Characterization of *Streptococcus pneumoniae* enoyl-(acyl-carrier protein) reductase (FabK)

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The enoyl-(acyl-carrier protein) (ACP) reductase catalyses the last step in each cycle of fatty acid elongation in the type II fatty acid synthase systems. An extensively characterized NADH-dependent reductase, FabI, is widely distributed in bacteria and plants, whereas the enoyl-ACP reductase, FabK, is a distinctly different member of this enzyme group discovered in *Streptococcus pneumoniae*. We were unable to delete the *fabK* gene from *Strep. pneumoniae*, suggesting that this is the only enoyl-ACP reductase in this organism. The FabK enzyme was purified and the biochemical properties of the reductase were examined. The visible absorption spectrum of the purified protein indicated the presence of a flavin cofactor that was identified as FMN by MS, and was present in a 1:1 molar ratio with protein. FabK specifically required NADH and the protein activity was stimulated by ammonium ions. FabK also exhibited NADH oxidase

activity in the absence of substrate. *Strep. pneumoniae* belongs to the *Bacillus/Lactobacillus/Streptococcus* group that includes *Staphylococcus aureus* and *Bacillus subtilis*. These two organisms also contain FabK-related genes, suggesting that they may also express a FabK-like enoyl-ACP reductase. However, the genes did not complement a *fabI*(Ts) mutant and the purified flavoproteins were unable to reduce enoyl-ACP *in vitro* and did not exhibit NAD(P)H oxidase activity, indicating they were not enoyl-ACP reductases. The restricted occurrence of the FabK enoyl-ACP reductase may be related to the role of substrate-independent NADH oxidation in oxygen-dependent anaerobic energy metabolism.

Key words: FabI, fatty acid biosynthesis, flavoprotein.

INTRODUCTION

Fatty acid synthesis in bacteria is carried out by a set of individual enzymes that are collectively known as the type II, or dissociated, fatty acid synthase (see for reviews [1,2]). The *trans*-2-enoyl-(acyl-carrier protein) (ACP) reductase component of the type II system catalyses the last step in each elongation cycle and plays a key role in the regulation of the pathway [3,4]. In *Escherichia coli*, there is a single NADH-dependent isoform of this enzyme, FabI, which is essential for cell survival [3,5,6]. FabI is a distant member of an extended superfamily of proteins, termed the short-chain alcohol dehydrogenases/reductases (SDRs) [7]. FabI is atypical in that the key residues are a diad consisting of a Tyr-(Xaa)₆-Lys motif [7,8] in contrast with the Tyr-(Xaa)₃-Lys in prototypical SDRs [7]. Genes encoding FabI homologues are readily identified in the available bacterial and plant genomic databases based on their high degree of overall identity and the conserved spacing of the Tyr-Lys diad. Until recently, this widely distributed FabI structure was thought to be the only type of enzyme catalysing enoyl reduction in the type II fatty acid synthase.

Investigation of the molecular basis for the different sensitivities of Gram-positive and Gram-negative bacteria to triclosan led to the identification of two additional enoyl-ACP reductase

enzymes. Triclosan is a broad-spectrum anti-bacterial agent that targets the FabI protein by forming a tight, non-covalent ternary complex with the oxidized cofactor in the active site [9–14]. *Staphylococcus aureus* possesses a minimum inhibitory concentration (MIC) for triclosan of less than 0.06 µg/ml, whereas other Gram-positive bacteria such as *Bacillus subtilis* and *Streptococcus pneumoniae* have MICs of 2 µg/ml [15–17,26]. *B. subtilis* has a *fabI* gene, but also expresses a second enoyl-ACP reductase encoded by the *fabL* gene [17]. FabL is only distantly related to FabI, but does have the Tyr-(Xaa)₆-Lys active-site motif and is a member of the SDR superfamily. FabL does not form a high-affinity complex with triclosan and its expression accounts for the higher MIC exhibited by *B. subtilis* for this drug. *Strep. pneumoniae* is unusual in that it does not contain a *fabI* gene, but rather the enoyl-ACP reductase step is encoded by a novel gene termed *fabK* that encodes a protein with no sequence similarity to FabI or the SDR superfamily [18]. FabK is a flavoprotein that requires NADH for activity and is completely refractory to triclosan inhibition [18]. Other bacteria, such as *Enterococcus faecalis* and *Clostridium acetobutylicum*, also possess genes with a high degree of identity with FabK (68 and 58% respectively), and are thus strongly predicted to encode FabKs. In the present study, we report on the biochemical properties of *Strep. pneumoniae* FabK and analyse two of the related FabK-like proteins

Abbreviations used: ACP, acyl-carrier protein; DTT, dithiothreitol; MIC, minimum inhibitory concentration; SDR, short-chain alcohol dehydrogenase/reductase.

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from *Staph. aureus* and *B. subtilis* (37 and 34% identity respectively) to ascertain whether these more distantly related FabK-like proteins function as enoyl-ACP reductases.

EXPERIMENTAL

Materials

The chemical reagents, nucleotide cofactors, CoA thioesters and crude ACP were purchased from Sigma (St. Louis, MO, U.S.A.), molecular biology reagents from Gibco BRL (Rockville, MD, U.S.A.), DNA preparation kits and Ni^{2+} -agarose resin from Qiagen, [^{14}C]malonyl-CoA (specific radioactivity 56 mCi/mmol) was from Pharmacia-Amersham, triclosan from KIC Chemicals (Aramonk, NJ, U.S.A.), pBluescript from Stratagene (La Jolla, CA, U.S.A.), and pET vectors and expression strains were from Novagen (Madison, WI, U.S.A.). The myristoyl-ACP was prepared by the acyl-ACP synthetase method [19,20]. Purified His-tagged FabD and FabB proteins were obtained as described previously [3,21]. Proteins were stored in 50% (w/v) glycerol at -20°C . Protein concentration was determined using the Bradford method with γ -globulin as the standard [22] and by amino acid analysis. Column chromatography was performed on an AKTA system (Pharmacia-Amersham) with Pharmacia-Amersham Hi-load columns. SDS/PAGE analyses were performed on 4–12% Bis-Tris gels (Novex, San Diego, CA, U.S.A.). The N-terminal amino acid sequence of the purified enzyme was deduced by automated Edman degradation with a Hewlett Packard model G1000A sequencer.

Essentiality testing of the *fabK* gene

Essentiality testing was performed as described by Throup et al. [23]. Chromosomal DNA fragments flanking the *fabK* gene were amplified by PCR from chromosomal DNA of *Strep. pneumoniae* 0100993 and purified. These were then used to make a cassette by overlap PCR in which they flanked an erythromycin resistance gene, *ermAM*. Expression of the *ermAM* gene was driven from its native promoter and all transcriptional terminator sequences were removed to facilitate the expression of genes downstream of the insertion site. Attempts were made to introduce the allelic replacement cassette into *Strep. pneumoniae* R6 by transformation. Competent cells were prepared according to published methods [24]. DNA was introduced into the cells by incubation of 500 ng of allelic replacement cassette with 10^6 cells at 30°C for 30 min. The cells were transferred to 37°C for 90 min to allow expression of the erythromycin resistance gene [24]. Cells were plated in rich agar medium containing $1\ \mu\text{g}/\text{ml}$ of erythromycin. In control experiments, the non-essential *malC* gene was deleted by allelic exchange. Transformation efficiencies were 10^2 – 10^3 transformants/ μg of control DNA.

Expression of FabK

Strep. pneumoniae fabK was amplified by PCR from strain 0100993 using the forward/reverse primer pairs 5'-AGGTTGG-AGGCCATATGAAACGCGTATT-3' and 5'-GGCGGATC-CTTAGTCATTTCTTACAACCTC-3' respectively. An *NdeI* site was integrated into the forward primer and a *BamHI* site into the reverse primer for cloning into pET-24b(+). The PCR product was digested with the restriction endonucleases *NdeI* and *BamHI* and then ligated into pET-24b(+) (also digested with *NdeI* and *BamHI*). The resulting plasmid was transformed into subcloning efficiency DH5 α cells. The sequence of the pET24bSpfabK expression construct was confirmed by DNA sequencing and the plasmid was transformed into electrocompetent BL21 (DE3) cells harbouring the tRNA vector pRR692. Alternately, the

FabK protein was expressed as a His $_6$ -tag fusion protein as described previously [18].

Purification

Cells (1 litre) containing the native FabK expression construct were grown to an absorbance A_{600} 0.6. Expression was induced with 0.1 mM isopropyl β -D-thiogalactoside and the cells were grown for a further 3 h and then harvested. The cell pellet was resuspended in 10 ml of 50 mM Tris/HCl (pH 7.5), 1 mM PMSF, 1 mM benzamidine and 1 mM dithiothreitol (DTT) (buffer A) and lysed by sonication. Cell debris was removed by centrifugation. The supernatant was loaded on to a Hi-load Q (16/10) column equilibrated in buffer A. Protein was eluted over a 200 ml gradient of 0–100% buffer B, where buffer B is buffer A containing 1 M KCl. Fractions containing FabK were identified by their A_{460} values and by their FabK activity and pooled. Ammonium sulphate was added to the pooled fractions (final concentration, 1.5 M), and these were then loaded on to a Hi-load phenyl-Sepharose (16/10) column equilibrated in 50 mM Tris/HCl (pH 7.5), 1 mM PMSF, 1 mM benzamidine, 1 mM DTT and 1.5 M ammonium sulphate. Proteins were eluted with a gradient of ammonium sulphate (1.5–5 M) over 200 ml. Fractions containing FabK were identified as described above and pooled. The pooled fractions were buffer-exchanged into 100 mM Tris/HCl (pH 7.5), 2 mM DTT, and glycerol was then added to 50%. The protein was stored in this buffer at -20°C . Activity of the enzyme was monitored throughout purification. Alternatively, the His-tagged FabK was purified by affinity chromatography [21]. The identity of the FabK protein was confirmed by N-terminal sequencing and matrix-assisted laser-desorption ionization-MS. Final protein concentration was determined by amino acid analysis.

Identification of cofactor and determination of stoichiometry

UV-visible spectral characterization was performed with a Hewlett Packard 8453 spectrophotometer. The absorbance spectrum of FabK was obtained at a concentration of $30\ \mu\text{M}$ in 25 mM Tris/HCl (pH 7.5), 50 mM NH_4Cl , 1 mM DTT and 50% (w/v) glycerol. A $30\ \mu\text{M}$ sample of FMN in the same buffer was analysed as a reference. Fluorescence excitation and emission spectra were collected on $200\ \mu\text{l}$ samples containing $65\ \mu\text{M}$ FabK or FMN in buffer containing 25 mM Tris/HCl (pH 7.5), 50 mM NH_4Cl , 1 mM DTT and 50% (w/v) glycerol using a Fluorolog-3 (Jobin Yvon, Edison, NJ, U.S.A.) fluorimeter and masked microcuvettes having a 3 mm path length in each direction. For the excitation spectra, scans ranged from 300 to 525 nm, entrance and exit slits were set to 2 and 1 mm respectively, and the emission monochromator was set to 550 nm. The emission spectra were collected from 450 to 700 nm with the entrance and exit slits both set to 1 mm and the excitation monochromator set to 425 nm.

The cofactor was removed from FabK by heating a $65\ \mu\text{M}$ sample for 10 min at 100°C followed by centrifugation at $16000\ g$ for 10 min. The released cofactor was subjected to liquid chromatography-MS as described below. A $10\ \mu\text{l}$ sample was injected on to a Baker Wide Pore Octadecyl (C_{18} , $4.6\ \text{mm} \times 250\ \text{mm}$) column connected to a Shimadzu HPLC system (Columbia, MD, U.S.A.) with LC-10ADvp pumps, an SCL-10Avp controller and a dual-wavelength UV-visible detector SPD-10Avp. The mobile phase consisted of solvent A (25 mM aq. ammonium bicarbonate) and solvent B (90% aq. CH_3CN) at a flow rate of 0.5 ml/min. The cofactor was eluted using a linear gradient of 0–50% B spanning 25 min (2%/min). The flavin was detected by monitoring the effluent at 460 nm, and a portion, approx. $10\ \mu\text{l}/\text{min}$ after the

column, was diverted to a Micromass (Wythenshaw, Manchester, U.K.) LCT-MS equipped with an electrospray ionization source. Using negative-ion detection, the deprotonated molecular ion ($M-H$)⁻ of the flavin was recorded for molecular-mass confirmation. The electrospray needle was operated at 3.0 kV. Nitrogen was used as the nebulizing and desolvation gas. Using a continuum acquisition mode, time-of-flight data were acquired from 400 to 900 Da with a 1.0 s cycle time and a data accumulation of 0.9 s/spectrum.

The stoichiometry of the FMN was estimated from the absorbance of the released cofactor after treatment of FabK with guanidinium chloride as follows: a 26 mg/ml (760 μ M as determined by amino acid analysis) sample of FabK was diluted 1:10 with 25 mM Tris/HCl (pH 7.5), 50 mM NH₄Cl, 1 mM DTT, 50% (w/v) glycerol and mixed with an equal volume of 7.5 M guanidinium chloride in 25 mM Tris/HCl (pH 7.5). The absorbance of the sample was then determined at 448 nm using a Hewlett Packard 8453 diode array spectrophotometer and compared with a standard curve (0–25 nmol) prepared with identically treated commercial FMN.

Preparation of crotonoyl-ACP

Crotonoyl-ACP was synthesized using *Strep. pneumoniae* ACP synthase to catalyse the addition of a crotonoyl group from crotonoyl-CoA to *E. coli* apo-ACP. To a reaction vessel containing 500 mg (58 μ mol) of *E. coli* apo-ACP in 20 mM Bis-Tris (pH 6.8) and 5 mM MgCl₂, 76 mg (81 μ mol) of crotonoyl-CoA and 5 mg of *Strep. pneumoniae* ACP synthase were added. The final volume and pH were adjusted to 100 ml and 6.8 respectively. The pH of the reaction was maintained at 6.8 with NaOH and monitored for completion by MS. Conversion was complete within 150 min with no detectable by-products. The reaction mixture was loaded at 10 ml/min on to a Q-Sepharose FF column (5 cm $\times$ 16 cm) pre-equilibrated with 20 mM Bis-Tris (pH 6.8). Crotonoyl-ACP was eluted over 2200 ml using a 0.2–0.6 M NaCl gradient at a flow rate of 20 ml/min. Fractions were monitored by MS for identity and purity. The appropriate fractions were pooled and concentrated using a YM-3 ultra-filtration membrane (Millipore).

Assay procedures

FabK catalyses the reduction of enoyl-ACPs with the concomitant oxidation of NADH. Crotonoyl-ACP can be prepared as described above. The reduction of crotonoyl-ACP to butyryl-ACP can be monitored by following the change in absorbance at 340 nm as NADH is oxidized. Standard assays performed during purification and for studying activation contained 50 μ M NADH and 25 μ M crotonoyl-ACP. Assays were performed in Costar 3696 half-area plates in a final assay volume of 150 μ l on a Spectramax platereader. NADH and crotonoyl-ACP were incubated with FabK enzyme in 100 mM *N*-(2-acetamido)-2-iminodiacetic acid (pH 6.5), 100 mM NH₄Cl and 4% (w/v) glycerol at 30 °C, and the reaction monitored at 340 nm. For kinetics measurements, 1.5 nM FabK was used in 100 mM 2-morpholinoethanesulphonic acid, 51 mM diethanolamine, 51 mM triethanolamine, 5% (w/v) glycerol (pH 7.5) and 100 mM NH₄Cl. Concentrations of substrates were varied from 5 to 160 μ M for crotonoyl-ACP, with NADH being held at 50 μ M and varied from 10 to 200 μ M for NADH, with crotonoyl-ACP held at 25 μ M.

The coupled assay for reduction of short-chain enoyl-ACPs (*trans*-2-butenoyl-ACP) consisted of a reaction mixture (40 μ l) containing 100 μ M ACP, 1 mM 2-mercaptoethanol, 100 μ M acetyl-CoA, 50 μ M [2-¹⁴C]malonyl-CoA (56 mCi/mmol), 1 mM

NADH, 1 mM NADPH and 25 μ g/ml each of FabD, FabH, FabG and FabZ in 0.1 M sodium phosphate (pH 7.0). The mixture was incubated at 37 °C for 20 min to generate the *trans*-2-enoyl-ACP substrate before being aliquoted into individual reaction tubes to which FabK proteins were added to the final amount of 0.5 μ g. The same assay conditions were used for reduction of *trans*-2-hexadecenoyl-ACP, except that 50 μ M myristoyl-ACP replaced the acetyl-CoA and 25 μ g/ml each of FabD, FabB, FabG, and 12.5 μ g/ml of FabA and FabZ were used. Reactions were then incubated for 20 min at 37 °C and were stopped by placing into an ice slurry. Gel-loading buffer was added, and the entire sample loaded on to a 13% (for short-chain) or 15% (for long-chain) polyacrylamide gel containing 0.5 M (for short-chain) or 2.5 M (for long-chain) urea [4].

NADH oxidase activity of *Strep. pneumoniae* FabK

Spectrophotometric assays were performed using 20 μ g of protein in 100 mM sodium phosphate buffer (pH 7.5) in a total volume of 300 μ l. The enzyme was incubated with buffer and the reaction started by adding 200 μ M NADH. The oxidation of NADH was followed at 340 nm on a Shimadzu UV-visible spectrophotometer UV-1601.

Cloning of *B. subtilis* and *Staph. aureus* *fabK*-like genes

The predicted open reading frame (*yrpB*; accession no. Z99117) for the *B. subtilis* *fabK*-like gene was amplified from genomic DNA using primers bs-nde (5'-CATATGAATGAATTTATG-AAAAAGTT) and bs-bam (5'-GGATCCTTGCTCTATCAAT-GAGGTGC), and then subcloned into the *Nde*I and *Bam*HI sites of the plasmid pET-15b (Novagen) to form pET-15bYrpB for inducible expression of the protein with an N-terminal His₆ tag. The predicted *Staph. aureus* *fabK*-like open reading frame (BAB42020; accession no. AP003131) was amplified from genomic DNA using the primers sa-xho (5'-CTCGAGTGGAATAA-GAATCGACTTACTCA) and sa-bam (5'-GGATCCTGTGG-ATTGCGGTCGATTATTTA) and subcloned into the *Xho*I and *Bam*HI sites of the plasmid pET-15b to form pET-15bBAB42020. The genes were sequenced to verify that the expected sequence had been obtained. Plasmids pET-15bYrpB and pET-15bBAB42020 were digested with *Xba*I and *Bam*HI and the open reading frames subcloned into pBluescript KSII(+) (Stratagene) digested with the same enzymes to create *pyrpB* and *pBAB42020* respectively to express constitutively the His-tagged FabK-like proteins. *E. coli* strain RJH13 [*fabI*(Ts)] was transformed with pBluescript (empty vector), *pfabI* [9], *pfabK* (*Strep. pneumoniae*), *pfabL* [17], or *pyrpB* (*B. subtilis*) and *pBAB42020* (*Staph. aureus*). Cells were grown at the permissive temperature for the host strain (30 °C), and then individual colonies spotted on to Luria-Bertani agar plates and incubated at 42 °C.

RESULTS

Essentiality of *fabK*

We attempted to inactivate the *fabK* gene from *Strep. pneumoniae* by allelic replacement. An allelic replacement cassette was constructed, consisting of the regions upstream and downstream of the *fabK* gene flanking the erythromycin resistance gene. This cassette was used to transform *Strep. pneumoniae* R6, but no transformants were obtained in multiple-transformation experiments. These results suggest that the *fabK* gene is essential for *Strep. pneumoniae* and, thus, is probably the only enoyl-ACP reductase activity in this organism.

Figure 1 Excitation and emission spectra of 64.4 μ M FMN (---) and FabK (—)

Spectra were recorded as described in the Experimental section. The spectral broadening and shift in λ_{max} of the excitation spectrum for FabK relative to free FMN (A) are characteristic of a protein-bound flavin. The fluorescence of the bound flavin (B) is highly quenched relative to free FMN.

Identification of the *Strep. pneumoniae* FabK cofactor

The UV-visible spectrum of the protein was characteristic of flavoproteins, showing absorbance maxima at 361 and 455 nm. The normalized fluorescence excitation spectrum of FabK showed a peak distribution similar to that observed in the absorbance spectrum (Figure 1A). The emission spectrum of FabK was shifted only slightly relative to the flavin (λ_{max} at 523 and 526 nm for FabK and the flavin respectively), and the flavin was quenched by at least 90% on binding to the protein (Figure 1B), as is often the case for flavoproteins. The significance of the small spectral shift is not known, since it is not clear whether the residual fluorescence of the flavin was the result of incomplete quenching by the protein or whether it represented a small amount of free (unbound) flavin.

The cofactor was released by heat denaturation of the protein and analysed by HPLC coupled with MS (Figure 2). The cofactor had the same retention time and mass-spectrometric characteristics as the FMN standard. The ratio of FMN/FabK was 0.97 ± 0.01 , calculated based on the A_{455} value of the FMN released after treatment of a known mass of FabK with 3.75 M guanidinium chloride compared with a standard sample curve of commercial FMN. The MS analyses of the flavin establish that

Figure 2 Identification of the cofactor in FabK by liquid chromatography/MS

A 2.2 mg/ml (64.4 μ M) sample of FabK was heated for 10 min at 100 $^{\circ}$ C and then subjected to liquid chromatography/MS as described in the Experimental section. UV (A) and mass (B) chromatograms at 460 nm and m/z 455 respectively for the released cofactor; (C) mass spectrum taken at retention time 13.80 min for the released cofactor. Retention time and spectral data were identical with that obtained using a commercial sample of FMN.

the cofactor for FabK is FMN and not FAD, as was described previously [18].

Activation of FabK by univalent cations

Purification of FabK was performed in two steps, an anion-exchange step followed by a hydrophobic interaction step. The activity of the enzyme preparation was evaluated at each step in the purification, and after the second hydrophobic interaction step, the activity of the enzyme had increased dramatically to approx. 70-fold when compared with the original. However, once the pooled protein was exchanged into the storage buffer [50 mM Tris/HCl (pH 7.5), 1 mM DTT, 50% (w/v) glycerol], the specific activity diminished to similar levels to those seen originally. Supplementing the enzyme assay buffer with

Figure 3 NADH oxidase activity of *Strep. pneumoniae* FabK compared with the *Bacillus* (YrpB) and *Staphylococcus* (BAB42020) FabK-like proteins

The substrate-independent oxidation of NADH was monitored at 340 nm for *Strep. pneumoniae* FabK (●), *Bacillus* YrpB (○) and *Staphylococcus* BAB42020 (▲) proteins using 20 µg of protein in 100 mM sodium phosphate buffer (pH 7.5) in a total volume of 300 µl as described in the Experimental section.

ammonium sulphate restored the enzyme activity to its higher levels. We therefore concluded that the increase in activity after hydrophobic chromatography was due to the ammonium sulphate present in the purification buffers.

The stimulation of the enzyme by a set of univalent cations was investigated to determine the specificity of activation. The assay buffer was supplemented with the chloride salts of different univalent cations, and NH_4^+ was the most potent activator, giving a 15-fold activation. The optimum concentration for this cation was 100 mM. Activation appeared to be dependent on the ionic radius of the cation, with Na^+ ($0.95 \text{ \AA}$; $1 \text{ \AA} = 10^{-10} \text{ m}$) $< \text{K}^+$ ($1.33 \text{ \AA}$) $< \text{NH}_4^+$ ($1.45 \text{ \AA}$) $> \text{Rb}^+$ ($1.48 \text{ \AA}$) $> \text{Cs}^+$ ($1.69 \text{ \AA}$). These experiments were performed with no preincubation of the enzyme with the univalent cation. Subsequently, it was determined that a preincubation of 5 min caused an even greater activation, and kinetic analysis of the enzyme was performed under these conditions. Bivalent cations were also tested and had no effect on activity. Removal of all cations from buffers caused rapid loss of the cofactor from the enzyme, which could be observed as the protein lost its yellow colour. The cations appear to stabilize the conformation of the enzyme. Subsequent purifications incorporated 50 mM NH_4Cl into the lysis, anion-exchange and storage buffers.

Specificity of the nucleotide cofactor

FabK was highly selective for NADH over NADPH. No reductase activity was detected in the presence of NADPH, tested at ten times the K_m for NADH (200 µM). One specific feature of the *Strep. pneumoniae* FabK is that this enzyme slowly oxidized NADH, but not NADPH, in the absence of substrate (Figure 3). The NADH oxidase activity of FabK is not a universal property of flavoproteins, and this property may be

Table 1 Occurrence of FabI- and FabK-related proteins in micro-organisms

Organism	% identity to*	
	FabI	FabK
<i>Escherichia coli</i>	100	—
<i>Streptococcus pneumoniae</i>	—	100
<i>Aquifex aeolicus</i>	49	—
<i>Archaeoglobus fulgidis</i>	—	37
<i>Bacillus subtilis</i>	51	34
<i>Bordetella pertussis</i>	65	—
<i>Chlamydia pneumoniae</i>	34	—
<i>Clostridium acetobutylicum</i>	—	58
<i>Enterococcus faecalis</i>	47	68
<i>Haemophilus influenzae</i>	75	—
<i>Helicobacter pylori</i>	45	31
<i>Mycobacterium tuberculosis</i>	33	31
<i>Neisseria gonorrhoeae</i>	61	—
<i>Neurospora crassa</i>	—	31
<i>Pseudomonas aeruginosa</i>	69	33
<i>Rickettsia prowazekii</i>	43	—
<i>Saccharomyces cerevisiae</i>	—	13
<i>Staphylococcus aureus</i>	43	37
<i>Thermatoga maritima</i>	—	48
<i>Williopsis saturnus</i>	—	24

* The *E. coli* FabI and *Strep. pneumoniae* FabK protein sequences were used to search the unfinished and complete microbial genomes database at www.tigr.org for open reading frames encoding homologous proteins. The number of identical residues was then scored. —, homologous genes were not detected.

important in integrating the activity of FabK with the overall anaerobic metabolism of the organism.

Kinetics of FabK under activating conditions

The kinetics of FabK was investigated under activating levels of NH_4Cl (100 mM). The kinetic parameters indicated that the K_m value for crotonyl-ACP was 9 ± 1 and $23 \pm 4 \text{ µM}$ for NADH. The k_{cat} value was $70 \pm 4 \text{ s}^{-1}$, a significant increase compared with that obtained when no univalent cations were included in the assay mixture (approx. 0.016 s^{-1}).

FabK-related proteins in other organisms

A search of the databases revealed that FabK-like proteins are predicted to be present in a variety of micro-organisms including bacteria, Archae, yeast and fungi (Table 1). Since the Streptococci, Enterococci, Bacilli and Staphylococci are taxonomically grouped together in the *Bacillus/Lactobacillus/Streptococcus* group of bacteria, we have concentrated on the FabK homologues from these organisms. The alignment of the predicted FabK-like proteins from this subset of organisms (Figure 4) demonstrates that they possess a conserved nucleotide-binding domain in the N-terminus and a conserved flavin-binding domain at the centre of the protein. The sequences diverge in the C-terminal regions. The *Ent. faecalis* protein is 68% identical with the *Strep. pneumoniae* FabK, strongly suggesting that these two proteins share a common substrate. However, the *Staph. aureus* and *B. subtilis* proteins, although being 41% identical with each other, were only approx. 30% identical with the other two. A major distinction between the two apparent classes is two insertions of approx. 10 and 8 amino acids in the *Staph. aureus* and *B. subtilis* proteins. In fact, the *Staph. aureus* and *B. subtilis* proteins more closely resemble a fungal protein annotated as a 2-

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S. pneumoniae. ~~~MKTRITELLKIDYPIFOGGMW.VADGDLGAVSKAGGLGIIGGNAPK.EVVKANIDKIKSLTDKPFQVNMIMLLS....P
Ent. faecalis   ~~~MNQELCELLGINYPFOGGMW.VADASLASAVSNAGGLGIAGGNAPK.EVVKKEIKKVKELTEQPFQVNMIMLLS....P
S. aureus      ~~~MWNKRLTQMLSTIEYPIFOAGMAGS.TTPKIVASVSNAGGLGTICAGYF.NTQOLEDEIDYVROLTSNPFQVNVFVPQQSYTS
B. subtilis    ~~~MNEFMKKFSLTKPIFOAPMAGGITKPRLASAVSNQCALGSLASCYL.TPDLLLEQQIKETIFELTDAPFQIVFVPLGLEMPF

FVVD.....IVDLVIEEGVKVVTTCAGNPSKY.MERFHEAGIIVIPVPSVALAKRMEKIGADAVIAEGMEAGG
FADE.....IVDLVCEEQVFPVTTGAGNPAKY.MARFKEHNIKVIPVPSVALAKRMEKIGADAVIFEGMEAGG
SQIENMNAWLKPYRRALHLEEPVVKITEE.QQFKCHIDTILKKOVPCCTFTIPSEQIISRLKANVKLIGTATSVDEATANEKAGMDAIVACSEAGG
K..DIKKWKENIPLANQVNO.FTSVQEEWDDFYQKIDILILKYVKACSFITDLPPEDAVKELKTACCLIGTASTVEEALLMBERGMIVVLOGSEAGG

Flavin Domain
HIGK.....LTTMTLVROVATNISIPVIAAGGIADCEGAAGFMLGAEAVOVCTRFVVAKESNAHPNYKEIKLKARDIDTITSAOHFGHAVRAIK
HIGK.....LTTMSGLEIVDAVSIPIVIAAGGIDGGRMAAFMLGAEAVOLSTRFLIAKENVHEDYKQKVLKARDLDAVITCOHFGHFVRTLK
HRSFLKPKNQPLPMVGTISLVPQIVDVVSIPIVIAAGGIMDGRGVLASIVLGAEVOMGTAFLTSDSNASELLRDAITNSKETDVTITKAFSGKLARGIN
HRC AFLPSKGE.SAVGLMALIPQADALSVPVIAACCMIDHRCVKAALTILGACQVOIGSAFLICHESNAHFVHKQKILEANEADTKLTTLFSCKEARGIV

NQLTRDFELAEKDAFKQBDPDLLEIFBOMGACALAKAVVHGDVDCGSVMACQIAGLVSKETABEILLDLYYGAAKKIQEASRWTVGVVRND
NKLTAAQYNQLEKQELQKHPDLEMFEEKIGOGALRKAVVDGDMVGSVMAGQIAGLIKKETAOEIIDSLMSECKAIVHKMNQRWG~~~~~
NRFIEEMSQYEGE....IPDYPIQNELTSSIRKAAANIGKELIHMWSQSPRLATTH.PANTIMSNINQINQIMOYK~~~~~
NKWMEENQOFETQ....TLPYPQNTLTAKMROKASLQNNHDOMSLWAGGIRSLTEB.IS...VKQLNLQLCQ.EDIKI~~~~~

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Figure 4 Superimposition of *Strep. pneumoniae* FabK with FabK-like proteins in other bacterial species

The FabK protein sequence from *Strep. pneumoniae* was aligned with the predicted sequences of the FabK protein from the related organism *Ent. faecalis*, and two closely related genes from *B. subtilis* (YrpB) and *Staph. aureus* (BAB42020; accession no. AP003131). Amino acids that are identical with the residues in *Strep. pneumoniae* are highlighted. The central region of the proteins containing a consensus flavin-binding site is indicated.

Figure 5 Conformationally sensitive gel assay of the enoyl-ACP reduction activity of *Strep. pneumoniae* FabK and FabK-like proteins from other bacterial species

A coupled enzyme system was employed to assay FabK by combining the purified *E. coli* proteins required to reconstitute a cycle of fatty acid synthesis with the exception of the enoyl-ACP reductase. Reaction mixtures (see the Experimental section) were first incubated to generate *trans*-2-butenoyl-ACP (A) or *trans*-2-hexadecenoyl-ACP (B) and then 0.5 µg of the indicated enoyl reductase was added. Products were separated by conformationally sensitive gel electrophoresis and the bands visualized and quantified using a PhosphorImager. ecFabI, *E. coli* FabI; bs, *B. subtilis* FabI, YrpB or FabL; spFabK, *Strep. pneumoniae* FabK; sa, *Staph. aureus* FabI or BAB 42020.

nitropropane reductase, although the physiological substrate for this enzyme is not known [25]. Thus it appears that the FabK-like proteins may fall into two subsets of activities. To test this hypothesis experimentally, we cloned and purified the FabK-like proteins from *Staph. aureus* and *B. subtilis*, and compared their activities with the previously cloned [18] FabK from *Strep. pneumoniae*.

Enoyl-ACP reduction activity of *Strep. pneumoniae* FabK and other bacterial FabK-like proteins

An *in vitro* coupled enzyme system was employed to assay FabK by combining the purified *E. coli* proteins required to reconstitute a cycle of fatty acid synthesis with the exception of the enoyl reductase. Reaction mixtures were first incubated to generate the short-chain *trans*-2-butenoyl-ACP (C-4) or the long-chain *trans*-2-hexadecenoyl-ACP (C-16) substrates and then equal amounts of FabK from different sources were added. The enoyl-ACP reductases FabI from *E. coli*, *B. subtilis* and *Staph. aureus*, as well as FabL from *B. subtilis* [17], were used as positive controls. The products formed (butyryl-ACP or palmitoyl-ACP) using FabI, FabL or FabK were separated by conformationally sensitive gel electrophoresis and quantified (Figures 5A and 5B). The enoyl-ACP reduction activity measured for *Strep. pneumoniae* FabK was similar to those measured for the FabI or FabL enzymes. In contrast, no significant enoyl-ACP activity for the *B. subtilis* and *Staph. aureus* proteins could be detected using either *trans*-2-butenoyl-ACP or *trans*-2-hexadecenoyl-ACP as substrate. These results, together with the absence of any NADH oxidase activity (Figure 3), strongly suggested that the YrpB and BAB42020 proteins were not enoyl-ACP reductases.

Functional replacement of the FabI enoyl reductase *in vivo*

FabI is the only enoyl reductase in *E. coli*, and strain RJH13 harbours a temperature-sensitive allele *fabI*(Ts) and fails to grow at 42 °C [10]. Transformation of strain RJH13 with a plasmid that constitutively expresses the *Strep. pneumoniae* FabK (pfabK) or the *B. subtilis* (pyrpB) and *Staph. aureus* (pBAB42020) counterparts was performed to determine if the latter proteins

Table 2 The ability of genes from different species to complement the *E. coli fabI*(Ts) growth phenotype and involvement of these proteins in triclosan resistance

Plasmid* ...	Origin of the gene							
	None	<i>E. coli</i>	<i>B. subtilis</i>			<i>Strep. pneumoniae</i>	<i>Staph. aureus</i>	
	Control	<i>pfabI</i>	<i>pfabI</i>	<i>pyrpb</i>	<i>pfabL</i>	<i>pfabK</i>	<i>pfabI</i>	pBAB42020
Complementation	No	Yes	Yes	No	Yes	Yes	Yes	No
Triclosan MIC† (μg/ml)	0.2	2	4	0.2	> 2000	> 2000	4	0.2

* The same vector [pBluescript KSII (+)] and construction method were used to express constitutively the His-tagged enoyl reductases FabI, FabK, YrpB, BAB42020 and FabL. *E. coli* strain RJH13 [*fabI*(Ts)] was transformed with pBluescript (empty vector), *pfabI* [10], *pfabK* (*Strep. pneumoniae*), *pfabL* [17] or *pyrpb* (*B. subtilis*) and pBAB42020 (*Staph. aureus*). Cells were grown at the permissive temperature for the host strain (30 °C), and then individual colonies spotted on to Luria–Bertani agar plates and incubated at 42 °C and scored for complementation.

† MICs for *E. coli* W3110 strains transformed with the indicated plasmids were tested by spotting six single colonies on to a series of Luria–Bertani agar plates containing different concentrations of triclosan. The MIC reported in each case is the concentration of triclosan at which no growth was observed in at least three separate experiments.

could complement the growth phenotype. In contrast with the *Strep. pneumoniae* FabK, which restored growth of the RJH13 strain at the non-permissive temperature (Table 2), the expression of the *B. subtilis* (YrpB) and *Staph. aureus* (BAB42020) FabK-like proteins failed to complement the *fabI*(Ts) phenotype, indicating that these proteins are not able to substitute for the function of FabI in fatty acid elongation. This same set of plasmids was also introduced into the wild-type *E. coli* strain W3110. Strain W3110 was sensitive to triclosan, and the presence of the *pfabI* plasmid from *E. coli*, *B. subtilis* or *Staph. aureus* increased triclosan resistance, shifting the MIC from 0.2 to 2 $\mu\text{g/ml}$ as a result of overexpression of FabI (Table 2). The introduction of the *Strep. pneumoniae* *pfabK* plasmid into strain W3110 shifted the MIC for triclosan to greater than the solubility of triclosan in the growth medium (2000 $\mu\text{g/ml}$) (Table 2). The much greater increase in this case is due to lack of inhibition of FabK by triclosan [18]. The triclosan MIC was unchanged after the introduction of the *pyrpb* or pBAB42020 plasmids. Thus these latter FabK-like proteins did not confer triclosan resistance by either mechanism, providing further evidence that these gene products are not involved in fatty acid biosynthesis.

DISCUSSION

Sequence analysis of the *Strep. pneumoniae* genome indicates that this organism utilizes FabK and not FabI to catalyse the last step in the fatty acid biosynthesis elongation cycle, the reduction of enoyl-ACP. Some organisms such as *Ent. faecalis* appear to possess genes encoding FabI and FabK, whereas others such as *E. coli* possess only FabI (Table 1). Our results point to *fabK* as an essential gene in *Strep. pneumoniae*, consistent with the bio-informatic analysis that this is the only enoyl-ACP reductase activity in this organism. The FabK protein sequence has a flavin-binding motif, and we identified this flavin to be FMN. FabK is significantly activated by univalent cations. The mechanism of this activation may reflect stabilization of the FMN binding to the enzyme. Removal of univalent cations from buffers resulted in the release of the cofactor from FabK and a concomitant loss of enzymic activity, perhaps the result of a destabilization of the enzyme structure in the absence of cations. It remains to be seen whether other FabK enzymes will show the same activation or whether this is unique to the *Strep. pneumoniae* enzyme.

Many organisms contain proteins with N-terminal (nucleotide-binding) and central (flavin-binding) domains that are similar to the corresponding domains in FabK (Figure 4; Table 1). Our direct analysis of two of these proteins from *Staph. aureus* and *B.*

subtilis with 37 and 34 % identity with FabK demonstrates that they do not possess enoyl-ACP reductase activity. *Staph. aureus* has a low MIC for triclosan, similar to the Gram-negative bacteria [16], and, accordingly, contains a FabI (Table 1). Mode of action and resistance studies demonstrate that FabI is the essential enoyl-ACP reductase in *Staph. aureus* [26,27]. These observations are consistent with the conclusion that the BAB42020 FabK-like protein in *Staph. aureus* is not involved in the reductase step of fatty acid elongation. *B. subtilis* is also relatively resistant to triclosan, but in this case a different triclosan-resistant enoyl-ACP reductase (FabL) is involved in fatty acid biosynthesis [17]. Again, the FabK-like YrpB does not play a role in the last step of the elongation cycle of fatty acid biosynthesis. Based on these observations, other proteins with highly related nucleotide and flavin-binding domains are unlikely to have a FabK function if they have low identities with FabK in the central and C-terminal regions.

We also noted the ability of FabK to oxidize NADH either via fatty acid synthesis or via a substrate-independent oxidase mechanism. This provides a possible physiological rationale for the existence of FabK in *Streptococcus/Enterococcus/Clostridium* species. These organisms lack cytochromes and generate ATP primarily by the anaerobic catabolism of glucose. NADH oxidase supplies the oxidized pyridine nucleotides for glycolysis by generating two molecules of NAD^+ by reducing O_2 to H_2O [28,29]. These NADH oxidases are related to FabK in that both enzymes have similar NADH- and flavin-binding domains. The fact that cells lacking NADH oxidase have severely impaired growth only under certain environmental conditions led to the conclusion that there are other enzymes capable of fulfilling that function [28]. The NADH oxidase activity of FabK (Figure 3) makes this protein a candidate allowing FabK to sustain glycolysis either by the synthesis of fatty acids or in the absence of enoyl-ACP substrate via the oxidation of NADH. All other enoyl reductases of fatty acid synthesis do not have a flavin cofactor and it will be interesting to determine if the FMN of FabK is involved in enoyl reductase reaction or whether it is present to execute the NADH oxidase function.

The expression of structurally different enoyl-ACP reductases in bacteria has important suggestions for anti-bacterial drug development. As a result of the structural differences between FabI and FabK, we predict it would be a challenge to identify compounds with inhibitory activity against both types of enoyl-ACP reductase. However, specific FabI and FabK inhibitors could have utility against specific pathogens and such compounds could be combined to combat a broad spectrum of FabI- and FabK-utilizing organisms.

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