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

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ABSTRACT Enoyl-acetyl 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 Enterococcus 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.
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. coli strains are resistant to virtually all clinically useful antibiotics (36). Fatty acid synthesis inhibitors are 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) grew at 42°C in the presence of IPTG.

In contrast, strains carrying EnfabI or EnfabK (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 EnfabI and EnfabK 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 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)
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 plasmids grew in liquid medium in the presence or absence of arabinose (Ara). Symbols are as described for panel B. OD<sub>600</sub>, optical density at 600 nm.

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 EnFabK, EnFabI, or Ec fabI (pZL30, pZL31, or pZL32, respectively) or the vector plasmid, pHSG575. (B) Growth of E. coli strain JP1111 carrying plasmids with EnFabI, EnFabK, 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 pZL32); ◼, plasmid carrying EnFabK (pZL31 or pZL24). (C) Growth of E. coli strain JP1111 with plasmids carrying En fabI, EnFabI, or EnFabK (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<sub>600</sub>, optical density at 600 nm.

### 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  | EnFabK    | 20                    |
| W3110/pZL31  | Empty vector | 0.2                   |
| W3110/pBAD24M| Ec fabI   | 2                     |
| W3110/pZL18  | EnFabK    | 3                     |
| W3110/pZL23  | EnFabK    | 20                    |
| W3110/pZL24  | EnFabK    | 120                   |
| E. faecalis   |           |                       |
| FA2-2        |           | 10                    |
| FAZL1(ΔfabI) |           | 7.5<sup>a</sup>       |
| FAZL1(ΔfabI)/pZL023 | EnFabI | 10                    |
| FAZL1(ΔfabI)/pZL024 | EnFabK | 10                    |
| FAZL2(ΔfabK) |           | 10                    |
| FAZL2/pZL024 | EnFabK    | 10                    |

<sup>a</sup>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).

Expression, purification, and characterization of the E. faecalis FabI and FabK proteins. The E. faecalis fabI and fabK 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-hydroxybutyril-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-hydroxybutyril-ACP was converted to butyryl-ACP.

To test the activities 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).

**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 (Enl check UP and Enl check DOWN) amplified a 1.25 kb *ΔfabI*-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 *ΔfabK* 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 (*ΔfabI*) on AC medium was tested, and although strain FAZL1 (*ΔfabI*) was viable, the growth was less than that of wild-type strain FA2-2 (wild type) and strain FAZL3 (*ΔfabK*) (Fig. 5A). The function of fabI in *E. faecalis* fatty acid synthesis was tested by measuring *de novo* fatty acid synthesis by [1-14C]acetate incorporation into membrane phospholipids. The *ΔfabI* 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 *ΔfabI* strain, the level of FabK ENR activity could not support sufficient fatty acid synthesis for growth and thus that the *ΔfabI* strain, the level of FabK ENR activity could not support sufficient fatty acid synthesis for growth and thus that the *ΔfabI*
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 fabI carried by vector pBM02) into the ΔfabI 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 ΔfabI 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 ΔfabI 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 ΔfabI mutant strain was investigated.

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).
tant was examined by addition of 1-14C-labeled octanoic, deca-noic, 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 \( \text{p} \text{fabI} \) 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 efficient incorporation into phospholipids.

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 \( \text{Δ} \text{fabK} \) strains elongated dodecanoic acid mainly to palmitic acid (C16), whereas the \( \text{Δ} \text{fabI} \) strain failed to extend the acid. These data indicated that fatty acid synthesis of the \( \text{Δ} \text{fabI} \) 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 \( \text{Δ} \text{fabI} \) mutant on fatty acid-depleted AC medium and found that the wild type grew well on this medium, whereas the \( \text{Δ} \text{fabI} \) strain mutant failed to grow (Fig. 6A). The growth defect of \( \text{Δ} \text{fabI} \) 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 \( \text{Δ} \text{fabI} \) strain did not give improved growth, in-

**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 IC50 and Kᵢ values were determined from Dixon plots. It should be noted that much lower Kᵢ 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 [1-14C]acetate-labeled phospholipids. Lanes 1, 2, and 7 represent the \( \text{Δ} \text{fabK} \) strain carrying either an *EnfabK* plasmid (pZL36, lane 1) or no plasmid. Lanes 3, 4, and 8 represent the \( \text{Δ} \text{fabI} \) 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 \( \text{Δ} \text{fabI} \) 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 \( \text{Δ} \text{fabI} \) strain was due to the low levels of unsaturated fatty acids present in AC medium.
FabK to rescue loss of FabI is due to low enzyme activity, indicating that, upon overproduction, E. faecalis strain was 1.38, but for the wild-type strain this ratio increased to 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 ΔfabI and ΔfabK strains. The MIC for triclosan for the ΔfabK strain was the same (10 μg/ml) as that for the wild-type strain (Table 1), whereas the MIC for the ΔfabI 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 ΔfabI or ΔfabK 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 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, ΔfabI, and ΔfabK strains were tested for the ability to synthesize fatty acids in vitro. Incubation of a cell-free extract of the wild-type strain with [2,14C]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 ΔfabI 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 ΔfabI 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 ΔfabI strain resulted in increased production of saturated fatty acids, whereas addition of FabI to the ΔfabI 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 ΔfabK 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.
pfabK denotes the strain carrying plasmid pZL36. The pfabI designation indicates the strain carrying plasmid pZL35, whereas growth of deletion mutants of rum were highly effective sources of fatty acids that supported these diverse bacteria were essentially identical. Brinster and co-workers (49) reported E. faecalis strain shows that antibacterial compounds targeted at the fatty acid synthesis 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.

**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.

**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; K2HPO4, 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 EnFabl activities in vitro.** To test ENR function in the first cycle of fatty acid synthesis, EnFabK and EnFabl 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 MgSO4, 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, partial 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 cm⁻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 Kₘ determinations, the concentration of the enoyl-ACP substrate was adjusted. In triclosan inhibition experiments, the enoyl substrate was crotonyl-ACP (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 chloride-substituted aromatic rings with the nicotinamide ring of the NAD⁺ product of the ENR reaction, the enoyl-thioester is used 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 [1-¹⁴C]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 [1-¹⁴C]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 1-¹⁴C-labeled octanoyl, decanoyl, or dodecanoyl 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 1-¹⁴C-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).

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