**Vibrio cholerae** FabV Defines a New Class of Enoyl-Acyl Carrier Protein Reductase*5

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Enoyl-acyl carrier protein (ACP) reductase catalyzes the last step of the fatty acid elongation cycle. The paradigm enoyl-ACP reductase is the FabI protein of *Escherichia coli* that is the target of the antibacterial compound, triclosan. However, some Gram-positive bacteria are naturally resistant to triclosan due to the presence of the triclosan-resistant enoyl-ACP reductase isoforms, FabK and FabL. The genome of the Gram-negative bacterium, *Vibrio cholerae* lacks a gene encoding a homologue of any of the three known enoyl-ACP reductase isoforms. FabV, FabK and FabL. The genome of the Gram-negative bacterium, *Vibrio cholerae* lacks a gene encoding a homologue of any of the three known enoyl-ACP reductase isoforms, FabK and FabL. The native FabV protein has been purified to homogeneity and is active with both crotonyl-ACP and the model substrate, crotonyl-CoA. In contrast to FabL and FabB, FabV shows a very strong preference for NADH over NADPH. Expression of FabV in *E. coli* results in markedly increased resistance to triclosan and the purified enzyme is much more resistant to triclosan than is *E. coli* FabI.

Fatty acid synthesis is essential for the formation of membranes and hence for the viability of all cells excepting the Archaea. The bacterial fatty acid synthesis system (FAS II) differs significantly from the mammalian and fungal system (FAS I). Whereas FAS I systems use complex multifunctional proteins to synthesize fatty acids, bacteria use separate discrete proteins for each step of the biosynthesis pathway (1, 2). In the FAS II systems of bacteria, chloroplasts, apicoplasts, and mitochondria, each acyl intermediate is channeled among enzymes as an acyl carrier protein (ACP) thioester. The differences between the FAS I and FAS II systems make the FAS II enzymes good targets for antibacterial inhibitors (3–6). The paradigm FAS II system is that of *Escherichia coli* and this has provided an excellent model system. Most FAS II enzymes are relatively conserved among bacteria and some domains of the FAS I proteins are clearly derived from FAS II proteins (7). An exception is the last step of the elongation cycle (Fig. 1) formation of a saturated acyl-ACP by an NAD(P)H-dependent reduction of the enoyl-ACP double bond. In *E. coli* this reaction is catalyzed by the product of the fabI gene, which was discovered as the target for the antibacterial action of a set of diazaborine compounds. FabI was later shown to also be the site of action of triclosan (8), an antibacterial compound used in hand soaps and a large variety of other everyday products. Although FabI homologues are widely distributed in bacteria and other FAS II-containing organisms, the existence of a number of bacterial species naturally resistant to triclosan was soon recognized. In these bacteria triclosan resistance was due to the presence of other enoyl-ACP reductase isoforms of varying resistance to triclosan. *Bacillus subtilis* contains two isoforms, a FabI homologue and a somewhat triclosan-resistant isozyme called FabB (9) that, like FabI, is a member of the short chain dehydrogenase reductase superfamily. *Streptococcus pneumoniae* contains a single enoyl-ACP reductase, FabK, that is refractory to triclosan and is a flavoprotein unrelated to the short chain dehydrogenase reductase isoforms (10).

*Vibrio cholerae* is a Gram-negative bacterium that causes cholera in humans. When we began our work only a single genome sequence was available for this organism (11) and this sequence argued that *V. cholerae* did not encode a convincing homologue of any of the known enoyl-ACP reductase isoforms (FabL, FabK, or FabB). This conclusion was recently confirmed by shotgun genome sequences of a large number of *V. cholerae* strains now available from the NCBI genomes data base. Moreover, the genome sequences of other *Vibrio* species also show the lack of known enoyl-ACP reductase homologues. The lack of a FabI homologue was especially surprising because otherwise the *Vibrio* FAS proteins are very similar to those of *E. coli*. Indeed, the major FAS II gene clusters of *E. coli* and *V. cholerae* have almost identical organizations. We report the isolation of the gene that encodes a fourth enoyl-ACP reductase isozyme that we have named FabV and some properties of the new enzyme.

**EXPERIMENTAL PROCEDURES**

*Construction of Bacterial Strains and Plasmids—* E. coli strain JP1111, which carried the temperature-sensitive (Ts)

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2. The abbreviations used are: FAS, fatty acid synthesis; ACP, acyl carrier protein; ORF, open reading frame; SDR, short chain dehydrogenase reductase.
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FIGURE 1. The elongation cycle of fatty acid biosynthesis. Each elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats. The elongation cycle is initiated by the condensation of malonyl-ACP with acyl-ACP carried out by one of the 3-ketoacyl-ACP synthases. The second step is the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP followed by the dehydratation of the resulting 3-hydroxyacyl-ACP and then 2-unsaturated acyl-ACP. The product is then dehydrogenated by 3-ketoacyl-ACP reductase to acetyl-CoA and then the cycle repeats.

The fabl392 allele, was obtained from the Coli Genetic Stock Center, Yale University. The mutation, which will be called fabl(Ts), allows growth at 30 °C but blocks growth at 42 °C. The temperature-sensitive activity of the mutant enzyme has been directly demonstrated (12) and is due to a single point mutation (13). Strain EPI300 (genotype recA1 endA1 araD139 Δ(ara,leu)7697 trfA, with the trfA gene being under arabinose regulation) (14) was made temporarily proficient in homologous recombination by introducing plasmid pEAK2, which carries a functional recA gene on an unstable plasmid (15). To move the fabl(Ts) mutation from JP1111 into this strain a closely linked Tn10 transposon insertion was introduced into the JP1111 chromosome by transduction to tetracycline resistance at 30 °C with a phage P1vir stock grown on the trpC::Tn10 strain CAG18455 (16, 17) followed by screening for recombinants that remained temperature sensitive. A phage P1vir stock grown on one of these recombinants was then used to transduce the EPI300 (pEAK2) strain to tetracycline resistance at 30 °C with screening for temperature sensitivity and loss of the pEAK2 antibiotic resistance determinant to give strain PMT03. For plasmid constructions the recombinants were end-repaired before ethanol precipitation in the presence of glycogen. Fragments larger than 3 kb were gel-purified, cloned into the complementing cosmids. The resulting DNA fragments were end-repaired to 5‘-phosphorylated blunt ends and then separated on a 0.8% low melting point agarose gel run overnight at 30 V. Sheared DNA fragments of about 40 kb were size selected, gel-purified, ligated to the dephosphorylated pCC1FOS vector, and then packaged into A phage particles. This was used to transduce the fabl(Ts) E. coli recipient strain PMT03 to chloramphenicol resistance at 30 °C under conditions where the entering plasmids would replicate at single copy. The resulting transductants were then tested for the ability to grow at 42 °C, the nonpermissive temperature of the fabl(Ts) allele. A complementing clone that grew well at 42 °C was obtained. The purified complementing cosmids was again transformed into the fabl(Ts) strain PMT03 to confirm the complementation. This strain was then induced with arabinose to induce the cosmids to high copy number and large quantities of the complementing cosmids DNA were obtained using the Qiagen Large-construct Kit (Valencia, CA). The cosmids was then partially sequenced using vector-specific primers to localize the insert DNA on the V. cholerae genome. To obtain smaller complementing clones 50 µg of the 40-kb cosmids was sonicated for 5 s, and the DNA fragments were end-repaired before ethanol precipitation in the presence of glycogen. Fragments larger than 3 kb were gel-purified, cloned into the pCC1FOS vector. The resulting plasmids were then transformed into mutant strain PMT03 and screened for complementation at 42 °C and for tetracycline resistance at 30 °C. Each complementing subclone was completely sequenced and its genomic location identified by a BLAST search against the V. cholerae El Tor N16961 genome (11) using the TIGR Comprehensive Microbial Resource website (cmr.tigr.org).

Enzyme Purification—Primers were designed for cloning the candidate gene into a pET101 expression vector from the Invitrogen Champion pET101 Directional TOPO Expression Kit (Carlsbad, CA). Six histidine codons were added at the 3’-end of the gene to produce a His tag at the C-terminal end of the protein. The gene was PCR-amplified using Pfu Turbo DNA polymerase from Stratagene (La Jolla, CA). The PCR fragment was cloned into pET101, sequenced, then transformed into BL21 Star (DE3) chemically competent cells from Invitrogen. The expression of the cloned fragment was induced with 150 µM isopropyl β-D-thiogalactopyranoside. The crude extract was then applied to a nickel-nitrilotriacetic acid spin column from Qiagen (Valencia, CA), washed
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with the lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole), and the reductase eluted with 250 mM imidazole in the same buffer. Similarly, the native protein (lacking a histidine-tag) was also cloned and overexpressed from the pET101 vector. Purification was first performed through a Vivapure D maxi H spin column from Vivascience (Sartorius). The protein was washed with 4 mM Tris-HCl, 1 mM EDTA (pH 8) lysis buffer, discontinuously eluted with a 50 mM Tris-HCl (pH 7.5) buffer containing 100, 200, 300, 400, 500, 700, or 1000 mM NaCl. Most of the reductase was eluted with 400 mM NaCl. The fractions eluted at 400 and 500 mM NaCl were pooled, then desalted by overnight dialysis before passing through a Blue Sepharose column from using the ÄKTA purification apparatus (GE Healthcare). Elution from the affinity column was achieved using a 2 M LiCl buffer solution after washing with 15 column volumes of 20 mM sodium phosphate buffer (pH 7). Finally the protein was concentrated by ultra centrifugation using a 30,000 M/WCO Amicon Ultra centrifugal filter device from Millipore.

**Fatty Acid Synthesis Assays**—The ability of the new gene to restore in vivo fatty acid synthesis was tested by transforming the fabI(Ts) mutant strain, JP1111 with a plasmid in which the gene encoding the putative *V. cholerae* enoyl-ACP reductase had been inserted into the arabinose inducible pBAD322 vector (18). The cultures were grown at permissive temperature, induced with arabinose, shifted to 42 °C, and then [1-14C]acetate (specific activity, 55 mCi/mmol) was added. Following incubation at 37 °C for 45 min (19), the reductase activity in the fractions was measured spectrophotometrically by decrease in absorbance at 340 nm using an NADH extinction coefficient of 6220 M⁻¹ cm⁻¹. Each 100-µl reaction was performed in disposable UV-transparent microcuvettes obtained from Brand-Tech Scientific, Inc. The activity assays contained varying concentrations of NADH, 1 pg of the purified native FabV, varying substrate concentrations (crotonyl-CoA or crotonyl-ACP), and 0.1 M LiCl in a 0.1 M sodium phosphate buffer (pH 7). Kinetic constants were determined using GraphPad PRISM version 4 software. The Kₘ values for NADH and NADPH were determined at a crotonyl-ACP concentration of 80 µM. The Kₘ values for crotonyl-ACP and crotonyl-CoA was determined using 150 µM NADH. Tricosan was purchased from KIC Chemicals, Inc. (Armonk, NY).

**Size Exclusion Chromatography**—The native FabV was chromatographed on a Superdex 200 HR 10/30 column in a 0.15 M NaCl, 50 mM sodium phosphate buffer at pH 7 on the AKTA purifier (GE Healthcare). The calibration curve was prepared using chymotrypsinogen, ovalbumin, bovine serum albumin, aldolase, ferritin, and thyroglobulin as standards ranging from 25,000 to 669,000 Da. The FabV peak was detected by absorbance at 280 nm and the reductase activity in the fraction was confirmed by spectrophotometric assay and the size of the monomer was confirmed by 8% SDS-PAGE of the peak fractions.

**RESULTS**

Identification of a *V. cholerae* Gene Encoding a Novel Enoyl-ACP Reductase—A cosmid was selected from the *V. cholerae* genomic library that consistently complemented growth of the fabI(Ts) strain PMT03 at 42 °C (Fig. 2A). The large *V. cholerae* genomic fragment (about 40 kb) carried by the cosmid was fragmented and the fragments were cloned into the same vector to obtain smaller complementing plasmids. Four of these subclones were able to complement growth of the fabI(Ts) mutant strain at 42 °C and only those clones also allowed growth on tricosan at 30 °C (Fig. 2B). Upon partial sequencing, all four clones were found to contain a common genomic region that contained only three open reading frames (ORFs) by comparison with the genome.

3 J. Thomas and J. Cronan, unpublished data.
sequence of *V. cholerae* O1 biovar El Tor. N16961, the only sequenced *V. cholerae* genome then available. One of the three ORFs (VC1737) was annotated as encoding protein synthesis initiation factor IF-1, whereas the other two ORFs, VC1738 and VC1739, were annotated as hypothetical proteins (supplemental Fig. 1S). The latter two ORFs had been predicted to be in a single transcriptional unit (www.BioCyc.org). To identify these ORFs, a BLAST search of each of the ORFs was performed against all of the bacterial genomes in the data base. Both VC1738 and VC1739 showed homology to a single ORF found in a number of bacterial genomes. These were (accession number follows the species name) *Xanthomonas campestris* (YP361851) *Vibrio parahemolyticus* (NP797610), *Vibrio vulniificus* (YP130609), *Vibrio fischeri* (NP640497), *Vibrio cholerae* N16961, the only recently sequenced *V. cholerae* which is predominately found in human pathogens and others infecting marine organisms. There may be bacteria that have both FabV and FabK such as various of the human pathogens and others infecting marine organisms. There may be bacteria that have both FabV and FabK such as various of the

![Figure 2](image2.png)

**FIGURE 2.** The cosmid complements growth of an *E. coli fabI* (Ts) strain and also imparts resistance to triclosan. *Panel A* shows the growth of the *E. coli fabI* (Ts) mutant strain PTO3 at either the permissive temperature of 30 °C or the nonpermissive temperature of 42 °C when transformed with either the fabV cosmid pMT18 or the vector pCC1 fos. *Panel B* shows the growth of the *E. coli fabI* (Ts) mutant strain PTO3 at the permissive temperature of 30 °C in the presence or absence of 2 μg/ml triclosan. The medium used was LB medium containing 12 μg/ml chloramphenicol.

![Figure 3](image3.png)

**FIGURE 3.** Alignments of FabV with FabI and FabL. The *V. cholerae* FabV sequence (top line) is that determined in this work, whereas the FabI (middle line) and FabL (bottom line) sequences are from *E. coli* and *B. subtilis*, respectively. The active site tyrosine and lysine residues are marked with asterisks.

![Alignments of FabV with FabI and FabL](image4.png)
Clostridia such as Clostridium tetani. However, like FabV various putative FabK homologues may not possess enoyl-ACP reductase activity (23).

FabV, like FabI and FabL, is a member of the SDR superfamily (24, 25) although FabV aligns only weakly with FabI or FabL even when many gaps are allowed (Fig. 3). FabV does not contain the Tyr-(Xaa)₅-Lys motif typical of most members of the SDR superfamily. FabI and FabL also lack this motif and instead use a Tyr-(Xaa)₆-Lys active site motif. In FabV another variation is seen, a Tyr-(Xaa)₈-Lys motif (Fig. 3). Given the conservation of residues that neighbor the key tyrosine and lysine residues this seems likely to be the FabV active site although this remains to be tested by mutagenesis. Despite the low homology scores FabV can be modeled on the crystal structures of known SDR enoyl-ACP reductases by the Geno3D server (geno3d-pbil.ibcp.fr), which takes into account known protein structures as well as predicted geometrical restraints (26).

**The Enzymatic Activity of FabV**—Expression of FabV in a fabI(Ts) mutant strain restored de novo fatty acid synthesis at the non-permissive temperature to wild type levels (Fig. 4). Moreover, addition of purified FabV (see below) to cell extracts of fabI(Ts) mutant strains gave wild type rates of in vitro fatty acid synthesis (supplemental Fig. 2S). These early in vitro experiments were done with a version of FabV carrying a His₆ purification tag and purified by Ni²⁺-chelate chromatography (see below). Later we found that under conditions of low expression (where protein production is stochastic among cells (27, 28)) the survival of an E. coli fabI(Ts) mutant strain expressing this construct was much lower than that of the same strain expressing the native form of the protein (Fig. 5). Because the N-terminal His₆-tagged FabV

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**FIGURE 4.** Assay of complementation in vivo. Cultures of the E. coli fabI(Ts) strain JP1111 carrying either the vector or the complementing cosmid or the wild type (WT) strain MG1655 were labeled with [1-¹⁴C]acetate followed by extraction of the cellular phospholipid and analysis by TLC. The presence or absence of the complementing cosmid is denoted by plus or minus signs, respectively. An autoradiogram of the TLC plate is shown. The phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL).

**FIGURE 5.** Growth of the E. coli fabI(Ts) strain transformed with plasmids encoding three different forms of FabV. Each version of the fabV gene was cloned into the arabinose-inducible low copy vector, pBAD322, and these plasmids were transformed into the fabI(Ts) strain JP1111. Serial 10-fold dilutions of each culture were spotted (from top to bottom) onto plates of LB medium supplemented with 0.2% glucose to repress the arabinose promoter. The plates were incubated at 42 °C.
also gave inefficient complementation (albeit to a much lesser extent), we produced the native FabV at high levels in E. coli and purified it from that source by two chromatographic steps (Fig. 6). The most effective step in the purification of the native protein was chromatography on Blue Sepharose CL-6B where the immobilized Cibacron Blue F3G-A dye mimics adenylate-containing cofactors such as NAD (29). A similar step was used in the purification of E. coli FabI (30).

The native FabV was active on both crotonyl-CoA and crotonyl-ACP using the standard NADH oxidation spectrophotometric assay and thus commercially available crotonyl-CoA was often used to assay the enzyme (Figs. 7 and 8). The native FabV was much more active than the C-terminal His<sub>6</sub>-tagged form (Fig. 8A) and showed a very strong preference for NADH over NADPH when assayed with crotonyl-CoA (Fig. 8B). The $K_{\text{m}}$ for NADH was 60-fold higher than that for NADPH (Table 1). The experimentally observed maximal velocities of FabV with crotonyl-CoA and crotonyl-ACP were similar with the rate with the latter substrate being about 50% higher. The $K_{\text{m}}$ for crotonyl-ACP (195 $\mu$M) was 6-fold lower than that for crotonyl-CoA (1178 $\mu$M) (Table 1). It should be noted that the ACP used was that of E. coli rather than that of V. cholerae. However, the ACP of V. cholerae is 96% identical to that of Vibrio harveyi, which has been shown to functionally replace the ACP of E. coli in vivo (31) (the ACPs of V. cholerae and E. coli are 84% identical).

FabV is a monomeric protein in solution as determined by size exclusion chromatography (Fig. 6). This is in contrast to FabI, which is a homotetramer. The solution structure of FabI has not been reported, although it has been crystallized (32).

**Triclosan Resistance of FabV—** Early in our work we found that expression of FabV in E. coli imparted resistance to triclosan (Fig. 2B). A similar picture was seen in an E. coli in vitro fatty acid synthesis system in which the inactivated mutant FabI was replaced with FabV (supplemental Fig. 3S) or when an in vitro fatty acid synthesis system prepared from V. cholerae cells was treated with triclosan (data not shown). Moreover, growth of the wild type V. cholerae strain ATCC 14547 was 20-fold more resistant to triclosan than was the wild type E. coli strain MG1655 (supplemental Fig. 4S). These observations argued that FabV might be significantly more resistant to triclosan than is E. coli FabI. Two modes of triclosan inhibition of enoyl-ACP reductases have been observed. Some triclosan-sensitive enoyl-ACP reductases, such as FabI, are irreversibly inhibited by formation of a dead-end reductase-NAD<sup>+</sup>-triclosan ternary complex, whereas other reductases such as FabI show reversible inhibition without formation of a ternary complex. Moreover, under the usual assay conditions triclosan is a slow binding inhibitor of FabI-type enzymes because the reductase-NAD<sup>+</sup> complex must be formed before the inhibitor can bind. Triclosan inhibition of FabV is very weak (Fig. 9). However, the degree of FabV inhibition increased as the reaction proceeds indicating that a product, presumably NAD<sup>+</sup>, may be involved in the inhibition. Indeed, triclosan inhibition of FabV was potentiated by preincubation with NAD<sup>+</sup> (Fig. 9). However, even in the presence of NAD<sup>+</sup>, triclosan was not a potent inhibitor of FabV.

**DISCUSSION**

We have identified a new enoyl-ACP reductase isoform encoded by V. cholerae. The lack of sequence alignment with
The catalytic efficiency of each enzyme was determined under pseudo first-order conditions with NADH in excess and crotonyl-ACP (or crotonyl-ACP) concentrations under $K_{m}$ values (100 and 15 μM, respectively). Under these conditions, the first-order rate constant $k_{cat}$ was determined over time and catalytic efficiency was determined as $k_{cat}/[E]_{T} = K_{cat}/K_{m}$. The enzyme concentrations were 2.61 pM for native FabV and 25 nM for the C-terminal His6-tagged (C-tag) FabV. The C-terminal-tagged FabV was active site and much greater size indicates that the V. cholerae enzyme should be considered a member of a new class of enoyl-ACP reductases having homologues in both closely and distantly related species of bacteria. We have named this fourth enoyl-ACP reductase isoform, FabV for *Vibrio* a species in which this is the only enoyl-ACP reductase now predictable by sequence alignment. Like its Fabl and FabL functional homologues, it is a member of the SDR superfamily. This very large family of NAD(P)-dependent oxidoreductases shows a significantly conserved structure despite little sequence homology (15–30%) among members. The largely conserved SDR folding pattern allows specific sequence motifs to be assigned, with those for the coenzyme-binding and active site regions being the most definitive. A recent bioinformatic analysis of the SDR superfamily placed the enzymes into five families (25). One of these families (called divergent) is composed of the Fabl-type enoyl-ACP reductases of bacteria and plants (25). FabV does not fall cleanly into this or any of the other four families. FabV is 60% larger than the typical SDR family member (which are generally about 250 residues in length) and the spacing between the putative FabV active site tyrosine and lysine residues is eight residues, two more than Fabl and FabL and one more than the maximum reported for other SDR proteins. Conversely the coenzyme-binding site of FabV has the classical Rossman fold motif like that of FabL, whereas the Fabl coenzyme-binding fold departs from that motif. Persson and co-workers (25) have reported that the coenzyme preference for the SDR enzymes can be predicted with >93% accuracy based on the presence or absence of key residues. The very strong preference of FabV for NADH over NADPH fits the predictive rules of Persson and co-workers (25). These rules also correctly predict the coenzyme preferences of *B. subtilis* FabL (NADPH strongly preferred) (9) and *E. coli* Fabl (little or no preference) (12).

In vivo and in vitro assays demonstrate that expression of FabV restores the otherwise defective *de novo* fatty acid synthesis of the *E. coli fabI392*(Ts) mutant at the nonpermissive temperature to normal (Figs. 2B and 4). Because

| Enzyme | $K_m$ (μM) | Catalytic efficiency $k_{cat}/[E]_T = K_{cat}/K_{m}$ | $K_m$ (μM) |
|--------|------------|--------------------------------|------------|
| FabV C-tag | 900 ± 64 | 1.2 x 10⁷/ND | 52 ± 14 |
| FabV Native | 1178 ± 471 | 9 x 10⁶/4.1 x 10⁷ | 53 ± 17 | 3043 ± 1713 |

*ND, not determined.*
FabI is the sole enoyl-ACP reductase of E. coli and is known to reduce enoyl-ACPs of acyl chain length from C2 to C18 (9, 33), the complementation data indicate that FabV is active with this range of acyl chain length substrates. Moreover, expression of FabV confers triclosan resistance to E. coli (Figs. 2B and supplemental Fig. 4S) as previously seen for FabK and FabL. The resistance imparted by FabV is also observed in cell extracts and for the purified enzyme. Therefore, FabV is the first intrinsically triclosan-resistant enoyl-ACP reductase found in a Gram-negative bacterium. Indeed, FabV shows little inhibition at triclosan concentrations approaching the solubility limit of the compound. Moreover, expression of FabV from a single copy vector in E. coli made the host more resistant to triclosan than was the V. cholerae strain from which the encoding gene was derived. Two of the possible explanations for this unexpected result are that V. cholerae may have a second target for triclosan, either another enoyl-ACP reductase or a FAS II-independent target (34, 35) or that FabV expression is negatively regulated in V. cholerae, but not in E. coli.

Triclosan is a very weak inhibitor of FabV even when inhibition is potentiated by addition of NAD$^+$. For example, FabL shows 50% inhibition by 12 $\mu$M triclosan (9), whereas the same extent of inhibition of FabV requires 136 $\mu$M triclosan in the absence of added NAD$^+$ (Fig. 9). Interpretation of the effects of NAD$^+$ on triclosan inhibition is complicated by the finding that NAD$^+$ was a much stronger inhibitor of FabV than triclosan (5 $\mu$M NAD$^+$ inhibited similarly to 168 $\mu$M triclosan). This is not the case for FabI. NAD$^+$ does not inhibit FabI and indeed FabI fails to bind NAD$^+$ unless triclosan is present (9, 36–38). Triclosan increases the affinity of FabI for NAD$^+$ by 1000-fold such that the inhibitory ternary complex can be formed (9, 36–38). FabV may also form an inhibitory ternary complex. If so, the triclosan-mediated increase in the affinity of FabV for NAD$^+$ would be very modest relative to that seen with E. coli FabI and would require much higher levels of triclosan. In the presence of NAD$^+$ E. coli FabI is completely inhibited by 2 $\mu$M triclosan (36), whereas complete inhibition of FabV in the presence of NAD$^+$ required about 300-fold higher levels of triclosan (Fig. 9). In any event triclosan is such a weak inhibitor of FabV that further investigation of the mode of inhibition seems of little practical use. In contrast to FabI and FabL, the reversible triclosan inhibition of FabV does not seem to involve its interaction with NAD$^+$ (9).

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**FabV Enoyl-ACP Reductase**

The diversity of the bacterial enoyl-ACP reductases relative to the lack of structural and mechanistic diversity seen in the other enzymes of the FAS II elongation cycle argues that naturally occurring compounds exist that selectively inhibit one or another of these enzymes. This hypothesis has recently been confirmed by the discoveries of natural enoyl-ACP reductase inhibitors of fungal origin that specifically target FabI and FabK (Atromentin and Leucemone) (40).
FabV Enoyl-ACP Reductase

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