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Fatty Acid Biosynthesis in *Pseudomonas aeruginosa*: Cloning and Characterization of the *fabAB* Operon Encoding β-Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabA) and β-Ketoacyl-Acyl Carrier Protein Synthase I (FabB)

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The *Pseudomonas aeruginosa fabA* and *fabB* genes, encoding β-hydroxyacyl-acyl carrier protein dehydratase and β-ketoacyl-acyl carrier protein synthase I, respectively, were cloned, sequenced, and expressed in *Escherichia coli*. Northern analysis demonstrated that *fabA* and *fabB* are cotranscribed and most probably form a *fabAB* operon. The FabA and FabB proteins were similar in size and amino acid composition to their counterparts from *Escherichia coli* and to the putative homologs from *Haemophilus influenzae*. Chromosomal *fabA* and *fabB* mutants were isolated; the mutants were auxotrophic for unsaturated fatty acids. A temperature-sensitive *fabA* mutant was obtained by site-directed mutagenesis of a single base that induced a G101D change; this mutant grew normally at 30°C but not at 42°C, unless the growth medium was supplemented with oleate. By physical and genetic mapping, the *fabAB* genes were localized between 3.45 and 3.6 Mbp on the 5.9-Mbp chromosome, which corresponds to the 58- to 59.5-min region of the genetic map.

The fatty acid synthetase system of *Escherichia coli* is the archetype of a type II or dissociated fatty acid synthetase system, meaning that the individual reactions are catalyzed by separate proteins that are encoded by separate genes (for reviews, see references 2 and 21). The type II system is found in most bacteria and plants.

In *E. coli*, fatty acid biosynthesis can be separated into two stages, initiation and cyclic elongation (for a comprehensive review, see reference 2). Each round of elongation requires four chemical reactions (11). Three β-ketoacyl-acyl carrier protein (ACP) synthetases, KAS I (FabB), KAS II (FabF), and KAS III (FabH), which are the products of the *fabB*, *fabF*, and *fabH* genes, respectively, play pivotal roles in fatty acid synthesis. Initiation requires malonyl coenzyme A (CoA) and malonyl-ACP. Malonyl-CoA is synthesized by acetyl-CoA carboxylase, and malonyl-ACP is derived from malonyl-CoA and ACP by the action of malonyl-CoA:ACP transacylase, the product of the *fabD* gene. The first cycle of elongation is initiated by KAS III (FabH), which condenses malonyl-ACP with acetyl-CoA. Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by KAS I (FabB) and KAS III (FabF). In the second step, the resulting β-ketoester is reduced to a β-hydroxyacyl-ACP by a single, NADPH-dependent β-ketoacyl-ACP reductase (FabG). The third step in the cycle is catalyzed by either the *fabA*- or the *fabZ*-encoded β-hydroxyacyl-ACP dehydratase. The fourth and final step in each cycle involves the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by a single NADH-dependent enoyl-ACP reductase (FabI).

FabA and FabB also play crucial roles in unsaturated fatty acid biosynthesis. FabA isomerizes *trans*-2 decenoyl-ACP to *cis*-3-decenoyl-ACP, which bypasses the FabI-catalyzed step, and is used by FabB for initiation of the first cycle of elongation of unsaturated fatty acid synthesis. The FabA dehydrase is a bifunctional enzyme, catalyzing reactions of dehydration and of double-bond isomerization on 10-carbon thiol esters of ACP at an unusual bifunctional active site at the interface of a symmetric dimer (18).

Although some steps can be catalyzed by multiple enzymes, each of the enzymes plays a distinct role in the pathway, which is probably reflective of different substrate specificities and/or physiological function. In case of the condensing enzymes, FabH seems to catalyze the initial condensation in the pathway, FabF is responsible for the temperature-dependent alterations in fatty acid composition (7), and FabB is required for the catalysis of a unique step in unsaturated fatty acid biosynthesis. Similarly, although FabA and FabZ function interchangeably in the cycles of fatty acid elongation up to 10 carbons, FabA is more active in the dehydration of β-hydroxydecanoyl-ACP and, unlike FabZ, is required for the formation of *cis*-3-decenoyl-ACP (11), a prerequisite for unsaturated fatty acid biosynthesis. Conversely, even though both FabA and FabZ function equally in the cycles leading to the formation of saturated fatty acid biosynthesis, FabZ is probably the primary dehydratase that participates in the elongation cycles of unsaturated fatty biosynthesis (11).

The *E. coli* structural genes for all of the enzymes described above have been cloned and sequenced. Whereas the *fabD*, *fabF*, *fabG*, and *fabH* genes are located within a cluster of fatty acid biosynthetic genes at 24.8 min on the *E. coli* map, the *fabA* (21.9 min), *fabB* (52.6 min), *fabI* (29.2 min) and *fabZ* (4.4 min) genes all lie at distant sites (2).

Until the present studies were initiated, there had been few reports about fatty acid synthesis, composition, and function in *Pseudomonas* spp. This is perhaps surprising given that many biologically important and even essential functions are dependent on fatty acid biosynthesis. These include (i) production of increased levels of the unsaturated fatty acids hexadecenoate and octadecenoate and reduced levels of the corresponding saturated fatty acids in response to lowering of the growth temperature (16); (ii) synthesis of the protein-bound coenzymes biotin and lipoic acid (3); and (iii) lipopolysaccharide, lipoprotein, and lipid biosynthesis (2). In many gram-negative

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or origin
P. aeruginosa		
PAO1	Prototroph	B. H. Holloway
PAO2	*ser-3*	B. H. Holloway
PAO191	PAO1 with *fabA*::Gm^r	This study
PAO192	PAO1 with *fabB*::Gm^r	This study
PAO194	PAO1 with *fabA*(Ts)	This study
PAO716	*cys-54 rif-96*	B. H. Holloway
E. coli		
DH5αF′	[F$^+$ φ80Δ*lacZ*ΔM15] Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1 gyrA relA1*	20
BL21(DE3)	*E. coli* B F$^-$ *ompT* r_B^- m_B^- (λDE3)	36
CY50	F$^-$ *fabA2*(Ts) *his trp gal mtl xyl rpsL*	J. Cronan
CY274	*fabB*(Ts) *arg pro* Tc^r	J. Cronan
Plasmids		
pGEM-T	Ap^r; TA-cloning vector	Promega
pRO271	Cb^r Hg^r; Cma$^+$	24
pUC18	Ap^r	43
pUCP20/21	Ap^r; broad-host-range cloning derivatives of pUC18/19	33
pUCP20/21T	Ap^r; mobilizable derivatives of pUCP20/21	33
pPS744	Ap^r; PCR-amplified 193-bp genomic segment from PAO1 cloned into pGEM-T	This study
pPS752	Ap^r; *orfS*$^+$ *fabA*$^+$ *fabB*$^+$ (4.8-kb chromosomal *Bam*HI-*Eco*RI fragment cloned between the same sites of pUC18)	This study
pPS755	Ap^r; *fabB*$^+$ (2,348-bp *Pst*I fragment from pPS752 cloned into the *Pst*I site of pUC18)[a]	This study
pPS770	Ap^r; *fabA*$^+$ *fabB*$^+$ (ligation of 0.5-kb *Kpn*I-*Sma*I fragment from pPS752 between the same sites of pPS755	This study
pPS771	Ap^r; *fabA*$^+$ (deletion of the 1.86-kb *Sph*I fragment from pPS770)	This study
pPS790	Ap^r; *fabA*$^+$ *fabB*$^+$ (2,039-bp PCR-amplified *Bam*HI-*Kpn*I fragment from pPS752 between the same sites of pUC19)	This study
pPS823	Ap^r; *orfS*$^+$ *fabA*$^+$ *fabB*$^+$ (4.8-kb chromosomal *Bam*HI-*Eco*RI fragment cloned between the same sites of pUCP21T)	This study

[a] Unless noted otherwise, the *fab* genes were in the same transcriptional orientation as the *lac* operon promoter.

pathogenic bacteria, the same pathway is probably also required for the synthesis of the acylated homoserine lactones used to monitor cell density and to regulate many virulence factors by quorum sensing and response (8, 17, 25, 26). It has recently been shown that acylated homoserine lactone synthesis in *Vibrio fischeri* requires *S*-adenosylmethionine and acylated ACP (30). Other cellular responses of *Pseudomonas* species that suggest the importance of fatty acid synthesis include (i) *cis-trans* isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* strains in the presence of toxic concentrations of toluene (39), (ii) the effect of lipid acyl chain length on the activity of sodium-dependent leucine transport in *P. aeruginosa* (38), and (iii) possible involvement of a fatty acid desaturase of *P. syringae* pv. phaseolicola in facilitating phaseolotoxin secretion at the low temperatures normally required for its synthesis (9).

However, in the absence of knowledge about the molecular architecture, biochemical function, and regulation of expression of the *Pseudomonas* fatty acid synthesis genes, the importance of this biosynthetic pathway in various cellular processes, as well as its exploitation as a target for novel antimicrobial agents that specifically inhibit the bacterial Fab pathway, including thiolactomycin (10, 23), cannot be properly evaluated. In this study, we characterized the genes encoding two key enzymes in saturated and unsaturated fatty acid synthesis, namely, *fabA* and *fabB.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains used in this study are listed in Table 1. Also listed in Table 1 are plasmids that are pertinent to this study and are not described in the text. The details underlying the construction of pPS752 (see Fig. 1) are as follows. The 193-bp biotinylated chromosomal fragment from pPS744 was used to identify a ca. 4.8-kb chromosomal *Bam*HI-*Eco*RI fragment by genomic Southern analysis. For cloning of this DNA fragment, *Bam*HI-*Eco*RI-digested PAO1 chromosomal DNA was electrophoresed on a 1% agarose gel in 0.5× Tris-borate-EDTA (TBE) and the fragments from the 4- to 5-kb region of the gel were eluted by the GeneClean procedure (Bio101, San Diego, Calif.). After ligation into *Bam*HI-*Eco*RI-digested pUC18 (43), ampicillin-resistant (Ap^r) DH5αF′ colonies were selected. Plasmid DNA was isolated and subjected to Southern analysis with the above described probe. A representative plasmid, designated pPS752, containing the cloned 4.8-kb *Bam*HI-*Eco*RI fragment was retained for further studies. Luria-Bertani (LB) medium (29) was used as the rich medium for both *E. coli* and *P. aeruginosa*. M9 medium (29) and VBMM (31) were used as the minimal media for *P. aeruginosa*. The Fab phenotypes were assessed on RB medium (12). Fatty acids, neutralized with KOH, were added to RB medium at a final concentration of 0.1% and solubilized by the addition of Brij 58 to a final concentration of 0.1 to 0.2%. Antibiotics were used in selection media at the following concentrations: for *E. coli*, 100 μg of ampicillin per ml and 10 μg of gentamicin per ml; for *P. aeruginosa*, 500 μg of carbenicillin per ml, 200 μg of gentamicin per ml, and 200 μg of rifampin per ml.

DNA procedures. Restriction enzymes, calf intestinal phosphatase, T4 DNA polymerase, and T4 DNA ligase were used as recommended by the suppliers. DNA fragments were made blunt ended with T4 DNA polymerase in the presence of 100 μM deoxynucleoside triphosphates (29). Small-scale isolations of plasmid DNA from *E. coli* and *P. aeruginosa*, and DNA transformations were done as previously described (34). DNA restriction fragments were eluted from low-gelling-temperature agarose gels as described by Wieslander (41) for <500-bp fragments or by the GeneClean procedure for >500-bp fragments. For PCR amplification of a *fabA* fragment from chromosomal DNA, two oligonucleotides, P1 (5′-CC[C/G] GC[C/CG CC[C/G] A[A/C]C ATG CT[C/G] ATG ATG) and P2 (5′-TAG AAG CC[C/G] AC[C/G] AGC TGC CAC AT) (marked in Fig. 2) were used to prime synthesis from PAO1 chromosomal DNA as previously described (13). The 193-bp PCR fragment was ligated to the pGEM-T cloning vector, by the method provided by the supplier (Promega, Madison, Wis.), to form pPS744. For genomic Southern analysis, chromosomal PAO1 DNA was digested with various restriction endonucleases, electrophoresed on a 1% agarose gel in 0.5× TBE, and transferred to Photogene nylon membranes (Gibco BRL, Gaithersburg, Md.) as described by Sambrook et al. (29). Plasmid DNA was biotinylated by random hexamer priming by the NEBlot Phototype kit method (New England Biolabs, Beverly, Mass.). Following transfer and UV fixation (29), the membranes were probed with the biotinylated DNA fragment as specified in the Phototype kit protocol. Sequences of double-stranded plasmid DNA templates were determined with the Taq FS ready reaction kit (Applied Biosystems, Foster City, Calif.) on a PTC-100 PCR system (MJ Research, Watertown, Mass.). Extensions were primed with the commercially available 24-nucleotide (nt) pUC/M13 reverse and forward sequencing primers, respectively. Labeled samples were analyzed on an ABI 377 PRISM sequencer at the Colorado State University Macromolecular Resource Facility. Both strands were entirely sequenced from plasmids obtained by deletion and subcloning of restriction fragments into the pUC18/19 vectors (43). Sequences were analyzed with the MacDNAsis (Hitachi Software Engineering, San Bruno, Calif.), SeqEd (Applied Biosystems), and SeqVu (Garvan Medical Institute, Sydney, Australia) programs. GenBank homology searches were performed with the online BLAST facilities of the National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.).

RNA procedures. Total RNA was isolated from ~10^9 cells (1.5-ml exponentially growing cultures; absorbance at 540 nm, ~0.8 to 1.0) of *P. aeruginosa* PAO1 with the RNAeasy kit (Qiagen, Chatsworth, Calif.). For Northern analysis, 30 μg of total RNA was electrophoresed on a 1.2% agarose gel prepared in 2.2 M formaldehyde–20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7)–8 mM sodium acetate–1 mM EDTA and submerged in the same buffer. The blots were processed and probed exactly as described above for the Southern blots, with a few modifications specified for RNA blots in the NEBlot Phototype kit protocol. The *fabA*- and *fabB*-specific probes were a 487-bp *Pst*I-*Sph*I fragment and a 730-bp *Sph*I fragment, respectively (see Fig. 2). These fragments were gel purified and then biotinylated by random hexamer priming by the NEBlot kit method.

Mutant isolation. For isolation of *fabA* and *fabB* mutants of *P. aeruginosa*, a blunt-ended 830-bp Gm^r-conferring fragment was ligated to *Not*I- or *Bgl*II-digested pPS752 DNA (see Fig. 1) to yield pPS798 (*fabA*::Gm^r) and pPS794 (*fabB*::Gm^r). Care was taken that in all cases the Gm^r-conferring *aacC1* gene was inserted in the same transcriptional orientation as the *fabAB* genes to ensure that the Gm^r insertions were nonpolar on downstream sequences. The mutated regions were then subcloned as blunt-ended *Bam*HI-*Eco*RI fragments into the *Sma*I site of the gene replacement vector pEX100T (32). This procedure yielded plasmids pPS815 (*fabA*::Gm^r) and pPS816 (*fabB*::Gm^r). For gene replacement in strain PAO1, the previously described *sacB*-based strategy was used (32). Sucrose-resistant colonies were obtained on RB medium in the presence of 0.1% oleate and then tested for unsaturated fatty acid auxotrophy. The mutations in strains PAO191 (*fabA*::Gm^r) and PAO192 (*fabB*::Gm^r) obtained by this procedure were verified by genomic Southern analyses (data not shown). Mutant PAO194 containing a *fabA*(Ts) allele was obtained as follows. A mutagenic primer (5′-GC<u>GCGGCCGC</u>GGtCGGGGTTGCCCT) was synthesized which is complementary to nt 496 to 513 and contains the *Not*I site located within *fabA* (underlined; see Fig. 2) plus a single mismatch (lowercase t) that results in a G101D change. This primer and the M13 reverse sequencing primer were used to prime synthesis from pPS782 DNA that contains the *fabA* region on the 1,067-bp *Kpn*I-*Sal*I fragment from pPS752 (Fig. 1) cloned between the same sites of pUC18, with Taq$^+$ polymerase (Sangon, Scarborough, Ontario, Canada) under previously described conditions (13). The amplified ~1,150-bp fragment was digested with *Eco*RI-*Not*I and ligated to *Eco*RI-*Not*I-cleaved pPS782 DNA, yielding pPS785, which is the same as pPS752 (see Fig. 1) but contains a *fabA* gene encoding a protein with the desired G101D change. For transfer of the G101D allele into the PAO1 chromosome, a blunt-ended 1.1-kb *Eco*RI-*Sal*I fragment from pPS785 was inserted into the *Sma*I site of pEX100T. After conjugal transfer into PAO1, sucrose-resistant and carbenicillin-sensitive colonies were selected at 30°C and screened for their inability to grow at 42°C in the absence of oleate supplementation.

Genetic mapping. The chromosome mobilizing pRO271 was conjugally transferred from PAO2 into PAO191 as previously described (24). The new donor strain PAO191/pRO271 was crossed with the recipient PAO716 by mixing 0.3 ml of donor cells (absorbance at 540 nm, 0.5 to 1; grown in LB medium with 500 μg of carbenicillin per ml and 0.1% oleate) with 0.6 ml of recipient cells (grown to the same optical density in LB–0.1% oleate medium) in a final volume of 1.8 ml of LB–0.1% oleate broth. After 2 h at 37°C, the cells were harvested in a microcentrifuge and washed once in 1 ml of M9 medium. The cells were suspended in 1 ml of M9 medium, and 0.2-ml samples were plated on LB–0.1% oleate medium with 200 μg each of gentamicin and rifampin per ml. Colonies growing on these selective plates were tested for the inheritance of the *cys* marker by patching on VBMM plates containing 0.1% oleate and 200 μg each of gentamicin and rifampin per ml, with or without 50 μg of cysteine per ml.

Expression of plasmid-encoded proteins. To demonstrate proper expression of the products of *fabA* and *fabB* in *E. coli*, the chromosomal inserts from pPS755, pPS770, and pPS771 (Fig. 1) were cloned as *Eco*RI-*Hin*dIII fragments into the expression vector pT7-5 (37) to yield plasmids pPS777 (*fabA*$^+$*B*$^+$), pPS779 (*fabB*$^+$), and pPS778 (*fabA*$^+$), respectively. Plasmid-encoded polypeptides were overexpressed and identified with the bacteriophage T7 RNA polymerase-promoter system (36). The host strain for T7 promoter-containing plasmids was BL21(DE3), which contains the gene for T7 RNA polymerase under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV* promoter (36). The products of the cloned genes were selectively labeled with [^{35}S]methionine as previously described (31). The proteins were separated on a 0.1% sodium dodecyl sulfate–10% polyacrylamide gel (pH 9.2) (22). The destained gels were dried, and the labeled proteins were visualized by autoradiography.

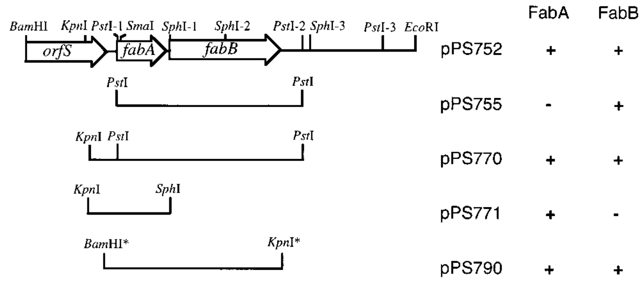

FIG. 1. Restriction maps of recombinant plasmids and location of the *fabA* and *fabB* genes. The + and − symbols denote the Fab phenotypes conferred on strain CY50 (*fabA*) or CY274 (*fabB*) by the indicated plasmids. In all constructs, the transcriptional orientation of *fabA* and *fabB* is the same as that of the *lac* promoter. Only selected restriction enzyme cleavage sites are shown (note that there are additional *Pst*I sites in the *fabA* upstream region [see Fig. 2], which were omitted from this figure for the sake of clarity). For details about pPS790, see Fig. 2.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been assigned GenBank accession no. U70470.

RESULTS AND DISCUSSION

Cloning of the region containing *fabA*. The partial *fabA* coding sequence was successfully amplified from *P. aeruginosa* chromosomal DNA by using two oligonucleotide primers (P1 and P2; marked in Fig. 2) with minor degeneracy modeled after conserved amino acid sequence regions found in the *E. coli* and *Haemophilus influenzae* FabA homologs. With the PCR-generated, cloned DNA fragment as a probe, a restriction map of the chromosomal *fabA* region was constructed and the entire *fabA* gene was cloned on a 4.8-kb *Bam*HI-*Eco*RI fragment by the strategy described in Materials and Methods. The physical maps of some representative clones are shown in Fig. 1. When *E. coli* CY50 [*fabA*(Ts)] and CY274 [*fabB*(Ts)] were transformed with pPS752, both strains were able to grow on RB medium at 42°C in the absence of oleate supplementation. The same strains harboring the vector control pUC18 did not grow at 42°C on RB medium unless it was supplemented with oleate. These results indicated that the chromosomal insert of pPS752 contained not only the *P. aeruginosa* *fabA* homolog but also a gene capable of complementing the *E. coli fabB*(Ts) allele. Subcloning and deletion analysis further localized the respective complementing sequences. Subcloning of a 2.35-kb *Pst*I fragment (pPS755) abolished *fabA*-complementing activity but complemented *fabB* normally, with *fabB* being transcribed from the *lac* promoter (Table 1). The addition of a 0.5-kb *Kpn*I-*Sma*I fragment from pPS752 to pPS755 restored the ability of the resulting pPS770 to complement both *fabA* and *fabB*, localizing both complementing activities on a 2.8-kb *Kpn*I-*Pst*I fragment. Finally, deletion of a 1.9-kb *Pst*I-*Sph*I fragment (pPS771) led to loss of *fabB* complementation activity without affecting the complementation of *fabA*, thus localizing *fabB* at least partially to the right of the *Sph*I-1 site as indicated in Fig. 1.

These results indicated that in contrast to *E. coli*, where *fabA* and *fabB* map at distant sites (2), the *P. aeruginosa* homologs were physically closely linked.

Identification and nucleotide sequence analysis of *fabA* and *fabB*. To verify that the ability of pPS770 to complement *E. coli fabA* and *fabB* was due to the presence of the corresponding *P. aeruginosa* homologs, its entire 2.8-kb insert was sequenced. The 2,771-nt sequence (part of which is presented in Fig. 2 and

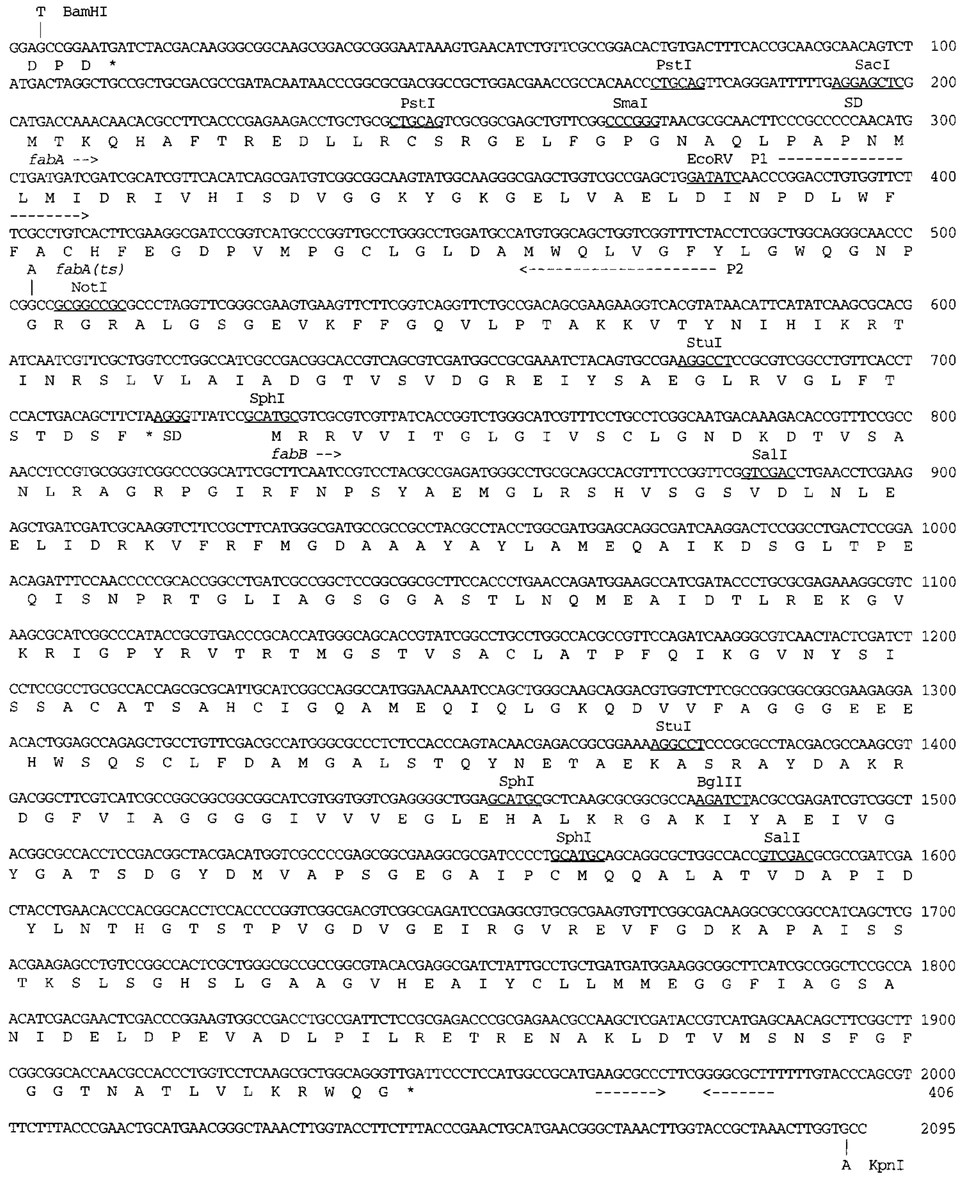

FIG. 2. Nucleotide sequence of the *fabAB*-containing region. The nucleotide sequence shown is part of a longer sequence that was assigned GenBank accession no. U70470. Listed below the sequence are the deduced amino acid sequences of FabA, FabB, and the extreme C terminus of the sensor/response regulatory protein OrfS. The G-to-A change introduced by site-directed mutagenesis and leading to a *fabA*(Ts) allele (for details, see Materials and Methods) is shown above the nucleotide sequence. The proposed consensus Shine-Dalgarno (SD) sequences are underlined. An inverted repeat sequence that may act as a Rho-independent transcriptional terminator is shown by convergent arrows. The locations and lengths of primers used for PCR amplification of partial FabA from genomic DNA are underlined with broken arrows labeled P1 and P2. The *Bam*HI and *Kpn*I sites of pPS790 that are marked with an asterisk in Fig. 1 were generated during PCR amplification of the *fabAB*-containing sequences with primers containing the indicated single-base changes. Only selected restriction enzyme cleavage sites are marked.

is available in its entirety from GenBank under accession no. U70470) contained three open reading frames with a codon usage that is typical for *P. aeruginosa* (40) and with significant homologies to proteins in GenBank.

The first of these, designated OrfS, extended from the *Bam*HI site of clone pPS752 (Fig. 1) to nt 10 of the sequence shown in Fig. 2 (data not shown). OrfS showed significant homology to the sensory transduction histidine kinases from various bacteria, most notably LemA from *P. syringae* (15), although it was not identical to the *lemA* homolog from *P. aeruginosa* (42).

The *fabA* gene is separated from the OrfS-encoding gene by 187 nt. It comprises 515 nt and is preceded by a good consensus Shine-Dalgarno sequence (AGGAG). The deduced amino acid sequence of *fabA* defines a protein of 171 residues with a calculated molecular weight of 18,747. This protein is very similar in size to and shares significant homology with *E. coli* FabA (79% similarity and 68% identity), as well as the putative *H. influenzae* FabA protein (77% similarity and 64% identity (Fig. 3A).

The *fabB* gene was found to start 12 nt downstream of *fabA* and is preceded by a reasonable Shine-Dalgarno sequence (AGGG) which overlaps the *fabA* termination codon. The 1,214-nt gene encodes a protein of 405 amino acids with a calculated molecular weight of 42,687. The FabB protein is highly homologous to its counterparts from *E. coli* (77% sim-

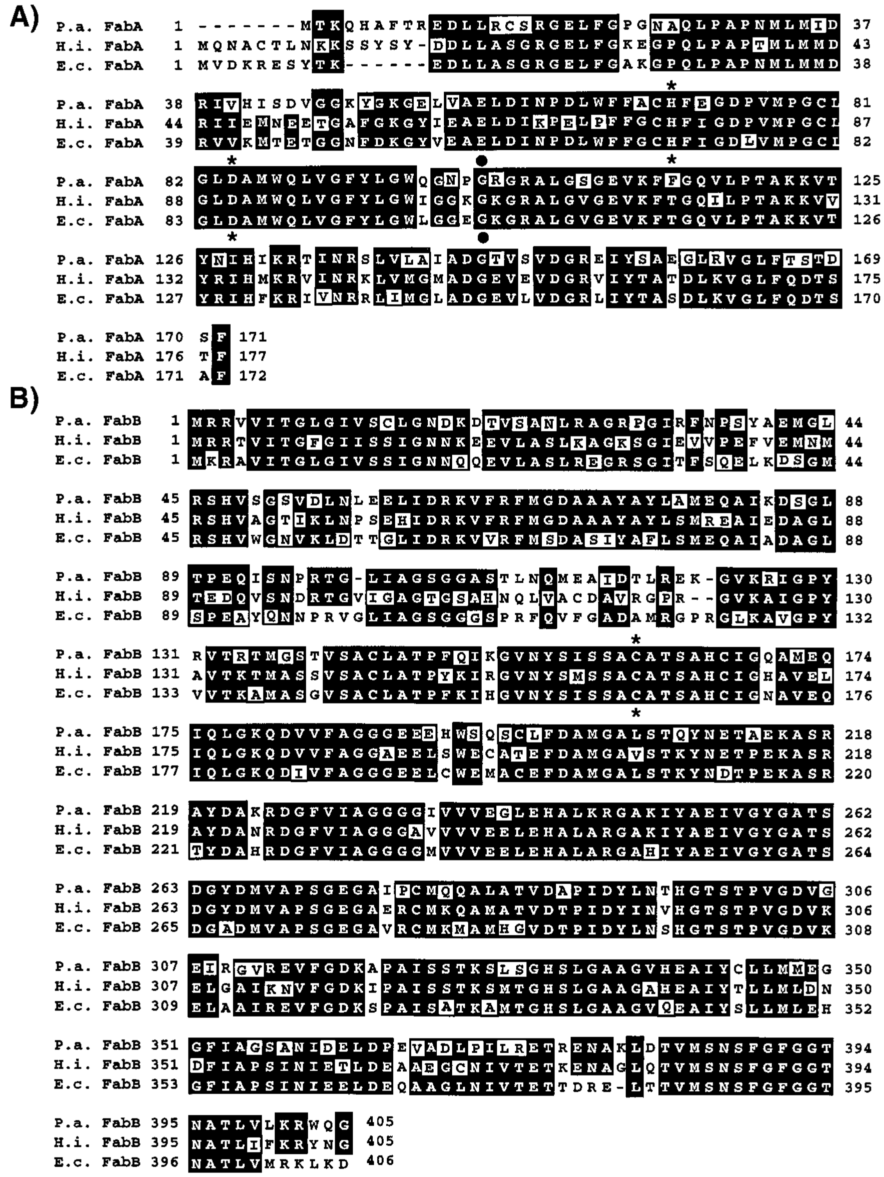

FIG. 3. Alignments of gram-negative FabA and FabB proteins. Alignments of *P. aeruginosa* (P.a.) FabA (A) and FabB (B) with their homologs from *E. coli* (E.c.) and *H. influenzae* (H.i.) are shown. Identical residues are indicated by white letters. The active-site residues, histidine and aspartic acid in FabA and cysteine in FabB, are marked with asterisks. The glycines whose changes to an aspartic acid residue in *E. coli* and *P. aeruginosa* resulted in temperature-sensitive FabA phenotypes are marked with solid dots. The homologies over the entire lengths of the proteins shown were as follows: FabA, 79% (P.a./E.c.) and 77% (P.a./H.i.); and FabB, 77% (P.a./E.c.) and 76% (P.a./H.i.).

ilarity and 65% identity) and *H. influenzae* 76% similarity and 67% identity) (Fig. 3B). The region downstream of *fabB* contained a sequence with characteristics of a Rho-independent transcription termination signal ($\Delta G = -18.6$ kcal/mol; calculated with the MacDNAsis program from Hitachi Software Engineering, San Bruno, Calif.) (28).

Overall, the sizes and amino acid compositions of FabA and FabB in the two bacteria are very similar, including conservation of the FabA active-site His-69 (1) and Asp-84 (18) (Fig. 3A) and of the FabB active-site Cys-161-containing sequence (Fig. 3B) (21).

Transcriptional organization of *fabA* and *fabB*. The organization of *fabA* and *fabB* predicted by nucleotide sequence analysis, i.e., separation of the two genes by only 12 bp and location of a putative terminator downstream of *fabB*, indicated that these two genes may be cotranscribed and form a *fabAB* operon. This notion was supported by the results of Northern analyses, which indicated a single ~1.7- to 1.8-kb transcript when total PAO1 RNA was probed with *fabA*- and *fabB*-specific probes, respectively (Fig. 4).

The finding that *P. aeruginosa fabA* and *fabB* form a single transcriptional unit may have important ramifications in terms of coordination of the regulation of expression of these two genes or may simply reflect the tendency of *P. aeruginosa* to cluster functionally related genes on the chromosome. However, the latter scenario probably does not offer the sole explanation, since none of the other *fab* genes were found in the vicinity of *fabAB*. In *E. coli*, transcription of the unlinked *fabA* and *fabB* genes is positively regulated by FadR, which also acts as a repressor of many unlinked genes and operons encoding

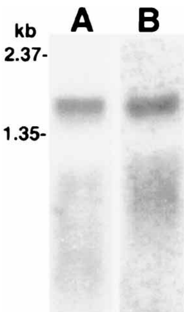

FIG. 4. Northern blot analysis of the *fabA* and *fabB* transcript. Two 30-μg samples of total *P. aeruginosa* PAO1 RNA were electrophoresed in a 1.2% formaldehyde–agarose gel, blotted, and probed with biotinylated *fabA* (lane A)- and *fabB* (lane B)-specific probes. The positions of the 2.37- and 1.35-kb RNA markers of the 0.24- to 9.5-kb RNA ladder from Gibco-BRL (Gaithersburg, Md.) are shown on the left.

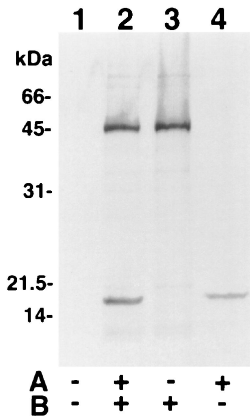

FIG. 5. Expression of FabA and FabB in *E. coli*. Proteins were selectively labeled with [^{35}S]methionine, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography as described in Materials and Methods. Lanes: 1, pT7-5; 2, pPS777; 3, pPS779; 4, pPS779. The *fabA* and *fabB* genotypes of the plasmids are indicated at the bottom. The positions of molecular mass markers (Bio-Rad, Richmond, Calif.) for (top to bottom) bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme are indicated on the left.

proteins involved in long-chain fatty acid transport, activation, and β-oxidation (4, 12). FadR also seems to play a role in stasis survival and growth-phase-dependent regulation of expression of several genes, including the universal stress protein-encoding gene *uspA* and the *fad*, *fabA*, and *fabB* genes (5). Analysis of the untranslated *P. aeruginosa fabA* upstream region did not reveal DNA sequences with significant homology to the FadR operator consensus sequence (5). In addition, in an *E. coli* system, transcription of a $fabA_{P.a.}$'-'*lacZ* transcriptional fusion was not regulated by FadR (data not shown). A more detailed transcriptional analysis of the transcriptional organization of *fabAB* and their mode(s) of regulation is under way.

Expression of FabA and FabB in *E. coli*. Although functional complementation already indicated expression of the products of *fabA* and *fabB* in *E. coli*, a T7 expression system was used to demonstrate the expression of gene products of the predicted sizes. To this end, pPS777 (*fabA*$^+$*B*$^+$), pPS779 (*fabB*$^+$), and pPS778 (*fabA*$^+$) were transformed into strain BL21(DE3). Following induction and expression, the radiolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. The results of the expression experiment are shown in Fig. 5. Whereas cells containing pPS777 expressed two IPTG-inducible proteins with estimated molecular masses of 44 and 19 kDa (lane 2), cells containing pPS779 or pPS778 expressed the 44- or the 19-kDa protein, respectively (lanes 3 and 4). Neither polypeptide was expressed in vector-containing cells (lane 1). The observed sizes (44 and 19 kDa) of the *P. aeruginosa* FabB and FabA polypeptides compare very favorably with the molecular weights deduced from the nucleotide sequence (42,687 for FabB and 18,747 for FabA), as well as the predicted molecular weights of the corresponding *E. coli* (42,611 and 18,984 [GenBank accession no. J03186 and M24427, respectively]) and putative *H. influenzae* (42,534 and 19,464 [GenBank accession no. L45260]) homologs.

Successful expression of the *P. aeruginosa* FabA and FabB proteins in the T7 expression system is an important step toward the generation of His-tagged proteins in the T7 promoter-based pET system, which is currently being used for overproduction, affinity purification, and biochemical characterization of the FabA and FabB enzymatic activities (11).

Isolation of *P. aeruginosa fabA* and *fabB* mutants. To confirm that *fabA* and *fabB* encode the *P. aeruginosa* homologs of its *E. coli* counterparts, nonpolar chromosomal *fabA* and *fabB* mutations were generated by insertion of a Gm^r-conferring element and the mutant constructs were returned into the chromosome by allelic exchange. The insertions were confirmed by genomic Southern analyses (data not shown). The *fabA* and *fabB* mutants were unsaturated fatty acid auxotrophs. The *fabA*$^+$*B*$^+$ pPS823 complemented both mutants. Analysis of the nature of the *fabA*(Ts) mutation in *E. coli* CY50 revealed a single G102D change, which results in a temperature-sensitive FabA enzyme (27). Since the *P. aeruginosa* FabA sequence contains a glycine in the corresponding position 101, in vitro site-directed mutagenesis was used to introduce a G101D change and the mutation was returned to the PAO1 chromosome by allelic exchange. The resulting mutant, PAO194, exhibited a temperature-sensitive growth phenotype; i.e., it did not grow at 42°C unless the medium was supplemented with oleate.

The observation that *fabA* and *fabB* mutants are unsaturated fatty acid auxotrophs demonstrated the conserved roles of FabA and FabB in unsaturated fatty acid biosynthesis. The same mutant analysis also indicates the presence of more than one fatty acid synthetase activity, e.g., FabF and/or FabH, since *fabB* mutants are saturated fatty acid prototrophs. The availability of an unmarked, defined *fabA* mutant will facilitate future studies of the role of this gene and its product in *P. aeruginosa* fatty acid biosynthesis. Furthermore, the demonstration that the introduction of a single base change into *P. aeruginosa fabA*, similar to that found in a temperature-sensitive *E. coli fabA* mutant, leads to the same temperature-sensitive phenotype not only proved that *E. coli* and *P. aeruginosa* FabA possess similar secondary structures, as already predicted by the protein similarities, but also demonstrated the importance of the affected glycine residue in assembly of a functional FabA enzyme. Analysis of the position of the affected glycine residue in the secondary structure indicates that the protein has less freedom to accommodate changes in the side chains without breaking hydrogen bonds in the secondary structure (18, 35). Thus, the glycine-to-aspartic-acid change may not allow for stable monomer folding or for stable dimerization at high temperatures.

Chromosomal mapping of the cloned *fabAB* genes. The *fabAB* genes were located on the PAO1 chromosome by hybridization of the biotin-labeled *Bam*HI-*Eco*RI fragment from pPS752 (Fig. 1) to Southern blots of *Dpn*I- and *Spe*I-digested genomic DNA, which previously had been separated by pulsed-field gel electrophoresis (reference 19 and data not shown). In blots of *Spe*I-digested chromosomal DNA, the *fabAB* sequences hybridized to a restriction fragment of approximately 150 kb, corresponding to restriction fragment *Spe*I-R (6). Similarly, the same probe hybridized to a *Dpn*I fragment of approximately 700 kb, which corresponded to *Dpn*I-C. Thus, the *fabAB* genes were mapped between 3.45 and 3.6 Mbp on the 5.9-Mbp chromosome, which corresponds to the 58- to 59.5-min region of the genetic map (14). In agreement with the physical mapping data, the *fabA*::Gm^r mutation was genetically (50%) closely linked to *cys-54* in pRO271-mediated chromosome mobilization experiments.

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