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Identification, Substrate Specificity, and Inhibition of the *Streptococcus pneumoniae* β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH)*

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In the bacterial type II fatty acid synthase system, β-ketoacyl-acyl carrier protein (ACP) synthase III (FabH) catalyzes the condensation of acetyl-CoA with malonyl-ACP. We have identified, expressed, and characterized the *Streptococcus pneumoniae* homologue of *Escherichia coli* FabH. *S. pneumoniae* FabH is ~41, 39, and 38% identical in amino acid sequence to *Bacillus subtilis*, *E. coli*, and *Hemophilus influenzae* FabH, respectively. The His-Asn-Cys catalytic triad present in other FabH molecules is conserved in *S. pneumoniae* FabH. The apparent K_m values for acetyl-CoA and malonyl-ACP were determined to be 40.3 and 18.6 μM, respectively. Purified *S. pneumoniae* FabH preferentially utilized straight short-chain CoA primers. Similar to *E. coli* FabH, *S. pneumoniae* FabH was weakly inhibited by thiolactomycin. In contrast, inhibition of *S. pneumoniae* FabH by the newly developed compound SB418011 was very potent, with an IC_{50} value of 0.016 μM. SB418011 also inhibited *E. coli* and *H. influenzae* FabH with IC_{50} values of 1.2 and 0.59 μM, respectively. The availability of purified and characterized *S. pneumoniae* FabH will greatly aid in structural studies of this class of essential bacterial enzymes and facilitate the identification of small molecule inhibitors of type II fatty acid synthase with the potential to be novel and potent antibacterial agents active against pathogenic bacteria.

Fatty acid biosynthesis in bacteria, plants and animals is carried out by the ubiquitous fatty acid synthase (FAS)[1] system. In the type I system of animals, including humans, FAS is a homodimer of two large polypeptides, each comprised of several distinct enzyme domains and an integral acyl carrier protein (ACP) (1, 2). In the type II systems of bacteria (3), plants (4), and protozoa (5), the FAS components, including the ACP, exist as discrete proteins. The corresponding enzymes of the type I and II FASs are related in structure and function but generally lack overall sequence homology. The essentiality of type II FAS for bacterial viability together with major differences between it and type I FAS suggest that broad-spectrum anti-bacterial drugs may be obtained by screening for inhibitors of the bacterial components (6–8).

In the type II FAS system, β-ketoacyl-ACP synthase (KAS) enzymes are central to the initiation and elongation steps and play a pivotal role in the regulation of the entire pathway (9). KAS I (FabB), II (FabF), and III (FabH) catalyze the condensation of malonyl-ACP with either acetyl-CoA (in the case of FabH) or the growing ACP-linked acyl chain to form the corresponding β-ketoacyl-ACP substrate for the subsequent reduction step in the elongation cycle catalyzed by FabG. FabH acts *via* a ping-pong mechanism (10, 11) and is unique among KAS in that it utilizes acetyl-CoA as an acyl group donor whereas FabB (12) and FabF (13) both utilize acyl-ACPs as primers. Also, unlike FabB and FabF, which are sensitive to both cerulenin and thiolactomycin (TLM), FabH is insensitive to cerulenin and much less sensitive to TLM (3, 12, 14, 15). FabH also possesses acetyl-CoA:ACP transacylase activity *in vitro*, albeit at a rate ~200-fold lower than that of the condensing activity. The physiological role of the transacylase reaction is unknown.

The crystal structure of *Escherichia coli* FabH was recently solved (16–18) and shown to display a five-layered core structure that, despite the lack of overall sequence similarity, is similar to that of FabF (19) and other condensing enzymes of known structure (20, 21). Consistent with our biochemical data (22), both in the presence and absence of acetyl-CoA, the structure has a quasi-2-fold symmetry (16, 17). In addition, co-crystallization of FabH with acetyl-CoA provided direct evidence that Cys-112 is the catalytic nucleophile (16, 17).

Interestingly, despite the similar folds, the substrate specificities for FabH from various bacterial species appear to be quite different. *E. coli* FabH was most selective for acetyl-CoA and inactive with longer chain acyl-CoAs (23). In contrast, *Bacillus subtilis* FabH displayed low activity with acetyl-CoA but was active with 4- to 8-carbon straight-chain acyl-CoA and all branched-chain acyl-CoA molecules (24), whereas the order of reactivity of *Streptomyces glaucescens* FabH was butyryl-CoA→acetyl-CoA→isobutyryl-CoA (25). Recently, Choi *et al.* (26) demonstrated that the FabH homolog of *Mycobacterium tuberculosis* prefers long-chain (C10-C16) acyl-CoA substrates over either short- or branched-chain primers, thus distinguish-

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The nucleotide sequence of S. pneumoniae FabH reported in this paper has been submitted to the GenBank™ with accession number AF384041.

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[1] The abbreviations used are: FAS, fatty acid synthase; ACP, acyl carrier protein; KAS, β-ketoacyl-ACP synthase; TLM, thiolactomycin; PCR, polymerase chain reaction; contig, group of overlapping clones; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; CD, circular dichroism; GdnHCl, guanidine hydrochloride; TLM, thiolactomycin.

TABLE I
Primers used in the present study

PCR primer	Sequence
EKG8	AGAGGGTTTCACTACCAATC
EKG10	GACTGTGCGTCCATCCATCT
FabH3′	CGCGGATCCACAATGTGTTCACCACATGATTAC
FabH5′	TGCGGAATTCCATATGGCTTTTGCAAAAATAAGTCAGGT
SPYOH1	GACGTCCTTCAAGTAATTCC
SPYOH2	AAAGACTGGCAGGGTTACGCTA

ing it from the other FabH molecules.

The role of FabH in fatty acid biosynthesis in *Streptococcus pneumoniae*, a clinically relevant Gram-positive bacterium, has been poorly understood. We have recently identified the *S. pneumoniae* homologue of *E. coli* FabH, and in this paper we describe its cloning and characterization. Our results show that *S. pneumoniae* FabH is able to utilize short (C2-C4) straight- and branched-chain acyl-CoAs, although activity with the straight-chain acyl-CoA was significantly higher. In addition, our studies have identified SB418011 as an inhibitor of *S. pneumoniae* FabH that is significantly more potent than TLM and cerulenin. SB418011 also inhibited *E. coli* and *Hemophilus influenzae* FabH but not human FAS. The availability of purified and well characterized *S. pneumoniae* FabH will greatly facilitate structural and kinetic studies and the discovery of potent and broad-spectrum antibacterial inhibitors.

MATERIALS AND METHODS

Reagents—Purified human testes FAS and chicken FAS proteins used for specificity assays were obtained from Dr. Zining Wu (Glaxo SmithKline, King of Prussia, PA) and Dr. Salih Wakil (Baylor College of Medicine), respectively. *E. coli* FabH was purified as described before (22). Hexahistidine (His_6)-tagged *H. influenzae* FabH was expressed and purified from the *E. coli* cell lysate[2] Reagents otherwise noted were purchased from Sigma.

Cloning of S. pneumoniae fabH in E. coli—The *S. pneumoniae fabH* gene was cloned *via* polymerase chain reaction (PCR) taking advantage of the conserved genomic structure between *S. pneumoniae* and *Streptococcus pyogenes*. Inspection of the publicly available *S. pyogenes* sequence revealed the presence of a contig containing the N-terminal coding region of the *S. pyogenes fabH* gene along with considerable upstream sequence. This contig was used as a query to search the *S. pneumoniae* sequence database, and a *S. pneumoniae* contig that was likely to lie upstream of *fabH* was identified. The primers SPYOH1 and SPYOH2 (Table I) were designed from the upstream contig that shared homology with the *S. pyogenes* contig whereas the primers EKG8 and EKG10 were designed from the already available partial *fabH* gene sequence. PCR of *S. pneumoniae* genomic DNA using all four possible primer combinations yielded products around the expected size of 1.0 to 1.2 kilobase pairs. Sequencing of the PCR products confirmed the presence of the N terminus of *fabH*. In this way, two of the *S. pneumoniae* contigs were joined, and the full-length sequence of *fabH* was obtained.

Expression and Overproduction of S. pneumoniae FabH—A plasmid directing the overproduction of *S. pneumoniae* FabH with a His_{10} tag (decahistidine tag) and thrombin cleavage site (LVPRGS) at its N terminus was constructed. Primers fabH5′ and fabH3′ (Table I) were used to amplify *fabH* from *S. pneumoniae* genomic DNA. After purification from free primers, the PCR product was digested with *Nde*I and *Bam*HI and gel-purified. The gel-purified product was then ligated with gel-purified pET16b digested with *Nde*I and *Bam*HI. Candidate plasmids containing correctly sized inserts were screened for over-production of *S. pneumoniae* FabH by SDS-PAGE. The inserts of plasmids shown to produce FabH were sequenced to ensure that no mutations were introduced by the PCR process. To obtain soluble protein, *E. coli* strain BL21 (DE3) was transformed with vector pFabH1 containing the *fabH* gene. Transformed cells were grown at 37 °C to an optical density ($A_{600\ nm}$) of 1.0 and then were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for additional 2.5 h (optical density of 2.6). Cells were harvested by centrifugation at 15,000 × g for 30 min, and the resultant cell pellet was stored at −70 °C until further use.

Purification of S. pneumoniae FabH—All lysis and purification steps were carried out at 4 °C. 10 g of *E. coli* cells over-expressing tagged *S. pneumoniae* FabH were resuspended in 300 ml of lysis buffer (buffer A) containing 20 mM Tris-HCl (pH 8.0), 300 mM sodium chloride, 0.2 mM phenylmethylsulfonyl fluoride, 40 mM imidazole, and 5 mM 2-mercaptoethanol. Cells were lysed twice at 10,000 p.s.i. using a microfluidizer (Microfluidics Corporation). Cell debris were removed by centrifugation (Sorvall RC-5B) at 35,000 × g for 30 min. The supernatant was applied to a 15-ml Ni-NTA metal affinity column (Qiagen) at 2 ml/min equilibrated in buffer A. The column was washed with 10-column-volumes of buffer A and eluted with 20 mM Tris-HCl (pH 8.0), 300 mM sodium chloride, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, and 300 mM imidazole. Fractions containing FabH were pooled, dialyzed against 4 liters of buffer B containing 20 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol, and applied to a 15-ml Source Q column (Amersham Pharmacia Biotech) column equilibrated in buffer B. The column was washed with 10-column-volumes of buffer B and then eluted with a 20-column-volume linear gradient of 0–1.0 M sodium chloride in buffer B. FabH, which eluted at 300–400 mM NaCl, was next applied to a Superdex 200 size exclusion column (2.6 × 60 cm, Amersham Pharmacia Biotech) equilibrated in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5 mM DTT. Yield of purified protein was ~10 mg/gm wet weight of *E. coli*. SDS-PAGE, native isoelectric focusing (IEF), N-terminal sequence, and amino acid composition analyses were performed as described (22).

Size Exclusion Chromatography—The apparent molecular weight of *S. pneumoniae* FabH was determined by size exclusion chromatography on analytical Superdex 200 or Superdex 75 (1.6 × 30 cm, Amersham Pharmacia Biotech) column equilibrated in 20 mM HEPES (pH 7.0), 300 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. The column was calibrated with the following molecular mass standards (Bio-Rad): thyroglobulin (658 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1 kDa). A 200-μg (3 mg/ml) sample of purified *S. pneumoniae* FabH was injected at a flow rate of 0.5 ml/min.

Enzyme Assays—S. pneumoniae FabH-directed acetoacetyl-ACP formation was specifically measured using [^{3}H]acetyl-CoA and malonyl-ACP. Malonyl-ACP was generated *de novo* by malonyl-CoA: acyl carrier protein transacylase (FabD) (12). The substrate [^{3}H]acetyl-CoA is soluble in 10% trichloroacetic acid whereas the resulting [^{3}H]acetoacetyl-ACP is not. Final reaction conditions in 25 μl were typically as follows: 100 mM sodium phosphate (pH 7.0), 1 mM DTT, 175 μM ACP, 200 μM malonyl-CoA, 15 ng of purified *S. aureus* FabD,[3] 40 μM acetyl-CoA, 0.5 μM [^{3}H]acetyl-CoA (specific activity 7 Ci/mmol, Moravek Biochemicals), and 4 nM *S. pneumoniae* FabH. ACP, malonyl-CoA, and FabD were preincubated with buffer and DTT for 10 min at 33 °C prior to addition of acetyl-CoA and FabH. FabH was added last to start the reaction, and reactions were then incubated at 33 °C for 10–15 min. 10% trichloroacetic acid was added to stop the reaction, and stopped reactions were incubated on ice for 20 min prior to filtration. Reaction mixtures were filtered and washed twice with 10% trichloroacetic acid on Packard GF/C filter plates using a Packard Filtermate harvester. The filter plates were then dried completely at 60 °C, and the radioactivity was quantified using Wallac Supermix scintillation mixture and a Wallac Microbeta 1450 liquid scintillation counter.

Malonyl-ACP formation was determined in the filtration assay format as described above for FabH. Final reaction conditions typically were 100 mM sodium phosphate (pH 7.0), 1 mM DTT, 50 μM malonyl-CoA, 8 μM [^{14}C]malonyl-CoA (specific activity 56 mCi/mmol, Moravek Biochemicals), 15 ng of FabD, and 25 μM ACP. Reactions were stopped, filtered, and quantified as for the FabH filtration assay.

To assay different non-radiolabeled acyl-CoAs as primers for FabH, a spectrophotometric assay was employed. α-Ketoglutarate dehydrogenase has been used previously to specifically quantify free CoA (27) and was used as coupling enzyme to follow FabH activity. Reaction conditions in 50 μl of final volume were as follows: 100 mM sodium phosphate (pH 7.0), 1 mM DTT, 500 μM NAD, 1 mM α-ketoglutaric acid, 0.025 units of α-ketoglutarate dehydrogenase, 75 μM various acyl-CoAs, 20 μM malonyl-ACP, and 4 nM *S. pneumoniae* FabH. Assays were started by the addition of FabH and followed spectrophotometrically at 340 nm.

For inhibition studies, cerulenin (0.011–666 μM), TLM (0.0034–200 μM) and SB418011 (0.0003–200 μM) were dissolved in dimethyl sulfoxide (Me_2SO) and tested for their ability to inhibit *S. pneumoniae*, *E. coli, H. influenzae* FabH, and human FAS proteins using the filtration assay as described above. IC_{50} values were determined by using GraFit version 4.09 fitting the data to the equation: $v = V_m/(1 + I/IC_{50})$. The

[2] D. Gentry, M. Grooms, S. Khandekar, and J. Lonsdale, unpublished results.

[3] G. Van Aller, S. Khandekar, and J. Lonsdale, unpublished data.

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                 sssssssss   sssshhhhh                                hhhhhhhh   ssss      hhhhhhhhhhhhhhhh   hhh       sssss
S. pneumoniae    MAFAKISQVAHYVPEQVVTNHDLAQIMDTN--------------------------DEWISSRTGIRQRHISRTE-STSDLATEVAKKLMAKAGITGEEL-----DFIILA   79
B. subtilis      -MKAGILGVGRYIPEKVLTNHDLEKMVETS--------------------------DEWIRTRTGIRERRIAADDVFSSHMAVAAAKNALEQAEVAAEDL-----DMILVA   79
E. coli          -MYTKIIGTGSYLPEQVRTNADLEKMVDTS--------------------------DEWIVTRTGIRERHIAAPNETVSTMGFEAATRAIEMAGIEKDQI-----GLIVVA   79
H. influenzae    -MNSRILSTGSYLPSHIRTNADLEKMVDTS--------------------------DEWIVTRSGIRERRIAAEDETVATMGFEAAKNAIEAAQINPQDI-----ELIIVA   79
H. sapiens       MEEVVIAGMFGKLPESENLQEFWDNLIGGVDMVTDDDRRWKAGLYGLPRRSGKLKDLSRFDASFFGV-HPKQAHTMDPQLRLLLEATYEAIVDGGINPDSLRGTHTGVWVGV  111
G. gallus        MEDVVIAGIAGKLPESENLQEFWENLLNGVDMVTEDDRRWKPGIYGLPKRNGKLKDIKKFDASFFGV-HPKQAHTMDPQLRLLLEVSYEAILDGGINPTALRGTDTGVWVGA  111

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S. pneumoniae    TITPDS------------------MMPSTAARVQANIGANKAFAFDLTAACSGFVFALSTAEKFIASGRFQKGLVIGSETLSKAVD---------WSDRSTAVLF-------  157
B. subtilis      TVTPDQ------------------SFPTVSCMIQEQLGAKKACAMDISAACAGFMYGVVTGKQFIESGTYKHVLVVGVEKLSSITD---------WEDRNTAVLF-------  157
E. coli          TTSATH------------------AFPSAACQIQSMLGIKGCPAFDVAAACAGFTYALSVADQYVKSGAVKYALVVGSDVLARTCD---------PTDRGTIIIF-------  157
H. influenzae    TTSHSH------------------AYPSAACQVQGLLNIDDAISFDLAAACTGFVYALSVADQFIRAGKVKKALVIGSDLNSRKLD---------ETDRSTVVLF-------  157
H. sapiens       SGSETSEALSRDPETLVGYSMVGCQRAMMANRLSFFFDFRG-PSIALDTACSSSLMALQNAYQAIHSGQCPAAIVGGINVLLKPNTSVQFLRLGMLSPEGTCKAFDTAGNGY  222
G. gallus        SGSEALEALSQDPEELLGYSMTGCQRAMLANRISYFYDFTG-PSLTIDTACSSSLMALENAYKAIRHGQCSAALVGGVNILLKPNTSVQFMKLGMLSPDGACKAFDVSGNGY  222

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S. pneumoniae    --GDGAGGVLLE-ASEQEHFLAESLNSDGSRSECLTYGHSGLHSPFSDQESADSFLKMDGRTVFDFAIRDVAKSIKQTIDESPIEVTDLDYLLLHQANDRILDKMARKIGVD  266
B. subtilis      --GDGAGAAVVGPVSDDRGILSFELGADGTGGQHLYLNEKR-------------HTIMNGREVFKFAVRQMGESCVNVIEKAGLSKEDVDFLIPHQANIRIMEAARERLELP  254
E. coli          --GDGAGAAVLA-ASEEPGIISTHLHADGSYGELLTLPNAD-----RVNPENSIHLTMAGNEVFKVAVTELAHIVDETLAANNLDRSQLDWLVPHQANLRIISATAKKLGMS  261
H. influenzae    --GDGAGAVILE-ASEQEGIISTHLHASADKNNALVLAQPE------RGIEKSGYIEMQGNETFKLAVRELSNVVEETLLANNLDKKDLDWLVPHQANLRIITATAKKLEMD  260
H. sapiens       CRSEGVVAVLLTKKSLARKVYTTILN---KGTNTDGFK--------------------EQGVTFP--QDIQEQPIRSLYQSAGVAPESFEYIEAHGPGTKVGDPQER-NGIT  308
G. gallus        CRSEAVVVVLLTKKSMAKRVYATIVN---AGSNTDGFK--------------------EQGVTFP-SGEMQQQLVGSLYRECGIKPGDVEYVEAHGTGTKVGDPQEV-NGIV  309

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S. pneumoniae    RAK---------LPANMMEYGN---TSAASIPILLSECVEQGLI--------------------------PLDGSQTVLLSGFG-GGLTWGTLILTI-------        324
B. subtilis      VEK---------MSKTVHKYGN---TSAASIPISLVEELEAGKI--------------------------KDG-DVVVMVGFG-GGLTWGAIAIRWGR-----        312
E. coli          MDN---------VVVTLDRHGN---TSAASVPCALDEAVRDGRI--------------------------KPG-QLVLLEAFG-GGFTWGSALVRF-------        317
H. influenzae    MSQ---------VVVTLDKYAN---NSAATVPVALDEAIRDGRI--------------------------QRG-QLLLLEAFG-GGWTWGSALVRF-------        316
H. sapiens       RALCATRQEPLLIGSTKSNMGHPEPASGLDALAKVLLSLEHGLWAPNLHFHSPNPEIPALLDGRLQVVDQPLPVRGAGNVGINSFGFGGSNMHIILRPNTQSAPAP        413
G. gallus        NVFCQCEREPLLIGSTKSNMGHPEPASGLAALAKVILSLEHGLWAPNLHFNSPNPDIPALHDGSLKVVCKPTPVKG-GLVSINSFGFGGSNAHVILRPNEKKCQ--        412
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FIG. 1. **Alignment of the *S. pneumoniae* FabH protein with bacterial and eukaryotic homologues.** Amino acid sequence of *S. pneumoniae* FabH is compared with amino acid sequences of *B. subtilis*, *E. coli*, and *H. influenzae* and with the corresponding eukaryotic FAS domains from *H. sapiens* and *G. gallus*. The conserved cysteine (Cys-112), histidine (His-249), and asparagine (Asn-279) active site residues are in *bold face*. Secondary structure labeling is based on the *E. coli* crystal structure (16). *s*, similar; *h*, homologous.

concentration of FabH in the assay reactions was: 4 nM *S. pneumoniae* FabH, 0.5 nM *E. coli* FabH, and 1 nM *H. influenzae* FabH, respectively. The concentrations of ACP (75 μM for *S. pneumoniae*, 20 μM for *E. coli*, and 62.5 μM for *H. influenzae* FabH, respectively), malonyl-CoA (100 μM for *S. pneumoniae*, 40 μM for *E. coli*, and 90 μM for *H. influenzae* FabH, respectively), and acetyl-CoA (40 μM for *S. pneumoniae*, 10 μM for *E. coli*, and 18 μM for *H. influenzae* FabH, respectively) were based on the turnover ratio of FabD and K_m values for malonyl-ACP and acetyl-CoA.

IC_{50} values against human FAS were determined spectrophotometrically by monitoring the oxidation of NADPH to NADP at 340 nm. The reaction mixture contained 50 mM potassium phosphate (pH 6.5), 4 mM DTT, 11 μM acetyl-CoA, 190 μM NADPH, and 10 nM human FAS. The reaction was started by the addition of 75 μM malonyl-CoA and incubated for 20 min at 25 °C.

Circular Dichroism (CD) Structure and Stability Analysis—Far-UV CD spectra were recorded on a Jasco J-710 CD instrument using a 0.1-cm path length cuvette cell. Data (seven accumulations) were collected using a time constant of 4 s, at 50 nm/min, and with a 1-nm spectral bandwidth. A buffer-only spectrum was subtracted from the FabH spectrum, and the resulting ellipticity data, θ (in degrees), were converted to difference molar extinction coefficient data as described (28). Concentration of residues was determined by absorbance at 210 nm using an extinction coefficient of 22 ml/mg and an average molecular mass of 110/residue. The data were analyzed for secondary structure prediction by Softsec for Windows Version 1.2 software provided by the manufacturer (Jasco). Guanidine hydrochloride (GdnHCl) denaturation was carried out while monitoring the secondary structure of FabH by CD at 220 nm. Purified FabH was diluted into denaturant to a 0.2 mg/ml final concentration at 20 °C and incubated for 10 min before taking the measurements. Dynamic light scattering analysis was performed on purified protein (1–6 mg/ml) using a DynaPro-MSTC instrument (Protein Solutions, Inc.) as described previously (22).

RESULTS AND DISCUSSION

Sequence Analysis of the fabH Gene and Its Product—The *S. pneumoniae fabH* gene was cloned via PCR taking advantage of conserved genomic structure between *S. pneumoniae* and *S. pyogenes* as described under "Materials and Methods." PCR of *S. pneumoniae* genomic DNA using all four possible primer combinations yielded products around the expected size of 1.0–1.2 kilobase pairs. Sequencing of the PCR products confirmed the presence of the N terminus of *fabH*. In this way two *S. pneumoniae* contigs were joined and the full-length sequence of *fabH* was obtained. The open reading frame encodes a protein of 324 amino acids (Fig. 1). The calculated molecular mass of the encoded protein is 34,901 Da, and the isoelectric point is 4.9.

The alignment in Fig. 1 was manually constructed with the aid of the sequence homology search algorithm PSI-BLAST. A FabH sequence profile was generated via five iterations of PSI-BLAST using the *S. pneumoniae* amino acid sequence as a query against an all-bacteria protein database. The profile was then used to search a *Homo sapiens*-specific protein database. The alignment was optimized by manual inspection using the x-ray crystal structure of *E. coli* FabH (16) and the predicted secondary structure of the eukaryotic FAS-β-ketoacyl synthase domain. The species shown are a subset of the alignment containing 43 members of the FabH family representing 38 different species including green plants and *Plasmodium falciparum*.

S. pneumoniae FabH is ~41, 39, and 38% identical to *B. subtilis*, *E. coli,* and *H. influenzae* FabH, respectively and only 19% identical to the β-ketoacyl synthase domain of human and chicken FAS molecules. The active site of *E. coli* FabH comprises Cys-112, His-244, and Asn-274 (16, 17). These residues are conserved in *S. pneumoniae* FabH (Cys-112, His-249, and Asn-279) (Fig. 1, highlighted in *bold*) as well as in other bacterial species (24, 26). Interestingly, Asn-343 is conserved across all bacterial FabH species, but is replaced by His-330 in eukaryotic FAS proteins.

With the exception of Phe-157, the hydrophobic residues that make up the active site pocket in the *E. coli* structure, including Phe-87′ that protrudes into the active site of the opposite dimer-pair, are not rigorously conserved in *S. pneumoniae*

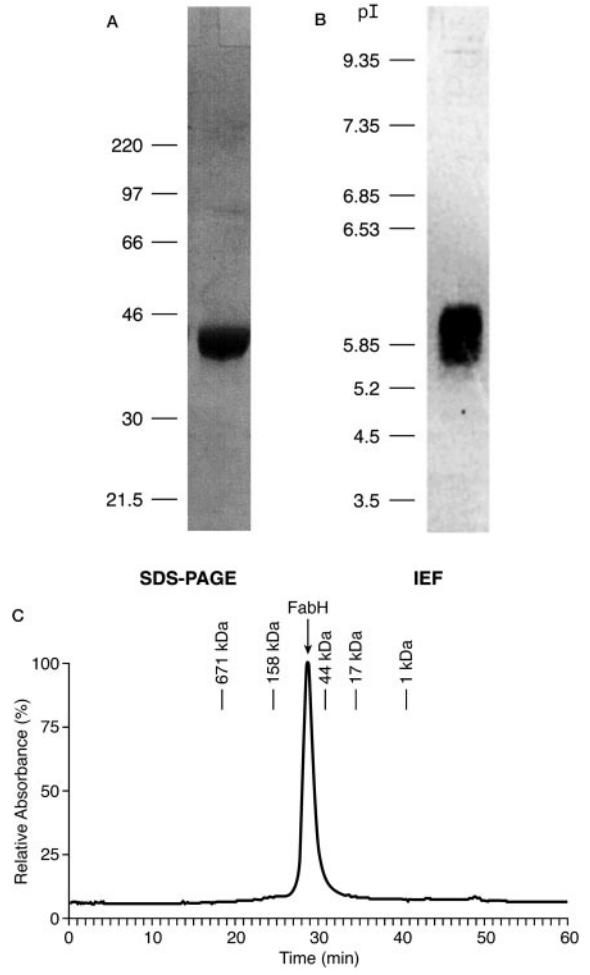

FIG. 2. **Characterization of purified *S. pneumoniae* FabH.** *A,* SDS-PAGE analysis. Purified FabH (20 μg) was subjected to 10% SDS-PAGE under reducing conditions. The band was visualized with Coomassie Blue stain. *B,* IEF analysis. Purified FabH (25 μg) was analyzed by IEF on a Novex (pH 3–10) gel at room temperature under native conditions. *C,* size exclusion chromatography. 300 μg of purified FabH (3 mg/ml) was analyzed on a Superdex 200 column under the conditions described under "Materials and Methods." Molecular mass standards (Amersham Pharmacia Biotech) used were: thyroglobulin (666 kDa), β-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1 kDa). FabH eluted with apparent molecular mass of ~60 kDa.

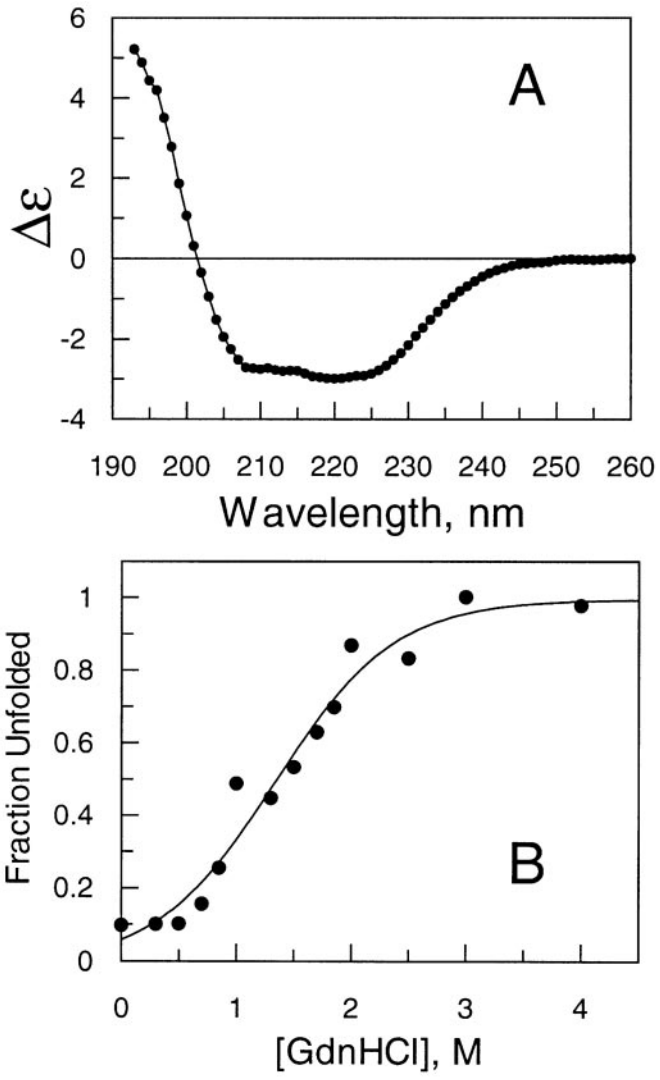

FIG. 3. **Circular dichroism analysis of the structure and stability of *S. pneumoniae* FabH.** *A,* far-UV circular dichroism spectrum of purified *S. pneumoniae* FabH shown as difference molar extinction coefficient/mole of peptide bond *versus* wavelength. Conditions: 3 mM sodium phosphate (pH 7.5), 25 mM NaCl, 0.1 mg/ml FabH, and 20 °C. *B,* GdnHCl denaturation (0–4 M) of purified *S. pneumoniae* FabH under conditions described under "Materials and Methods."

(Leu-142, Phe-157, Leu-189, Leu-205, and Phe-87′). The *E. coli* FabH residues, Trp-32 and Arg-151, are invariant within the FabH family, presumably preserving their side-chain interactions with the adenine ring of CoA as suggested in *E. coli* but different in human and chicken FAS. In addition, the residues that form hydrogen bonds to pantotheinate, Gly-209 and Asn-247, are invariant in the FabH family but again are different in eukaryotic FAS.

Although the high degree of sequence conservation within the bacterial FabH protein family easily distinguishes this member of the β-ketoacyl synthase superfamily from FabF and the eukaryotic fatty acid synthases, distant similarities can be found using sensitive search algorithms such as PSI-BLAST. Structural similarities between FabF and FabH are easily recognizable and, from this analysis, one would predict a similar structure for the corresponding domain in eukaryotic FAS.

Purification and Characterization of FabH S. pneumoniae—FabH was expressed in *E. coli* as an N-terminal decahistidine-tagged fusion protein. FabH expressed well and was purified from *E. coli* cell lysate using a combination of Ni-NTA, anion exchange, and preparative Superdex 200 size exclusion chromatography as described under "Materials and Methods." The overall yield of the purified FabH was ~10 mg/gm (wet weight) of *E. coli* cells.

TABLE II
Substrate specificities of S. pneumoniae FabH

Specific activity was determined using the spectrophotometric assay as described under "Materials and Methods." Values represent the mean ± standard deviation, $n = 3$. ND, not detectable.

Substrate	Enzyme activity
	nmoles/min/μg
Acetyl-CoA	5.04 ± 0.50
Butyryl-CoA	3.66 ± 0.32
Isobutyryl-CoA	1.38 ± 0.19
Isovaleryl-CoA	0.71 ± 0.12
Lauroyl-CoA	ND
Palmitoyl-CoA	ND

Purified FabH was nearly homogeneous as judged by SDS-PAGE (Fig. 2*A*). N-terminal sequence analysis confirmed the 34-kDa band as N-terminal decahistidine-tagged *S. pneumoniae* FabH. The amino acid composition was in complete

TABLE III
Substrate specificities of various bacterial FabH homologs

Specific activity for *S. pneumoniae* FabH was determined using the spectrophotometric assay as described under "Materials and Methods." Values represent the mean ± standard deviation, $n = 3$. ND, not determined.

Acyl CoA substrate	*E. coli* FabH (Ref. 24)	*B. subtilis* FabH1 (Ref. 24)	*S. glaucescens* FabH (Ref. 25)	*M. tuberculosis* FabH (Ref. 26)	*S. pneumoniae* FabH (present study)
	nmol/min/mg	*nmol/min/mg*	*pmol/min/mg*	*pmol/min/μg*	*nmoles/min/μg*
Acetyl	465 ± 62	24 ± 0.9	11.5	<0.001	5.0 ± 0.5
Butyryl	1 ± 0.1	8 ± 0.8	20.5	<0.001	3.7 ± 0.3
Lauroyl	ND	ND	ND	130.0 ± 2.6	<0.1
Palmitoyl	ND	ND	ND	12.5 ± 0.3	<0.1
Isobutyryl	<1	58 ± 2	3.4	ND	1.4 ± 0.2
Isovaleryl	<1	16 ± 0.8	ND	<0.001	0.7 ± 0.1

agreement with the predicted composition based upon the primary amino acid sequence deduced from the nucleotide sequence of the expressed protein (not shown). Purified FabH migrated predominantly with a pI of ~6.1 under native conditions (Fig. 2*B*), which is in close agreement to its theoretical pI of 6.02 calculated from the amino acid sequence of decahistidine-tagged *S. pneumoniae* FabH.

Our previous studies showed that the molecular mass of *E. coli* FabH by size exclusion chromatography and dynamic light scattering performed under nondenaturing conditions was ~65 kDa, implying the non-covalent dimeric nature of the protein (22). In agreement with the solution studies, the crystal structure of the *E. coli* FabH in the presence and absence of acetyl-CoA revealed a non-covalent homodimer (16, 17). The homodimeric nature of the *E. coli* FabH was the same as that reported for the *E. coli* FabB (29) and FabF (19, 29) proteins and for the FabH of *S. glaucescens* (25).

To determine whether *S. pneumoniae* FabH has similar properties, purified protein was subjected to size exclusion chromatography and dynamic light scattering techniques. On an analytical Superdex S200 column *S. pneumoniae* FabH eluted as a single species with a molecular mass of ~60 kDa (Fig. 2*C*); similar results were obtained using a Superdex S75 column (not shown). The size exclusion chromatography profile and SDS-PAGE analysis indicate a non-covalent homodimeric structure for *S. pneumoniae* FabH. *S. pneumoniae* FabH was monodisperse at lower concentration as judged by dynamic light scattering (not shown). However, unlike the *E. coli* FabH, at higher concentrations (>3 mg/ml), the *S. pneumoniae* FabH appeared to form soluble aggregates as evident from an increased polydispersity index (not shown). Similar results were obtained with purified untagged *S. pneumoniae* FabH indicating that the decahistidine tag was not the cause of the aggregation.[4] It may be that the hydrophobic residues of *S. pneumoniae* FabH that are exposed to solvent are responsible for its aggregation. Further work, including modeling and site-directed mutagenesis, may help to determine whether the aggregation is an intrinsic property of *S. pneumoniae* FabH.

CD Spectroscopy—Our previous circular dichroism spectroscopic studies showed the presence of both α-helix and β-sheet elements in purified *E. coli* FabH (22). Consistent with these results, the crystal structure of *E. coli* FabH displayed a five-layered core structure, α-β-α-β-α, where each α comprises two α-helices and each β consists of a five-stranded, mixed β-sheet (16, 17). To determine its secondary structure and stability, *S. pneumoniae* FabH was subjected to CD spectroscopy (Fig. 3). The far-UV circular dichroism spectrum of *S. pneumoniae* FabH is shown as difference molar extinction coefficient/mole of peptide bond *versus* wavelength (Fig. 3*A*). Softsec computer software analysis predicted a α-helix content of 26% and a β-sheet content of 31%. These values are comparable with

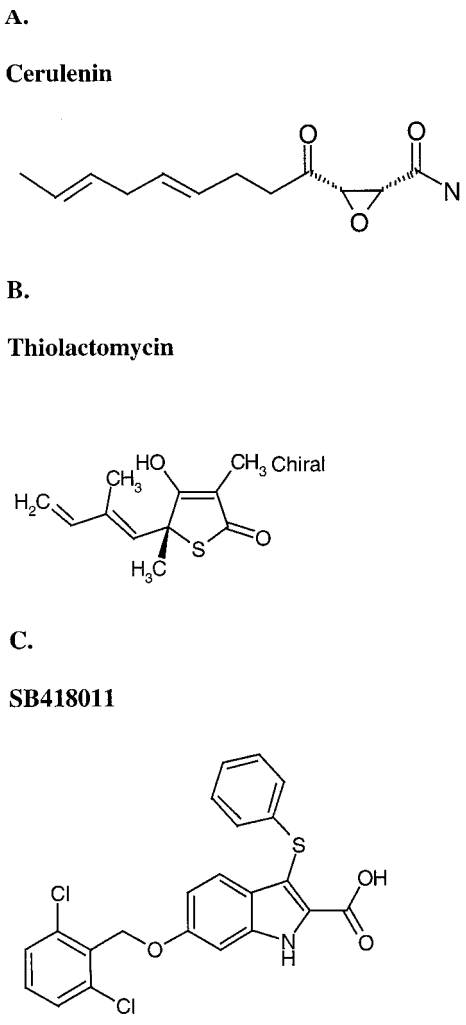

FIG. 4. **Structures of inhibitors used.** *A*, cerulenin; *B*, thiolactomycin (TLM); and *C*, SB418011.

those obtained for *E. coli* FabH based on its crystal structure (39% α-helix, 28% β-sheet; Ref. 16) and indicate the presence of a similar core structure despite the overall low homology between these two enzymes. To determine its stability, GdnHCl denaturation was carried out, monitoring the secondary structure of *S. pneumoniae* FabH using the CD signal at 220 nm as indicator. Purified FabH was diluted into denaturant to a 0.2 mg/ml final concentration at 20 °C and incubated for 10 min before taking measurements. The denaturation midpoint (1.3 M GdnHCl), *m* value (1.14 kcal/mol) and ΔG of unfolding (1.53 kcal/mol) revealed that purified *S. pneumoniae* FabH is moderately stable (Fig. 3*B*) and unfolds in a manner consistent with a single cooperative unfolding transition. In contrast, the

[4] C. Silverman, S. Khandekar, unpublished data.

TABLE IV
Inhibition of S. pneumoniae FabH and its homologues

IC_{50} values were determined as described under "Materials and Methods."

Source	SB418011	TLM	Cerulenin
	μM	μM	μM
S. pneumoniae FabH	0.016 ± 0.003	7.9 ± 1.1	>200
E. coli FabH	1.20 ± 0.40	32.0 ± 12.0	>200
H. influenzae FabH	0.59 ± 0.05	5.8 ± 1.6	>200
Human FAS	>200	>200	45 ± 8

denaturation midpoint (2.1 ± 0.1 M GdnHCl), *m* value (0.55 ± 0.21 kcal/mol) and ΔG of unfolding (1.2 ± 0.6 kcal/mol) revealed that purified *E. coli* FabH unfolds in a substantially less cooperative manner (not shown). These results imply that despite their overall similar core structures, *E. coli* and *S. pneumoniae* FabH have different structural folds. These differences may account for the aggregation of *S. pneumoniae* FabH observed at high concentrations. The crystal structure of *S. pneumoniae* FabH will greatly aid in dissecting the differences.

Enzyme Kinetics—The kinetic constants for acetoacetyl-ACP synthase activity were determined using the filtration assays described under "Materials and Methods." The malonyl-ACP concentration in the FabH filtration assay was estimated to be 25% of the total ACP added based on the percent conversion calculated from the FabD assay alone. Using this method the apparent K_m values for acetyl-CoA and malonyl-ACP were determined to be 40.3 ± 2.3 μM and 18.6 ± 1.5 μM, respectively. The maximum velocity of the reaction was 7.1 nmoles/min/μg. The affinity of acetyl-CoA for *S. pneumoniae* FabH was nearly identical to that determined for *E. coli* FabH (23). In contrast, *S. glaucescens* FabH exhibited higher affinity for acetyl-CoA, with K_m of 2.4 μM.

Substrate Specificity—FabH species purified from Gram-negative and Gram-positive bacteria, despite their overall similar catalytic mechanism, have displayed significantly different substrate specificities. To determine substrate specificity of Gram-positive *S. pneumoniae* FabH, purified protein was assayed spectrophotometrically as described under "Materials and Methods," and the results are summarized in Table II. Purified *S. pneumoniae* FabH was able to utilize short straight- and branched-chain acyl-CoAs. Of the CoA substrates examined, the relative order of activity was determined to be acetyl→butyryl→isobutyryl→isovaleryl. Longer-chain substrate such as, lauroyl-CoA and palmitoyl-CoA, showed no detectable activity in this assay.

The substrate specificity of various FabH molecules are summarized in Table III. *E. coli* FabH preferred short-chain acyl-CoA primers, among which acetyl-CoA was the most preferred substrate (23, 24). The enzyme was inactive with longer-chain (longer than C4) primers as well as all branched-chain CoA primers. The smaller binding pocket observed in the crystal structures of *E. coli* FabH (16, 17) and FabH-CoA complex (16) support this finding. Additionally, observing CoA in the crystal indicated that the reaction product has a notable affinity for FabH (16). The *B. subtilis* FabH homologs, in contrast, were active with 4- to 8-carbon straight-chain acyl-CoAs and all branched-chain acyl-CoAs (24). Similarly, the *S. glaucescens* FabH efficiently utilized both butyryl-CoA and isobutyryl-CoA (25). Interestingly, FabH of *M. tuberculosis* (26) preferred only long-chain (C8-C20) acyl-CoA substrates indicating that its substrate-binding pocket is significantly different from those of other FabH molecules. Based on our results and those summarized in Table III, the binding site pocket of *S. pneumoniae* FabH appears to be more similar to those of *S. glaucescens* and *B. subtilis* FabH than those of *E. coli* and *M. tuberculosis* FabH molecules. Availability of a FabH crystal structure from a Gram-positive organism will be helpful in comparing the substrate binding pockets to account for the differences in the substrate specificity of various primers.

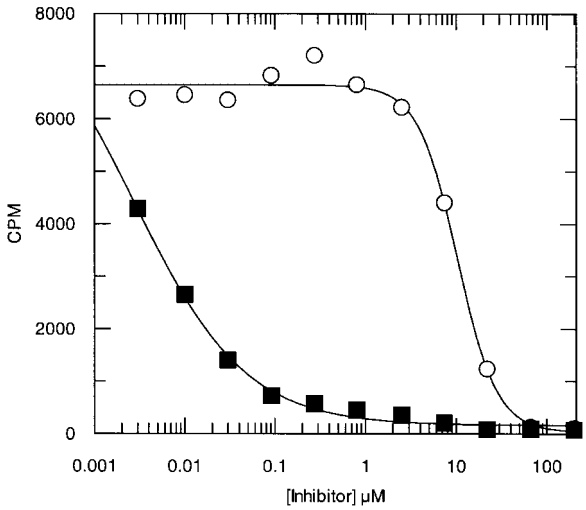

FIG. 5. **Inhibition of *S. pneumoniae* FabH.** *Curves* represent inhibition profiles by thiolactomycin (○) and SB418011 (■). The compounds (n = 4) were assayed (200 μM to 3 nM in Me_2SO) using the filtration assay as described under "Materials and Methods."

The fatty acid composition of bacteria appear to vary from species to species. For example, Gram-negative *E. coli,* in which FabH cannot use branched-chain acyl-CoA primers, does not produce branched-chain fatty acids (30). In contrast, Gram-positive bacilli and streptomycetes, whose FabH components are capable of efficiently utilizing branched-chain acyl-CoAs as substrates, produce significant amounts of odd and even carbon-number branched-chain fatty acids (24, 31). Interestingly, *S. pneumoniae*, also a Gram-positive organism, does not produce significant amounts of branched-chain fatty acid structures (30, 31). In agreement with the fatty acid composition of *S. pneumoniae*, the activity of *S. pneumoniae* FabH with the branched-chain CoAs was significantly weaker than that with the straight-chain CoAs (Table II). Thus, as surmised by Choi *et al*. (24), substrate specificity of the FabH enzymes appears to be the determining factor in the biosynthesis of branched- or straight-chain fatty acids by type II fatty acid synthase.

Enzyme Inhibition—There is considerable potential for selective inhibition of the bacterial FAS by broad-spectrum antibacterial agents. The broad-spectrum antibiotic triclosan (6, 32) inhibits the FabI (enoyl-ACP reductase) component of bacterial FAS; the anti-tuberculosis drug isoniazid may inhibit both InhA and a β-ketoacyl-ACP synthase (7, 8, 33). *E. coli* FabB and F are sensitive to both cerulenin (29, 34) and TLM (14, 34), whereas the human FAS is sensitive to cerulenin but not TLM (9). *E. coli* FabH, unlike the other *E. coli* KAS enzymes, is insensitive to cerulenin and sensitive, albeit weakly, to TLM (12, 35). Likewise, *M. tuberculosis* FabH is inhibited by TLM but not by cerulenin (26). Earlier, TLM was shown to inhibit mycolic acid synthesis in mycobacteria (36) and the FabH of *S. glaucescens* (25).

To extend these studies to purified *S. pneumoniae* FabH and its *E. coli* and *H. influenzae* FabH homologues, conditions were set at K_m concentrations for the substrates as described under "Materials and Methods." The structures of the compounds tested are shown in Fig. 4 and the IC_{50} values are summarized in Table IV. In agreement with the earlier reports (14, 34), *E. coli* FabH in our studies was sensitive, albeit weakly, to TLM with an IC_{50} of 32.0 ± 12.0 μM, and likewise to those studies, cerulenin did not inhibit *E. coli* FabH in our hands (Table IV). In addition, similar to *E. coli* FabH, *H. influenzae* and *S.*

pneumoniae FabH were not inhibited by cerulenin (Table IV). However, *S. pneumoniae* and *H. influenzae* FabH were comparatively more sensitive to TLM with IC_{50} values of 7.9 ± 1.1 μM and 5.8 ± 1.6 μM, respectively.

Recently, crystal structures of *E. coli* FabB-TLM and FabB-cerulenin binary complexes were reported (34). Unlike FabH, which contains a His-Asn-Cys catalytic triad, FabB contains a His-His-Cys triad in its active site. Using site-directed mutagenesis studies, Price *et al.* (34) showed that the two-histidine active site architecture of FabB is critical to cerulenin and TLM binding and inhibition. In our studies, we found that despite the conservation of His-Asn-Cys triad *H. influenzae* and *S. pneumoniae* FabH were 4- to 5-fold more sensitive to TLM than to *E. coli* FabH. It appears that in addition to the catalytic triad, other yet unidentified factors may be responsible for the inhibition of *S. pneumoniae* and *H. influenzae* FabH by TLM.

Given the resurgence of antibiotic resistance in pathogenic bacteria, there is an urgent need for novel broad-spectrum antibacterial agents. In bacteria, FabH appears to play a major role in the regulation of the overall rate of fatty acid biosynthesis (9, 17). Because none of the available small molecule inhibitors of bacterial FAS appear to be potent against FabH, we have initiated the development of high throughput screening assays to identify potent, small molecule broad-spectrum inhibitors FabH. The inhibition profile of one such inhibitor, SB418011 (Fig. 4), against *S. pneumoniae* is shown in Fig. 5, and its IC_{50} values against purified preparations *E. coli*, *H. influenzae,* and *S. pneumoniae* FabH are summarized in Table IV. From Fig. 4 it is apparent that SB418011 is highly potent against *S. pneumoniae* FabH, with an IC_{50} of 0.016 ± 0.003 μM (Table IV). SB418011 appears to be a broad-spectrum inhibitor as it also inhibited *E. coli* and *H. influenzae* FabH with IC_{50} of 1.2 ± 0.40 and 0.59 ± 0.05 μM, respectively (Table IV). Compared with TLM and cerulenin, SB418011 was significantly more potent against all three bacterial species. Importantly, SB418011 was inactive against human FAS with an IC_{50} of >200 μM (Table IV). Together, these results strongly suggest that it is indeed feasible to obtain highly potent and broad-spectrum inhibitor(s) of this physiologically important enzyme target (17, 23).

In conclusion, we have cloned and characterized the *S. pneumoniae* homologue of *E. coli* FabH. The availability of purified *S. pneumoniae* FabH will be of great utility in kinetic and structural studies as well as in developing high throughput screening assays to identify and develop small molecule inhibitors of pathogenic bacteria.

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