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