

Characterization of *Pseudomonas aeruginosa* Enoyl-Acyl Carrier Protein Reductase (FabI): a Target for the Antimicrobial Triclosan and Its Role in Acylated Homoserine Lactone Synthesis

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The *Pseudomonas aeruginosa* *fabI* structural gene, encoding enoyl-acyl carrier protein (ACP) reductase, was cloned and sequenced. Nucleotide sequence analysis revealed that *fabI* is probably the last gene in a transcriptional unit that includes a gene encoding an ATP-binding protein of an ABC transporter of unknown function. The FabI protein was similar in size and primary sequence to other bacterial enoyl-ACP reductases, and it contained signature motifs for the FAD-dependent pyridine nucleotide reductase and glucose/ribitol dehydrogenase families, respectively. The chromosomal *fabI* gene was disrupted, and the resulting mutant was viable but possessed only 62% of the total enoyl-ACP reductase activity found in wild-type cell extracts. The *fabI*-encoded enoyl-ACP reductase activity was NADH dependent and inhibited by triclosan; the residual activity in the *fabI* mutant was also NADH dependent but not inhibited by triclosan. An polyhistidine-tagged FabI protein was purified and characterized. Purified FabI (i) could use NADH but not NADPH as a cofactor; (ii) used both crotonyl-coenzyme A and crotonyl-ACP as substrates, although it was sixfold more active with crotonyl-ACP; and (iii) was efficiently inhibited by low concentrations of triclosan. A FabI Gly⁹⁵-to-Val active-site amino acid substitution was generated by site-directed mutagenesis, and the mutant protein was purified. The mutant FabI protein retained normal enoyl-ACP reductase activity but was highly triclosan resistant. When coupled to FabI, purified *P. aeruginosa* *N*-butyryl-L-homoserine lactone (C₄-HSL) synthase, RhlI, could synthesize C₄-HSL from crotonyl-ACP and *S*-adenosylmethionine. This reaction was NADH dependent and inhibited by triclosan. The levels of C₄-HSL and *N*-(3-oxo)-dodecanoyl-L-homoserine lactones were reduced 50% in a *fabI* mutant, corroborating the role of FabI in acylated homoserine lactone synthesis *in vivo*.

The important opportunistic pathogen *Pseudomonas aeruginosa* contains a type II or dissociated fatty acid synthetase system, in which the individual reactions are catalyzed by separate proteins (22, 24). Although the overall organization of the *P. aeruginosa* *fab* genes is similar to that in *Escherichia coli* (for reviews see references 9 and 28), some potentially significant differences exist. First, the *P. aeruginosa* *fabA* and *fabB* genes, encoding β -hydroxyacyl-acyl carrier protein (ACP) dehydratase and β -ketoacyl-ACP synthase I, form an operon (22), whereas in *E. coli* these genes map to separate genetic loci. Second, unlike in *E. coli* and several other gram-negative bacteria, the *fabH* gene, encoding β -ketoacyl-synthase III, is absent from the *fabD-fabG-acpP-fabF* gene cluster, encoding malonyl-coenzyme A (CoA):ACP transacylase, β -ketoacyl-ACP reductase, ACP, and β -ketoacyl-ACP synthase II (25). Searches of the *P. aeruginosa* genome database revealed several potential *fabH* homologs located elsewhere on the genome.

Our current model for fatty acid biosynthesis in *P. aeruginosa* is shown in Fig. 1. Three β -ketoacyl-ACP synthases, KAS I (FabB), KAS II (FabF), and KAS III (FabH), play pivotal roles in fatty acid synthesis. Initiation requires malonyl-CoA and malonyl-ACP. Malonyl-CoA is synthesized from acetyl-

CoA via the acetyl carboxylase reaction (5). Malonyl-ACP is derived from malonyl-CoA and ACP by malonyl-CoA:ACP transacylase (FabD) (24, 25). Although a *P. aeruginosa* *fabH* homolog has only tentatively been identified, the first cycle of elongation is probably initiated by KAS III (FabH), which condenses malonyl-ACP with acetyl-CoA. Subsequent cycles are then initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by KAS I (FabB) for saturated fatty acid substrates and KAS II (FabF) for unsaturated fatty acid substrates. In the second step, the resulting β -ketoester is reduced to a β -hydroxyacyl-ACP by a NADPH-dependent β -ketoacyl-ACP reductase (FabG). It has recently been shown that *P. aeruginosa* contains a FabG homolog, RhlG, which presumably functions as a NADPH-dependent β -ketoacyl-ACP reductase specific for rhamnolipid synthesis (6). The third step in the cycle is catalyzed by either the *fabA*- or *fabZ*-encoded β -hydroxyacyl-ACP dehydratase. The final step in each cycle involves conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by NADH-dependent enoyl-ACP reductase (FabI). Physiologically, FabI is an important enzyme because (i) reduction of enoyl-ACP derivatives is thought to regulate the ratio of saturated to unsaturated fatty acids and to coordinate fatty acid and phospholipid syntheses (16, 23); and (ii) FabI plays a determinant role in completing cycles of fatty acid elongation (15). FabI belongs to the short-chain alcohol dehydrogenase family and in *E. coli* is the target of a group of antibacterial compounds, the diazaborines (44) and triclosan (19, 33). In *Mycobacterium tuberculosis*, the FabI homolog InhA is one of the targets for the clinically used antimycobac-

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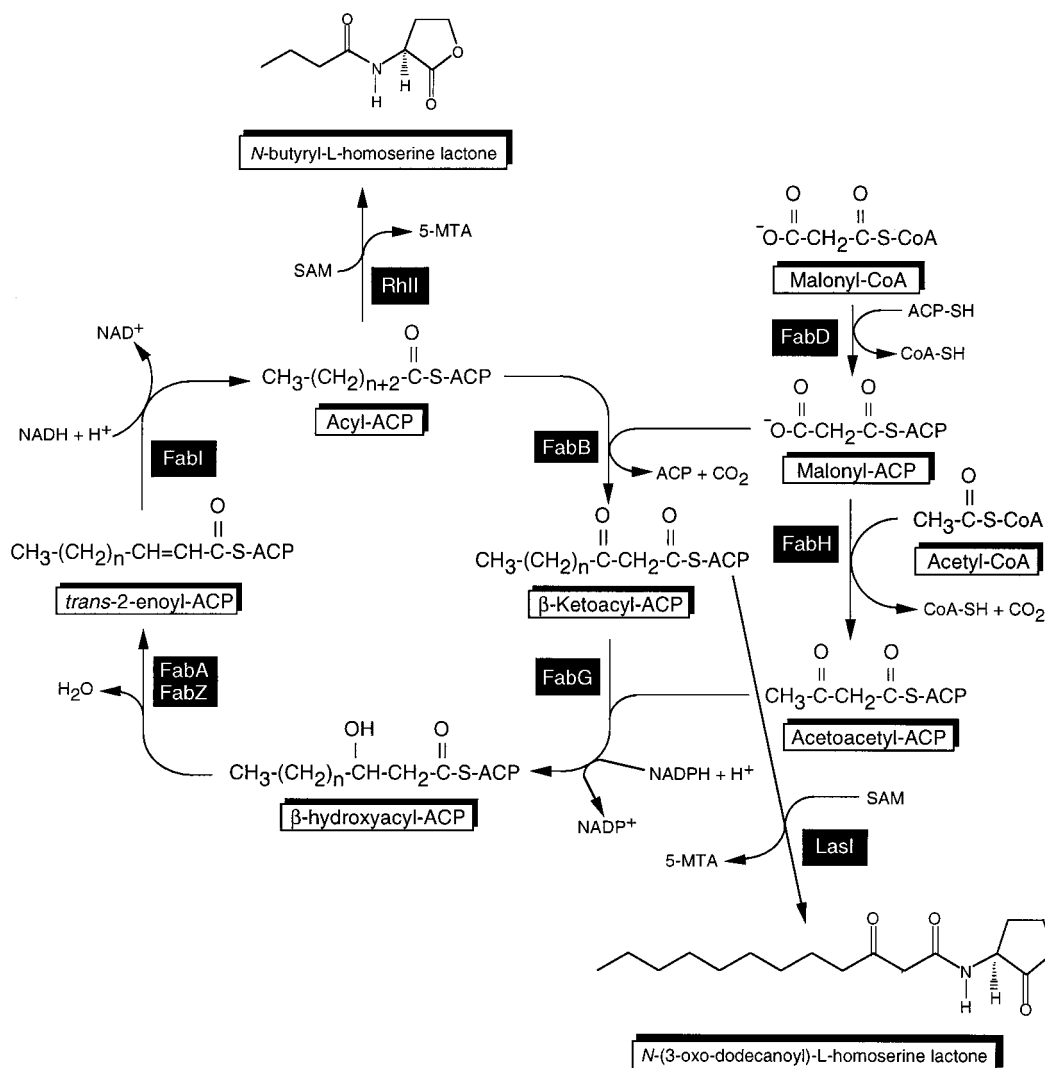


FIG. 1. Current model for fatty acid biosynthesis in *P. aeruginosa* and proposed role of butyryl-ACP as acyl donor in *N*-buteryl-L-homoserine lactone synthesis. The genes for all enzymes except one (*fabH*) have been identified and characterized; for *fabH*, only a tentative identification has been made among several paralogs. Abbreviations: ACP, acyl carrier protein; ACP-SH, ACP with 4'-phosphopantetheine co-factor; CoA-SH; coenzyme A; FabA (and FabZ), β-hydroxyacyl-ACP dehydratase; FabB, β-ketoacyl synthase I; FabD; malonyl-CoA:ACP transacylase; FabF, β-ketoacyl synthase II; FabG, β-ketoacyl-ACP reductase; FabH, β-ketoacyl synthase III; FabI; enoyl-ACP reductase; 5-MTA, 5-methylthioadenosine; SAM, *S*-adenosylmethionine; LasI, *N*-(3-oxo)-dodecanoyl homoserine lactone synthase; RhII, *N*-buteryl homoserine lactone synthase.

terial isoniazid (2), and genetic evidence was obtained that *M. smegmatis* InhA is a triclosan target (32). More recently, it has been shown that *E. coli* mutants resistant to the antiseptic triclosan contain *fabI* mutations (33), and a subsequent study confirmed that triclosan and other related compounds indeed target FabI (19). Although *P. aeruginosa* PAO1 is resistant to triclosan due to constitutive expression of the *mexAB-oprM*-encoded efflux pump, $\Delta(mexAB-oprM)$ mutants are susceptible to triclosan (41). The *E. coli* FabI protein has been cocrystallized with NADH and thienodiazaborine (18), or NAD⁺ (26), and these studies allowed definition of the enzyme active site and amino acid residues important in substrate and drug interactions.

Besides providing fatty acid intermediates for a multitude of cellular constituents (phospholipids [9], lipid A [37], etc.), the Fab pathway has been implicated in providing the acyl groups for the acylated homoserine lactones (HSLs) that are the sig-

naling molecules in quorum sensing (14, 35, 39, 45). In *P. aeruginosa*, quorum sensing is a mechanism that regulates virulence factor gene expression (46), biofilm formation in vitro (10) and in natural settings (31), twitching motility (13), and other important cellular processes. According to the current model, quorum sensing in *P. aeruginosa* involves two separate systems, LasR-LasI and RhlR-RhII. These proteins are encoded by two separate, tandemly arranged transcriptional units. In these two systems, the LasI and RhII proteins are HSL synthases that direct the synthesis of *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (3-oxo-C₁₂-HSL) and *N*-buteryl-L-homoserine lactone (C₄-HSL), respectively. According to the current model, the synthases react with acyl-ACPs and *S*-adenosylmethionine (SAM) to form the cognate HSLs, with concomitant release of 5-methylthioadenosine (35, 39). For C₄-HSL synthesis, the *rhlI*-encoded *P. aeruginosa* HSL synthase RhII catalyzes the formation of C₄-HSL in a reaction

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence ^a	Reference or source
<i>E. coli</i>		
DH5 α F'	[F ⁺ ϕ 80 <i>lacZ</i> Δ M15] Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> [<i>r_K</i> ⁻ <i>m_K</i> ⁺] <i>supE44 thi-1 gyrA relA1</i>	27
JP1111	Hfr <i>galE45 fabI392</i> (Ts) <i>relA1 spoT1</i>	44
BL21(DE3)	<i>E. coli</i> B; F ⁻ <i>ompT r_B</i> ⁻ <i>m_B</i> ⁻ (λ DE3)	43
<i>P. aeruginosa</i>		
PAO1	PAO1	B. H. Holloway
PAO200	PAO1 with Δ (<i>mexAB-oprM</i>)	41
PAO234	PAO1 with <i>fabI::Gm^r-FRT</i>	This study
PAO235	PAO1 with <i>fabI::FRT</i>	This study
Plasmids		
pET-15b	Ap ^r ; hexahistidine fusion and expression vector	Novagen
pWSK29/30	Ap ^r ; low-copy-number cloning and T7 expression vectors	47
pFLP2	Ap ^r ; source of Flp recombinase	21
pPS856	Ap ^r Gm ^r ; source of Gm ^r - <i>FRT</i> cassette	21
pUCP21T	Ap ^r ; broad-host-range cloning vector	42
pEX18Tc	Tc ^r ; <i>sacB</i> -based gene replacement vector	21
pPS922	Ap ^r <i>fabI</i> ⁺ (ligation of a 880-bp <i>Bam</i> HI- <i>Hind</i> III PCR fragment between the same sites of pWSK30; <i>fabI</i> -transcription driven by P _{<i>lac</i>})	This study
pPS925	Ap ^r <i>fabI::Gm^r-FRT</i> (ligation of a 1,053-bp blunt-ended <i>Sac</i> I fragment from pPS856 into the <i>Sma</i> I site of pPS922)	This study
pPS933	Ap ^r <i>sacB</i> ⁺ <i>fabI::Gm^r-FRT</i> (ligation of a 1.95-kb <i>Bam</i> HI- <i>Hind</i> III fragment between the same sites of pEX18Tc)	This study
pPS935	Ap ^r ; H ₆ - <i>FabI</i> expression vector (PCR-amplified 0.85-kb <i>Nde</i> I- <i>Bam</i> HI fragment cloned between same sites of pET-15b)	This study
pPS967	Ap ^r <i>fabI</i> ⁺ (pUCP21T with <i>Bam</i> HI- <i>Hind</i> III fragment from pPS922; <i>fabI</i> transcription driven by P _{<i>lac</i>})	This study
pPS1098	Ap ^r ; H ₆ - <i>FabI</i> expression vector (PCR-amplified 0.85-kb <i>Nde</i> I- <i>Bam</i> HI fragment cloned between same sites of pET-15b); expresses H ₆ - <i>FabI</i> mutant protein	This study
Primers		
FabIU	<i>Hind</i> III-GCAAGCttGCGGAAAACCTGAGTAGCAGA	
FabID	<i>Bam</i> HI-ATGGatCCGCTTGAAGCGGCATTTTTC	
FabI-Nde	<i>Nde</i> I-GGcatATGGGATTTCTCACAGGAAAAC	
FabI G>V	CCACTCCGTCGtATTCGCTCCAG	

^a P_{*lac*} *E. coli lac* operon promoter. Primer sequences are printed 5' to 3'; lowercase letters indicate nonmatching oligonucleotides used to either form the indicated motif as underlined or introduce other nonmotif changes indicated in lowercase letters.

utilizing butyryl-ACP as the acyl donor and SAM as the nucleophile in a lactonization reaction (35, 39).

In this report, we describe the characterization of the *P. aeruginosa fabI* gene and its product, show evidence for the presence of at least one other enoyl-ACP reductase in this bacterium, provide biochemical evidence that *FabI* is a tri-closan target, and demonstrate its role in C₄-HSL synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The relevant strains, plasmids, and primers are described in Table 1. LB (Luria-Bertani) medium (34) was routinely used as the rich medium for all bacterial strains. For growth of the *fabI*(Ts) mutant JP1111, the NaCl concentration of LB medium was reduced to 0.05%. For some experiments, RB medium (20) was used instead of LB. The minimal medium used for growth of *P. aeruginosa* was VBMM (40). The antibiotics used in selection media were as follows: for *E. coli*, ampicillin (100 μ g/ml) and gentamicin (15 μ g/ml); for *P. aeruginosa*, carbenicillin (500 μ g/ml) and gentamicin (200 μ g/ml).

General DNA procedures. Routine DNA were performed as previously described (21). The details underlying construction of pPS921 were as follows. For PCR amplification of a fragment from chromosomal DNA, two mutagenic oligonucleotides were synthesized. The *FabIU* and *FabID* primers were designed to introduce a *Hind*III site upstream of *fabI* and a *Bam*HI site downstream of *fabI*, respectively (Table 1). These oligonucleotides were used to prime synthesis from PAO1 chromosomal DNA. PCRs were performed on a PTC-100 PCR system thermocycler (MJ Research, Watertown, Mass.) as previously described (21, 22). The ca. 870-bp PCR fragment was ligated to *Bam*HI-*Hind*III-digested pWSK30

(47) DNA to form pPS921. Nucleotide sequences were determined and analyzed as previously described (22). Motif searches were performed by using the on-line E-Motif program provided by Stanford University, Palo Alto, Calif.

A two-step method was used for site-directed mutagenesis for *fabI*. First, PCRs were set up as described above but with pPS935 DNA as the template, and DNA synthesis was primed with the T7 terminator primer (Novagen, Madison, Wis.) and a mutagenic primer, *FabI* G>V (Table 1), that was designed to (i) introduce a single G-to-T change which leads to a Gly⁹⁵-to-Val amino acid change and (ii) yield a PCR product that would properly prime to pPS935 DNA in a second round of PCR even when T-tailed by *Taq* DNA polymerase. The single 1,046-bp PCR product was digested with *Bam*HI plus *Nde*I, and the resulting 840-bp *Bam*HI-*Nde*I fragment was gel purified and ligated to *Bam*HI-*Nde*I-digested pET-15b DNA. The presence of the single G-to-T change was verified by nucleotide sequence analysis.

Disruption of the *fabI* gene. The *fabI* strain PAO235 was isolated in several steps. A plasmid-borne *fabI* mutation was constructed by insertion of a blunt-ended 1,053-bp gentamicin resistance (Gm^r)-*FRT* cassette from pPS856 (21) into the *Sma*I site located within the *fabI* coding sequence. The resulting mutation was returned to the *P. aeruginosa* chromosome after subcloning into the *sacB*-based suicide vector pEX18Tc (21) to derive the *fabI::Gm^r-FRT* mutant PAO234. From PAO234, the unmarked *fabI::FRT* mutant PAO235 was then derived by in vivo excision of the Gm^r-*FRT* cassette with Flp recombinase (21). The mutants were confirmed by performing colony PCR (21) utilizing the *FabIU* and *FabID* primers.

Purification of a polyhistidine-tagged *FabI* fusion protein. A hexahistidine-*FabI* (H₆-*FabI*) expression vector was constructed by PCR amplifying the *fabI* coding sequence from PAO1 genomic DNA with primers *FabI*-Nde, creating a *Nde*I site at the *fabI* ATG initiation codon, and *FabID*, which creates a *Bam*HI site immediately downstream of *fabI*. The PCR fragment was digested with *Nde*I and *Bam*HI, and the resulting 840-bp *Bam*HI-*Nde*I fragment was ligated between the same restriction sites of pET-15b to form pPS935, which was then trans-

formed into *E. coli* BL21(DE3). Expression of H₆-FabI, cell lysis, and purification of the soluble fusion protein on a Ni²⁺-agarose column (Qiagen) were performed as previously described (17) except that the cells were grown in LB medium containing ampicillin (LB-Ap medium). The same procedure was used for purification of a mutant FabI protein.

Protein concentrations were determined by using the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, Calif.) and bovine serum albumin as the standard. Proteins were analyzed by electrophoresis on 0.1% sodium dodecyl sulfate–10% polyacrylamide gels (SDS-PAGE) as previously described (29). The proteins were visualized by quick staining with Coomassie brilliant blue R-250 (7).

Enoyl-ACP reductase assays. *P. aeruginosa* cell extracts were prepared from exponentially growing cells (A_{540} of ~0.8 to 1.0). Cells grown in LB medium at 37°C were harvested by centrifugation and then suspended and washed in 0.1 M sodium phosphate buffer (pH 6.5). Cell lysates were prepared by passing the cell suspensions three times through a French pressure cell (19,000 lb/in²). Cell debris was removed by ultracentrifugation for 1 h at 260,000 × g, and the supernatants were saved as cell extracts.

Enoyl-ACP reductase activity of purified H₆-FabI, as well as in cell extracts, was determined by using crotonyl-ACP as the substrate. This was done by measuring spectrophotometrically the decrease in absorbance at 340 nm at room temperature due to consumption of NADH (or NADPH) during reduction of crotonyl-ACP (3). In 500 μl, a typical assay mixture contained 0.1 M sodium phosphate (pH 7.5), 0.1 mM NADH, and 1 μg of purified H₆-FabI. The reactions were initiated by addition of 20 μM crotonyl-ACP. In some instances, 0.1 mM NADPH was used as the reductant. For some experiments, enzyme and substrate concentrations were varied as indicated in the figure legends. A stock solution of triclosan was prepared in 95% ethanol, and appropriate amounts of ethanol were added to control experiments to assess possible inhibitory effects of the solvent on enzyme activities.

Crotonyl-ACP was prepared by using a previously published procedure (48), with slight modifications. Briefly, 20 mg of purified *P. aeruginosa* ACP was precipitated as previously described (8) except that the hydroxylamine step was omitted. After precipitation of the protein with 10% trichloroacetic acid for 30 min on ice, the precipitate was pelleted in a microcentrifuge and then washed once with 0.2 M citrate buffer (pH 4.0) and once with water. The protein was resuspended in 0.1 M sodium phosphate buffer (pH 8.0) containing 2 μmol of dithiothreitol and reacted with a 10-fold molar excess of crotonic anhydride (Aldrich, Milwaukee, Wis.) for 30 min at 4°C with constant stirring. The reaction mixture was loaded on a PD10 column (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with 0.1 M sodium phosphate buffer (pH 7.5), and the crotonyl-ACP was eluted with 3.5 ml of the same buffer. The crotonyl-ACP concentration was determined by measuring the difference in the number of free thiol groups of an untreated versus a hydroxylamine-treated sample by the Ellmann technique (12). The treatment of crotonyl-ACP (20 μl of the column eluate) was performed for 5 min at room temperature in a 1 M hydroxylamine solution in 0.1 M sodium phosphate buffer (pH 7.5). For thiol group determinations, hydroxylamine-treated or untreated crotonyl-ACP was reacted for 30 min at room temperature with 0.15 mM 5,5'-dithiobis (2-nitrobenzoic acid) (Aldrich) in a final volume of 0.8 ml of 0.1 M phosphate buffer (pH 7.5), and the absorption was measured at 400 nm. According to this determination, 80% of the ACP was acylated.

Determination of acyl HSL concentrations in culture fluid. Acyl HSLs were detected in culture fluids of LB-grown *P. aeruginosa* strains as previously described (36), using *E. coli* XL-1 Blue/pECP61.5 for detection of C₄-HSL and *E. coli* MG4/pKDT17 for detection of 3-oxo-C₁₂-HSL. Standard curves were prepared by using synthetic HSLs to ensure that bioassays were always performed in the linear range.

C₄-HSL assays. The in vitro C₄-HSL synthesis reactions contained (in 500 μl) Moré buffer (10 mM Tris-HCl [pH 7.4], 330 mM NaCl, 15% glycerol, 0.7 mM dithiothreitol, 2 mM EDTA, 25 mM MgSO₄, 0.1 mM FeSO₄) (35), 0.1 mM SAM, 0.2 mM NADH, 1 μg of purified H₆-RhlI (details to be published elsewhere), and various amounts of H₆-FabI. The reaction mixtures were incubated at 37°C for 1 h and then extracted three times with 250 μl of ethyl acetate. The ethyl acetate extracts were vacuum dried, and the residue was suspended in 20 μl of high-pressure liquid chromatography-grade acetonitrile (Fisher Scientific, Fair Lawn, N.J.). For detection of C₄-HSL, the *Chromobacterium violaceum* bioassay was used (30). Aliquots (5 μl) of the extracted reactions were spotted onto a Whatman (Clifton, N.J.) C₁₈ reverse-phase thin-layer chromatography (TLC) plate and allowed to air dry. The solvent used for chromatography was 60% methanol–40% water (vol/vol). For detection, 2.5 ml of an overnight culture of strain CV026 grown at room temperature in LB medium was diluted into 25 ml of 0.3% LB agar kept at 45°C, and the agar mixture was used to overlay the dried TLC plate. After solidification of the overlay, the TLC plate was incubated overnight at room temperature in a wet box. The presence of C₄-HSL in the extracted reactions was scored by appearance of a violet spot on the TLC plates.

Nucleotide sequence accession number. The DNA sequence of the *fabI* region has been deposited in GenBank under accession no. AF104262.

RESULTS

Cloning of the *fabI* gene of *P. aeruginosa*. At the onset of this project, an examination of the incomplete *Pseudomonas* genome database release revealed two contigs which contained sequences that in BLAST searches showed extensive similarities to *E. coli fabI*. The amino and carboxy termini were located on two different contigs. The putative *fabI* coding sequence was successfully amplified from *P. aeruginosa* chromosomal DNA by using two oligonucleotide primers, FabIU and FabID (Table 1). These two primers were designed to introduce a unique *Hind*III site upstream of *fabI* and its putative ribosome binding site and to introduce a unique *Bam*HI site downstream of *fabI*. PCR amplification was very specific and yielded a 880-bp product that was digested with *Bam*HI plus *Hind*III and ligated to similarly digested pWSK30 DNA to form pPS922. This procedure placed *fabI* under transcriptional control of the *lac* promoter (P_{lac}) since the cloned *fabI* gene presumably lacks its own promoter. The low-copy-number (five to eight copies per cell) cloning vector pWSK30 (47) was used to avoid potential toxicity problems that we had observed during the cloning of other *fab* genes (25). The *fabI*(Ts) *E. coli* strain JP1111 does not grow at 42°C and has a low-salt tolerance at the nonpermissive temperature; e.g., it does not grow on regular LB medium containing 0.5% NaCl. This strain was transformed with pPS922 DNA, and Ap^r transformants were selected at 30°C on LB-Ap medium. The transformants were then transferred to LB-Ap medium with and without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 42°C. Within 24 h, only the colonies plated on LB-Ap medium with IPTG grew under these conditions, indicating that the cloned *fabI* (i) complemented the temperature-sensitive (Ts) and low-salt-tolerance phenotype of the *E. coli fabI*(Ts) mutant and (ii) does not contain its own promoter since transcription from P_{lac} requires IPTG induction in the *lac* repressor producing strain JP1111. When JP1111 was transformed with pPS967, a multi-copy plasmid expressing *fabI* from P_{lac} (Table 1), complementation was observed in the absence of IPTG since the amounts of chromosomally encoded *lac* repressor were insufficient to fully repress *fabI* transcription.

Sequence analysis of the *fabI* gene. Since the *fabI* gene sequence in the incomplete genome database was contained on two separate contigs, we sequenced the cloned 874-bp *Hind*III-*Bam*HI fragment which contained a single open reading frame (ORF), and BLAST searches revealed significant identity of the protein to other FabI proteins. Alignments showed the greatest similarities with FabI proteins from *E. coli* and *Salmonella typhimurium* (69% identity; 81% similarity) and *Haemophilus influenzae* (63% identity; 76% similarity). This similarity also extended to FabIs from gram-positive bacteria, including *Bacillus subtilis* (49% identity; 66% similarity).

Analysis of the deduced FabI amino acid sequence using the E-Motif program revealed a glucose/ribitol dehydrogenase family signature motif (P..[KR]....[DE][FILVY]....[FWY].[AST]), as well as a signature motif for the FAD-dependent pyridine nucleotide reductase family (D..[FILMV]..[IV]G..P...L). The latter sequence includes Gly⁹⁵, which is equivalent to a glycine found in position 93 of *E. coli* FabI. In *E. coli*, Gly⁹³-to-Ser and Gly⁹³-to-Ala changes, which lead to resistance to diazaborine (4) and triclosan (33), respectively, map close to the nucleotide binding site.

Examination of the latest *P. aeruginosa* genome database revealed a complete *fabI* gene sequence. Our sequence was identical to the database sequence with the exception of an additional G residue. This discrepancy arose from a mistake in the previous contig sequence after which our primer FabID

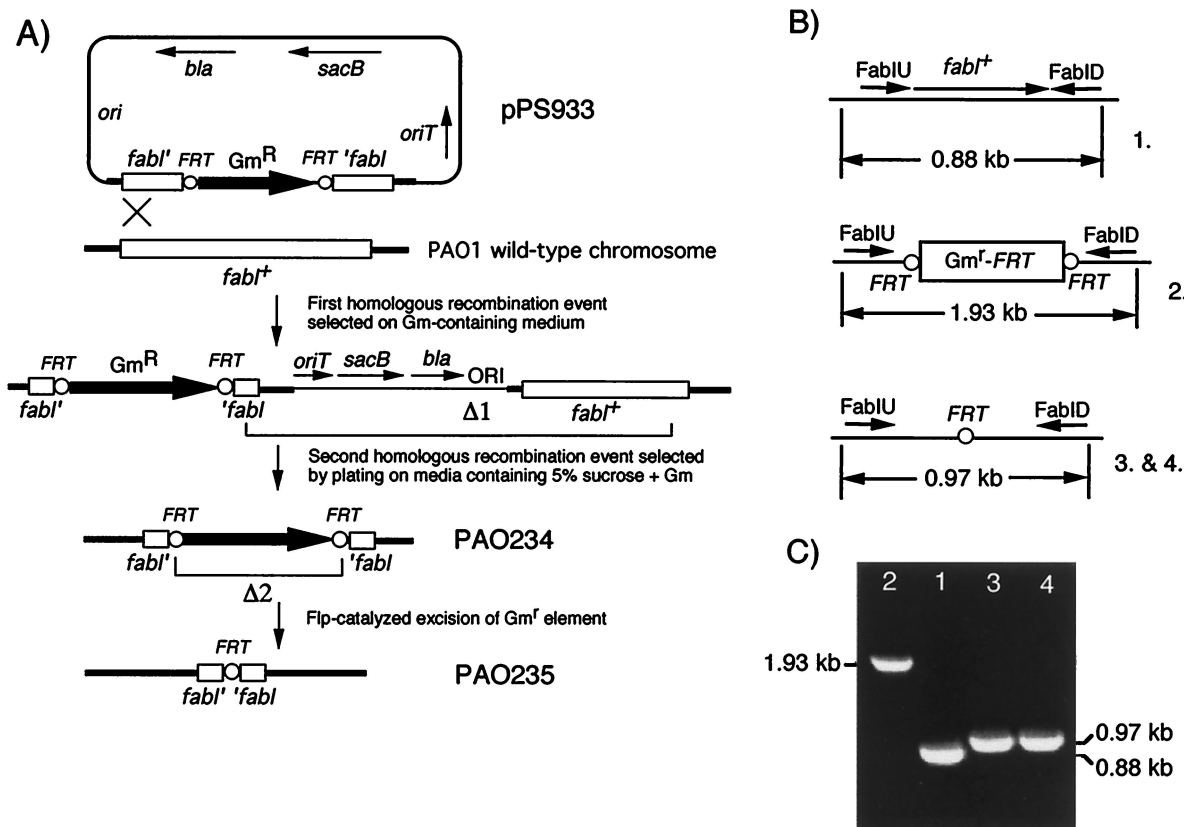


FIG. 2. (A) Strategy for isolation of an unmarked chromosomal *fabI* mutation via *sacB*-counterselected gene replacement ($\Delta 1$) and Flp-mediated excision of the Gm^r marker ($\Delta 2$). (B) Genetic organization of the *fabI* region in wild-type PAO1 (1.), the *fabI::Gm^r-FRT* insertion mutant (2.), and the unmarked *fabI* mutant after Flp-mediated excision of the Gm^r marker (3. and 4.). (C) PCR analysis of genomic DNA from the strains depicted in panel B. The PCRs were primed with the primers FabIU and FabID (B). Abbreviations: *bla*, β -lactamase-encoding gene; *FRT*, Flp recombinase target sites; Gm^r , Gm^r marker; *ori*, pMB1 origin of replication; *oriT*, origin of transfer; *sacB*, levansucrase-encoding gene.

was modeled. The *fabI* gene is followed by a sequence that could assume a stem-loop structure ($\Delta G = -48.2$ kcal/mol) indicative of Rho-independent transcriptional terminators (38). Analysis of the *fabI* upstream sequence did not reveal any obvious promoter/regulatory sequences. Instead, the *fabI* gene is separated by 21 nucleotides from another ORF whose gene product exhibits significant similarity to many bacterial ATP-binding proteins that are components of ABC transporters; this ORF is 58% identical and 74% similar to the hypothetical *E. coli* ABC transporter ATP-binding protein YejF (GenBank accession no. U00008).

Disruption of the chromosomal *fabI* gene. A defined pPS933-borne *fabI::Gm^r* insertion was constructed as described in Table 1, and the deletion was returned to the *P. aeruginosa* chromosome as illustrated in Fig. 2A. After conjugal transfer of the nonreplicative pPS933 from *E. coli* SM10 into PAO1, merodiploids were obtained by selecting for Gm^r . From these, colonies having undergone $\Delta 1$ were selected as sucrose resistant, Gm^r , and carbenicillin resistant. The unmarked *fabI::FRT* mutant PAO234 was then derived from PAO234 by Flp-catalyzed excision of the Gm^r marker. During its expression in the recipient, Flp recombinase acted at the *FRT* sites to catalyze excision of the Gm^r element (labeled $\Delta 2$ in Fig. 2A), leaving behind a short *FRT*-containing sequence.

Successful execution of the steps labeled $\Delta 1$ and $\Delta 2$ in Fig. 2A was monitored by colony PCR analysis using the FabIU and FabID primers. As expected, the primers amplified a

880-bp *fabI*-containing fragment from wild-type PAO1 DNA (Fig. 2C, lane 1). In PAO234 (lane 2), this fragment was increased to 1.93 kb by insertion of the 1,053-bp Gm^r -*FRT* cassette. After Flp-mediated excision of the Gm^r marker, the PCR fragment was reduced to ~ 970 bp (880 bp and one 86-bp *FRT*-containing sequence from pPS856) (lanes 3 and 4). These events were further verified by genomic Southern analyses utilizing Gm^r and *fabI*-specific DNA segments as the probes (data not shown).

***fabI* mutants contain reduced levels of enoyl-ACP reductase activity.** The growth rates of wild-type PAO1 and the *fabI* mutant PAO235 were superimposable when grown in RB medium and other media (data not shown). Since PAO235 contains only a 86-bp nonpolar insertion at a *SmaI* site that is located late in the *fabI* gene, we considered the possibility that this strain could still express a functional FabI protein. To rule out this possibility, we PCR amplified the *fabI* region from PAO235 by using the primers FabIU and FabID and subcloned the resulting *Bam*HI-*Hind*III fragment into pWSK30. Under inducing conditions, this DNA segment no longer complemented the Ts and low-salt-tolerance phenotypes of *E. coli fabI(Ts)* strain JP1111.

Determination of enzyme activities in cell extracts of wild-type and mutant PAO235 confirmed the existence of residual NADH-dependent activity in the mutant that amounted to 62% of the total activity observed in PAO1 extracts (Fig. 3). Whereas total NADH-dependent enoyl-ACP reductase in

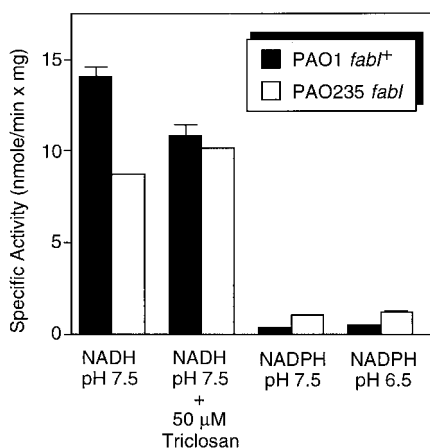


FIG. 3. Enoyl-ACP reductase activities in cell extracts of wild-type PAO1 and the *fabI* mutant PAO235. The 500- μ l reactions contained in phosphate buffer with the indicated pH, 0.1 mM NADH or NADPH, and 50 to 100 μ g of PAO1 extract or 300 μ g of PAO235 extract for determination of NADH- or NADPH-dependent enoyl-ACP reductase activities, respectively. The reactions were initiated by addition of 13 μ M crotonyl-ACP, and the decrease in absorbance at 340 nm was recorded. The results shown represent the means plus standard deviations of three experiments.

PAO1 was inhibited by the addition of 50 μ M triclosan, this inhibitor had no effect on the residual activity found in PAO235, even when its concentration was increased to 100 μ M (data not shown). Cell extracts from both strains showed very little NADPH-dependent enoyl-ACP reductase activity when assayed at pH 7.5 or 6.5.

Purification and properties of purified FabI. An expression vector was constructed, and FabI was purified as an H₆-FabI protein. When transformed into *E. coli* JP1111, the expression vector could complement the *fabI*(Ts) mutation of this strain, indicating expression of a functional H₆-FabI protein. The purified FabI protein (Fig. 4A) could utilize crotonyl-CoA and

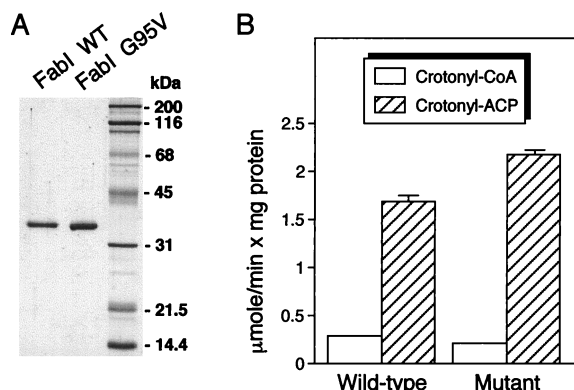


FIG. 4. (A) SDS-PAGE of purified wild-type (WT) FabI and FabI G95V mutant proteins; (B) substrate preference and specific activities of wild-type and mutant FabI proteins. (A) The proteins were expressed as H₆-tagged proteins and purified by Ni²⁺ affinity chromatography. One microgram of each protein was analyzed by SDS-PAGE, and proteins were detected by Coomassie blue staining. The protein standards (Bio-Rad Laboratories) in the rightmost lane were (top to bottom) myosin, β -galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme; molecular masses are shown on the right. (B) Enoyl-ACP reductase activities were measured with 20 μ M crotonyl-CoA or 13 μ M crotonyl-ACP as substrate and 0.1 mM NADH as cofactor. The reaction mixture contained either 1 μ g of wild-type FabI or 1 μ g of mutant FabI protein. The results shown represent the means plus standard deviations of three experiments.

crotonyl-ACP as substrates (Fig. 4B), although it was 5.9-fold more active with crotonyl-ACP as the substrate. The enzyme was unable to utilize NADPH as cofactor (not shown), which may be a consequence of its purification at alkaline pH, conditions that may inactivate its NADPH-dependent activity, as has been observed with the *E. coli* enzyme (3).

The enoyl-ACP reductase activity was inhibited by very low concentrations of triclosan (Fig. 5). Triclosan-resistant *E. coli* strains contain changes in amino acid residues that are thought to line the enzyme active site (1, 33), and the most resistant strains contained Gly⁹³-to-Val substitutions (33). We therefore changed the corresponding Gly⁹⁵ in the *P. aeruginosa* enzyme to a Val and purified the resulting H₆-tagged mutant protein. The mutant protein (Fig. 4A) was slightly less active than wild-type FabI with crotonyl-CoA as the substrate and slightly more active with crotonyl-ACP as the substrate (Fig. 4B). The mutant enzyme was significantly more resistant to triclosan (Fig. 5).

Role of FabI in HSL synthesis in vitro and in vivo. Reduction of crotonyl-ACP by FabI to form butyryl-ACP is the last step in the first cycle of fatty acid elongation following condensation of malonyl-ACP with acetyl-CoA (Fig. 1). Theoretically, butyryl-ACP generated by FabI could serve as a substrate for transacylation of RhlI, followed by a lactonization reaction utilizing SAM as the substrate to form C₄-HSL, and inhibition of FabI or mutational inactivation should therefore lead to reduced levels of HSL production.

To test this hypothesis, we first set up an in vitro C₄-HSL synthesis system with purified H₆-FabI and H₆-RhlI. In this system, RhlI synthesized C₄-HSL from crotonyl-ACP in the presence of FabI, NADH, and SAM (Fig. 6, lane g). Under the same conditions, C₄-HSL synthesis was completely inhibited by 25 μ M triclosan (lane b), and this inhibition was partially relieved by increasing concentrations of FabI (lane c to f). Inhibition of C₄-HSL synthesis by triclosan could not be relieved by increasing the RhlI concentration sevenfold (lane h).

To confirm the in vivo role of *fabI* in HSL synthesis, HSL levels were measured in culture fluids of wild-type PAO1 and *fabI* mutant PAO235. The *fabI* mutant produced only 50% of the 3-oxo-C₁₂-HSL and C₄-HSL levels of wild-type PAO1 (Fig. 7), paralleling the reduction of FabI enoyl-ACP reductase activity observed in this mutant (Fig. 3).

DISCUSSION

The *fabI* gene encodes an enoyl-ACP reductase. We have cloned and characterized a gene from *P. aeruginosa*, encoding an enoyl-ACP reductase, by PCR amplification from genomic DNA and complementation of a defined *E. coli fabI*(Ts) mutant. Sequence analysis revealed that this gene may be transcriptionally linked to an upstream ORF encoding an ATP-binding protein of an ABC transporter of unknown function, and the presence of strong downstream Rho-independent transcriptional terminator indicated that it is probably the last gene in a transcriptional unit.

Although our experiments showed that *P. aeruginosa* contains several enoyl-ACP reductases, several lines of evidence strongly support the notion that the cloned gene is *fabI*. First, the deduced FabI sequence is 69% identical to FabI sequences from *E. coli* and *S. typhimurium* and contains the signature motif (D..[FILMV]..[IV]G.P...L) for the FAD-dependent pyridine nucleotide reductase family. Second, this motif contains the amino acid residues forming the enzyme active site that when mutationally altered confer resistance to specific inhibitors of FabI enoyl-ACP reductase activity. This included Gly⁹⁵, which is equivalent to Gly⁹³ found in *E. coli* FabI. In *E.*

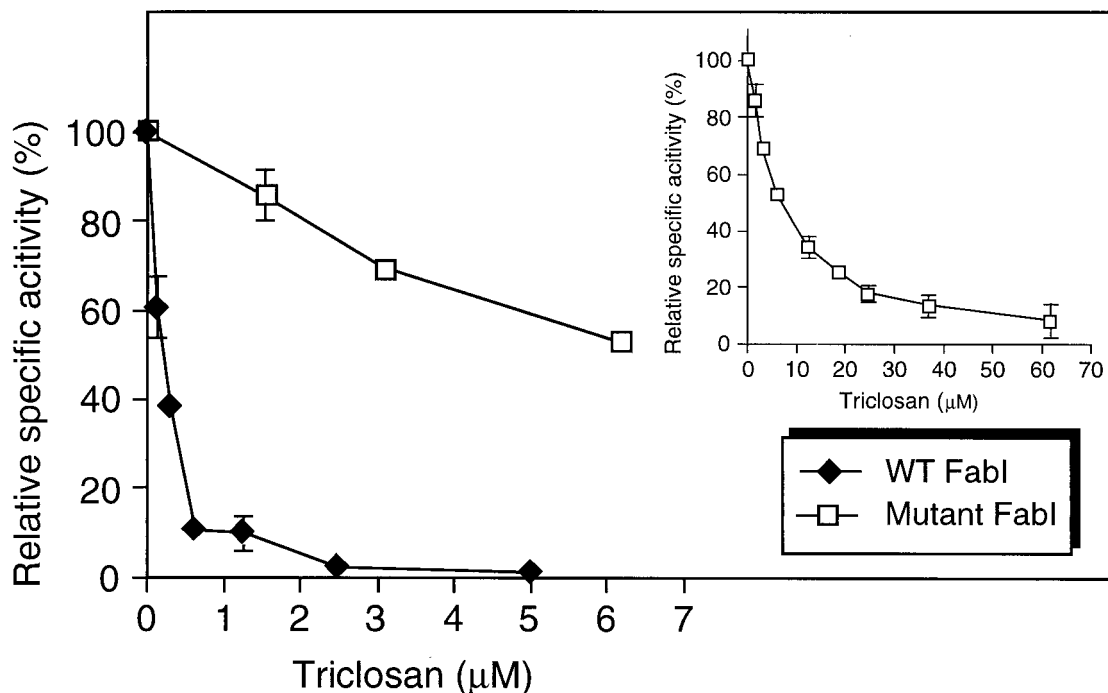


FIG. 5. Inhibition of FabI activity by triclosan. The reaction mixtures (500 μ l) in phosphate buffer (pH 7.5) contained either 1 μ g of purified wild-type (WT) FabI or 1 μ g of mutant FabI protein, 0.1 mM NADH, 13 μ M crotonyl-ACP, and increasing concentrations of triclosan. The insert shows the inhibition curve for the mutant FabI protein over a wider triclosan range; the first four values are identical to the ones plotted in the larger graph. Each value shown represents the mean plus standard deviation of three independent enzymatic reactions.

coli, Gly⁹³-to-Ser and Gly⁹³-to-Ala changes led to resistance to diazaborine (4) and triclosan (33), respectively. In *M. tuberculosis*, a Ser⁹⁴-to-Ala mutation, which leads to isoniazid resistance, lies in the same region of the mycobacterial FabI homolog, InhA (11).

In *E. coli*, *fabI* is an essential gene and *fabI* knockout mutants are not viable. Mutants containing single-amino-acid substitutions can be isolated, but they grow at reduced rates (33) or contain Ts alleles (4) that grow only at the permissive temperature. We were able to disrupt the chromosomal *fabI* gene and showed that the disrupted gene no longer complemented an *E. coli fabI*(Ts) allele. This was expected since the insertion at a *SmaI* site disrupted amino acid residues that form an integral part of the enzyme active site (18, 26). Since we could isolate a *fabI* insertion mutant and since this mutant was not altered in its growth rate, we concluded that unlike *E. coli*, *P. aeruginosa* must contain more than one enoyl-ACP

reductase activity. This was corroborated by the presence of significant levels of NADH-dependent enoyl-ACP reductase activities in cell extracts of the *fabI* mutant PAO235 (Fig. 3). Since the activity found in the mutant amounted to 62% of the total activity found in the wild type, we concluded that FabI contributes a substantial part of the total enoyl-ACP reductase activity found in *P. aeruginosa*. This was corroborated by triclosan inhibition studies which indicated that ~23% of the total enoyl-ACP reductase activity in the wild type was inhibited by this drug, whereas the residual activity in PAO235 was

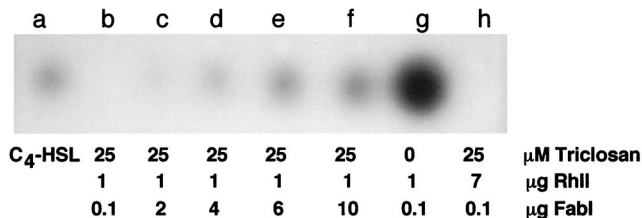


FIG. 6. In vitro synthesis of C₄-HSL and inhibition by triclosan. In vitro synthesis reaction mixtures containing the indicated components plus 20 μ M crotonyl-ACP, 0.1 mM SAM, and 0.2 mM NADH were extracted with ethyl acetate, and portions of the concentrated residues were separated by TLC. C₄-HSL in the samples was detected by overlaying the TLC plate with the *C. violaceum* indicator strain CV026. Lane a contained 50 pmol of synthetic C₄-HSL.

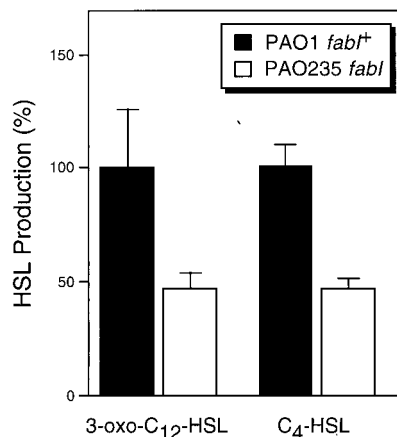


FIG. 7. HSL levels in wild-type and a *fabI* *P. aeruginosa* mutant. HSLs were extracted from overnight culture supernatants, and relative levels were determined by using *E. coli*-based C₄-HSL and 3-oxo-C₁₂-HSL bioassays. Values were determined in triplicate, and the values shown represent the means plus standard deviations.

triclosan insensitive. Searches of the *P. aeruginosa* genome database revealed at least 18 possible paralogs exhibiting on average 25% identity and 40% similarity to FabI, but none of them contained the signature motifs found in FabI proteins.

In cell extracts prepared at pH 6.5, *E. coli* FabI accepted both NADH and NADPH as cofactors, but when cell extracts were prepared at 7.5, only the NADH-dependent enoyl-ACP reductase activity remained (3). However, the NADPH-dependent FabI activity amounted to only 14 to 22% of the NADH-dependent enzyme. Although we isolated our cell extracts at pH 6.5, the NADPH-dependent FabI activity in wild-type or mutant cell extracts was very low, even at pH 6.5, and required three to six times more enzyme for detection (Fig. 3). Under these conditions, the NADPH-dependent activities were 3.5 or 10% of the NADH-dependent activities observed with wild-type and mutant extracts, respectively. We do not yet know what proportion, if any, of this activity is attributable to FabI, since our purified FabI protein does not exhibit NADPH-dependent enoyl-ACP reductase activity (see below).

To assess the substrate specificity of FabI, we expressed and purified FabI as an H₆-tagged protein. As its *E. coli* counterpart, the purified FabI protein could use both crotonyl-CoA and crotonyl-ACP as substrates, although it was much more active with crotonyl-ACP (Fig. 4B). The purified enzyme could not use NADPH as cofactor (data not shown). We do not yet know whether this reflects an intrinsic property of the *P. aeruginosa* FabI enzyme or whether the NADPH-dependent activity was lost during the purification steps that involve buffers with alkaline pH. We were unable to differentiate between these two possibilities since H₆ fusion proteins cannot be purified at pH 6.5.

FabI is a triclosan target. It has recently been shown that in *E. coli* triclosan targets lipid synthesis, and since triclosan-resistant mutants harbor mutations in *fabI*, it was concluded that the most likely target is enoyl-ACP reductase (33); this was subsequently confirmed in assays using purified FabI (19). To examine whether *P. aeruginosa* FabI is a triclosan target, we used the purified H₆-FabI in triclosan inhibition studies (Fig. 5). The purified protein was efficiently inhibited by very low concentrations of triclosan, showing 50% inhibition (50% inhibitory concentration [IC₅₀]) with 0.2 μM triclosan. McMurry et al. (33) and Heath et al. (18, 19) showed that their most triclosan-resistant mutants contained Gly⁹³-to-Val substitutions. To assess whether changing a glycine residue at the equivalent position 95 in *P. aeruginosa* FabI would lead to synthesis of a triclosan-resistant FabI enzyme, we introduced a Gly⁹⁵-to-Val change by site-directed mutagenesis and then expressed and purified the mutant H₆-FabI protein. Although the mutant protein exhibited a slightly increased enoyl-ACP reductase activity (Fig. 4B), it showed an IC₅₀ of 7 μM for triclosan and was not yet completely inhibited by the highest concentration (62 μM) tested in our experiments (Fig. 5, insert). While our studies neared completion, Heath et al. (18, 19) arrived at similar results after comparing the purified *E. coli* FabI with Gly⁹³-to-Ser and Gly⁹³-to-Val mutant enzymes; the triclosan IC₅₀s were 2 μM for FabI, 8 μM for the Gly⁹³-to-Ser mutant enzyme (19), and 10 μM for the Gly⁹³-to-Val mutant enzyme (18). Although both proteins were purified by using the same expression vector and same NH₂-terminal H₆ extension, *P. aeruginosa* FabI was ~10-fold more sensitive than the *E. coli* enzyme to triclosan. Although we do not know the reason(s) for this observation, the differences may be partially due to the different enoyl-ACP reductase assays used for the inhibition studies. We used the natural substrate crotonyl-ACP, whereas Heath et al. (18, 19) used the synthetic substrate *trans*-2-octadecenoyl-*N*-cysteamine, and the latter assay re-

quired substantially more FabI (12 μg, versus 1 μg in our assays). The recent determination of the X-ray structure of the *E. coli* FabI-NAD⁺-triclosan complex confirmed that triclosan interacts with both the NAD⁺ and the protein via hydrogen and hydrophobic bonds, forming a stable ternary complex (18, 26). Mutations in the FabI active site interfere with the formation of a stable FabI-NAD⁺-triclosan ternary complex and thus confer resistance to the drug.

We have previously shown that *P. aeruginosa* PAO1 is resistant to triclosan due to efflux by the tripartite MexAB-OprM multidrug efflux system, but Δ(*mexAB-oprM*) mutants are susceptible to triclosan (41). The same efflux system seems also partially responsible for diazaborine resistance. Both wild-type PAO1 and *fabI* mutant PAO235 showed high levels of resistance to diazaborine (the MIC in both strains was >160 μg/ml), and the MIC for diazaborine was significantly lower (80 μg/ml) in the Δ(*mexAB-opM*) mutant PAO200.

FabI participates in homoserine lactone synthesis. According to the current model, butyryl-ACP serves as the most likely acyl donor in C₄-HSL synthesis. To confirm that FabI can indeed provide butyryl-ACP for C₄-HSL synthesis by RhlI, we set up an in vitro synthesis system containing purified FabI, RhlI, the cofactor NADH, and the substrates crotonyl-ACP and SAM. This reconstituted system allowed synthesis of biologically active C₄-HSL that comigrated with synthetic C₄-HSL (Fig. 6). Synthesis of C₄-HSL was efficiently inhibited by triclosan. This inhibition was relieved by increasing concentrations of FabI but not RhlI, indicating that FabI and not RhlI is the triclosan target. Corroborating evidence that FabI plays a central role in HSL synthesis in vivo was provided by the observation that a *fabI* mutant produced only 50% of the HSLs found in wild-type cells. This is the first documented evidence that an enzyme of the fatty acid biosynthetic pathway plays a crucial role in HSL synthesis, and the establishment of an in vitro HSL synthesis system will facilitate the search for novel antimicrobials aimed at interfering with the pathways involved in HSL biosynthesis. The design and development of new FabI inhibitors will form an integral part of such strategies.

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