A New Mechanism for Anaerobic Unsaturated Fatty Acid Formation in *Streptococcus pneumoniae*

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The anaerobic pathway for unsaturated fatty acid synthesis was established in the 1960s in *Escherichia coli*. The double bond is introduced into the growing acyl chain by FabA, an enzyme capable of both the dehydration of β-hydroxydecanoyl-acyl carrier protein (ACP) to trans-2-decenoyl-ACP, and the isomerization of trans-2 to cis-3-decenoyl-ACP. However, there are a number of anaerobic bacteria whose genomes do not contain a fabA homolog, although these organisms nonetheless produce unsaturated fatty acids. We cloned and biochemically characterized a new enzyme in type II fatty acid synthesis from *Streptococcus pneumoniae* that carries out the isomerization of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP, but is not capable of catalyzing the dehydration of β-hydroxy intermediates. This tetrameric enzyme, designated FabM, has no similarity to FabA, but rather is a member of the hydratase/isomerase superfamily. Thus, the branch point in the biosynthesis of unsaturated fatty acids in *S. pneumoniae* occurs following the formation of trans-2-decenoyl-ACP, in contrast to *E. coli* where the branch point takes place after the formation of β-hydroxydecanoyl-ACP.

Unsaturated fatty acid (UFA) biosynthesis is essential for the maintenance of membrane structure and function in many groups of bacteria that embrace the anaerobic life style. In eukaryotes, olefin formation requires molecular oxygen (1), and double bonds are introduced into the fatty acids following the completion of their synthesis via the type I, multifunctional fatty acid synthase (2). In contrast, bacteria synthesize fatty acids using the dissociated, type II fatty acid synthase system in which each of the steps is catalyzed by distinct enzymes that are encoded by separate genes (3, 4). The key players in UFA synthesis were first defined by the isolation and characterization of UFA-auxotrophs (5). In the type II system, the double bond is introduced anaerobically into the growing acyl chain at the 10-carbon intermediate by β-hydroxydecanoyl-ACP dehydratase, FabA (6). FabA is capable of both the removal of water to generate trans-2-decenoyl-ACP and the isomerization of this intermediate to the cis-3-decenoyl-ACP (3, 7). However, FabA is not the only protein that is required for introduction of the double bond and does not catalyze the rate-limiting step in UFA formation (8). A second unsaturated fatty acid auxotroph was isolated that corresponds to the fabB gene, which encodes β-ketoacyl-ACP synthase I. In fabB mutants, saturated fatty acid synthesis persists due to the presence of the other elongation condensing enzyme in *Escherichia coli*, FabF (9, 10). Although FabF readily elongates 16:1 to 18:1 (10), the inability to support UFA synthesis in fabB mutants leads to the conclusion that FabF cannot elongate a key intermediate in UFA biosynthesis (3, 4). The analysis of fabB and fabF mutants, coupled with the catalytic properties of FabB and FabF in vitro supports a function for FabB in UFA synthesis and a role for FabF in the thermal modulation of membrane fatty acid composition (11–14).

The availability of numerous bacterial genomes sequences allows the reconstruction of type II fatty acid synthase in these organisms using standard bioinformatics analysis tools. It is notable that fabA and fabB genes occur together in most bacteria that produce UFA (15). However, many anaerobes that synthesize UFA, such as the Streptococci and Clostridia, do not have a recognizable fabA homolog in their genomes, and also have a fabF rather than a fabB subtype of elongation condensing enzyme. Clearly, UFA are synthesized by a distinct biochemical mechanism in these organisms, and the goal of this study was to identify the enzyme(s) responsible for olefin formation in *Streptococcus pneumoniae*. Like *E. coli*, *S. pneumoniae* produces straight-chain saturated and monounsaturated fatty acids predominately of 16 and 18 carbon chain lengths (16). Our experiments show that this organism does not utilize a FabA-like mechanism for introducing a double bond into the growing acyl chain, but rather accomplishes this task using a previously unknown enzyme, termed trans-2, cis-3-decenoyl-ACP isomerase (FabM). Reconstitution of the *S. pneumoniae* UFA synthetic pathway in vitro and in vivo lead to the conclusion that the branch point for UFA synthesis occurs at the enoyl-ACP intermediate, and the amount of UFA produced arises from the competition of FabM and FabK for enoyl-ACP.

**EXPERIMENTAL PROCEDURES**

Materials—Sources of supplies were: Amersham Biosciences, [2-14C]malonyl-CoA (specific activity, 56 mCi/mmol); Sigma, antibodies, acyl-CoA, ACP; Difco, microbiological media; Promega, molecular reagents and restriction enzymes; Invitrogen, T4 ligase; Novagen, pET vectors and expression strains; Qiagen, Ni²⁺-agarose resin. Protein was synthesized using a previously unknown enzyme, termed trans-2, cis-3-decenoyl-ACP isomerase (FabM). Reconstitution of the UFA synthetic pathway in vitro and in vivo lead to the conclusion that the branch point for UFA synthesis occurs at the enoyl-ACP intermediate, and the amount of UFA produced arises from the competition of FabM and FabK for enoyl-ACP.

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quantitated by the Bradford method (17). The Mycobacterium tuberculosis mmFabH, E. coli ecFabH, ecFabF, S. pneumoniae spFabH, and spFabF proteins were purified as described previously (18–22). All other chemicals were reagent grade or better.

Cloning and Purification of FabM—The fabM gene was amplified from genomic DNA from S. pneumoniae R6. The spfabM PCR primer pair consisted of 5′-AAATAAAGGAGGCCATATG and 5′-GGATCCGCGGATGCGAG. The primers introduced novel restriction sites for NdeI at the initiator methionine codon of the predicted coding sequence and BamHI downstream of the stop codon. The PCR products were ligated into the plasmid pCR2.1 and sequenced to verify the absence of PCR mutations. The plasmid was isolated and digested with NdeI and BamHI, and the gene fragment was isolated and ligated into plasmid pET28b+ digested with the same enzymes. The resulting plasmid was used to transform strain BL21(DE3) codonplus-RIL strain, and the protein was overexpressed and purified as described previously (20). Affinity chromatography was followed by gel filtration on Superdex-200 HR 26/60. The enzyme was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The apparent molecular weight of FabM was estimated by gel filtration chromatography using a Superdex-200 HR10/30 column calibrated with globular protein standards.

Measurement of FabM Isomerase Activity in Vitro—Cycles of fatty acid elongation were reconstituted in vitro to detect isomerase activity using the purified individual enzymes that catalyze the fatty acid biosynthesis cycle as essentially described previously (20, 23). The reaction mixtures contained 100 μM ACP, 10 mM dithiothreitol, 0.1 mM sodium phosphate buffer, pH 7.0, 100 μM NADPH, 100 μM NADH, 50 μM octanoyl-CoA, 100 μM [1-14C]octanoyl-CoA (specific activity, 56 mCi/μmol), mFabH (1 μg/reaction), eFabF (1 μg), eFabD (1 μg), spFabF (2.5 μg/reaction), eFabF (2 μg/reaction), spFabM (5 μg) in a final volume of 40 μl. The assay mixtures were incubated at 37 °C for 20 min and analyzed by conformationally sensitive gel electrophoresis in 15% polyacrylamide gels containing 2.5 M urea. Electrophoresis was performed at 25 °C and 32 mA/gel. The gels were dried, and the bands were quantitated using a phosphorimager screen. Specific activities were calculated from the slopes of the plot of product formation versus protein concentration in the assay. Bands were identified based on the generation of standards as described previously (20, 21). The substrate specificity of FabM was addressed by determining the relative sizes of the genes. The numbers above the arrows indicate the gene designations in the S. pneumoniae TIGR 4 data base, and the gene names below the arrows indicate the E. coli genes that correspond to the open reading frames in the S. pneumoniae cluster. fabM, 10:1Δ2o-octanoyl-ACP isomerase and the desalted protein was introduced by loop injection. Data were collected for an acetonitrile concentration of 50%. Mass measurements were performed using an LCT electrospray-time of flight spectrometer (Micromass Inc, Beverly, MA) equipped with a Z-spray electrospray interface formed using an LCT electrospray-time of flight spectrometer (Micromass Inc, Beverly, MA) equipped with a Z-spray electrospray interface.

RESULTS

Characteristics of FabM—An analysis of the type II fatty acid biosynthetic genes in S. pneumoniae show that they cluster at a single location within the genome (Fig. 1). A comparison of the predicted protein sequences of these open reading frames to the known enzymes of E. coli showed that the S. pneumoniae gene cluster lacks both FabI and FabA homologs. Recently, the open reading frame termed fabK (Fig. 1) was demonstrated to encode a novel flavoprotein enoyl-ACP reductase that replaces FabI in the S. pneumoniae type II system (27). There are two unknown genes at the end of this cluster of known fatty acid biosynthetic genes. One gene, spO416, is predicted to encode a helix-turn-helix DNA binding protein of the MarR family that may be a transcriptional regulator involved in controlling the expression of this gene cluster. Adjacent to the transcription factor is the spO415 open reading frame that is renamed in this work as fabM.
Further bioinformatics analysis of the gene cluster reveals a potential connection between the fabM, HTH (SP0416), and fabK genes. The MarR transcription factor is a dimer that utilizes a winged-helix motif to bind a DNA palindrome (28, 29). Often bacterial transcription factors are autoregulated, and their DNA binding motifs are located within their own promoter regions. A DNA palindrome was located in the promoter region of the putative SP0416 transcriptional regulator (32). PhaB (PaaB) is an enzyme essential for the catabolism of phenylacetic acid in P. putida and is thought to carry out either the hydroxylation or isomerization of the double bonds once the aromatic ring has been opened (33). This protein is part of a novel 3,2-enoyl-CoA isomerase involved in the biosynthesis of the cyclohexanecarboxylic acid moiety of the polyketide ansatrienin A (34). ChcB has 27% identity and 41% similarity to FabM. FadB is a multifunctional protein involved in fatty acid β-oxidation in E. coli (35), and contains as one of its activities a cis-3-to-trans-2-enoyl-CoA isomerase activity that the data base entry is referring to as an enoyl-CoA hydratase/isomerase. The hydratase/isomerase activity that the data base entry is referring to is associated with the enzymes responsible for either hydrating or isomerizing cis-3 to trans-2-enoyl-CoA intermediates in fatty acid β-oxidation. However, S. pneumoniae lacks cytochromes and does not possess enzymes of the β-oxidation pathway making it highly unlikely that FabM functions in this context (32). PhaB (PaaB) is an enzyme essential for the catabolism of phenylacetic acid in P. putida (36). ChcB is a novel 3,2-enoyl-CoA isomerase involved in the biosynthesis of the cyclohexanecarboxylic acid moiety of the polyketide ansatrienin A (34). ChcB has 27% identity and 41% similarity to FabM. FadB is a multifunctional protein involved in fatty acid β-oxidation in E. coli, and contains as one of its activities a cis-3-trans-2-enoyl-CoA isomerase (35). The section of this protein that aligns with FabM is the component of the complex thought to be responsible for the enoyl-CoA isomerase activity required in the degradation of unsaturated fatty acids and over this segment of the protein has a 30% identity and 46% similarity to FabM. These strong similarities to enzymes known to catalyze isomerizations of enoyl thioesters led us to test the hypothesis that FabM encodes a trans-2 to cis-3-decenoyl-ACP isomerase.

Expression and Purification of FabM—The FabM open reading frame specifies a protein that is a member of the hydratase/isomerase superfamily (Pfam 000378, Ref. 30). A comparison of the predicted protein sequence to three members of this superfamily and to the family consensus sequence is illustrated in Fig. 2. FabM has a strong similarity to the consensus sequence of Pfam 000378 (Fig. 2) exhibiting 28% identity over the 169-amino acid sequence. This family of enzymes catalyze a wide variety of reactions centered on double bond isomerizations and water addition and elimination at the α,β carbons of thioester substrates. The structures of family members show a common active site design that provides for CoA binding, an expandable acyl chain binding pocket, an oxyanion hole for polarizing the thioester carbonyl, and multiple active site stations for the positioning of acidic and basic amino acid side chains to facilitate proton shuffling (31).

Analysis of the purified FabM protein by SDS gel electrophoresis using a 12% polyacrylamide gel is shown in the left inset. His-tagged FabM was expressed and purified as described under “Experimental Procedures” and the purified 31-kDa product visualized by staining with Coomassie Blue. The protein was applied to a Superdex-200 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA and was eluted at a flow rate of 0.5 ml/min. FabM was monitored at 280 nm and eluted at 11.8 ml. The molecular mass was estimated to be 129 kDa by graphic analysis of a standard curve based on the elution volumes of protein molecular mass markers (Sigma) (right inset). Ferritin (440 kDa); catalase (292 kDa); aldolase (158 kDa); bovine serum albumin (66 kDa); ovalbumin (43 kDa); chymotrypsinogen (25 kDa); RNaseA (13.7 kDa) were used to calibrate the column.

Expression and Purification of FabM—The FabM open reading frame was cloned into pET-15b, and the His-tagged fusion protein purified by affinity chromatography and gel filtration as described under “Experimental Procedures.” The purified protein has a monomeric molecular size of 31 kDa (Fig. 3). Members of the hydratase/isomerase protein family are uni-
FabM catalyzes the formation of cis unsaturated acyl-ACP intermediates in vitro. Panel A, a reconstitution assay designed to demonstrate the catalytic properties of FabM, spFabF, and spFabZ within the context of the other enzymes of the type II fatty acid synthase cycle. The assay contained the indicated Fab enzymes and were initiated with octanoyl-CoA and [2-14C]malonyl-CoA. The reaction products were separated by conformationally-sensitive gel electrophoresis as described under “Experimental Procedures.” The appearance of either β-hydroxy-12:1(5c,2t)-ACP or 12:2(5c,2t)-ACP indicated the ability of the system to isomerize 10:1(3Δ2t)-ACP to 10:1(3Δ3t)-ACP, which can be elongated by spFabF. Panel B, the specific activity of FabM using either 10:1(3Δ2t)-ACP or 12:1(2Δ2t)-ACP as the substrate in the reconstitution assay. The FabM isomerase assay was performed using different concentrations of FabM protein and the collection of Fab enzymes and the collection of Fab enzymes to illustrate that spFabF is a dehydratase and that it is not capable of isomerizing the trans-2-enoyl-ACP to the cis-3 intermediate. The addition of FabM to the base reaction did not lead to the formation of enoyl-ACP (Fig. 4A, lane 6), and in combination with spFabF did not lead to the appearance of any additional products (lane 7). Thus, FabM lacked β-hydroxyacyl-ACP dehydratase activity. The combination of spFabZ and FabM led to the formation of enoyl-ACP (lane 8), but it was not possible to discern if the cis intermediate was formed in this reaction mixture. In the presence of spFabZ, spFabF, and FabM, a new product appeared indicating that cycles of elongation occurred (Fig. 4A, lane 9). This product(s) arises from the elongation of the cis-3 intermediate by spFabF and the dehydration by spFabZ. In contrast to FabA and like ecFabZ (20), spFabZ was capable of utilizing unsaturated β-hydroxy intermediates to form enoyl-ACPs. These experiments with the reconstituted fatty acid synthase enzymes establish that FabM is capable of isomerizing trans-2-enoyl-ACP to cis-3-acyl-ACP, but cannot dehydrate β-hydroxyacyl-ACP. In addition, spFabF is capable of elongating cis-3-acyl-ACP intermediates.

The reconstituted fatty acid synthase assay is a crude tool for detailed biochemical characterization of an individual enzyme specificity, but we employed this assay to evaluate the substrate specificity of FabM isomerase (Fig. 4, panel B). The mtFabH/FabG/spFabZ system was used to present FabM with different chain length enoyl-ACP substrates. The formation of an elongation product arising from FabM isomerase activity as a function of FabM protein in the assay was used to estimate the activity of FabM. FabM was most active when the 10-carbon enoyl-ACP was presented as the substrate exhibiting a specific activity under these defined conditions of 1.6 ± 0.09 pmol/min/μg. The 12-carbon enoyl-ACP was also utilized in vitro, albeit at a much lower rate (0.4 ± 0.08 pmol/min/μg). We examined 14- and 16-carbon enoyl-ACPs as substrate; however, there was no evidence for isomerization of these longer substrates (not shown). The low activity of mtFabH with hexanoyl-CoA (18) did not permit the analysis of FabM activity on trans-2-octenoyl-ACP using this assay. The specific activity of ecFabA for the 10-carbon substrate under these same assay conditions was 33 ± 1 pmol/min/μg. Thus, FabM was 20-fold less efficient than ecFabA in the formation of cis-double bonds in the in vitro fatty acid synthase assay reconstituted with the indicated constellation of enzymes and the E. coli ACP cofactor. These data are consistent with FabM, like ecFabA (20, 37), being most active on 10-carbon enoyl-ACP, but capable of isomerizing longer chain substrates at a lower rate.

Mass Spectrometry Analysis of FabM Products—The ACP thioester intermediates in the reconstitution assays (Fig. 4A) were analyzed by electrospray ionization mass spectrometry (ESI-MS) to confirm the identities of the products (Fig. 5). The

densizing enzyme, but the cis-3 intermediate can. Accordingly, the addition of spFabF, the elongation condensing enzyme of S. pneumoniae, condenses the cis-3-decenoyl-ACP with malonyl-ACP, and following reduction by ecFabG, gives rise to the accumulation of a new band on the gel corresponding to β-hydroxy-cis-5-dodecanoyl-ACP (Fig. 4A, lane 3). Since this reaction mixture did not contain an enoyl-ACP reductase, additional rounds of elongation cannot occur, and the product accumulates at the 12-carbon stage. Furthermore, ecFabA is characteristically inactive with unsaturated β-hydroxy intermediates (20), so there is little conversion of the β-hydroxy-cis-5-dodecanoyl-ACP to the trans-2 intermediate. The addition of spFabZ also converts the β-hydroxydodecanoyl-ACP to the enoyl-ACP (Fig. 4A, lane 4); however, the addition of spFabF to this reaction did not lead to the appearance of the elongated 12-carbon unsaturated intermediate (Fig. 4, lane 5). These data illustrate that spFabZ is a dehydratase and that it is not possible of isomerizing the trans-2-enoyl-ACP to the cis-3 intermediate. The addition of FabM to the base reaction did not lead to the formation of enoyl-ACP (Fig. 4A, lane 6), and in combination with spFabF did not lead to the appearance of any additional products (lane 7). Thus, FabM lacked β-hydroxyacyl-ACP dehydratase activity. The combination of spFabZ and FabM led to the formation of enoyl-ACP (lane 8), but it was not possible to discern if the cis intermediate was formed in this reaction mixture. In the presence of spFabZ, spFabF, and FabM, a new product appeared indicating that cycles of elongation occurred (Fig. 4A, lane 9). This product(s) arises from the elongation of the cis-3 intermediate by spFabF and the dehydration by spFabZ. In contrast to FabA and like ecFabZ (20), spFabZ was capable of utilizing unsaturated β-hydroxy intermediates to form enoyl-ACPs. These experiments with the reconstituted fatty acid synthase enzymes establish that FabM is capable of isomerizing trans-2-enoyl-ACP to cis-3-acyl-ACP, but cannot dehydrate β-hydroxyacyl-ACP. In addition, spFabF is capable of elongating cis-3-acyl-ACP intermediates.

Enzymatic Activity of FabM—The ability of FabM to act as an isomerase was tested in a reconstituted fatty acid biosynthetic system designed to detect isomerase activity (Fig. 4). Cycles of fatty acid elongation were reconstituted in vitro using purified enzymes as described under “Experimental Procedures.” The assay employed the FabH enzyme from M. tuberculosis to generate β-keto[14C]decanoyl-ACP starting with octanoyl-CoA and [2-14C]malonyl-ACP (via ecFabD) as substrates. The NADPH-dependent ecFabG reduced the intermediate to the initial substrate for the assays, β-hydroxy[14C]decanoyl-ACP (Fig. 4A, lane 1). The addition of ecFabA (lane 2) results in the conversion of the β-hydroxy intermediate to a mixture of trans-2- and cis-3-decenoyl-ACPs. These isomeric forms are not distinguished on the gel, but previous work suggests that the trans intermediate would predominate (36). The enoyl-ACP cannot be elongated by a conditionally multimeric proteins with hexamers of identical subunits being a common configuration, although some members are dimers and tetramers. We therefore examined the size of native FabM by gel filtration chromatography to estimate its aggregation state (Fig. 3). These results are consistent with FabM existing as a tetramer of identical subunits in solution.
FIG. 5. Electrospray ionization-mass spectrometry of precursor and products of the FabM-catalyzed reaction. Panel A, mass spectrum of the reaction mixture in lane 1 showing the β-hydroxydecanoyl-ACP substrate. Panel B, mass spectrum of the products of the isomerization of 2-trans-decenoyl-ACP by FabM to 3-cis-decenoyl-ACP (C10:1, 3Δc-ACP) (9001), 3Δc,3Δc-2t-dodecadienoyl-ACP (C12:2, Δ5c,3Δc-2t-ACP) (9027), and to 7-cis,5-cis,2-trans(3-cis)-tetradecatrienoyl-ACP (C14:3, Δ7c,5c,2t/3c-ACP) (9053). In both spectra, the M+131 ions represent the molecular ions of ACP molecules that retained the aminoterminal methionine residue.

major mass peak for the ACP starting material occurred at 8849 with a minor peak at mass 8980 corresponding to the ACP molecules that retained the aminoterminal methionine residue. A new mass peak of 9019 with the expected mass increase of 170 appeared in the β-hydroxydecanoyl-ACP starting material (lane 1). The trans-2-and cis-3-decenoyl-ACP mixture (lane 2) formed after dehydration by ecFabA led to the appearance of a new peak at mass 9001 (ACP+152). The elongated product of cis-3-decenoyl-ACP by spFabF was predicted to be the β-hydroxy-cis-5-dodecenoyl-ACP (lane 3), and accordingly the mass spectrum of the mixture contained a new peak at 9044 corresponding to ACP+196. Samples from the reactions in lanes 4 and 5 contained trans-2-decenoyl-ACP and displayed the expected mass peak at 9001. The β-hydroxydecanoyl-ACP precursor (lanes 6 and 7) was not converted to other products by FabM, and the predominant acyl-ACP peak in these reactions mixtures was 9019, diagnostic for β-hydroxydecanoyl-ACP (Fig. 5A). Lane 8, trans-2- and/or cis-3-decenoyl-ACPs were revealed by the appearance of a characteristic mass peak at 9001. The FabM-catalyzed isomerization of the trans-2 to cis-3-decenoyl-ACP was revealed by the appearance of acyl-ACP products elongated by spFabF (lane 9). Two products were detected by the appearance of a peak for cis-5, trans-2-C12:2-ACP (ACP+178) as well as cis-7, cis-5, trans-2- C14:3-ACP (ACP+204) with predicted and observed molecular masses of 9027 and 9053, respectively (Fig. 5B). These data confirm the identities of the labeled intermediates indicated in Fig. 4A and substantiate the conclusion that FabM was capable of isomerizing both C10 and C12 enoyl-ACPs in vitro.

Direct Demonstration of FabM Isomerase Activity—We employed a direct assay for isomerase activity using substrate analogs to complement reconstituted fatty acid synthase systems. Although the enzymes of E. coli fatty acid synthesis are highly specific for ACP thioester substrates, in all cases these enzymes utilize substrate analogs (either CoA or NAC thioesters) presented in high concentrations in biochemical assays (38, 39). Thus, we developed an assay employing the substrate analog trans-2-octenoyl-NAC that was modeled on the spectrophotometric assay using NAC substrate analogs to detect the isomerization activity of ecFabA (40). Enoyl-thioesters absorb at 283 nm (24), and this absorption is lost upon conversion to the cis-3-thioester. FabM was capable of isomerizing trans-2-octenoyl-NAC (Fig. 6). FabM activity was dependent on time and protein concentration (Fig. 6, panel A) and increased with increasing substrate concentration (Fig. 6, panel B). FabM activity was linear for the first minute, and although relatively high concentrations of the substrate analog were employed, it was clear from panel B that the experiments were performed at octenoyl-NAC concentrations that were below saturation. The specific activity calculated using 200 μM trans-2-octenoyl-NAC was 87 ± 2.8 pmol/min/μg. The importance of these studies with a substrate analog was that they provided a direct demonstration that FabM alone is capable of catalyzing the isomerization of enoyl-thioesters.

Activity of FabM in E. coli—We cloned fabM, spfabZ, and ecfabA into pBluescript vectors and used these constructs to test whether the gene could complement the temperature-sensitive growth phenotype of strain JT60 (fabA(Ts)). As expected, the fabA construct permitted growth at 42 °C; however, the constructs engineered to express fabM or spfabZ did not. All strains grew at 42 °C on plates supplemented with oleate. We also cloned fabM into a plasmid that places it under arabinose regulation. However, complementation was not observed at any concentration of arabinose tested (data not shown). These data illustrate that fabM isomerase activity alone cannot substitute for fabA within the context of the E. coli fatty acid synthase system.

We attributed this result to the differences in the overall organization of the UFA biosynthetic pathway in E. coli and S. pneumoniae (Fig. 7). The idea was that the complementation experiments described above failed because of the inability of FabM to successfully compete with FabI for the available enoyl-ACP within the context of the E. coli fatty acid synthase system. In S. pneumoniae, FabI is absent and FabM competes with FabK. We hypothesized that FabM would be capable of complementing fabA(Ts) mutants if the cells expressed FabK, and the endogenous FabI activity was eliminated. This was accomplished by introducing FabM and FabK expression using plasmids and ablating FabI activity with the potent inhibitor triclosan. FabI is exquisitely sensitive to triclosan (38, 41), whereas FabK is not affected by this drug (27). If this hypothesis was correct, then fabM would only be able to complement the fabA(Ts) temperature-sensitive growth defect in the presence of fabK and triclosan.

The results from this complementation experiment are summarized in Table I. At 42 °C in the absence of triclosan, only strains expressing the fabA gene were able to grow. In the

2 Fatty acid abbreviations: 10:1(AΔ2t), number of carbon atoms: number of double bonds. A is double bond, number is the location of the double bond, and c or t is the cis or trans configuration.
purified FabM. Reactions were started by the addition of 10 or 40 pmol/min/μg.

**DISCUSSION**

The study of the mechanism of oxygen-independent double bond formation was brought to the forefront by Konrad Bloch's research group (42) whose interest in the anaerobic bacteria in the clostridium family was piqued by the presence of unsaturated fatty acid synthesis in vivo.

**FIG. 6.** Direct detection of FabM isomerase activity. Incubation mixtures contained trans-2-octenyl-NAC (C8:1-NAC) in 150 μl of 10 mM potassium phosphate, pH 7.0. Reactions were started by the addition of the indicated amounts of FabM and followed at 263 nm for 3 min. **Panel A**, time course of the isomerization of trans-2-octenyl-NAC by purified FabM, and the conversion of trans-2-octenyl-NAC (100 μM) to cis-3-octenyl-NAC was monitored by the decrease in absorbance at 263 nm. The controls without substrate and without enzyme showed no significant change in absorbance. The curves have been adjusted to the same zero time absorbance in the figure. **Panel B**, initial rate of conversion of trans-2-octenyl-NAC by FabM. Reactions containing trans-2-octenyl-NAC (100 or 200 μM) were started by the addition of increasing amounts of FabM. The FabM specific activity calculated was 87 ± 2.8 pmol/min/μg.

The presence of triclosan at 42°C, the only strains that grew were those harboring the pFabK plasmid in conjunction with either the pFabA or pFabM plasmids. Thus, FabM was capable of complementing fabA(Ts) mutants only when FabI activity was abolished and in the presence of FabK. These data corroborate the conclusion that FabM functions as an isomerase in type II fatty acid synthase in vivo.

**FIG. 7.** Comparison of the fatty acid biosynthetic pathway in *E. coli* and *S. pneumoniae*. Left panel, the branch point in saturated/unsaturated fatty acid synthesis in *E. coli* occurs at the dehydratase step. This bacterium contains two dehydratases: FabZ, which functions on all chain-lengths (20, 47), and FabA, which not only dehydrates the β-hydroxy intermediates, but also isomerizes the double bond to produce a mixture of C10:1Δ2t-ACP and C10:1Δ3c-ACP (6, 7, 48). SFA biosynthesis proceeds by the action of FabM on the trans-2 intermediate followed by further elongation cycles initiated by either FabB or FabF condensing enzymes. C10:1Δ3c-ACP is the least abundant product of FabA at equilibrium (49), thus UFA requires FabB to efficiently utilize the cis-3 intermediate and initiate the elongation cycles that form the major long-chain unsaturated fatty acids. Accordingly, the FabB transcription factor controls the cellular content of UFA primarily by altering the expression of fabF (44). Right Panel, in *S. pneumoniae* there is only a single dehydratase, FabZ, that forms the C10:1Δ2t-ACP intermediate. SFA are formed by the action of the FabK enoyl-ACP reductase followed by further elongation cycles initiated by FabF. UFA arise from the isomerization of the trans-2 intermediate by FabM followed by further elongation cycles initiated by FabF. The branch point in *E. coli* is based on the competition of the two dehydratases for the β-hydroxyacyl-ACP intermediate, and the activity of FabB, which pulls the FabA product down the UFA branch of the pathway. In *S. pneumoniae* the branch point is at the enoyl-ACP level and the proportion of products is determined by competition between enoyl-ACP reductase II, FabK, and FabM, the trans-2, cis-3-decenoyl-ACP isomerase.

*E. coli* contains neither FabA nor FabB, although they have an fatty acid synthase in these organisms. Bloch pursued his investigation of the unsaturated fatty acid pathway in *E. coli* culminating in the discovery of FabA, and in the 40 years since this discovery, FabA in combination with FabB, became the paradigm for anaerobic olefin formation (see Introduction). The advent of genome sequencing and bioinformatics genomic analysis reveals the surprising result that several groups of bacteria contain neither FabA nor FabB, although they have an anaerobic metabolism and synthesize unsaturated fatty acids. Specifically, *S. pneumoniae* possess only the FabZ dehydratase, and lacks both FabA and FabB enzymes (Fig. 1). Thus, this bacterium must employ a different mechanism for the formation of unsaturated fatty acids. The first possibility was that the FabZ-related protein expressed in this organism (Fig. 1) not only dehydrates β-hydroxyacyl-ACP, but like FabA, also isomerizes enoyl-ACP to 3-cis-acyl-ACP. However, spFabZ clearly is only capable of catalyzing the dehydration reaction of the type II synthase (Fig. 4). Thus, a previously undiscovered enzyme must be present to carry out an isomerization reaction and genomic analysis reveals a candidate gene associated with the fatty acid biosynthetic gene cluster termed FabM (Fig. 1). Indeed, reconstitution of fatty acid synthesis *in vitro* with FabM in combination with spFabZ and spFabF resulted in the
A FabK in this context, the finding of similar transcription factor binding sites within the promoters of the fabM and fabK gene in S. pneumoniae (Fig. 1) suggests that these two partners in UFA biosynthesis are coordinately regulated, reminiscent of the coordinate regulation of fabA and fabB in E. coli (15, 44, 45).

FabM homologs are only found in the Streptococcus species and thus the discovery of FabM does not explain how all anaerobic bacteria synthesize UFA. Other genera, like the Clos- tridia, contain UFA, but do not possess either a FabA or a FabM homolog in their genomes. Thus, it is likely that organisms in this group utilize another mechanism to introduce a double bond into the growing acyl chain of type II fatty acid synthase.

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FabM trans-2, cis-3-Decenoyl-ACP Isomerase

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