The genome of a Bacteroidetes inhabitant of the human gut encodes a structurally distinct enoyl-acyl carrier protein reductase (FabI)

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3The abbreviations used are: FabI, enoyl-(acyl-carrier-protein) reductase; ACP, acyl carrier protein; FabK, enoyl-ACP reductase II; ENR, enoyl-ACP reductase; FASII, bacterial type II fatty acid synthesis; RMSD, root-mean-square deviation; TBLASTN, translated nucleotide databases; InhA, M. tuberculosis enoyl-(acyl-carrier-protein) reductase; MIC, minimum inhibitory concentration

Enoyl-acyl carrier protein reductase (FabI) catalyzes a rate-controlling step in bacterial fatty-acid synthesis and is a target for antibacterial drug development. A phylogenetic analysis shows that FabIs fall into four divergent clades. Members of clades 1–3 have been structurally and biochemically characterized, but the fourth clade found in members of phylum Bacteroidetes is uncharacterized. Here, we identified the unique structure and conformational changes that distinguish clade 4 FabIs. Alistipes finegoldii is a prototypical Bacteroidetes inhabitant of the gut microbiome. We found that A. finegoldii FabI (AfFabI) displays cooperative kinetics and uses NADH as a cofactor, and its crystal structure at 1.72 Å resolution showed that it adopts a Rossmann fold as do other characterized FabIs. It also disclosed a C-terminal extension that forms a helix–helix interaction that links the protomers as a unique feature of AfFabI. An AfFabI•NADH crystal structure at 1.86 Å resolution
revealed that this feature undergoes a large conformational change to participate in covering the NADH-binding pocket and establishing the water channels that connect the active site to the central water well. Progressive deletion of these interactions led to catalytically compromised proteins that fail to bind NADH. This unique conformational change imparted a distinct shape to the AfFabI active site that renders it refractory to a FabI drug that targets clade 1 and 3 pathogens. We conclude that the clade 4 FabI, found in the Bacteroidetes inhabitants of the gut, have several structural features and conformational transitions that distinguish them from other bacterial FabIs.

Introduction

Bacterial fatty acid biosynthesis (FASII) consists of a conserved collection of individual proteins that produce the fatty acids required for membrane phospholipid biogenesis (1). The FASII substrates are linked to acyl carrier protein (ACP) and undergo successive rounds of condensation, reduction, dehydration, and reduction to extend the acyl chain by two carbons with each elongation cycle (Fig. 1A). The final reduction step of each elongation cycle is catalyzed by enoyl-ACP reductase (ENR) (Fig. 1A). There are two widely distributed, distinct ENR protein families, FabI and FabK (2-5). FabI proteins use NAD(P)H as the reductant and belong to the short-chain dehydrogenase superfamily of enzymes that use a Rossmann fold to bind the nucleotide cofactor (6,7). Most short-chain dehydrogenases have an active site dyad consisting of Tyr-Xaaα-Lys, but the bacterial FabI subfamily is distinguished by having a differently spaced catalytic dyad consisting of Tyr-Xaaα-Lys (8,9). FabK is a flavoprotein (FMN) that belongs to the NAD(P)H-dependent flavin oxidoreductase family (4). FabK adopts an overall TIM barrel fold (4,5) and requires NAD(P)H as a reductant (2), but how the NADP(H) and FMN cofactors collaborate in catalysis is not clear (3,5). There are also two FabI-like ENRs (FabL and FabV) with a limited distribution in bacteria (10-14). These two reductases also belong to the short-chain dehydrogenase superfamily but are distantly related to FabI. The function of ENR in the FASII pathway is to pull each cycle of elongation to completion and is the rate-determining step of fatty acid elongation (15).

The role of ENR as a pacemaker of the essential FASII pathway has made it the subject of intense investigation as a target for antibiotic drug discovery (16,17). AFN-1252 (afabicin, Debio1452) is the most clinically advanced of these compounds and is designed to specifically target Staphylococcal FabI (18-21). Most commonly used, broad-spectrum antibiotics not only attack the pathogen but also devastate the commensal gut microbiome leading to multiple complications (22,23). As a pathogen-specific antibiotic (16), AFN-1252 has the desirable property of having no effect on the size or composition of the gut microbiome during therapy (24). Firmicutes and Bacteroidetes are the two most abundant bacterial phyla in the gut microbiome. Most Firmicutes (85%) are in Class Clostridia that encode fabK and not fabI (25), therefore these organisms are expected to be refractory to growth inhibition by FabI-targeted drugs (2,3). The Bacteroidetes occupants of the gut microbiome are more diverse. Some contain only a fabI homolog, some contain only a fabK homolog and some contain both a fabI and fabK (Fig. 1B). This bioinformatic analysis suggests that some commensal Bacteroidetes may be susceptible to FabI-targeted therapeutics. We constructed a phylogenetic tree to understand the evolutionary relationships between the FabIs expressed in bacteria (Fig. 1C). This analysis shows that there are four distinct FabI clades. Representative FabIs from clades 1-3 have been biochemically and structurally characterized. Bacteroidetes/Chlorobi phyla are in the fourth FabI clade suggesting that Bacteroidetes FabI may have unique features not found in structures from the other clades.

The goal of this study was to structurally and functionally characterize the FabI from Alistipes finegoldii (AfFabI), a human Bacteroidetes commensal anaerobe containing a clade 4 FabI. Like other FabIs, AfFabI is a tetramer and adopts a Rossmann fold to bind the nucleotide cofactor. The unique feature of AfFabI is the carboxy-terminal α9 helix that forms a coiled-coil structure with the α9 helix of...
an adjacent protomer to form a protomer-protomer contact that is absent in the other 3 FabI clades. Upon NADH binding, the intertwined α-α helices unravel to form a structured loop that is involved in sealing the lid over the active site of the opposite protomer. Deletion of the unique carboxy-terminal domain results in a folded, but inactive enzyme due to severely compromised NADH affinity. The AfFabI active site uses the same Tyr-Xaa-Lys catalytic dyad as other FabIs, but the surrounding residues create a unique active site environment that renders AfFabI refractory to a FabI therapeutic (AFN-1252) that effectively targets the clade 1 and 3 FabIs.

**Results**

**Bioinformatics**

The distribution of species in Bacteroidetes taxa encoding FabI, FabK, or both was determined by counting the high homology FabI and FabK TBLASTN hits for each unique species entry in the RefSeq Representative Genomes Database (12/22/2019) using the Alistipes FabI and FabK sequences and an e-value cut-off of 1e-100 for each hit. Environmental Bacteroidetes species encode for FabI only, FabK only, or both (not shown). Likewise, the individual species in the five major human-associated Bacteroidetes genera (Alistipes, Bacteroides, Parabacteroides, Porphyromonas, and Prevotella) encode for one of the three possible combinations of FabI and FabK (Fig. 1B). Prevotella, a prominent genus of the gut microbiome, had the highest number of species that encode only a FabI, but all three possible combinations were found in the individual species from all the commensal Bacteroidetes genera. A phylogenetic analysis was conducted to understand the evolutionary relationships between the FabIs (Fig. 1C). Clade 1 FabIs are encoded in the Proteobacteria and Firmicute phyla and are represented by the prototypical E. coli (26) and S. aureus (27) crystal structures. Clade 2 are the Mycobacterial enzymes typified by the structure of InhA of Mycobacterium tuberculosis (28). Clade 3 FabIs consist of the plastid FabIs and include the structurally characterized FabIs of Chlamydia trachomatis (29) and Plasmodium falciparum (30). The FabI sequences from the closely related Bacteroidetes and Chlorobi phyla form a distinct fourth clade (Fig. 1C). The phylogenetic relationship between the various Bacteroidetes/Chlorobi FabIs sequences is in overall agreement with the phylogenetic relationships between the organisms based on their 16S RNA sequences (31). These data show that Bacteroidetes/Chlorobi clade 4 FabIs have a divergent evolutionary history from the other clades suggesting that there may be structural and/or functional differences between clade 4 FabIs and the other three characterized clades.

**A. finegoldii FabI and FabK**

We selected *A. finegoldii* as a prototypical gut commensal bacterium to examine the properties of the clade 4 FabIs. *A. finegoldii* is predicted to have both a FabI and FabK. *A. finegoldii* FabI (AfFabI) (Uniprot ID: A0A174E195) has 32% identity with *Neisseria gonorrhoeae* FabI. The *A. finegoldii* FabK (AfFabK) (Uniprot ID: I3Y165) has 42% identity with *Streptococcus pneumoniae* FabK and contains the signature FMN-binding motif. The ENR functions of AfFabI and AfFabK were verified by determining whether plasmids directing their synthesis would complement *E. coli* strain JP1111 (fabI(Ts)) (Fig. 2A & 2B). The positive controls for the experiment were strain JP1111 carrying plasmids expressing *E. coli* FabI (EcFabI), *C. trachomatis* FabI (CtFabI), and *C. acetobutylicum* FabK (CaFabK), and the negative control was the expression vector lacking a gene insert. All strains grew at the permissive temperature (30°C) (Fig. 2A). Strain JP1111 containing the empty vector failed to grow at the non-permissive temperature (42°C) (Fig. 2B). These data show that *A. finegoldii* encodes both a FabI and FabK ENR as predicted from the bioinformatic analyses.

AFN-1252 is a FabI inhibitor that is known to inhibit clade 1 and clade 3 FabIs (29,32,33). The sensitivity of AfFabI (clade 4) to AFN-1252 was compared to other FabIs using *E. coli* strain ANS1 (ΔtolC) expressing a series of FabIs as previously described (33). Strain ANS1 was transformed with a series of plasmids expressing different FabI and FabK
enzymes and the AFN-1252 minimal inhibitory concentrations were determined using a microbroth dilution assay (Fig. 2C). Growth of strains ANS1/pEcFabI (clade 1) and ANS1/pCFFabI (clade 3) were both inhibited by AFN-1252 whereas all strains expressing a FabK were refractory to AFN-1252 growth inhibition. A key result was that strain ANS1/pAFFabI was refractory to AFN-1252 inhibition indicating that clade 4 Fabls may have an active site that is distinct from the clade 1 and clade 3 proteins.

**Biochemical properties of AFFabI**

An amino-terminal His-tagged version of AFFabI was expressed in *E. coli* and purified by affinity and gel filtration chromatography to obtain a homogeneous 34 kDa protein based on SDS gel electrophoresis (Fig. 3A, inset). AFFabI eluted as a single species on the calibrated XBridge BEH SEC column (Fig. 3A). Its Stokes radius was consistent with a molecular weight of 145 kDa (Fig. 3A, inset) indicating that like other characterized Fabl proteins AF FabI exists as a tetramer (theoretical molecular weight 136 kDa) (7). Sedimentation velocity analysis confirmed AFFabI exists as a homotetramer in solution with an S20 value of 6.59 S corresponding to 141 kDa protein (Fig. 3B). AFFabI exhibited high-affinity, cooperative binding to NADH. The Kd was estimated by surface plasmon resonance to be 225 nM with a Hill coefficient of 1.6 (Fig. 3C). The AF FabI affinity for its reaction product NAD+ was estimated in similar surface plasmon resonance experiments to be orders of magnitude lower (Kd = 1.623 ± 0.008 mM) than for NADH (not shown). NADH increased the stability of AFFabI to thermal denaturation by 7°C (Fig. 3D). These data indicated that NADH binding results in a more stable protein structure.

AF FabI enzymatic activity of NADH oxidation to NAD+ was monitored by spectrophotometry at 340 nm. AFFabI catalyzed the oxidation of NADH to NAD+ in the presence of crotonyl-CoA (Fig. 4A). The NADH apparent K0.5 was 22.3 ± 4.6 μM at 1.25 mM crotonyl-CoA and NADH exhibited positive cooperative behavior with a Hill number of 2.4 ± 0.2 (Fig. 4B). NADPH did not support the reaction (Fig. 4A, inset). Cooperative binding of the reduced nicotinamide substrate is characteristic of Fabls and has been studied in detail with *S. aureus* FabI (34). The crotonyl-CoA K0.5 was 457 ± 45 μM and exhibited some cooperative behavior with a Hill number of 1.5 (Fig. 4C). Mass spectrometry analysis confirmed that butyryl-CoA was the product in the enzymatic reaction (Fig. 4C, inset). These data demonstrate AF FabI catalyzes the FASII ENR reaction and that AF FabI exhibits kinetic properties that are like other characterized Fabl enzymes. Although triclosan inhibited AF FabI with an IC50 of 1.76 ± 0.57 μM, which was similar to triclosan inhibition of EcFabI (35), AF FabI was refractory to AFN-1252 inhibition (Fig. 4D) suggesting its active site was significantly different than the active sites in the clade 1 and clade 3 Fabls that are sensitive to this drug.

**The AF FabI crystal structure**

The 1.72 Å AF FabI structure was refined to R/Rfree 20.6/24.5 (Table 1). Two protomers were in the asymmetric unit and are similar to each other with a Cα RMSD of 0.34 Å across 260 residues. The protomers interact with two symmetry-related protomers around a crystallographic two-fold axis to create the tetramer. The presence of tetramers in the crystal is consistent with the gel filtration and sedimentation experiments. The unique AF FabI protomer feature is α9, a carboxy-terminal helix that protrudes from the α/β core structure that is absent in the clade 1-3 enzymes (Fig. 5A). Structural alignment of AF FabI protomer (lacking α9) with EcFabI (PDB ID: 5CFZ) (36) showed the two structures are near identical with a Cα RMSD of 1.48 Å across 448 residues. Like the clade 1-3 Fabls (7), the AF FabI protomer consists of a seven-stranded parallel β-sheet flanked by three α-helices on either side that adopts a Rossman fold for dinucleotide substrate binding (Fig. 5A). The signature Tyr-(Xaa)6-Lys FabI catalytic dyad places the active site in the same location as other Fabls (8,9). FabI is a dimer of dimers with three molecular two-fold noncrystallographic symmetry axes (P, Q, and R) along the subunit interfaces (37-39) (Fig. 5B). Each protomer has a total surface area of ~13,400 Å², and ~4,800 Å² (~36%) is buried upon
tetramerization. These values are similar to other FabIs (28,36,40-42). There are two disordered regions in the structure. The residues constituting the putative α6 active site lid (T197 to G209) were expected to be absent from the model because the flexible lid is only resolved in FabI•NAD(P)H binary complexes in other FabI clades (37,43,44). The second unstructured region is the final 13 amino acids of the carboxy-terminus.

FabI tetramers are stabilized by extensive intermolecular interactions along the protomer interfaces with conserved dimerization and tetramerization interfaces (37,38,45). Like clade 1-3 FabIs, AfFabI dimerization occurs along the P axis interface and consists of interacting helices α8 and strands β7 linking the A/B or C/D protomers (Fig. 6A). The AfFabI tetramerization domain links protomers along the Q axis interface and consists of an antiparallel four-helical bundle composed of interacting helices α4 (kinked) and α5 linking the A/C or B/D protomers (Fig. 6B). AfFabI α9-α9 interaction domain is a unique feature of clade 4 FabIs and links the A/D or B/C protomers along the R axis (Fig. 6C). Helix α9 extends from S261 to E275 and is amphipathic with the hydrophobic side chains forming the α9-α9 contact interface and the polar side chains extending into solvent.

The AfFabI•NADH binary complex

The 1.86 Å AfFabI•NADH complex structure was refined to R/Rfree 15.8/19.7 (Table 1). The asymmetric unit contained one homotetramer. The protomers are nearly identical with a Cα RMSD of 0.13 Å across 282 residues and the NADH molecules were well resolved in all four active sites. The planarity of the cofactor carboxamide relative to its attached pyridine ring is indicative of the NADH redox state (46). The C2N-C3N-C7N-O7N torsion angles in the NADH molecules were between 150.8° and 162.5° and are consistent with reduced NADH being present in the crystals. NADH binding increases the overall protomer surface area to ~15,000 Å², and the buried surface area of the subunit interfaces to ~5,400 Å² (~36%). The active site lid containing α6 was not resolved in the AfFabI structure but was clearly seen in the AfFabI•NADH electron density map. The lid forms several hydrogen bond interactions with the pyrophosphate and nicotinamide of NADH (Fig. 7A). Nucleotide binding proteins often stabilize pyrophosphate binding using a positive helix dipole (47). Helix α1 creates this helix dipole in AfFabI and hydrogen bonds between NADH and lid residues T199 side chain and A201 backbone amide fix the cofactor in space. K167 on α5 is part of the Tyr-Xaa6-Lys catalytic dyad and donates two hydrogen bonds to the nicotinamide ribose to position and stabilize the nicotinamide (Fig. 7A). In addition, the nicotinamide moiety is also stabilized by hydrogen bonds that form between lid residue T197 and the nicotinamide ring. The adenine moiety sits in a solvent-exposed hydrophobic pocket in the active site cavity. Hydrogen bonds between the adenine nitrogen N1 and A67 backbone carbonyl, and the adenine ribose and N41 and S45 side chains orient the adenine moiety in this cavity. The locations and interactions between AfFabI and NADH are very similar to other FabI proteins (48-51).

The NADH-induced conformational change creates four water-filled channels that connect the active sites to a central water well located in the core of the tetramer (Fig. 7B). These water channels enable efficient proton transfer to the active sites for catalysis (52). The resolution of the AfFabI•NADH crystal structure enabled the visualization of ordered water molecules within the individual channels making hydrogen bond interactions with the peptide backbone (Fig. 7C). The individual water channels exit the active sites, narrow into tunnels that are formed by residues along the R axis of the promoter interfaces and empty into the water well at the center of the protein (Fig. 7C). The active site cavity is hydrated in the AfFabI crystal structure, and few ordered water molecules are detected because the water channels are only partially formed and the active site lid is open to solvent. As with other FabIs (52), the lid isolates the active site from bulk solvent to create an environment that promotes hydride transfer from NADH.

Helix α9 and the carboxy terminus

A unique feature of AfFabI is how the clade 4-specific α9 helix and disordered carboxy-
terminal tail participate in the conformational change required for NADH binding (Fig. 8). In the example shown, helix $\alpha_9^C$ and $\alpha_9^B$ intertwine to connect the C/B partner protomers in AfFabl and the 13 residues of the protein attached to carboxy-terminus of $\alpha_9$ are disordered (Fig. 8A). In the AfFabl•NADH structure, the interacting $\alpha_9$ helices unwind, and along with 10 of the 13 disordered carboxy-terminal amino acids (V277 to E286), form an ordered loop feature that extends to the active site lid of the partner protomer (Fig. 8B). The amino terminus of $\alpha_7^A$ lies at the base of the $\alpha_9$-$\alpha_9$ interaction domain and forms the corner of the active site cavity. The conformational change relocates the C$\alpha$ chain of $\alpha_9$ up to 20 Å toward the opposite active site and drives the formation of a new protomer-protomer interaction interface that closes the active site and creates the water channels.

The active site is a large solvent-exposed crevice in the AfFabl structure (Fig. 9A). In this conformation, N213$^C$ and N217$^C$ from the last two turns of $\alpha_7^C$ form hydrogen bonds with Y268$^B$ from $\alpha_9^B$. Upon NADH binding, the lid consisting of $\alpha_6$ and adjacent residues closes over NADH to convert the solvent exposed crevice into an enclosed active site (Fig. 9B). D210$^C$ from $\alpha_7^C$ forms a hydrogen bond with T267$^B$ from $\alpha_9^B$ (Fig. 9B) as the unwound $\alpha_9^B$ residues slide along $\alpha_7^C$. Lid closure leaves the adenine moiety surface-exposed and buries the pyrophosphate and nicotinamide regions of NADH. The solvent accessible surface area of the active site crevice decreases from ~500 Å$^2$ to ~300 Å$^2$. Every residue in the first turn of $\alpha_9$ in AfFabl forms a new hydrogen bond interaction in the AfFabl•NADH complex (Fig. 9C). The R262$^B$ backbone amide nitrogen forms a hydrogen bond interaction with the Y179$^A$ side chain from $\alpha_5^A$ in the tetramerization domain. The R263$^B$ side chain forms hydrogen bond interactions with backbone carbonyls from S219$^B$, E216$^B$, and N217$^B$ from $\alpha_7^B$ in the corner of the protomer B active site. The A264$^B$ backbone amide forms a hydrogen bond interaction with the S261$^B$ side chain at the base of $\alpha_9^B$, and the backbone carbonyls from R263$^B$ and M265$^B$ form hydrogen bond interactions with the K266$^B$ side chain from $\alpha_9^C$. Two residues in the second turn of $\alpha_9$ form new hydrogen bond interactions in AfFabl•NADH. In addition to the T267$^B$-D210$^C$ interaction shown in Fig. 9B, the side chain of K266$^B$ forms hydrogen bonds with R263$^C$ and M265$^C$ from $\alpha_9^C$ (Fig. 9D). There are no protein-protein interactions formed between the loop residues derived from the third turn of $\alpha_9$ in AfFabl and the protomers in the AfFabl•NADH structure. This loop extends away from the surface as seen in Fig. 8B.

Ten carboxy-terminal disordered residues after $\alpha_9^B$ in the AfFabl structure become ordered to create a channel connecting the active site to the water well in the AfFabl•NADH structure (Fig. 9E). These interactions form ‘latches’ along the R axis that create the surface enclosing a water channel. The first latch is formed by E275$^B$ backbone amide and D276$^B$ side chain hydrogen bonds to R183$^A$ and Y158$^C$ side chains (Fig. 9E). D276$^B$ is oriented to coordinate a water molecule in the channel. R262$^B$ in the first turn of $\alpha_9^B$ forms a planar stacking interaction (53) between the guanidinium group in the side chain and the aromatic ring of Y158$^C$. A van der Waals contact distance is formed between lid residue L206$^C$ side chain and E275$^B$ backbone carbonyl. The second latch is formed by H278$^B$ backbone amide hydrogen bonding to the T106$^D$ and D108$^D$ side chains, and Q279$^B$ backbone carbonyl and side chain hydrogen bonds to the R134$^A$ backbone carbonyl and side chain (Fig. 9E). A van der Waals contact is formed between the V277$^C$ and I180$^A$ side chains (not shown in Figure 9). The molecular latches convert a region of the tetramer that freely exchanges with bulk solvent in AfFabl into an enclosed channel that connects the active site with the central water well in AfFabl•NADH. These data indicate that the conformational changes associated with $\alpha_9$ and the carboxy terminus are important for catalysis because they participate in the formation of the catalytic center and the water channel that connects the active site to the water well.

**Function of the carboxy-terminus**

We constructed a series of truncation mutants to test the role of $\alpha_9$ and the disordered carboxy terminus in catalysis. All the truncated mutant enzymes had similar
turnover numbers \((V_{\text{max}}/[E_1])\) but had reduced catalytic efficiencies \((V_{\text{max}}/[E_1]/K_{0.5})\) that were driven by defects in the apparent \(K_{0.5}\) for NADH (Table 2). \(Af\)FabI(1-273) lacked the disordered carboxy-terminal residues in the \(Af\)FabI structure and eliminated the interactions contributing to sealing the water channels in the \(Af\)FabI•NADH structure illustrated in Fig. 9E. \(Af\)FabI(1-273) was a tetramer that was stabilized by NADH, but the catalytic efficiency of the enzyme was reduced by 3-fold driven by an increase in the apparent \(K_{0.5}\) for NADH (Table 2). Although \(Af\)FabI(1-273) was catalytically compromised, it was able to complement the growth of the \(fabl(Ts)\) mutant when overexpressed. \(Af\)FabI(1-269) removed four residues (1 turn) from \(\alpha9\) in the \(Af\)FabI structure. This region did not form any specific protein contacts in the \(Af\)FabI•NADH structure because it loops away from the protein and the side chains point toward solvent (Fig. 8B, arrows). Thus, the biochemical properties of \(Af\)FabI(1-269) were similar to the properties of \(Af\)FabI(1-273). The \(Af\)FabI(1-265) truncation removed the second turn of the \(\alpha9\) helix and the important interactions depicted in Figs. 9B and 9D. These two interactions are key to closing one side of the lid that lays over the active site in the \(Af\)FabI•NADH structure and would be predicted to compromise NADH binding. \(Af\)FabI(1-265) was a thermally stable tetramer but was not stabilized by the addition of NADH (Table 2). The apparent \(K_{0.5}\) for NADH increased 6-fold and the catalytic efficiency dropped by 5.5-fold leading to a protein that could not complement the \(fabl(Ts)\) mutant even when overexpressed (Table 2). \(Af\)FabI(1-261) removed the remaining turn of the \(\alpha9\) helix and the multiple interactions depicted in Fig. 9C. These interactions connect protomers A and C with protomer B and form a junction with the NADH lid. \(Af\)FabI(1-261) reduces \(Af\)FabI to the core clade 1 structure. Like clade 1 enzymes it is stable and cooperative but had a 23-fold increase in the NADH apparent \(K_{0.5}\) and was 22.5-fold less catalytically efficient (Table 2). These data show the importance of \(\alpha9\) and the carboxy terminus in creating the substrate binding lid and the water channels required for efficient catalysis.

Discussion

This work defines the function of the structural features that are unique to clade 4 Fabls. The four clades of bacterial Fabls diverged from their common ancestor in the distant past and each have a long and unique evolutionary history. All Fabls have common features like a Rossmann fold, cofactor-induced conformational change, Tyr-Xaa-Lys catalytic dyad, and dimerization and tetramerization interfaces. These basic structural features are embodied in the prototypical clade 1 EcFabl structure depicted in Fig. 10 (Upper panel). Using the clade 1 enzymes as the prototypical comparator, each of the other three clades possesses a unique structural feature. clade 2 Fabls are the Mycobacterium InhA enzymes that have a deeper active site crevice and longer \(\alpha6\) lid than the other Fabl clades. These features are thought to enable InhA to bind very long-chain enoyl-ACP substrates for the synthesis of cell wall mycolic acids (28,54). Clade 3 Fabls have an insertion between \(\beta3\) and \(\alpha3\) that creates peripheral loops that are related by the Q axis interface. A functional or structural role is yet to be described for this feature in the plastid Fabls. Clade 4 Fabls have an extension of the carboxy-terminus that provides a new protomer-protomer interaction surface that links protomers along the R axis. This study shows that the carboxy terminal extension is critical for catalysis in clade 4 Fabls by participating in the conformational change that creates the NADH binding pockets and water channels that feed the active site. This unique feature is present in all the Bacteroidetes Fabls and has evolved into a domain that is essential for catalysis in clade 4 enzymes.

Further our data explains why a potent antimicrobial drug that targets the Fabl of clade 1 and 3 pathogens does not impact a major constituent of the microbiome. AFN-1252 is a S. aureus selective (Fig. 10, Lower panel) Fabl inhibitor that was developed as a pathogen-specific drug (16,17). Treatment of mice with AFN-1252 does not perturb the Bacteroidetes phylum in the gut microbiome, whereas broad-spectrum antibiotics devastate the microbiome (24). Bioinformatic analysis of the microbiome enoyl-ACP reductase distribution indicates the presence of FabK in the Clostridia and
commensal Bacteroidetes, and the expression of FabK would account for the resistance of organisms to AFN-1252. However, many Bacteroidetes genera only express a FabI (Fig 1B). Biochemical analysis shows that clade 4 FabIs are resistant to AFN-1252 explaining why Bacteroidetes genera are not impacted by AFN-1252 therapy. AFN-1252 is a rigid small molecule that contains an oxotetrahydrodronaphthyridine and 3-methylbenzofuran moiety connected by a cis-amide. All three components of the drug form interactions with the protein in AFN-1252-sensitive FabIs in clades 1 and 3 (19,29). The AffabI•NADH crystal structure shows that clade 4 FabIs have a differently shaped active site cavity than clade 1 and 3 enzymes (Fig 10, Lower panel). In AffabI, the lid residue V205 side chain occupies the space where the 3-methylbenzofuran moiety should reside and the R102 side chain sterically clashes with the oxotetrahydrodronaphthyridine moiety. Similarly, modeling AFN-1252 in the clade 2 active site reveals a steric clash with I202. These considerations suggest that FabI-targeted drugs against clade 1 or 3 pathogens would have the benefit of having little to no impact on the gut microbiome sparing patients the complications that arise from current therapies that destroy the microbiome (55,56).

Experimental procedures
Materials
All chemicals and reagents were obtained from Sigma-Aldrich or Fisher unless otherwise indicated.

Phylogeny
Predicted FabI sequences were collected from the NCBI Reference Sequence Database to include characterized FabI sequences and their homologs. Maximum likelihood phylogenetic trees were constructed using the DECIPHER and phangorn packages in R (57,58). Briefly, sequences were aligned using the AlignSeqs and StaggerAlignment functions in DECIPHER. The LG + (4) + I model (59) was best fitting by the Bayesian information criteria from the model. Test function in phangorn evaluating the WAG, JTT, LG, and Dayhoff amino acid replacement matrix models with and without gamma distributed rate variation among sites [Γ] and invariant sites [I]). The initial neighbor-joining tree was constructed using the distance matrix, with the maximum likelihood tree generated from the neighbor-joining tree using the LG + Γ (4) + I model with stochastic branch rearrangement. Bootstrap method of 1000 replicate trees was used to determine the confidence of the tree topology. The maximum likelihood tree was visualized using ggtree (60).

Molecular biology
The predicted A. finegoldii FabI and FabK have been previously identified (61). Representative predicted A. finegoldii genes AffabI (Uniprot ID: A0A174E195) and AffabK (Uniprot ID: I3Y165) were optimized for gene expression in E. coli using GeneArt Gene Synthesis Technology (Life Technologies, Inc.). An NdeI restriction site was engineered at the 5'-end of the gene with the start codon in the Ndel site, whereas a stop codon and an EcoRI restriction site were sequentially engineered at the 3'-end of the gene. The genes were cloned into the pPJ131 plasmid (a modified version of the pBluescript plasmid with the multiple cloning site from pET28a) via the Ndel and EcoRI (New England Biolabs) restriction sites (29). To create the Affabl carboxy-terminal truncation mutant constructs, premature stop codons were engineered into the gene using the QuikChange II Site-directed mutagenesis kit (Agilent) and primers 5'-CAAGCATGGGTATGTCCATGATGACTCGT GCAATGAAAAC, 5'-TGTCACTGTCGTAATGTGATGAAAACC TATGAAAAGG, 5'-CAATGAAAAACCCTATGATGAAAAAGGTA TGCCTTTGA, and 5'-ATGAAAAAGGTATGCGCTGATGATTGAAA GATGTCACCA and their reverse complements. The amino acid numbering convention used assumes the initiating methionine of the native protein as residue 1. For protein purification, the Affabl constructs were cloned into the pET28b plasmid via the NcoI and EcoRI (New England Biolabs) restriction sites.

Complementation assay
The AffabK amino acid sequence contains an FMN-binding motif (5); the Affabl does not.
The trans-2-enoyl-ACP reductase function of AfFabI and AfFabK was confirmed using the previously established complementation method (3,29). Expression plasmids were constructed to overexpress nothing (negative control), E. coli FabI (EcFabI), C. trachomatis FabI (CtFabI), AfFabI, and its truncation mutants, C. acetobutylicum FabK (CaFabK), or A. finegoldii FabK (AfFabK) and then transformed into E. coli strain JP1111 (fabl(Ts)) and plated at 30°C on LB plates containing 100 \( \mu \)g/ml carbenicillin. Individual colonies were streaked on plates containing 100 \( \mu \)g/ml carbenicillin and incubated at 30°C and 42°C. Carbenicillin stress slows growth at 42°C. Strain JP1111 is nonviable at 42°C unless complemented with a functional trans-2-enoyl-ACP reductase, such as EcFabI or CaFabK that serve as positive controls.

**Minimum inhibitory concentration (MIC) analysis**

The MICs for AFN-1252 and triclosan against E. coli strain ANS1 ( ΔtolC) were determined using a broth microdilution method as described previously (62). ANS1 is a tolC knockout mutant that is used to eliminate the contribution of type 1 secretion systems to drug resistance (63). Briefly, pPJ131 expression plasmids containing nothing, EcFabI, CtFabI, AfFabI, CaFabK or AfFabK were transformed into ANS1, and these strains were grown to OD\(_{600}\)=0.6 in LB before being backdiluted 1:30,000 in 1% DMSO in 1% tryptone media. Diluted cells (100 \( \mu \)l) were added to each well of a U-bottom 96-well plate except the first column of wells. Diluted cells (200 \( \mu \)l) were mixed with 50 \( \mu \)M AFN-1252 or 50 \( \mu \)M triclosan was added to the first column of wells, and 100 \( \mu \)l was serially diluted through the plate excluding the last column of wells leaving 100 \( \mu \)l of cells in each well with the appropriate concentration of compound. The plate was incubated at 37°C for 24 hours and then read at 600 nm using a SPECTRAmax 340PC Microplate Reader. Cells from each strain grown with 0 \( \mu \)M compound were used as a reference (i.e., 100% fractional growth).

**AfFabI protein expression and purification**

BL21 (DE3) cells harboring the pET-AfFabI plasmid were grown in LB medium with 100 \( \mu \)g/ml carbenicillin at 37°C and 200 rpm shaking to OD\(_{600}\)=0.6. The culture was cooled to 16°C then induced with 1 mM isopropyl-1-thio-\( \beta \)-galactopyranoside overnight. Cells were centrifuged and pellets were resuspended in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM imidazole (30 ml/l culture). Cells were lysed via a cell disruptor and the amino-terminal His\(_6\)-tagged AfFabI was purified by standard nickel chelation chromatography (29). The protein was then gel filtered into 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM EDTA using a preparative Superdex 200 column with dimensions 16 mm x 60 cm. The AfFabI molecular weight was estimated using an analytical XBridge BEH SEC 200 Å 3.5 \( \mu \)m column with dimensions 7.8 mm x 150 mm. Approximately 26 mg of purified AfFabI was obtained per liter of culture. The same methods were used to overexpress and purify AfFabI mutant proteins.

**Analytical ultracentrifugation**

Sedimentation velocity experiments were conducted in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Indianapolis, IN) following standard protocols unless mentioned otherwise (64,65). Samples in buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM EDTA were loaded into cell assemblies comprised of double sector charcoal-filled centerpieces with a 12 mm path length and sapphire windows. The cell assemblies, containing identical sample and reference buffer volumes of 390 \( \mu \)l, were placed in a rotor and incubated at 20°C for 2 hours before being accelerated from 0 to 50,000 rpm. Rayleigh interference optical data as well as absorbance data at 280 nm were collected at 1-min intervals for 12 hours. The velocity data were modeled with diffusion-deconvoluted sedimentation coefficient distributions c(s) in SEDFIT (sedfit.sedphat.nibib.nih.gov) using algebraic noise decomposition and with signal-average frictional ratio and meniscus position refined with non-linear regression (66). The s-value was corrected for time and finite acceleration of the rotor was accounted for in the evaluation of Lamm equation solutions (67). Maximum entropy regularization was applied at
a confidence level of $p = 0.68$. The partial specific volumes of the proteins, based on its amino acid composition, was calculated in SEDFIT.

**Enzymology**

The $\text{AfFabI}$ enzymatic activity was determined by measuring the conversion of NADH to NAD$^+$ at 340 nm. The enzyme reactions were 100 µl in volume and monitored in Costar UV half-area 96-well plates with a SPECTRAmax 340PC instrument taking 340-nm readings at 10-s intervals at 30°C. In these experiments, enzyme concentrations are reported relative to monomer concentration. The velocity of the $\text{AfFabI}$ enzyme (100 nM) was measured by adding 1.25 mM crotonyl-CoA and 100 µM NADH in 20 mM Tris-HCl, pH 8.0. The apparent $K_{0.5}$ of crotonyl-CoA was determined by adding 100 nM $\text{AfFabI}$ to 200 µM NADH and 0, 0.05, 0.1, 0.25, 0.5, 1, 1.5, 2, or 2.5 mM crotonyl-CoA. The apparent $K_{0.5}$ of NADH was determined by adding 100 nM $\text{AfFabI}$ to 1.25 mM crotonyl-CoA and 0, 5, 10, 15, 20, 30, 40, 50, or 70 µM NADH. The reactions were mixed for 10 s by the mix function on the plate reader, and data were acquired at 10 s intervals for 30 min. Initial velocity was calculated from the linear phase of the progress curve and fit using the Hill equation to determine the apparent $K_{0.5}$ (68). The $IC_{50}$ of AFN-1252 and triclosan were measured above saturating substrate concentrations (100 nM $\text{AfFabI}$, 1.25 mM crotonyl-CoA, and 200 µM NADH in 20 mM Tris-HCl, pH 8.0) against 0, 0.15, 0.31, 0.62, 1.25, 2.5, 5, and 10 µM compound. Initial velocities comparing $\text{AfFabI}$ full length and truncated proteins were determined using a Waters e2695 Separations Module high performance liquid chromatography system. Protein (100 nM) was added to 1.25 mM crotonyl-CoA and 0, 5, 10, 15, 20, 30, 40, 50, 60, 125, 250, 500, 1000, 2000, 4000 µM NADH in 20 mM Tris-HCl, pH 8.0. The enzyme reactions were 40 µl in volume and incubated for 10 min at 30°C. Reactions were stopped by adding equal volume methanol and applied to a Gemini C18, 3 µm 100 Å, 4.6 mm x 150 mm column at a 0.5 ml/min flow rate. Butyryl-CoA product was separated from crotonyl-CoA substrate with the following program and monitored with a Waters 2489 UV/Vis detector at A$_{280}$. Buffer A is 50 mM KH$_2$PO$_4$, pH 4.6. Buffer B is acetonitrile. The program was: starting solvent mixture of 10% B, 0 to 2 min isocratic with 10% B, 2 to 6 min linear gradient from 10% to 15% B, 6 to 18 min concave gradient from 15% to 60% B, 18 to 23 min isocratic with 60% B, 23 to 25 min linear gradient from 60% to 10% B, and 25 to 30 min isocratic with 10% B. All kinetic experiments were run in triplicate.

**Surface plasmon resonance (SPR) experiments**

SPR experiments were conducted at 20°C using a ForteBio Pioneer optical biosensor (ForteBio). Poly-His tagged $\text{AfFabI}$ constructs were immobilized on polycarboxylate hydrogel-coated gold chips preimmobilized with nitrioltriacetic acid (HisCap chips; ForteBio). The chip was primed in chelating buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 µM EDTA, 0.005% Tween 20) and was preconditioned at 10 µl/min with three 60s injections of wash buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.05% Tween 20) and one 60s injection of chelating buffer before being charged with a 60s injection of 500 µM NiCl$_2$ in chelating buffer. After priming into binding buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.01% Brij-35, 5% DMSO), Fabs were injected until ~1500-1700 RU of protein were captured. One flow cell on the chip was charged with Ni$^{2+}$ without adding protein to be used as a reference cell.

NADH was prepared in binding buffer as a two-fold dilution series with maximum concentration of 4 µM for binding to $\text{AfFabI}$ and was injected in triplicate for each concentration at a flow rate of 30 µl/min. A series of buffer-only (blank) injections was included throughout the experiment to account for instrumental noise. NADH fully dissociated from the protein surfaces, eliminating the need for a regeneration step. The data were processed, double-referenced, microcalibrated and analyzed using the software package Qdat (version 4.3.1.2, ForteBio). Equilibrium binding levels were determined and exported to GraphPad Prism for fitting to the Hill equation.

**Protein thermal shift assays**
Protein thermal shift analysis was conducted to investigate if NADH enhanced the thermal stability of the AfFabI constructs. Solutions (30 μL) of AfFabI (10 μM) and AfFabII (10 μM) + NADH (100 μM) in 20 μM HEPES, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, and 2.5x Sypro Orange Dye were added to wells of ThermoGrid optically clear PCR plates (Denville Scientific). The plates were centrifuged at 1000xg for 5 min and then analyzed by the ABI 7300 real-time PCR system as described previously (29). The temperature was ramped from 25°C to 95°C at 1°C/min with the fluorescence read six times at each temperature ramp. The resulting data were fit to a Boltzmann sigmoidal equation to determine the melting point of each AfFab construct with and without substrate. Each enzyme or enzyme and substrate condition was replicated six times, and the thermal melting point of each replicate was determined independently. The melting points from each replicate were averaged to determine the reported thermal melting point. A representative thermal shift experiment and average thermal melting points from triplicate experiments are shown.

Mass spectrometry analysis of the AfFabI enzyme reaction

Samples were diluted with an equal volume of 80% acetonitrile + 15 mM ammonium hydroxide and were analyzed by direct injection to a QTRap 4500 equipped with a Turbo V ion source (Sciex). The QTRap 4500 was operated in the positive mode using neutral loss scanning, and the ion source parameters were: ion spray voltage, 5000 V; curtain gas, 15 psi; temperature, 275°C; collision gas, medium; ion source gas 1, 15 psi; ion source gas 2, 20 psi; neutral loss, 507.0 m/z; declustering potential, 50 V; and collision energy, 40 V. The system was controlled and analyzed by the Analyst® software (Sciex).

Crystallization and structure determination

The AfFabI protein was concentrated to 12 mg/ml for crystallization. Initial screening was performed at 20°C against the Protein Complex Suite (Qiagen) by hanging drop vapor diffusion method combining 300 nl protein and 200 nl precipitant. Diffraction quality crystals were obtained by combining 1.5 μl protein and 1 μl 1.2 M sodium potassium tartrate and 100 mM Tris-HCl, pH 8.0. Crystals were cryo-protected with 1.3 M sodium potassium tartrate, 100 mM Tris-HCl, pH 8.0, and 20% v/v glycerol, and then flash-frozen in liquid nitrogen for X-ray diffraction experiments. The diffraction datasets were collected at the SER-CAT beamline 22-ID at the Advanced Photon Source and processed using HKL2000 (69). The AfFabI structure was solved by the molecular replacement method using the program Phaser (70) and the coordinates of FabI from Aquifex aeolicus (PDB ID: 2P91) (71) as the search model. The AfFabI•NADH complex was achieved by incubating 5 mg/ml (150 μM) AfFabI with 500 μM NADH for 2 hours at room temperature prior to crystallization. Crystals were grown by hanging drop vapor diffusion by combining 1.5 μl of the protein•substrate mixture and 1.5 μl 12.5% PEG 1000, 200 mM NaCl and 100 mM MES pH 6.0. Crystals were cryo-protected with 12.5% PEG 1000, 200 mM NaCl, 100 mM MES, pH 6.0, and 25% glycerol, and then flash-frozen in liquid nitrogen for X-ray analysis. Diffraction datasets were collected at the NSLS-II NYX beamline (19-ID) and AMX beamline (17-ID-1) at the Brookhaven National Laboratory and processed using XDS (72). The structure of the AfFabI•NADH complex was solved by molecular replacement using the AfFabI structure lacking α9 as a search model. The structures were completed by iterative rounds of refinement using REFMAC (73) and manual rebuilding using Coot (74). The refinement was monitored by following the R_free value calculated from a random subset (5%) of omitted reflections. A tight turn resolved in the electron density map led to 0.2% of the atoms to be in the Ramachandran outliers (N161A in the AfFabI structure, and N161A, N161C in the AfFabI•NADH complex structure). A summary of the data processing and structure refinement statistics is provided in Table 1. The coordinates have been deposited in the Protein Data Bank (accession code 6V LX AfFabI; 6XLY AfFabI•NADH complex). The figures related to the protein structure were generated with PyMOL (75).

In-silico analyses
RMSD calculations were performed using SSM Superpose in Coot (74). Surface areas were calculated in PyMOL as previously described (76). The buried surface area by dimerization was calculated by subtracting the surface area of two protomers linked along the P axis from the combined surface area of the two individual protomers. The buried surface by tetramerization was calculated by subtracting the surface area of two protomers linked along the Q axis from the combined surface area of the two individual protomers. The buried surface area from the helix-helix interaction domain was calculated by subtracting the surface area of two protomers linked along the R axis from the combined surface area of the two individual protomers. The water channels and water well were visualized using the PyMOL surface view in Cavities and Pockets (Culled) mode. AFN-1252 binding visualization across FabI clades was done by structural alignment of the catalytic dyads from clade I EcFabI•NAD⁺•AFN-1252 (PDB ID: 4JQC) (77), clade II MtInhA•NAD⁺ (PDB ID: 4DRE) (78), clade III CtFabI•NADH•AFN-1252 (PDB ID: 4Q9N) (29), and clade IV AfFabI•NAD⁺ (PDB ID: 6VLY).

**Statistical analysis**

Statistical analysis (i.e., standard error, $K_{0.5}$) and mathematical modeling (i.e., Hill equation, variable slope nonlinear regression) were performed using GraphPad Prism software version 8.2.1.

**Data Aailability**

Coordinates and structure factors for AfFabI and AfFabI•NADH crystal structures have been deposited in the Protein Data Bank (PDB) under accession codes 6VLX and 6VLY, respectively. All remaining data are contained within the article.

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References

1. Parsons, J. B., and Rock, C. O. (2013) Bacterial lipids: Metabolism and membrane homeostasis. Prog. Lipid Res. 52, 249-276
2. Heath, R. J., and Rock, C. O. (2000) A triclosan-resistant bacterial enzyme. Nature 406, 145-146
3. Marrakchi, H., DeWolf Jr, W. E., Quinn, C., West, J., Polizzi, B. J., So, C. Y., Holmes, D. J., Reed, S. L., Heath, R. J., Payne, D. J., Rock, C. O., and Wallis, N. G. (2003) Characterization of Streptococcus pneumoniae enoyl-[acyl carrier protein] reductase (FabK). Biochem. J. 370, 1055-1062
4. Saito, J., Yamada, M., Watanabe, T., Iida, M., Kitagawa, H., Takahata, S., Ozawa, T., Takeuchi, Y., and Ohswava, F. (2008) Crystal structure of enoyl-acyl carrier protein reductase (FabK) from Streptococcus pneumoniae reveals the binding mode of an inhibitor. Protein Sci. 17, 691-699
5. Hevener, K. E., Santarsiero, B. D., Lee, H., Jones, J. A., Boci, T., Johnson, M. E., and Mehoob, S. (2018) Structural characterization of Porphyromonas gingivalis enoyl-ACP reductase II (FabK). Acta Crystallogr. F Struct. Biol. Commun. 74, 105-112
6. Oppermann, U., Filling, C., Hult, M., Shaqfet, N., Wu, X., Lindm, M., Shaqfet, J., Nordling, E., Kallberg, Y., Persson, B., and Jornvall, H. (2003) Short chain dehydrogenases/reductases (SDR): the 2002 update. Chem. Biol. Interact. 143-144, 247-253
7. White, S. W., Zheng, J., Zhang, Y.-M., and Rock, C. O. (2005) The structural biology of type II fatty acid biosynthesis. Annu. Rev. Biochem. 74, 791-831
8. Baker, M. E. (1995) Enoyl-acyl-carrier-protein reductase and Mycobacterium tuberculosis InhA do not conserve the Tyr-Xaa-Xaa-Xaa-Lys motif in mammalian 11β- and 17β-hydroxysteroid dehydrogenases and Drosophila alcohol dehydrogenase. Biochem. J. 309, 1029-1030
9. Parikh, S., Moynihan, D. P., Xiao, G., and Tonge, P. J. (1999) Roles of tyrosine 158 and lysine 165 in the catalytic mechanism of InhA, the enoyl-ACP reductase from Mycobacterium tuberculosis. Biochemistry 38, 13623-13634
10. Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000) The enoyl-[acyl-carrier-protein] reductases FabI and FabL from Bacillus subtilis. J. Biol. Chem. 275, 40128-40133
11. Kim, K. H., Ha, B. H., Kim, S. J., Hong, S. K., Hwang, K. Y., and Kim, E. E. (2011) Crystal structures of enoyl-ACP reductases I (FabI) and III (FabL) from B. subtilis. J. Mol. Biol. 406, 403-415
12. Massengo-Tiasse, R. P., and Cronan, J. E. (2008) Vibrio cholerae fabV defines a new class of enoyl acyl-carrier-protein reductase. J. Biol. Chem. 283, 1308-1316
13. Zhu, L., Lin, J., Ma, J., Cronan, J. E., and Wang, H. (2010) Triclosan resistance of Pseudomonas aeruginosa PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. Antimicrob. Agents Chemother. 54, 689-698
14. Hirschbeck, M. W., Kuper, J., Lu, H., Liu, N., Neckles, C., Shah, S., Wagner, S., Sotriffer, C. A., Tonge, P. J., and Kisker, C. (2012) Structure of the Yersinia pestis FabV enoyl-ACP reductase and its interaction with two 2-pyridone inhibitors. Structure 20, 89-100
15. Heath, R. J., and Rock, C. O. (1995) Enoyl-acyl carrier protein reductase (fabI) plays a determinant role in completing cycles of fatty acid elongation in Escherichia coli. J. Biol. Chem. 270, 26538-26542
16. Yao, J., and Rock, C. O. (2016) Resistance mechanisms and the future of bacterial enoyl-acyl carrier protein reductase (FabI) antibiotics. Cold Spring Harb. Perspect. Med. 6, a027045
17. Yao, J., and Rock, C. O. (2017) Bacterial fatty acid metabolism in modern antibiotic discovery. Biochim. Biophys. Acta 1862, 1300-1309
18. Kaplan, N., Awrey, D., Bardouniotis, E., Berman, J., Yethon, J., Pauls, H. W., and Hafkin, B. (2013) In vitro activity (MICs and rate of kill) of AFN-1252, a novel FabI inhibitor, in the presence of serum and in combination with other antibiotics. J. Chemother. 25, 18-25
19. Kaplan, N., Albert, M., Awrey, D., Bardouniotis, E., Berman, J., Clarke, T., Dorsey, M., Hafkin, B., Ramnauth, J., Romanov, V., Schmid, M. B., Thalakada, R., Yethon, J., and Pauls, H. W. (2012) Mode of action, in vitro activity, and in vivo efficacy of AFN-1252, a selective antistaphylococcal FabI inhibitor. Antimicrob. Agents Chemother. 56, 5865-5874

20. Hafkin, B., Kaplan, N., and Murphy, B. (2016) Efficacy and safety of AFN-1252, the first Staphylococcus-specific antibacterial agent, in the treatment of acute bacterial skin and skin structure infections, including those in patients with significant comorbidities. Antimicrob. Agents Chemother. 60, 1695-1701

21. Menetrey, A., Janin, A., Pullman, J., Overcash, J. S., Haouala, A., Leylavergne, F., Turbe, L., Wittke, F., and Nicolas-Metral, V. (2019) Bone and joint tissue penetration of the Staphylococcus-selective antibiotic afabicin in patients undergoing elective hip replacement surgery. Antimicrob. Agents Chemother. 63

22. Keeney, K. M., Yurist-Doutch, S., Arrieta, M. C., and Finlay, B. B. (2014) Effects of antibiotics on human microbiota and subsequent disease. Annu. Rev. Microbiol. 68, 217-235

23. Croswell, A., Amir, E., Teggatz, P., Barman, M., and Salzman, N. H. (2009) Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect. Immun. 77, 2741-2753

24. Yao, J., Carter, R. A., Vuagniaux, G., Barbier, M., Rosch, J. W., and Rock, C. O. (2016) A pathogen-selective antibiotic minimizes disturbance to the microbiome. Antimicrob. Agents Chemother. 60, 4264-4273

25. Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., and Mele, M. C. (2019) What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. Microorganisms 7, 14

26. Ward, W. H., Holdgate, G. A., Rowsell, S., McLean, E. G., Paupert, R. A., Clayton, E., Nichols, W. W., Collins, J. G., Minshull, C. A., Jude, D. A., Mistry, A., Timms, D., Camble, R., Hales, N. J., Britton, C. J., and Taylor, I. W. (1999) Kinetic and structural characteristics of the inhibition of enoyl (acyl carrier protein) reductase by triclosan. Biochemistry 38, 12514-12525

27. Schiebel, J., Chang, A., Lu, H., Baxter, M., Tonge, P., and Kisker, C. (2012) Staphylococcus aureus FabI: Inhibition, substrate recognition, and potential implications for in vivo essentiality. Structure 20, 802-813

28. Rozwarski, D. A., Vilcheze, C., Sugantino, M., Bittman, R., and Sacchettini, J. C. (1999) Crystal structure of the Mycobacterium tuberculosis enoyl-ACP reductase, InhA, in complex with NAD+ and a C16 fatty acyl substrate. J. Biol. Chem. 274, 15582-15589

29. Yao, J., Abdelrahman, Y. M., Robertson, R. M., Cox, J. V., Belland, R. J., White, S. W., and Rock, C. O. (2014) Type II fatty acid synthesis is essential for the replication of Chlamydia trachomatis. J. Biol. Chem. 289, 22365-22376

30. Belluti, F., Perozzo, R., Lauciello, L., Colizzi, F., Kostrewa, D., Bisi, A., Gobbi, S., Rampa, A., Bolognesi, M. L., Recanatini, M., Brun, R., Scapozza, L., and Cavalli, A. (2013) Design, synthesis, and biological and crystallographic evaluation of novel inhibitors of Plasmodium falciparum enoyl-ACP-reductase (PFabI). J. Med. Chem. 56, 7516-7526

31. Johnson, E. L., Heaver, S. L., Walters, W. A., and Ley, R. E. (2017) Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. J. Mol. Med. (Berl) 95, 1-8

32. Karlowsky, J. A., Kaplan, N., Hafkin, B., Hoban, D. J., and Zhanel, G. G. (2009) AFN-1252, a FabI inhibitor, demonstrates a Staphylococcus-specific spectrum of activity. Antimicrob. Agents Chemother. 53, 3544-3548

33. Yao, J., Maxwell, J. B., and Rock, C. O. (2013) Resistance to AFN-1252 arises from missense mutations in Staphylococcus aureus enoyl-acyl carrier protein reductase (FabI). J. Biol. Chem. 288, 36261-36271

34. Heath, R. J., Li, J., Roland, G. E., and Rock, C. O. (2000) Inhibition of the Staphylococcus aureus NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. J. Biol. Chem. 275, 4654-4659
35. Heath, R. J., Rubin, J. R., Holland, D. R., Zhang, E., Snow, M. E., and Rock, C. O. (1999) Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *J. Biol. Chem.* **274**, 11110-11114

36. Jordan, C. A., Sandoval, B. A., Serobyan, M. V., Gilling, D. H., Groziak, M. P., Xu, H. H., and Vey, J. L. (2015) Crystallographic insights into the structure-activity relationships of diazaborine enoyl-ACP reductase inhibitors. *Acta Crystallogr. F Struct. Biol. Commun.* **71**, 1521-1530

37. Schiebel, J., Chang, A., Lu, H., Baxter, M. V., Tonge, P. J., and Kisker, C. (2012) *Staphylococcus aureus* FabI: inhibition, substrate recognition, and potential implications for in vivo essentiality. *Structure* **20**, 802-813

38. Kim, H. T., Kim, S., Na, B. K., Chung, J., Hwang, E., and Hwang, K. Y. (2017) Structural insights into the dimer-tetramer transition of FabI from *Bacillus anthracis*. *Biochem. Biophys. Res. Commun.* **493**, 28-33

39. Kim, S. J., Ha, B. H., Kim, K. H., Hong, S. K., Shin, K. J., Suh, S. W., and Kim, E. E. (2010) Dimeric and tetrameric forms of enoyl-acyl carrier protein reductase from *Bacillus cereus*. *Biochem. Biophys. Res. Commun.* **400**, 517-522

40. Priyadarshi, A., Kim, E. E., and Hwang, K. Y. (2010) Structural insights into *Staphylococcus aureus* enoyl-ACP reductase (FabI), in complex with NADP and triclosan. *Proteins* **78**, 480-486

41. Rafferty, J. B., Simon, J. W., Baldock, C., Artymiuk, P. J., Baker, P. J., Stuitje, A. R., Slabas, A. R., and Rice, D. W. (1995) Common themes in redox chemistry emerge from the X-ray structure of oilseed rape (*Brassica napus*) enoyl acyl carrier protein reductase. *Structure* **3**, 927-938

42. Perozzo, R., Kuo, M., Sidhu, A. S., Valiyaveettil, J. T., Bittman, R., Jacobs, W. R., Jr., Fidock, D. A., and Sacchettini, J. C. (2002) Structural elucidation of the specificity of the antibacterial agent triclosan for malarial enoyl acyl carrier protein reductase. *J. Biol. Chem.* **277**, 13106-13114

43. Luckner, S. R., Liu, N., am Ende, C. W., Tonge, P. J., and Kisker, C. (2010) A slow, tight binding inhibitor of InhA, the enoyl-acyl carrier protein reductase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **285**, 14330-14337

44. Pidugu, L. S., Kapoor, M., Surolia, N., Surolia, A., and Suguna, K. (2004) Structural basis for the variation in triclosan affinity to enoyl reductases. *J. Mol. Biol.* **343**, 147-155

45. Ghosh, D., Wawrzak, Z., Weeks, C. M., Duax, W. L., and Erman, M. (1994) The refined three-dimensional structure of 3α,20β-hydroxysteroid dehydrogenase and possible roles of the residues conserved in short-chain dehydrogenases. *Structure* **2**, 629-640

46. Tanaka, N., Nonaka, T., Tanabe, T., Yoshimoto, T., Tsuru, D., and Mitsui, Y. (1996) Crystal structures of the binary and ternary complexes of 7α-hydroxysteroid dehydrogenase from *Escherichia coli*. *Biochemistry* **35**, 7715-7730

47. Hol, W. G., van Duijnen, P. T., and Berendsen, H. J. (1978) The α-helix dipole and the properties of proteins. *Nature* **273**, 443-446

48. Muench, S. P., Prigge, S. T., McLeod, R., Rafferty, J. B., Kirisits, M. J., Roberts, C. W., Mui, E. J., and Rice, D. W. (2007) Studies of *Toxoplasma gondii* and *Plasmodium falciparum* enoyl acyl carrier protein reductase and implications for the development of antiparasitic agents. *Acta Crystallogr. D Biol. Crystallogr.* **63**, 328-338

49. Qiu, X., Janson, C. A., RI, C., Smyth, M. G., Payne, D. J., and Abdel-Meguid, S. S. (1999) Molecular basis for triclosan activity involves a flipping loop in the active site. *Protein Sci.* **8**, 2529-2532

50. Dessen, A., Quemard, A., Blanchard, J. S., Jacobs, W. R., Jr., and Sacchettini, J. C. (1995) Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. *Science* **267**, 1638-1641
51. Mehboob, S., Hevener, K. E., Truong, K., Boci, T., Santarsiero, B. D., and Johnson, M. E. (2012) Structural and enzymatic analyses reveal the binding mode of a novel series of Francisella tularensis enoyl reductase (FabI) inhibitors. J. Med. Chem. 55, 5933-5941
52. Schiebel, J., Chang, A., Merget, B., Bommineni, G. R., Yu, W., Spagnuolo, L. A., Baxter, M. V., Tareilus, M., Tonge, P. J., Kisker, C., and Sotriffer, C. A. (2015) An ordered water channel in Staphylococcus aureus FabI: unraveling the mechanism of substrate recognition and reduction. Biochemistry 54, 1943-1955
53. Flocco, M. M., and Mowbray, S. L. (1994) Planar stacking interactions of arginine and aromatic side-chains in proteins. J. Mol. Biol. 235, 709-717
54. Rozwarski, D. A., Grant, G. A., Barton, D. H., Jacobs, W. R., Jr., and Sacchettini, J. C. (1998) Modification of the NADH of the isoniazid target (InhA) from Mycobacterium tuberculosis. Science 279, 98-102
55. Langdon, A., Crook, N., and Dantas, G. (2016) The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Med. 8, 39
56. Francino, M. P. (2015) Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. Front. Microbiol. 6, 1543
57. Schliep, K. P. (2011) Phangorn: phylogenetic analysis in R. Bioinformatics 27, 592-593
58. Wright, E. S. (2015) DECIPHER: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinformatics 16, 322
59. Le, S. Q., and Gascuel, O. (2008) An improved general amino acid replacement matrix. Mol. Biol. Evol. 25, 1307-1320
60. Yu, G. C., Smith, D. K., Zhu, H. C., Guan, Y., and Lam, T. T. Y. (2017) GGTREE: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. 8, 28-36
61. Radka, C. D., Frank, M. W., Rock, C. O., and Yao, J. (2020) Fatty acid activation and utilization by Alistipes finegoldii, a representative Bacteroidetes resident of the human gut microbiome. Mol. Microbiol. 00, 1-19
62. Parsons, J. B., Frank, M. W., Subramanian, C., Saenkham, P., and Rock, C. O. (2011) Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. Proc. Natl. Acad. Sci. U. S. A. 108, 15378-15383
63. Jackowski, S., Zhang, Y.-M., Price, A. C., White, S. W., and Rock, C. O. (2002) A missense mutation in the fabB (β-ketoacyl-acyl carrier protein synthase I) gene confers thiolactomycin resistance to Escherichia coli. Antimicrob. Agents Chemother. 46, 1246-1252
64. Zhao, H., Brautigam, C. A., Ghirlando, R., and Schuck, P. (2013) Overview of current methods in sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation. Curr. Protoc. Protein Sci. 71, 20.12.21-20.12.49
65. Schuck P., Z. H., Brautigam C. A., and Ghirlando R. (2015) Basic Principles of Analytical Ultracentrifugation, CRC Press, Inc., Boca Raton, FL.
66. Schuck, P. (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. Biophys. J. 78, 1606-1619
67. Zhao, H., Ghirlando, R., Alfonso, C., Arisaka, F., Attali, I., Bain, D. L., Bakhtina, M. M., Becker, D. F., Bedwell, G. J., Bekdemir, A., Besong, T. M., Birck, C., Brautigam, C. A., Brennerman, W., Byron, O., Bzowska, A., Chaires, J. B., Chaton, C. T., Colfen, H., Connaghan, K. D., Crowley, K. A., Curth, U., Daviter, T., Dean, W. L., Diez, A. I., Ebel, C., Eckert, D. M., Eisele, L. E., Eisenstein, E., England, P., Escalante, C., Fagan, J. A., Fairman, R., Finn, R. M., Fischle, W., de la Torre, J. G., Gor, J., Gustafsson, H., Hall, D., Harding, S. E., Cifre, J. G., Herr, A. B., Howell, E. E., Isaac, R. S., Jao, S. C., Jose, D., Kim, S. J., Kokona, B., Kornblatt, J. A., Kosek, D., Krayukhina, E., Krozizek, D., Kusznir, E. A., Kwon, H., Larson, A., Laue, T. M., Le, R. A., Leech, A. P., Lilie, H., Luger, K., Luque-Ortega, J. R., Ma, J., May, C. A., Maynard, E. L., Modrak-Wojcik, A., Mok, Y. F., Mucke, N., Nagel-Steiger, L., Narlikar, G. J., Noda, M., Nourse, A., Obsil, T., Park, C. K., Park, J. K., Pawelek, P. D., Perdue, E. E.,
Perkins, S. J., Perugini, M. A., Peterson, C. L., Peverelli, M. G., Piszczech, G., Prag, G., Prevelige, P. E., Raynal, B. D., Rezabkova, L., Richter, K., Ringel, A. E., Rosenberg, R., Rowe, A. J., Rufer, A. C., Scott, D. J., Seravalli, J. G., Solovyova, A. S., Song, R., Staunton, D., Stoddard, C., Stott, K., Strauss, H. M., Streicher, W. W., Sumida, J. P., Swygert, S. G., Szczepanowski, R. H., Tessmer, I., Toth, R. T., Tripathy, A., Uchiyama, S., Uebel, S. F., Unzai, S., Gruber, A. V., von Hippel, P. H., Wandrey, C., Wang, S. H., Weitzel, S. E., Wielgus-Kutrowska, B., Wolberger, C., Wolff, M., Wright, E., Wu, Y. S., Wubben, J. M., and Schuck, P. (2015) A multilaboratory comparison of calibration accuracy and the performance of external references in analytical ultracentrifugation. *PLoS ONE* **10**, e0126420.

Yao, J., Bruhn, D. F., Frank, M. W., Lee, R. E., and Rock, C. O. (2016) Activation of exogenous fatty acids to acyl-acyl carrier protein cannot bypass FabI inhibition in *Neisseria*. *J. Biol. Chem.* **291**, 171-181.

Otwinski, Z., and Minor, W. (1997) [20] Processing of X-ray diffraction data collected in oscillation mode. *Meth. Enzymol.* **276**, 307-326.

McCoy, A. J. (2007) Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallogr. D Biol. Crystallogr.* **63**, 32-41.

[dataset] Chen, L., Li, Y., Ebihara, A., Shinkai, A., Kuramitsu, S., Yokoyama, S., Zhao, M., Rose, J.P., Wang, B.C., Southeast Collaboratory for Structural Genomics (SECSG), and RIKEN Structural Genomics/Proteomics Initiative (RSGI). (2007) Crystal structure of enoyl-[acyl-carrier-protein] reductase (NADH) from *Aquifex aeolicus* VF5. Protein Data Bank. 2P91.

Kabsch, W. (2010) Xds. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125-132.

Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2196-2201.

DeLano, W. L. (2002) The PyMOL molecular graphics system. *DeLano Scientific, Palo Alto, CA*

Radka, C. D., Chen, D., DeLucas, L. J., and Aller, S. G. (2017) The crystal structure of the *Yersinia pestis* iron chaperone YiuA reveals a basic triad binding motif for the chelated metal. *Acta Crystallogr. D Struct. Biol.* **73**, 921-939.

[dataset] Subramanya, H., Rao, K.N., and Anirudha, L. (2013) Crystal structure of *E. coli* enoyl reductase in complex with NAD and AFN-1252. Protein Data Bank. 4JQC.

Hartkoorn, R. C., Sala, C., Neres, J., Pojer, F., Magnet, S., Mukherjee, R., Uplekar, S., Boy-Rottger, S., Altmann, K. H., and Cole, S. T. (2012) Towards a new tuberculosis drug: pyridomycin - nature’s isoniazid. *EMBO Mol. Med.* **4**, 1032-1042.

Bergler, H., Hogenaer, G., and Turnowsky, F. (1992) Sequences of the envM gene and of two mutated alleles in *Escherichia coli*. *J. Gen. Microbiol.* **138**, 2093-2100.

Lu, Y.-J., Zhang, Y.-M., Grimes, K. D., Qi, J., Lee, R. E., and Rock, C. O. (2006) Acyl-phosphates initiate membrane phospholipid synthesis in gram-positive pathogens. *Molec. Cell* **23**, 765-772.

Marrakchi, H., Choi, K.-H., and Rock, C. O. (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. *J. Biol. Chem.* **277**, 44809-44816.
| PDB ID       | 6VXL | 6VLY |
|-------------|------|------|
| Data collection |     |      |
| Space group  | $P2_12_1$ | $P2_12_12_1$ |
| Cell dimensions |     |      |
| $a, b, c$ (Å) | 60.6, 77.8, 127.6 | 75.3, 111.5, 150.5 |
| $\alpha, \beta, \gamma$ (°) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 35-1.72 (1.76-1.72)$^a$ | 29-1.86 (1.91-1.86) |
| $R_{merge}$ | 0.056 (0.483) | 0.077 (0.901) |
| $CC_{1/2}$ | 99.7 (90.9) | 99.7 (77.0) |
| $I/\sigma$ | 13.4 (1.8) | 12.6 (1.7) |
| Completeness (%) | 98.3 (87.7) | 94.4 (88.8) |
| Redundancy | 6.4 (5.4) | 4.9 (4.7) |
| Wilson B-factor (Å²) | 25.8 | 24.7 |
| Refinement |     |      |
| Resolution (Å) | 30-1.72 (1.76-1.72) | 29-1.86 (1.91-1.86) |
| No. reflections | 59692 (3142) | 100112 (5169) |
| $R_{work}$ (%) | 20.6 (33.5) | 15.8 (42.8) |
| $R_{free}$ (%)$^b$ | 24.5 (34.3) | 19.7 (44.3) |
| Number of non-hydrogen atoms |     |      |
| Protein | 4110 | 8862 |
| Ligand/ion | 6 | 225 |
| Water | 402 | 1046 |
| RMSD |     |      |
| Bond lengths (Å) | 0.006 | 0.010 |
| Bond angles (°) | 1.380 | 1.463 |
| Average $B$-factors (Å²) | 34.0 | 25.0 |
| Ramachandran plot |     |      |
| Favored (%) | 95.4 | 96.8 |
| Allowed (%) | 4.4 | 3.0 |
| Outliers (%) | 0.2 | 0.2 |

$^a$Statistics for the highest-resolution shell are shown in parentheses.

$^b$R$_{free}$ test set uses ~5% of the data.
Table 2
Properties of *A*fabl carboxy-terminal truncation mutants

| Property                      | *A*fabl<sup>a</sup> (1-289) | *A*fabl (1-273) | *A*fabl (1-269) | *A*fabl (1-265) | *A*fabl (1-261) |
|------------------------------|-----------------------------|----------------|----------------|----------------|----------------|
| Complementation              | Yes                         | Yes            | Yes            | No             | No             |
|     *fabl*(Ts)               |                             |                |                |                |                |
| Biophysical Properties       |                             |                |                |                |                |
|     **S**<sub>20</sub>       | 6.59                        | 6.40           | 6.37           | 6.18           | 6.11           |
| **Thermal Stability**        |                             |                |                |                |                |
|     Ligand-Free (°C)         | 47.9 ± 0.1                  | 46.0 ± 0.2     | 45.0 ± 0.1     | 48.8 ± 0.2     | 52.1 ± 0.4     |
|     + NADH (°C)              | 54.8 ± 0.2                  | 52.1 ± 0.2     | 50.5 ± 0.3     | 49.3 ± 0.2     | 51.4 ± 0.2     |
|     ΔT (°C)                  | 6.9 ± 0.2                   | 6.1 ± 0.3      | 5.5 ± 0.4      | 0.5 ± 0.4      | -0.7 ± 0.6     |
| Biochemical Properties       |                             |                |                |                |                |
|     NADH **K**<sub>0.5</sub> (µM) | 52.8 ± 3.0                 | 160.3 ± 22.2   | 127.1 ± 10.9   | 291.9 ± 31.8   | 1225.0 ± 47.5  |
|     h<sup>b</sup>            | 2.1 ± 0.2                   | 1.4 ± 0.2      | 1.3 ± 0.1      | 1.0 ± 0.1      | 2.6 ± 0.2      |
|     V<sub>max</sub>/[E<sub>T</sub>]<sup>c</sup> (min<sup>-1</sup>) | 6.45 ± 0.19                 | 6.57 ± 0.24    | 7.26 ± 0.19    | 6.46 ± 0.21    | 6.63 ± 0.18    |
|     V<sub>max</sub>/[E<sub>T</sub>]/**K**<sub>0.5</sub><sup>d</sup> (10<sup>-3</sup> µmol·min<sup>-1</sup>) | 122.12 ± 3.64             | 40.97 ± 1.51   | 57.13 ± 1.52   | 22.14 ± 0.71   | 5.41 ± 0.15    |

<sup>a</sup>*A*fabl(1-289) is the full-length, wild-type protein.

<sup>b</sup>Hill number.

<sup>c</sup>Turnover number. [E<sub>T</sub>] = total enzyme concentration.

<sup>d</sup>A measure of catalytic efficiency.
### Table 3
Bacterial strains and plasmids

| Strain      | Description                                                                 | Reference |
|-------------|-----------------------------------------------------------------------------|-----------|
| *E. coli* JP1111 | Hfr(PO1), galE45(GalS), λ, fabl392(Ts), relA1, spoT1, thiE1                | (79)      |
| *E. coli* ANS1  | metB1 relA1 spoT1 gyrA216 tolC::Tn10 λ− λ′ F−                             | (63)      |

| Plasmid     | Description                                                                 | Reference |
|-------------|-----------------------------------------------------------------------------|-----------|
| pPJ131      | *E. coli* expression vector                                                  | (80)      |
| pEcFabI     | *E. coli* fabI in pPJ131                                                    | (29)      |
| pCtFabI     | *Chlamydia trachomatis* fabI in pPJ131                                       | (29)      |
| pAfFabI     | *A. finegoldii* fabI (Uniprot ID: A0A174E195) in pPJ131                      | This study|
| pCaFabK     | *Clostridium acetobutylicum* fabK in pPJ131                                  | (81)      |
| pAfFabK     | *A. finegoldii* fabK (Uniprot ID: I3YI65) in pPJ131                          | This study|
| pET_AfFabI  | *A. finegoldii* fabl 1-289 (Uniprot ID: A0A174E195) in pET28b               | This study|
| pET_1-273   | *A. finegoldii* fabl 1-273 (Uniprot ID: A0A174E195) in pET28b               | This study|
| pET_1-269   | *A. finegoldii* fabl 1-269 (Uniprot ID: A0A174E195) in pET28b               | This study|
| pET_1-265   | *A. finegoldii* fabl 1-265 (Uniprot ID: A0A174E195) in pET28b               | This study|
| pET_1-261   | *A. finegoldii* fabl 1-261 (Uniprot ID: A0A174E195) in pET28b               | This study|
Figure 1. Enoyl-ACP reductases in the Bacteroidetes. A, a diagram of the four-step elongation cycle of bacterial fatty acid synthesis (FASII). The acyl chains are covalently bound to acyl carrier protein (ACP) and are extended by two carbons with each turn of the cycle. The final step is catalyzed by an enoyl-ACP reductase that catalyzes the reduction of trans-2-acyl-ACP to acyl-ACP. FabI and FabK are two widely distributed enoyl reductase protein families. B, the percentage of individual species encoding FabI, FabK or both in five abundant human associated Bacteroidetes genera (Alistipes (n=10), Bacteroides (n=41), Parabacteroides (n=7), Porphyromonas (n=15), Prevotella (n=38)). C, evolutionary relationship between the FabI family sequences. The distantly related FabL sequences were used to root the tree. FabI proteins fall into four clades: clade 1, prototypical Proteobacteria/Firmicute proteins; clade 2, Mycobacteria proteins; clade 3, plastidial enzymes; and clade 4, Bacteroidetes/Chlorobi enzymes.
Figure 2. *A. finegoldii* encodes two functional enoyl-acyl carrier protein reductases. 

**A**, growth of temperature-sensitive *E. coli* strain JP1111 (*fabI*(Ts)) mutant transformed with empty plasmid vector control (VC), or plasmids expressing either *E. coli* FabI (EcFabI), *Chlamydia trachomatis* FabI (CtFabI), *A. finegoldii* FabI (AfFabI; Uniprot ID: A0A174E195), *Clostridium acetobutylicum* FabK (CaFabK), or *A. finegoldii* FabK (AfFabK; Uniprot ID: I3YI65) under the growth permissive temperature (30°C). 

**B**, growth of the same strain set at the non-permissive temperature (42°C). Five biological replicates were tested for each strain. 

**C**, microbroth dilution assay to rank the sensitivities of the FabIs and FabKs to AFN-1252 by determining the minimal inhibitory concentrations for AFN-1252 against *E. coli* strain ANS1 (*ΔtolC*) (33) transformed with the same series of plasmids described in panel A. Mean ± SE (n=3).
Figure 3. AfFabI oligomerization and NADH binding. AfFabI was purified by affinity and gel filtration chromatography. 

A, AfFabI migrated as a single 145 kDa species ($V_e$=5.058 min) on a calibrated XBridge BEH SEC 200 Å 3.5 μm column (136 kDa theoretical mass). 

Insets: gel electrophoresis shows the purity of AfFabI (34 kDa) along with the indicated molecular weight standards (Std). The standard curve for the XBridge BEH SEC column used thyroglobulin (669 kDa), immunoglobulin (150 kDa), bovine serum albumin (66.4 kDa) and myoglobin (17 kDa). 

B, the sedimentation velocity profiles (fringe displacement) were fitted to a continuous sedimentation coefficient distribution model $c(s)$. AfFabI sedimented as a 141 kDa tetramer with a sedimentation coefficient of 6.59 S. 

C, surface plasmon resonance sensorgrams depicting one experiment to determine NADH binding to AfFabI (top panel). The data from three independent titrations were fit to the Hill equation using GraphPad Prism software to calculate the $K_D$ and Hill coefficient ($h$). Mean ± SE (n=3). 

D, protein thermal denaturation analysis was used to determine the stabilization of AfFabI by NADH. Assays contained 10 μM AfFabI (black) or 10 μM AfFabI + 100 μM NADH (red).
Figure 4. *AfFab* displays prototypical *Fab* kinetics. 

**A**, the rate of 200 μM NADH oxidation in 1.25 mM crotonyl-CoA, 20 mM Tris-HCl pH 8.0 as measured by the change in absorbance at 340 nm with (blue) or without (red) 100 nM *AfFab*. The slope of the linear phase of the progress curve was converted to specific activity using the NADH extinction coefficient of 6220 M⁻¹ cm⁻¹. *Inset*, NADPH did not support the reaction. 

**B**, specific activities were calculated as a function of NADH concentration. The behavior of *AfFab* with NADH is best described by the Hill equation (fitted line shown on graph). 

**C**, specific activities were calculated as a function of crotonyl-CoA concentration. The behavior of *AfFab* with crotonyl-CoA is best described by the Hill equation (fitted line shown on graph). *Inset*, mass spectrum analysis of the reaction mixture shows reduction of crotonyl-CoA (m/z = 836.2) to butyryl-CoA (m/z = 838.3) either with (blue) or without (red) *AfFab*. 

**D**, fractional activity of *AfFab* versus increasing concentrations of AFN-1252 or triclosan. Data points were fit to the [Inhibitor] vs. response -- Variable slope nonlinear regression equation using GraphPad Prism 8.2.1 software, and the fitted lines are shown on the graph. *AfFab* spectrophotometric assays were performed in triplicate as described under “Experimental Procedures.” Mean ± SE (n=3).
Figure 5. Overall crystal structure of A fabI. A, the A fabI monomer contains a Rossmann fold. The polypeptide chain is colored from blue (amino-terminus) to red (carboxy-terminus). The active site lid (α6) and terminal 13 residues are depicted as disordered (broken dashes) because these residues were not resolved in the electron density map. B, A fabI is a tetramer with three perpendicular 2-fold non-crystallographic symmetry axes P, Q, and R.
Figure 6. Subunit interface domains of AfFabl. Specific structural domains link the protomers along the P, Q, and R axes interfaces. A, the dimerization domain consists of interacting α8 helices and β7 strands that link protomers A/B and C/D along the P axis. B, the tetramerization domain is an antiparallel four-helix bundle composed of α4 and α5 that links protomers A/C and B/D along the Q axis. C, the carboxy-terminal α9 helix-helix interaction domain is a unique feature of AfFabl. This domain links protomers A/D and B/C along the R axis. Rotation of the tetramer by 90° around the Q axis enables clear visualization of the protomer-protomer interaction mediated by α9.
Figure 7. Active site and water channels in the AfFabI•NADH complex upon NADH binding.  
A, schematic diagram shows hydrogen bond interactions between the active site and NADH designated by purple lines. Lid residues that interact with NADH are shown by yellow highlight.  
B, molecular surface rendering of the central water well that connects to the four active sites was visualized in the AfFabI•NADH complex crystal structure along with the water channels that connect each active site with the water well using PyMOL. The molecular surface of the water channels and central water well are colored according to the contribution from each protomer. Segments of each water channel have more than one color because they are formed by the protomer interfaces along the R axis. The active sites in each protomer are designated with the letter A and the protomer superscript, and the letter W indicates the location of the central water well.  
C, each water channel contains ordered water molecules (red spheres) that form hydrogen bond interactions with each other and the peptide backbone. The line traces the chain of structured waters that begin at the carboxamide of NADH in the active site (A$^C$), exit into a narrow tunnel formed by the A/B/C protomer interfaces along the R axis and empty into the central water well (W).
Figure 8. Conformational change in helix α9 and the carboxy-terminus. A, in the AlfFabl crystal structure, the α9^C and α9^B helices are wound together connecting the C and B protomers and the last 13 residues of the protein are disordered. Broken dashes indicate the relative location of missing carboxy-terminus residues. B, in the AlfFabl•NADH crystal structure, the α9^C-α9^B interaction domain unwinds and 10 of the 13 carboxy-terminal residues form a structured loop feature that connects to the active site lid of the partner protomer. The arrows denote the location of residues (purple) in the α9 turn that are removed in the AlfFabl(1-269) truncation mutant.
Figure 9. Creation of the NADH binding site. The active site lid is disordered in the absence of NADH and helix α9B (red) is coiled around α9C (yellow) along the R axis. NADH (cyan) binding to the active site crevice orders the lid. A, Y268B in the second turn of helix α9 in the AfFabI structure forms hydrogen bond interactions with N217C and N213C in the second two turns of α7C. B, following NADH binding, the unwound α9B slips along α7C. The T267B side chain on α9B forms a hydrogen bond interaction with D210C in the first turn of α7C. C, residues in the first turn of α9 in the AfFab structure become an ordered loop in AfFab•NADH with multiple hydrogen bond interactions with the other protomer. D, K266B from the second turn of α9 in AfFab form hydrogen bond interactions with R263C and M265C from α9C in AfFab•NADH. E, hydrogen bond interactions between the disordered carboxy-terminus of AfFab with the other protomers in AfFab•NADH. The backbone amine of E275B forms a hydrogen bond with the Y158C side chain, the side chain of D276B forms a hydrogen bond to the side chain of R183A (on α4A), the backbone amine of H278B forms a hydrogen bond interaction with the T106C and D108C side chains and the backbone carbonyl and side chain from Q279B forms hydrogen bonds with the side chain and backbone carbonyl of R134A from α5A.
Figure 10. Unique structural features of the FabI clades. Representative models for the four FabI clades are compared using clade 1 FabI as the standard for comparison. Top panel, clade 1 EcFabI has the basic Rossmann fold and tetrameric organization common to all FabIs. The unique features in clades 2-4 are highlighted in blue. Clade 2 enzymes are represented by the Mycobacterial enoyl-ACP reductase (MtInhA). Clade 2 enzymes contain an extended active site lid to accommodate the long acyl-ACP substrates (28 carbons) that arise during the synthesis of mycolic acids. Clade 3 plastid enzymes are represented by the Chlamydial CfFabI and contain peripheral loops of unknown function. Clade 4 enzymes are presented by AfFabI and contain carboxy-terminal \( \alpha_9-\alpha_9 \) coiled coils that are required for high affinity NADH binding. Bottom panel, active site volumes for each clade are depicted by a surface mesh. The catalytic lysine and tyrosine residues are shown in orange. Ternary complex structures of clade 1 (PDB ID: 4JQC) (77) and 3 (PDB ID: 4Q9N) (29) enzymes with NAD(H) and AFN-1252 (shown in yellow) have been determined and contain active site volumes that bind AFN-1252. Binary complex structure of clades 2 (PDB ID: 4DRE) (78) and 4 (PDB ID: 6VLY) enzymes with NAD(H) have been determined and AFN-1252 was modeled in their active site volumes with respect to the tyrosine. In both cases, the 3-methylbenzofuran moiety of AFN-1252 extends outside the active site and the oxotetrahydronaphthyridine moiety clashes with amino acid residues (shown in black).
The genome of a Bacteroidetes inhabitant of the human gut encodes a structurally distinct enoyl-acyl carrier protein reductase (FabI)
Christopher D. Radka, Matthew W. Frank, Jiangwei Yao, Jayaraman Seetharaman, Darcie J. Miller and Charles O. Rock

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