

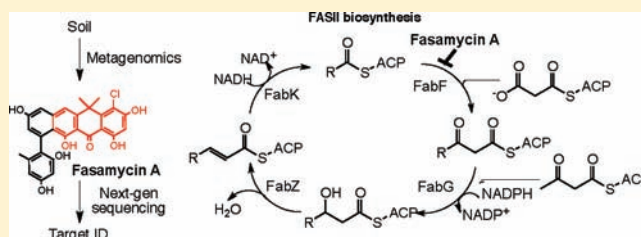
Environmental DNA-Encoded Antibiotics Fasamycins A and B Inhibit FabF in Type II Fatty Acid Biosynthesis

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Supporting Information

ABSTRACT: In a recent study of polyketide biosynthetic gene clusters cloned directly from soil, we isolated two antibiotics, fasamycins A and B, which showed activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. To identify the target of the fasamycins, mutants with elevated fasamycin A minimum inhibitory concentrations were selected from a wild-type culture of *E. faecalis* OG1RF. Next-generation sequencing of these mutants, in conjunction with *in vitro* biochemical assays, showed that the fasamycins inhibit FabF of type II fatty acid biosynthesis (FASII). Candidate gene overexpression studies also showed that fasamycin resistance is conferred by *fabF* overexpression. On the basis of comparisons with known FASII inhibitors and *in silico* docking studies, the chloro-*gem*-dimethyl-anthracenone substructure seen in the fasamycins is predicted to represent a naturally occurring FabF-specific antibiotic pharmacophore. Optimization of this pharmacophore should yield FabF-specific antibiotics with increased potencies and differing spectra of activity. This study demonstrates that culture-independent antibiotic discovery methods have the potential to provide access to novel metabolites with modes of action that differ from those of antibiotics currently in clinical use.



INTRODUCTION

Community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) infections are a growing concern to human health. As many as 30% of Enterococcal and 70% of *S. aureus* infections are reported to be resistant to first-line antibiotics.^{1,2} Structurally novel antibiotics with modes of action that differ from those in current clinical use are needed to combat these common drug-resistant phenotypes. Bacterial culture broth extracts have been very productive sources of new anti-infective agents. However, the vast majority of bacteria present in nature remain recalcitrant to culturing; as a result, most bacteria have not yet been explored for the production of novel antibacterial agents.³ Uncultured bacteria are likely the largest remaining pool of biosynthetic diversity that has not yet been examined for the production of medically relevant secondary metabolites. Exploiting this genetic diversity should prove to be a useful strategy for uncovering new bioactive metabolites that can serve as novel therapeutics.^{4,5}

The inability to culture many of the bacteria present within environmental samples renders these microbes incompatible with the most heavily relied upon techniques for characterizing bioactive natural products. Although it is still not possible to easily culture most bacteria in the environment, it is possible to extract microbial DNA directly from environmental samples and clone this DNA into model cultured bacteria where, for the first time, it can be functionally characterized. This general approach has been termed metagenomics,⁴ and its application

to the study of bacterial secondary metabolism is particularly appealing in light of the fact that all of the genes required for the biosynthesis of a natural product, including genes that code for biosynthetic enzymes, regulatory enzymes, and self-immunity enzymes, are typically clustered together on bacterial chromosomes.^{6,7} Heterologous expression of eDNA-derived secondary metabolite gene clusters has now led to the characterization of both interesting new biosynthetic enzymes and new bioactive small molecules.⁵

In a recent study of Type II (iterative, aromatic) polyketide (PK) biosynthetic gene clusters cloned directly from soil, we identified two antibiotics which show activity against methicillin-resistant *S. aureus* and vancomycin-resistant *E. faecalis*.⁸ These metabolites are related to the KB-3346-5 substances reported in the patent literature and have been given the trivial names fasamycin A (**1**) and fasamycin B (**2**) (Figure 1).⁹ Neither the mode of action of the fasamycins nor that of the KB-3346-5 substances had been described in the literature. Here we report on the use of next-generation sequencing of fasamycin-resistant mutants to elucidate the mode of action of the fasamycins.

MATERIAL AND METHODS

Isolation of Fasamycins A (1) and B (2). **1** and **2** were obtained using previously described methods.⁸ Briefly, *Streptomyces albus*

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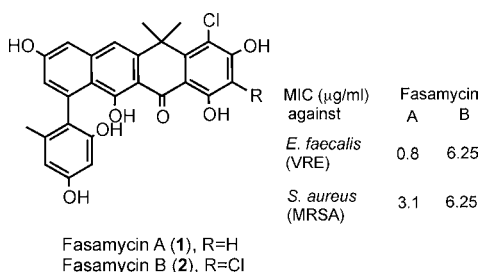


Figure 1. Fasamycins A (1) and B (2) were identified through the heterologous expression of an environmental DNA-derived type II polyketide gene cluster in *Streptomyces albus*.⁷

transformed with the eDNA-derived cosmid designated cosAZ154 was grown in ISP4 medium containing 5% HP-20 resin at 30 °C (200 rpm) for 12 days. The HP-20 resin was collected from the fermentation broth, washed with water, and then flushed with methanol. The methanol eluent was fractionated by silica gel flash chromatography using a CHCl_3 :MeOH (0.1% acetic acid) step gradient. Compounds 1 and 2 eluted from this column with the 95:5 CHCl_3 :MeOH fraction. Each metabolite was then purified by reversed-phase (XBridge C18, 10 \times 150 mm, 5 μm) HPLC (7 mL/min) using a linear gradient from 10:90 H_2O :MeOH (containing 0.1% formic acid) to 100% MeOH (containing 0.1% formic acid) over 30 min. Fasamycins A and B are produced at approximately 0.5 mg/L. BABX was purchased from Santa Cruz Biotechnology.

Selection of *Enterococcus faecalis* Strains Resistant to Fasamycin A. *E. faecalis* OG1RF was grown overnight in LB at 37 °C, and 100 μL of the overnight culture was spread on a LB agar plate containing 5 $\mu\text{g}/\text{mL}$ fasamycin A. The plate was incubated at 37 °C. Resistant colonies appeared after 18 h. Nine resistant colonies were picked and struck on selection plates, and then cultures of the mutants grown in the absence of fasamycin were used for sequencing.

Genome Sequencing and Analysis Methods. Library Preparation. Genomic DNA libraries were prepared using Illumina TruSeq library kits in accordance with the manufacturer's protocols. Briefly, genomic DNA was sheared using a Covaris S2 ultrasonicator, the resultant dsDNA fragments were end-repaired and A-tailed, and indexed adapters were ligated to the end-repaired DNA. PCR was performed for 10 cycles, and the libraries were quantitated using an Agilent Bioanalyzer 2100 and a High-Sensitivity DNA kit. Indexed adapters were used to allow for the multiplexing of samples at the sequencing stage.

Sequencing and Raw Data Analysis. Samples were sequenced on an Illumina HiSeq2000 for 101 cycles, with an additional 7 cycles for the indexing read. Sequencing was performed according to manufacturer's protocols using TruSeq chemistry. Image data were analyzed in real time by the onboard RTA software package. Bcl files produced by RTA were converted to qseq files by Illumina's OLB1.9 software package, and the qseq files were then converted to a fastq file for subsequent analysis.

Alignments and Variant Detection. Sequencing reads were aligned to the *E. faecalis* OG1RF genome assembly from Baylor University (http://www.hgsc.bcm.tmc.edu/microbial-detail.xsp?project_id=111) using the Stampy alignment package. The BWA aligner within Stampy was used to decrease processing time.^{10–12} BWA and Stampy allow for gapped alignment to facilitate the alignment of sequencing reads in situations where there is significant heterogeneity due to sequence insertions, deletions, or other lesions with regard to the reference genome. The alignment files were processed by Dindel and the Samtools mpileup program to yield potential variant calls consisting of single nucleotide polymorphisms, insertions, and deletions.^{13,14}

Confirmation of *fabT* Mutations. Sanger sequencing of *fabT* genes PCR amplified from resistant strains was used to verify each *fabT* mutation detected in the Illumina sequencing experiment (Supporting Information). Colony PCR was performed using whole cells, *fabT* flanking primers (*fabTF* TGCTTATTTACGATA-

TAGTTTG, *fabTR* TGAAGATTTTTTGTAGTGCTC), Phusion High-Fidelity DNA polymerase (New England Biolabs), and the following cycling parameters: denaturation (98 °C, 2 min), 10 touchdown cycles (98 °C, 20 s; 55 °C (dt -1 °C/cycle), 30 s; 72 °C, 30 s), 30 standard cycles (98 °C, 20 s; 45 °C, 30 s; 72 °C, 30 s), and a final extension step (72 °C, 7 min). Gel-purified amplicons were sequenced from both ends using the PCR primers.

Cloning FASII Genes from *E. faecalis* OG1RF. Individual genes in the *E. faecalis* FASII gene cluster were amplified by PCR using Phusion High-Fidelity DNA polymerase, *E. faecalis* OG1RF genomic

Table 1. Primers Used for Cloning *E. faecalis* FASII Genes

name	sequence ^a
FabHFor	GCGCCGGCCGATGAAGAATTATGCACGAATT
FabHRev	GCGCAGTACTTTTTACAGCGTTAGGAGCAG
acpPFor	GCGCCGGCCGATGGTATTTGAAAAAATTC
acpPRev	GCGCTCGCGACAGCCCCCTATTTTCATGTAT
FabKFor	GCGCCGGCCGATGAAGTGTACTTATCTTAGA
FabKRev	GCGCTCGCGATCACTTAGCCCCAACGCTGAT
FabDFor	GCGCCGGCCGATGAGGTGTCGTATGAAAACA
FabDRev	GCGCTCGCGACCATTTTTTACCTCCAGTAG
FabGFor	GCGCCGGCCGATGGAATTAACAGGGAAAAACG
FabGRev	GCGCTCGCGACCTTTTCGTTTATCCGTGCATG
FabFFor	GCGCCGGCCGATGAATCGAGTAGTTATTACCGG
FabFRev	GCGCTCGCGAAGTGCATGTTAATCTCCAG
accBFor	GCGCCGGCCGATGCAGTTAGAAGAAGTAAAGC
accBRev	GCGCTCGCGAGTTAATTTTCATGTTTTATTCTCC
FabZFor	GCGCCGGCCGATGAAATTAACAATTAACAGAAATTC
FabZRev	GCGCAGTACTCGAAAACATTTTTACCTATCC
accCFor	GCGCCGGCCGATGTTTTTCGAAAGTATTAATCGC
accCRev	GCGCAGTACTCTGCGCGTTCTTTCTTTAATG
accDFor	GCGCCGGCCGATGGCATTATTTTAAAGAAA
accDRev	GCGCTCGCGATTAGCCGCCACCTTCCAA
accAFor	GCGCCGGCCGATGAAAAGAAAACAGCCAATGA
accARev	GCGCTCGCGAGAGTAGTAACCTTCTAATATTGCG

^aRestriction sites added to each primer are underlined.

DNA as a template, and the primers listed in Table 1. PCR amplicons of the correct predicted size were double-digested with *EagI* (or *NofI*) and *NruI* (or *ScaI*) and ligated with the pMGS100 *E. faecalis* expression vector that had been double-digested with *EagI* and *NruI*.¹⁵ Each of the resulting expression constructs was transferred into *E. faecalis* by electroporation.¹⁶

Bioactivity: Growth on Elevated Concentrations of Fasamycin A. *E. faecalis* strains harboring FASII biosynthetic gene expression constructs were grown overnight (37 °C) in LB containing 12.5 $\mu\text{g}/\text{mL}$ of chloramphenicol. Overnight cultures were then diluted 10⁴-fold, and 5 μL aliquots of each diluted culture were inoculated onto a LB agar plate containing 12.5 $\mu\text{g}/\text{mL}$ of chloramphenicol and 5 $\mu\text{g}/\text{mL}$ of fasamycin A. Plates were incubated at 37 °C, and after 18 h, spots were scored for growth or no growth. Wild-type *E. faecalis* OG1RF and a mutant containing the M1 mutation were transformed with the empty pMGS100 expression vector and used as negative and positive controls, respectively.

Bioactivity: In Vivo Studies. *E. faecalis* strains harboring FASII gene expression constructs were checked for resistance to fasamycin A and other antibiotics. In these studies, wild-type *E. faecalis* harboring empty pMGS100 was used as a control. Overnight cultures grown in LB (12.5 $\mu\text{g}/\text{mL}$ chloramphenicol) were diluted 10⁶-fold. Fifty-microliter aliquots of the dilute cultures were added to individual wells of a 96-well plate. Compounds were resuspended in methanol at 5 mg/mL and then diluted 100-fold with culture medium. A 150 μL sample of this solution was added to the first well of the microtiter plate and then serially diluted across the plate. The plates were incubated at 30 °C for 24–48 h. Minimum inhibitory concentrations

(MICs) are reported as the lowest concentration at which no bacterial growth was observed after 24 h. MICs against other microorganisms were determined in the same manner. Yeast MIC studies were carried out using YPD broth.

Bioactivity: *In Vitro* Studies. *Acyl Carrier Protein (ACP) Cloning.* The ACP gene from *S. aureus* was amplified from *S. aureus* genomic DNA using the following primers and PCR conditions. Primers: forward StaphACPFHis, GCGCGGATCCGATGGAAAATTTTCGATAAAGTAA; reverse StaphACPR, GCGCAAGCTTATTTTCAAGACTGT-TAATA (restriction sites added for cloning are shown in bold). Cycling parameters: denaturation (98 °C, 2 min), 35 cycles (98 °C, 20 s; 55 °C, 30 s; 72 °C, 30 s), and a final extension step (72 °C, 7 min). 50 μ L reaction conditions: 10 μ L of 5 \times Phusion HF buffer, 1.5 μ L of DMSO, 0.3 μ L of each 100 mM oligonucleotide primer, 1 μ L of 10 mM dNTPs mix, 1 unit of Phusion High-Fidelity DNA polymerase and water as needed. Amplicons were doubly digested with *Bam*HI/*Hind*III, ligated into *Bam*HI/*Hind*III digested pColaDuet-1 (Novagen), and then transformed into *E. coli* BAP1 cells to yield pColaDuet:6His-SA-ACP. BAP1 is a BL21(DE3)-derived expression strain that contains an inducible phosphopantethinyl transferase (*spf* gene), which should assist with generating the desired holo-ACP.¹⁷

Expression and Purification of 6His-SA-ACP. Overnight cultures of BAP1/pColaDuet:6His-SA-ACP were used to inoculate 1 L cultures of LB (1:1000 dilution), which were grown at 37 °C until the OD₆₀₀ reached 0.6. The temperature was then reduced to 20 °C, and after 1 h, protein expression was induced with IPTG (0.5 mM). After an additional 16 h at 20 °C, the cultures were harvested by centrifugation (3200g for 15 min). The cell pellet was resuspended in 40 mL of lysis buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 10 mM imidazole), and the cells were lysed by sonication. Crude cell lysates were centrifuged at 25000g for 30 min, and the supernatant was incubated for 15 min (4 °C) with 1 mL of Ni-NTA resin (Qiagen). This slurry was washed with 20 mL of lysis buffer and then 20 mL of lysis buffer containing 50 mM imidazole. 6His-SA-ACP was subsequently eluted with 5 mL of lysis buffer containing 125 mM imidazole. 6His-SA-ACP was further purified by anion-exchange chromatography (Mono Q 10/100 GL) using a linear gradient from 0.1 M NaCl (10 mM Tris, pH 7.5) to 1 M NaCl (10 mM Tris, pH 7.5) over 120 min. Protein concentrations were determined with the Bradford assay. Aliquots of protein were flash-frozen in liquid nitrogen and stored at -80 °C.

FASII Enzyme Extract from *S. aureus*. The preparation of FASII enzymes from *S. aureus* has been described previously.¹⁸ Briefly, 6 L of *S. aureus* was grown (LB broth, 37 °C) to stationary phase. Cells were harvested by centrifugation (2500g, 10 min, 4 °C). The cell pellet was then washed twice with ice-cold FASII buffer (0.1 M sodium phosphate, pH 7, 1 mM EDTA, 5 mM β -mercaptoethanol) and resuspended in 0.5 L of this buffer. Washed cells were lysed by French press. Cell debris was removed by centrifugation (20 000 rpm, 15 min, 4 °C). The supernatant was collected, and ammonium sulfate was slowly added to reach 45% saturation. Precipitated protein was removed by centrifugation (10 000 rpm, 5 min, 4 °C), the supernatant was collected, and ammonium sulfate was then slowly added to reach 80% saturation. Precipitated protein was collected by centrifugation (10 000 rpm, 5 min, 4 °C). The resulting pellet was resuspended in 20 mL of FASII buffer and dialyzed (10 kDa MW cutoff) at 4 °C against four changes of buffer. This crude extract contains all of the factors necessary for reconstituting FASII elongation *in vitro*. Protein concentration measurements and protein storage were performed as described above.

FASII Elongation Assay. FASII inhibition assays were performed in 96-well phospholipid Flashplates (Perkin-Elmer).¹⁸ First, 0.4 μ g of FASII protein extract was added to 40 μ L of buffer [100 mM sodium phosphate (pH 7), 5 mM EDTA, 1 mM NADPH, 1 mM NADH, 150 μ M DTT, 5 mM β -mercaptoethanol, 20 μ M lauroyl-CoA, 4% Me₂SO, and 5 μ M DTT-pretreated 6His-SA-ACP] and preincubated with inhibitor at room temperature for 20 min. Reactions were initiated with the addition of 8 μ L of C-2 labeled [¹⁴C]-malonyl-CoA (Perkin-

Elmer 57.5 mCi/mmol) dissolved in water to a final concentration of 4 μ M. After 60 min at 37 °C, reactions were terminated with the addition of 80 μ L of 14% perchloric acid. The 96-well phospholipid Flashplates were sealed, incubated at room temperature overnight, and then read in a top count scintillation counter (Trilux Microbeta).

FASII Elongation and ACP Loading Assay. FASII elongation assays were carried out as described above with a few modifications. A 0.2 μ g sample of FASII extract was used in each 50 μ L reaction, and reactions were run in microcentrifuge tubes. After a preincubation with inhibitor at room temperature for 20 min, fatty acid elongation was initiated with the addition of 10 μ L of dissolved C-2 labeled [¹⁴C]-malonyl-CoA (Perkin-Elmer 57.5 mCi/mmol, final concentration 4 μ M), and then after 30 min at 37 °C, 15 μ L of native sample buffer was added to each reaction. Reactions were resolved on 16% polyacrylamide gels containing 4 M urea. Gels were blotted to polyvinylidene difluoride membrane (Bio-Rad) and imaged using a PhosphorImager.

Docking with AutoDock Vina. Crystal structures of *E. coli* FabF deposited in the PDB were used for docking studies (PDB entries 2gfv, 2gfw, and 2gfx). 2gfw is a native structure of wild-type FabF. 2gfv and 2gfx are both structures of C163Q active-site mutants. In the structure 2gfv, platensimycin is bound in the active site. All cofactors, ions, and water molecules were removed, and Pdbqt-formatted files were generated for each structure using AutoDock Tools 1.5.6.¹⁹ MM2-minimized fasamycin A, fasamycin B, and BABX were prepared using ChemBioDraw 3D (CambridgeSoft). Pdbqt-formatted files of these structures were used as ligands in the docking studies. Rigid docking was performed using AutoDock Vina in command line mode. We used the averaged position of the three C- α atoms in the catalytic triad as the center of the docking search and 25 Å³ space around this point as the search area. We performed three docking iterations for each FabF/molecule pair, and all three iterations were run using a different random seed. Low-energy docking solutions were then visually compared. 2gfw did not yield any low-energy solutions with antibiotics bound in the active site. When docking into 2gfv (C163Q mutant), the only chemically reasonable low-energy binding motif that was consistently observed and common to all three antibiotics is shown in Figure 7b. The absolute stereochemistry of BABX is not known. Only one enantiomer closely mimics the binding of the fasamycins in these studies. This enantiomer is shown in Figure 7b and assumed to be the naturally occurring (-)-BABX. Fasamycin A was docked into 2gfv (platensimycin bound to FabF) by first removing the bound platensimycin from the model (Figure 7c).

RESULTS AND DISCUSSION

Fasamycins A and B were heterologously expressed from an environmental DNA (eDNA) derived Type II PK gene cluster using *Streptomyces albus* as a host. Both metabolites are Gram-positive specific antibiotics (Table 2). Monohalogenated

Table 2. Fasamycins A (1) and B (2) Activity

organism	1 ^a	2
Gram-positive bacteria		
<i>Bacillus subtilis</i> BR151	3.1	6.25
<i>Staphylococcus aureus</i> 6538P	3.1	6.25
<i>Staphylococcus aureus</i> USA300 (MRSA)	3.1	6.25
<i>Enterococcus faecalis</i> OG1RF	0.8	6.25
<i>Enterococcus faecalis</i> EF16 (VRA)	0.8	6.25
Gram-negative bacteria		
<i>Escherichia coli</i> EC100	>100	>100
<i>Escherichia coli</i> BAS849	12.5	25
<i>Ralstonia metallidurans</i> CH34	>100	>100
<i>Burkholderia thailandensis</i> E264	>100	>100
Eukaryote		
<i>Saccharomyces cerevisiae</i> w303	>100	>100

^aMinimum inhibitory concentrations are reported as μ g/mL.

fasamycin A is consistently more active than dihalogenated fasamycin B against the Gram-positive bacteria we examined. While the fasamycins appear to be inactive against wild-type Gram-negative bacteria they do exhibit activity against membrane permeabilized *E. coli* (BAS849), suggesting that the absence of activity in Gram-negative bacteria is due to the inability of the fasamycins to access their target. At the highest concentrations tested, neither metabolite showed cytotoxicity against yeast.

Mutant bacterial strains that show decreased susceptibility to a small-molecule toxin can provide insight into the toxin's mode of action. In an effort to identify the target of the fasamycins using a resistance phenotype selection strategy, a single wild-type colony of *E. faecalis* OG1RF was grown to confluence overnight, and the resulting culture was then plated on LB agar containing ~6 times the MIC for fasamycin A (5 $\mu\text{g/mL}$).^{10,20} One in every 10^8 – 10^9 bacterial cells we plated grew at this elevated fasamycin A concentration. Genomic DNA isolated from nine unique resistant colonies as well as genomic DNA from the culture that gave rise to these mutants was sequenced using Illumina HiSeq2000 technology. The mutant genomes were then compared to our experiment-specific reference genome and queried for sequence differences. In each case, the fasamycin resistant mutant strains were found to contain mutations in the *fabT* gene. In total, five unique *fabT* mutations (Figure 2, M1–M5) were found among the nine

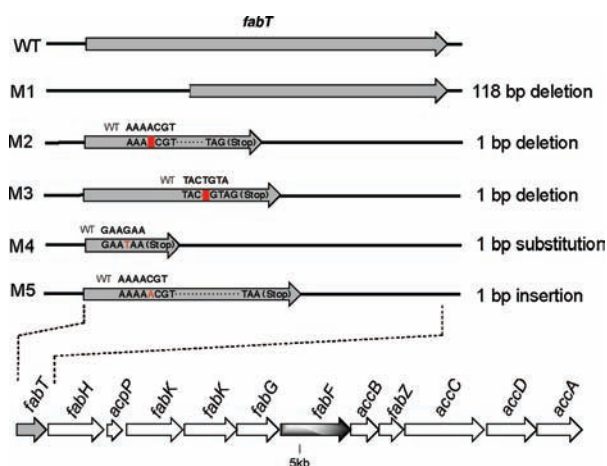


Figure 2. Genomes from nine fasamycin A resistant strains were sequenced. All contained mutations that disrupt the *fabT* gene. The five unique *fabT* mutations (M1–M5) that we observed are shown. FabT is a transcriptional repressor that regulates the expression of the FASII gene cluster in *E. faecalis*.

sequenced mutant strains, each of which is predicted to result in truncation of the *fabT* gene product (Figure 2). PCR amplification and resequencing of the *fabT* locus from each mutant confirmed the presence of these mutations. A small number of additional mutations were found in some genomes, but only the *fabT* mutations were common to all nine strains.

By selecting mutants from a natural population of cells instead of from cultures treated with mutagenizing agents, the number of mutations per genome is minimized. With only a small number of mutations per genome, it should be much easier to establish a functional link between a resistance phenotype and the specific mutation leading to this phenotype. In this case, the *fabT* gene is predicted to encode a MarR-like transcriptional repressor.^{21,22} In *E. faecalis*, *fabT* is found in a

gene cluster that encodes for all of the enzymes required for type II fatty acid biosynthesis (FASII) (Figure 2).²³ Disruption of the FabT transcriptional repressor is predicted to result in increased expression of this gene cluster. Constitutive over-expression of the FASII gene cluster could lead to an increased MIC for fasamycin A if the primary *in vivo* antibacterial target of the fasamycins is a FASII biosynthetic enzyme.

The activity of the fasamycins was tested in an *in vitro* fatty acid elongation assay. This assay employs crude FASII extract that contains all of the enzymes required for fatty acid elongation, long-chain acyl CoA (lauroyl-CoA) starter units, and [¹⁴C]-malonyl CoA that together are used to extend fatty acids on purified holo-ACP substrates. ACP-linked long-chain fatty acids are then captured using a phospholipid Flashplate, and ¹⁴C incorporation into newly generated fatty acids is read by a scintillation counter. In elongation assays with *S. aureus* FASII extract and recombinant *S. aureus* 6-His-ACP (6-His-SA-ACP), fasamycins A and B both inhibit fatty acid elongation (IC_{50} = 50 and 80 $\mu\text{g/mL}$, respectively) and show inhibition curves similar to those of other known FASII inhibitors (Figure 3).

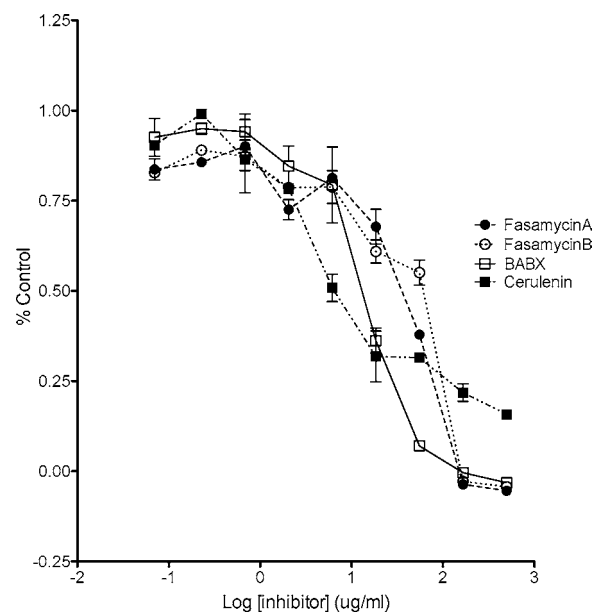


Figure 3. *In vitro* FASII inhibition using *S. aureus* 6His-SA-ACP and *S. aureus* FASII extract. Reactions were run with an excess of [¹⁴C]-malonyl-CoA and lauroyl-CoA. Elongation assays were run in duplicate.

Based on their ability to inhibit this particular configuration of the *in vitro* FASII elongation assay, only five FASII enzymes, FabD, F, G, K, and Z, remained candidate fasamycin targets (Figure 4). To distinguish among the inhibition of FabD, FabF, and the reductive enzymes of the elongation cycle (FabG, Z, and K), FASII elongation assays were examined using urea polyacrylamide gel electrophoresis. With urea gels it is possible to resolve malonyl-ACP, the product of FabD, from the longer chain acyl-ACPs generated in the FASII elongation cycle. As can be seen in Figure 5, fasamycin A does not inhibit FabD-dependent formation of malonyl-ACP (Figure 5, upper band) but does inhibit the formation of long-chain acyl-ACPs from the FabD product (Figure 5, lower bands). The absence of any labeled long-chain acyl-ACPs in the fasamycin A-containing reaction indicates that fasamycin A inhibits FabF, the initial

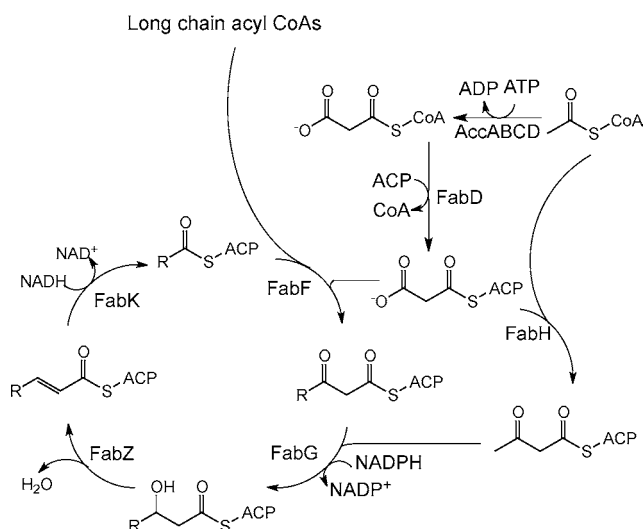


Figure 4. Schematic of FASII biosynthesis. *In vitro* fatty acid elongation assays use a long-chain acyl CoA starter and the incorporation of [14 C]-malonyl-CoA to measure FASII activity.

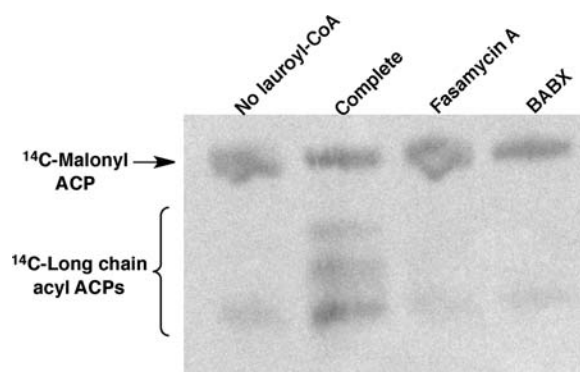


Figure 5. Fatty acid elongation assay with [14 C]-malonyl-CoA, lauroyl-CoA, 6His-ACP, and *S. aureus* FASII extracts. Reactions were run on a 16% polyacrylamide gel containing 4 M urea to resolve 14 C-labeled malonyl-ACP (upper band) from 14 C-labeled long-chain ACPs (lower bands). Fasamycin A inhibits fatty acid elongation but does not inhibit FabD-dependent formation of malonyl-ACP. Lane 1, control reaction without lauroyl-CoA; lane 2, complete reaction; lane 3, 200 μ g/mL fasamycin A; lane 4, 200 μ g/mL BABX.

condensation step of the elongation cycle, and not simply a reductive step (FabG, K, and Z) in this cycle. FabF is one of two FASII-specific condensation enzymes. It is specifically used in the fatty acid chain elongation cycle, while the second condensing enzyme, FabH, is used in fatty acid initiation (Figure 4). Our biochemical studies do not rule out the possibility that the fasamycins might also inhibit FabH.

The specific target of the fasamycins was further confirmed using a candidate gene overexpression strategy in which each FASII gene was individually overexpressed and assayed for the ability to confer fasamycin A resistance to *E. faecalis*. Both gene knockdown and gene overexpression strategies have been used to identify the protein targets of other FASII inhibitors.^{24,25} For this study FASII genes were PCR-amplified from wild-type *E. faecalis* genomic DNA and cloned into the *E. coli*-*E. faecalis* shuttle expression vector, pMGS100.¹⁵ Individual FASII gene expression constructs were then assayed for the ability to confer fasamycin A tolerance to wild-type *E. faecalis*. As would be expected for a FabF specific antibiotic, only *E. faecalis*

transformed with the FabF overexpression construct grew robustly in the presence of lethal concentrations of fasamycin A (Figure 6a). Cerulenin, a well-characterized FabF inhibitor,

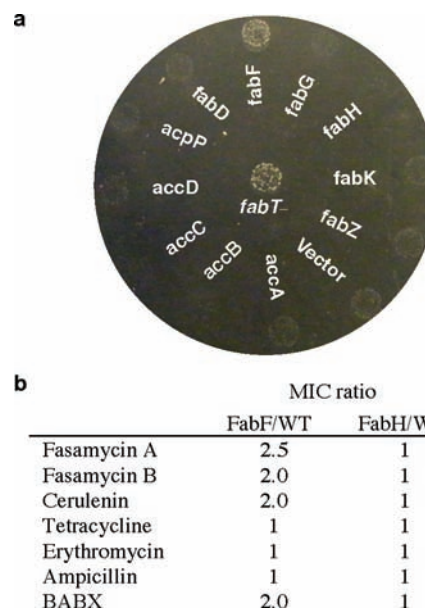


Figure 6. (a) A FabF knockout mutant with the M1 mutation and wild-type *E. faecalis* transformed with either a vector control or individual FASII overexpression constructs were inoculated onto LB agar containing 5 μ g/mL fasamycin A. (b) The MIC ratios for antibiotics measured against wild-type *E. faecalis* and *E. faecalis* transformed with either the *fabF* or *fabH* overexpression constructs are listed.

shows a similar MIC profile against these same FASII overexpression strains, while antibiotics with different FASII protein targets do not exhibit elevated MICs against FASII overexpression strains (Figure 6b).^{24,25}

The fasamycins share a common tricyclic chloro-*gem*-dimethyl-anthracenone substructure with one previously described FASII inhibitor, BABX (3) (Figure 7a).^{18,26} Based on data from *in vitro* fatty acid chain elongation assays, BABX was also predicted to target FabF.^{18,26} As with fasamycin A, BABX shows reduced potency against the FabF overexpression strain but not against other FASII gene overexpression strains (Figure 6b). BABX and fasamycin A also show very similar *in vitro* FASII and FabF inhibitory activities (Figures 3 and 5).

When the fasamycins and BABX were docked into the *E. coli* FabF crystal structure using AutoDock software, their common *gem*-dimethyl-anthracenone substructure was predicted to bind the FabF active site in the same position and orientation (Figure 7b).¹⁹ In the AutoDock models, the anthracenone ring systems extend into the back of the active site toward the catalytic triad while the variable regions extend out of the active-site pocket and make unique contacts with residues that line the opening of the active site (Figure 7b). Chemically reasonable docking was only observed when using X-ray structures of the FabF C163Q active-site mutant. This mutation is reported to mimic the active-site conformation of an acyl-enzyme intermediate and shows a 50-fold increase in apparent binding to the known FabF inhibitor platensimycin (4).¹⁹ Co-crystal structures of FabF with cerulenin and platensimycin show they bind distinct regions of the active site.^{24,25} The fasamycins dock into the malonyl binding portion of the active site, which is where platensimycin also binds (Figure 7b,c). The

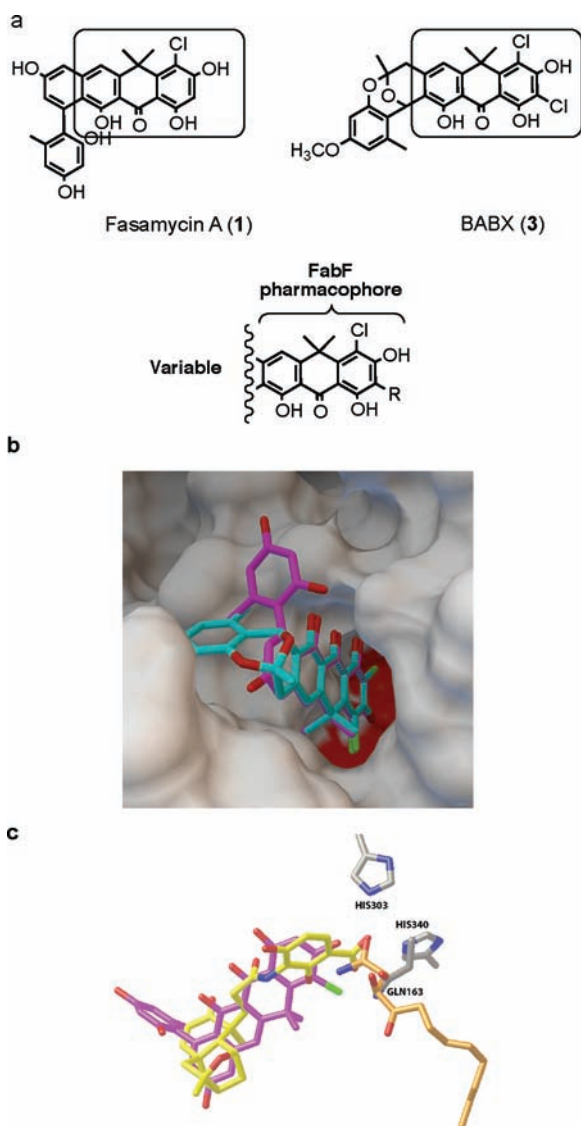


Figure 7. (a) The fasamycins and BABX share a chloro-*gem*-dimethyl-anthracenone substructure. (b) Fasamycin A (purple) and BABX (blue) were docked into the active site of *E. coli* FabF(C163Q) using AutoDock software and were predicted to share a common binding motif. The catalytic triad (H303, H340, C163Q) at the back of the active site is colored in red. (c) Fasamycin A (purple) was docked into the platensimycin (teal):*E. coli* FabF cocrystal structure. The catalytic triad is shown, and all residues predicted to be in van der Waals contact with both platensimycin and fasamycin A are labeled. Fasamycin A is predicted to bind in the same site as platensimycin. Fasamycin A (purple), platensimycin (yellow), and cerulenin (orange) are shown in the active site of *E. coli* FabF. Platensimycin (4) and cerulenin were placed according to their observed positions in cocrystal structures.^{14,15}

halogenated *gem*-dimethyl-anthracenone substructure that is common to both the fasamycins and BABX appears to represent a natural FabF-specific antibacterial pharmacophore (Figure 7a).

Bacterial FASII systems are composed of collections of discrete monofunctional enzymes that carry out individual transformations in fatty acid biosynthesis. Mechanistically, this process is identical to that used in humans and other higher eukaryotes; however, in higher eukaryotes, all reactions are carried out on a single multisynthase. Structural differences

between the two systems have made bacterial fatty acid biosynthesis an attractive drug development target.^{24,25,27–29} A recent study reported that some FASII knockouts can be competent for infection because they are able to uptake long fatty acids from serum, questioning the potential utility of FASII inhibitors as therapeutic agents.^{31,32} These results have been called into question in subsequent studies that show that FASII is essential in at least some Gram-positive pathogens. The effectiveness of the FabI inhibitor, isoniazid, against *Mycobacterium tuberculosis* implies that FASII inhibition is in fact a clinically relevant antibacterial target.

Only two FASII inhibitors, triclosan and isoniazid, both of which target the enoyl-acyl carrier protein reductase FabI, are used commercially as antibiotics. FabF-specific antibiotics like the fasamycins show activity against therapeutically relevant antibiotic resistant bacteria. In the case of the fasamycins and BABX, the evolutionary convergence to the same pharmacophore, on at least two separate occasions, suggests this may be a promising antibacterial lead structure. Optimization of the chloro-*gem*-dimethyl-anthracenone pharmacophore and the variable region that extends out of the FabF active site could yield additional FabF-specific antibiotics with increased potencies and potentially differing spectra of activity. In fact, the specific chlorination pattern on the *gem*-dimethyl-anthracenone substructure is clearly important for the fasamycins' activity against *E. faecalis*, as the monohalogenated analogue (1) is almost an order of magnitude more active against this pathogen than the dihalogenated derivative (2).

Researchers at Merck reported the identification of four novel FASII inhibitors from high-throughput screening efforts involving broth extracts derived from cultured bacteria [Figure 8, BABX (3), platensimycin (4), phomallenic acid C (5), and

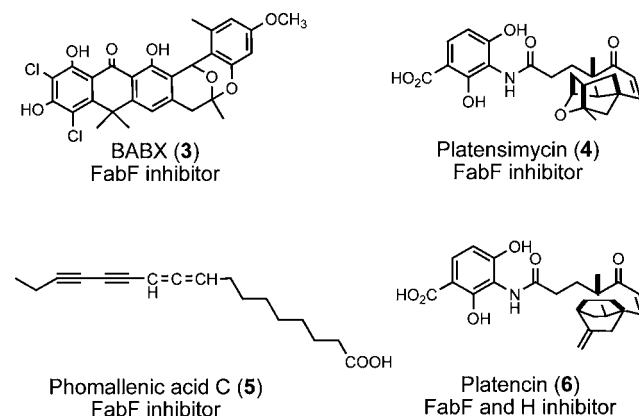


Figure 8. FASII inhibitors reported recently from the high-throughput screening of bacterial culture broth extracts.^{24–26,30} The specific protein target(s) of these metabolites are shown below each structure.

plantencin (6)].^{24–26,30} The fasamycins, on the other hand, were discovered from the examination of a small set of environmental DNA-derived type II PKS gene clusters that were selected for functional analysis because they differed from previously sequenced type II PKS gene clusters.⁸ Advances in both metagenomic methods and DNA sequencing technologies have the potential to invigorate the process of antibiotic discovery. Next-generation sequencing now makes it possible to routinely and rapidly inform on the mode of action of new antibiotics through the sequencing of resistant mutants, and metagenomics has the potential to access large numbers of

previously inaccessible natural antibiotics. In the case of the fasamycins, both their discovery and the identification of their *in vivo* target were made possible through the application of these rapidly advancing technologies to the problem of antibiotic discovery.

■ ASSOCIATED CONTENT

📄 Supporting Information

fabT mutant sequences. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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