Resistance to AFN-1252 Arises from Missense Mutations in *Staphylococcus aureus* Enoyl-Acyl Carrier Protein Reductase (FabI)*

Jiangwei Yao, John B. Maxwell and Charles O. Rock1

Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, 38105, USA.

Running title: Molecular Mechanism of AFN-1252 Resistance

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1To whom correspondence should be addressed: Department of Infectious Diseases, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105 USA. Tel: 901-595-3491; Fax: 901-595-3099; E-mail: charles.rock@stjude.org

2Abbreviations used are: ACP, acyl carrier protein; apoACP, ACP lacking the 4′-phosphopantetheine prosthetic group; FabI, enoyl-ACP reductase; MIC, minimum inhibitory concentration; LB, Luria-Bertani medium; FASII, bacterial type II fatty acid synthase; qRT-PCR, quantitative real-time polymerase chain reaction.

Keywords: *Staphylococcus aureus*, antibiotic resistance, AFN-1252, enoyl-ACP reductase, FabI, fatty acid synthesis.

Background: AFN-1252 is a FabI inhibitor developed to treat *Staphylococcus aureus*. A thorough screen for AFN-1252 resistant strains was undertaken to identify the spectrum of mechanisms for acquired resistance. A missense mutation in *fabI* predicted to encode FabI(M99T) was isolated 49 times, and a single isolate was predicted to encode FabI(Y147H). AFN-1252 only bound to the NADPH form of FabI and the close interactions between the drug and Met99 and Y147 explained how the mutations would result in resistant enzymes. The clone expressing FabI(Y147H) had a pronounced growth defect that was rescued by exogenous fatty acid supplementation, and the purified protein had less than 5% the enzymatic activity of FabI. AFN-1252 was indistinguishable from FabI. The AFN-1252 *K*_{app} increased from 4 nM in FabI to 69 nM in FabI(M99T) accounting for the increased resistance of the corresponding mutant strain. The low activity of FabI(Y147H) precluded an accurate *K*_{app} measurement. The strain expressing FabI(Y147H) was also resistant to triclosan; however, the strain expressing FabI(M99T) was more susceptible. Strains with higher levels of AFN-1252 resistance were not obtained. The AFN-1252 resistant strains remained sensitive to sub-micromolar concentrations of AFN-1252, which blocked growth through inhibition of fatty acid biosynthesis at the FabI step.

Enoyl-acyl carrier protein (ACP) reductase (FabI) catalyzes the NAD(P)H-dependent reduction of *trans*-2-enoyl-ACP to acyl-ACP, as one of the four essential steps required for every cycle of two carbon elongation in the biosynthesis of fatty acids in bacteria (1). The FabI of *Escherichia coli* plays a determinant role in pulling each round of elongation to completion (2). The discovery that the antibiotic...
activity of triclosan (Fig. 1A) against E. coli was due to the selective inhibition of FabI (3,4) validated FabI as a bona fide drug target, and stimulated the biochemical and structural characterization of FabI (5-12), as well as the development of multiple small molecule FabI inhibitors as potential antibacterial agents (11-15). It was initially thought that such inhibitors would be broad-spectrum antibacterials because triclosan is effective in killing almost all bacteria. However, FabI is not the only enoyl-ACP reductase isomerase in bacteria. Streptococcus pneumoniae uses a flavoprotein called FabK that is unrelated in structure to FabI and is refractory to triclosan inhibition (16,17). Thus, the inhibitory action of triclosan in this bacterium arises by an unknown mechanism unrelated to fatty acid synthesis. Two other FabI-related proteins also carry out enoyl-ACP reduction, FabL (5) and FabV (18). Although these enzymes are related to FabI, bacteria that express these proteins are resistant to triclosan (5,16,18). Despite the fact that FabI inhibitors will not have broad-spectrum activity, development of FabI-directed drugs has continued because FabI is essential in the important human pathogen Staphylococcus aureus (6). The rapid spread of S. aureus resistant to multiple drugs is a major threat to our health care system, and therapies directed against new targets are needed to combat this emerging pathogen (19,20).

AFN-1252 (Fig. 1A) was developed to selectively target S. aureus FabI through structure-guided optimization (21-24). AFN-1252 is a potent, orally administered anti-Staphylococcal drug. It inhibits FabI with an apparent IC$_{50}$ of about 10 nM and the drug has 4 ng/ml antibacterial potency against S. aureus (25,26). The advancement of AFN-1252 into human Phase II clinical trials emphasizes the importance of understanding the biochemical mechanism(s) of genetically acquired resistance. In previous work, bacterial clones with increased AFN-1252 resistance were isolated that contained missense mutations in the fabI gene predicted to encode FabI(M99T) and FabI(Y147H) proteins (25,26). This study conducts a thorough screen for AFN-1252 resistant mutants to identify all mechanisms for genetically acquired resistance, examines the biochemical properties of the resistant enzymes, and evaluates the effect of their expression on bacterial physiology. Although strains expressing FabI mutants had increased resistance to AFN-1252 inhibition, they still had sub-micromolar MICs for AFN-1252 due to on-target inhibition of FabI.

**EXPERIMENTAL PROCEDURES**

**Bacteria and Molecular Biology**—All strains used in this work were derivatives of S. aureus strain RN4220 (27). The MICs were determined by growing strains to an optical density at 600 nm (OD$_{600}$) of 1.0 and diluted 30,000-fold in Luria-Bertani (LB) medium. A 10 µl aliquot of diluted cells was added to each well of a U-bottom 96-well plate containing 100 µl of LB medium with the appropriate concentration of compound. The plate was incubated at 37°C for 20 h and cell growth was determined using a Fusion plate reader at 600 nm. Cells grown in medium containing dimethyl sulfoxide (DMSO; 1%) were used as the control for 100% growth. LB media containing 0.1% Brij-58 at 37°C with or without supplementation with 133 µM anteiso15:0, 66 µM anteiso17:0 and 10 µM lipoate was used to test the influence of exogenous fatty acids on growth.

The fabI gene from S. aureus was cloned into pET15b (Novagen), overexpressed, and purified as previously described (6). The plasmids expressing the FabI(M99T) and FabI(Y147H) mutants were generated by mutagenizing the pET15b-fabI plasmid using the QuickChange Mutagenesis Kit from Agilent Technology, following the manufacturer protocol. The mutagenesis primers for the M99T mutant were 5'-tgtatatcattcatcagttgttaatgcgaattcgccacctaaatgtgttgttgcaacaatgcta and 5'-ccgtagcttgttctggttaatatcgcatttgcta. The primers for the Y147H mutant were 5'-tagttgttaacacaaagaatgaatgcggccttttcgtttg and 5'-gattcttgtatatcattgcattgcatttgcttttcgtttgctttgccatgcagc. Expression and purification of the mutant proteins were the same as those used for the wild-type protein. The FabI(M99T, Y147H) double mutant was generated by sequential mutagenesis of the FabI(M99T) plasmid with the FabI(Y147H) primers. The wild-type and mutant fabI genes were cloned into the plasmid pCL15, a S. aureus expression vector, and the plasmids were transformed into strain RN4220 as previously described (26).

**Selection of AFN-1252 Resistant Mutants**—AFN-1252 resistant mutants were selected on LB-agar plates containing 40 ng/ml AFN-1252. An aliquot of 200 µl of OD$_{600}$ of 1 (1 x 10$^8$ cells) S. aureus strain RN4220 was spread on a 10 cm LB-agar plate containing AFN-1252. The plate was incubated at 37°C for 48 hours, and on average, 5-8 resistant colonies were found per plate. Colonies that grew on the plate were restreaked onto another AFN-1252-containing LB agar plate to purify individual clones. The sequences of the fabI genes of the confirmed mutants were amplified via PCR and sequenced. Strains RN4220, MWF32...
(FabI(M99T)) and MWF33 (FabI(Y147H)) were subjected to additional rounds of selection on LB-agar plates containing 0.2-1 µg/ml of AFN-1252 as described above. No resistant colonies were isolated at this AFN-1252 concentration.

Synthesis of Crotonyl-ACP—The *S. aureus* acpP gene was amplified via *taq* polymerase (Invitrogen) from *S. aureus* genomic DNA, and cloned into the pET-15b plasmid (Novagen) at the Ndel and BamHI restriction sites. The resulting plasmid was transformed into BL21 DE3 (Novagen) cells. Following a 3-h induction of log-phase cells, the cells were lysed and the amino-terminal 6x-histidine-tagged ACP was purified using Ni-NTA affinity chromatography. A yield of 40 mg pure apoACP was obtained from each liter of culture. The protein was dialyzed against 20 mM Tris, pH 8.0, to remove the imidazole from purification. The 6x-histidine tag was cleaved from apoACP by treatment with thrombin (5 unit per mg purified protein) overnight at 4°C. Thrombin and the cleaved histidine tag were separated from the ACP via size exclusion chromatography. The resulting apoACP had a GlySerHis addition at the amino-terminus of the protein. Crotonyl-ACP was biosynthesized by incubating 250 µM crotonyl-CoA with 200 µM apoSaACP, 10 mM MgCl₂, and 1 µM purified *Streptococcus pneumoniae* [ACP]synthase in 50 mM Tris-HCl, pH 7.0, at 30°C for 2 hours (28). Completion of the reaction was determined by analysis using a 15% polyacrylamide gel containing 0.5 M urea gel to separate apoACP from crotonyl-ACP. The His-tagged [ACP]synthase was removed using an Amicon Ultra Centrifugal Filter (3 kDa cutoff). The MgCl₂ and CoA were then removed via Ni-NTA affinity chromatography. A yield of 40 mg pure apoACP was purified using Ni-NTA chromatography over a 24 ml Superdex G200 column equilibrated with 20 mM Tris HCl, 150 mM NaCl, pH 7.8, at 1 ml/min, and 200 µl fractions collected. The fractions were added to 3 ml of ScintSafe scintillation solution and counted in a Beckman LS6500 Scintillation Counter.

Real Time PCR for Analysis of Gene Expression—The strains were grown to an optical density at 600 nm (OD₆₀₀) of 1.0. Cells were washed with 1 ml of RNAlater Solution from Ambion, and treated with Lysostaphin for 15 minutes at room temperature. Total RNA was isolated from the bacterial cells using the RNAqueous Kit (Ambion) per manufacturer’s instructions including the LiCl precipitation. The pelleted RNA was resuspended in nuclease-free water and a 10 µg aliquot was treated with Ambion’s Turbo DNA-free Kit to remove genomic DNA. First-strand cDNA was generated from 500 ng total RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System. Experimental samples were run in triplicate; negative controls (distilled water) and RNA samples without the reverse transcription step were run in duplicate. PCR reactions contained SYBR Green PCR Master Mix (Applied Biosystems), 150 nM of each primer and cDNA synthesized from 10 ng of total RNA. The expression level of various housekeeping genes (*proC*, *gyrB*, *gmk*, *glyA*, *rpoD*, *rho*, *recF* and *pyk*) was checked and *gyrB* was determined to be the calibrator least changed by AFN-1252 treatment and subsequently was used as the control to normalize mRNA levels between samples. Template curves of 7 points ranging from 50 ng to 50 pg of total RNA input were run for each primer set and analyzed for linearity and relative efficiency of the PCR reaction as compared to the control. Dissociation curves were generated after each real-time PCR run to check for the presence of non-specific amplification.

Analysis of [¹⁴C]AFN-1252 Binding to FabI—Gel filtration was used to determine which FabI form bound AFN-1252. [¹⁴C]AFN-1252 (specific activity 58.1 mCi/mmol) was a generous gift from Nachum Kaplan of Affinium Pharmaceuticals. [¹⁴C]AFN-1252 (25 µM) was incubated with FabI (25 µM), and either 200 µM NADP⁺, 200 µM NADPH, or no nicotinamides in 20 mM Tris HCl, pH 7.8, for 20 minutes at room temperature. The mixtures were then chromatographed over a 24 ml Superdex G200 column equilibrated with 20 mM Tris HCl, 150 mM NaCl, pH 7.8, at 4°C. Then the sample (100 µl) was applied to the column, the column was eluted with 20 mM Tris HCl, 150 mM NaCl, pH 7.8, at 1 ml/min, and 200 µl fractions collected. The fractions were added to 3 ml of ScintSafe scintillation solution and counted in a Beckman LS6500 Scintillation Counter.

ACP Immunoblotting— Cultures (50 ml) of strains RN4220, MWF32 and MWF33 were grown in LB media to OD₆₀₀ of 0.5 at 37°C. AFN-1252 (final concentration of 100 ng/ml for strain RN4220 and 2 µg/ml for the mutant strains) was added to the culture. Control samples had only DMSO (1%) used to dissolve the drug. The treated cultures were incubated for 30 minutes, the cells were harvested, lysed, and the cell extracts were fractionated on a 15% polyacrylamide/0.5 M urea gel, and immunoblotted using anti-ACP antibodies (29) to determine the ACP species (26). Protein from the same number of cells was loaded into each lane by normalizing the extract volume to the OD₆₀₀ of the culture.
Acetate Labeling—[14C]Acetate labeling experiments were conducted to measure the effect of AFN-1252 on lipid biosynthesis in strains RN4220, MWF32, and MWF33 when treated with AFN-1252. Starter cultures were grown overnight, and the starter culture was back diluted to an initial OD600 of 0.1 and grown until OD600 reached 0.5 at 37°C and 225 rpm shaking. The culture was split into 10 mL aliquots and each aliquot was incubated with the appropriate concentration of AFN-1252 and 10 µCi of [14C]acetate for a single doubling time (30 minutes for strains RN4220 and MWF32, 120 minutes for strain MWF33). The cells were collected by centrifugation and washed twice with phosphate-buffered saline. Lipids were harvested from pelleted cells using the method of Bligh and Dyer (30). [14C]Acetate incorporation was measured by counting the lipid extracts on a LS6500 Multipurpose Scintillation Counter. Measurements were made in duplicate and the averages with standard error were reported.

Analysis of FabI Mutants by Heterologous Expression in E. coli—The Met99 residues of the S. aureus fabI gene in the Bluescript vector (6) was mutagenized to other amino acids using the QuikChange Site-Directed Mutagenesis Kit (Agilent) and primers generated by the QuikChange Primer Design program (Agilent). The changes were verified by DNA sequencing. The Bluescript vectors harboring the mutant fabI genes were transformed into E. coli strain JP1111 (fabI(Ts)) via electroporation. Strain JP1111 harboring pBluescript expression vector containing the mutant fabI gene were streaked on LB-CBC plates at 42°C to determine if the S. aureus FabI mutants supported cell growth. These same vectors were also transformed into E. coli strain ANS1 (tolC) to rank the AFN-1252 resistance phenotype of the mutants by determining their ability to shift the MIC for AFN-1252 as previously described (26). Strain ANS1 cells harboring the empty vector without the S. aureus fabI gene was included as control.

FabI Enzymatic Assays—The FabI enzymatic progress was determined by measuring the conversion of NADPH to NADP⁺ at 340 nm. The enzyme reactions were performed in 100 µl volume in Costar UV half-area 96 well plates with a SpectraMax 340 instrument taking 340 nm readings at 10 sec intervals at 30°C. For velocity measurements, FabI enzyme buffered in 100 mM glutamate pH 7.8 was added to either 50 µM crotonyl-ACP or 200 µM crotonyl-CoA and 250 µM NADPH buffered in 20 mM Tris-HCl pH 7.8. Assays for E. coli FabI were the same, except NADH is substituted for NADPH instead. For determination of Kₘ of NADPH for the wild type and mutant FabI enzymes, 50 nM of enzyme was added to 50 µM crotonyl-ACP and 15 µM, 25 µM, 50 µM, 75 µM, 100 µM, 150 µM, or 250 µM NADPH. The reaction was mixed for 10 seconds by the plate reader and data was acquired at 10 sec intervals for 10 minutes. Initial velocities were calculated from the initial linear phase of the progress curve. All kinetic experiments were run in duplicates, and parameters were fit to the duplicate data sets. Determination of Kₘ of crot-ACP is similar, except with 200 µM NADPH, and 2.5 µM, 5 µM, 7.5 µM, 10 µM, 15 µM, 25 µM, and 50 µM of crotonyl-ACP.

The affinity of AFN-1252 for FabI was determined by measuring the initial velocity with 50 nM of enzyme, 200 µM NADPH, 50 µM crotonyl-ACP, and 15.6 nM, 31.2 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, or 1000 nM of AFN-1252. The affinity of triclosan for FabI was determined similarly, except FabI is incubated with triclosan and 200 µM NADP⁺ for 10 minutes before starting the assay to account for the slow binding nature of triclosan (3). Under standard steady state conditions, inhibitors are kept at concentrations 10-fold or more above the concentration of the enzyme, so that formation of the enzyme-inhibitor complex does not alter the concentration of free inhibitors. However, AFN-1252 had affinity values in the nanomolar range, while nanomolar concentrations of FabI are necessary to generate a detectable signal in our kinetic assay. Thus, the data was fit to the Morrison’s quadratic equation for fitting tight binding inhibitors (Equation 1), where v₀ is the velocity with no inhibitor, vi is the velocity at the given concentration of inhibitor, [E]T is the total concentration of FabI in the reaction, [I]T is the total concentration of inhibitor in the reaction, and solved for Kᵦ, the apparent dissociation constant of the inhibitor (31).

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\frac{v_i}{v_0} = 1 - \frac{([E]_T+[I]_T+K_i^{\text{app}})^2-4[E]_T[I]_T}{2[E]_T}\]

**Equation 1**

Briefly, the Morrison’s equation allows the determination of affinity in terms of free and bound concentrations of enzyme and inhibitor accounting for the impact of enzyme-inhibitor binding on the free concentration of inhibitor.

For determination of the slow binding mechanism of triclosan to FabI(M99T), 50 nM of enzyme was added to a solution of 400 µM NADPH, 120 µM crotonyl-ACP, and 0.25 µM, 0.5 µM, 0.75
Expression of either FabI(M99T) or FabI(Y147H) as the cellular target for the drug (Fig. 1). The wild-type FabI increased the MIC for AFN-1252 and strain RN4220 was transformed with these expression constructs. Plasmid-driven expression of FabI(Y147H) was 2-fold more resistant to AFN-1252 than FabI(M99T) in vivo. Together, the selection and expression experiments show that the expression of FabI(M99T) or FabI(Y147H) mutants were necessary and sufficient to confer increased resistance to AFN-1252. The cross-resistance of strains MWF32 and MWF33 against other FASII targeted drugs were tested (Fig. 1D). Strain MWF33 exhibited increased resistance to the prototypical FabI inhibitor triclosan compared to the wild-type strain (62.5 ng/ml to 500 ng/ml). This result was consistent with the isolation of a mutant fabI allele encoding FabI(Y147H) in a study of triclosan resistance (33). In contrast, strain MWF32 had increased sensitivity to triclosan (62.5 ng/ml to 3.9 ng/ml; Fig. 1D). AFN-1252 selection did not give rise to commonly found triclosan-resistant fabI alleles predicted to encode FabI(A95V), FabI(I192S), or FabI(F204S) (3, 9). The fact that triclosan had a MIC of 1 μg/ml against strain PS01 (∆accD) showed that triclosan also had a non-FASII target in S. aureus. The wild-type and AFN-1252-resistant strains MWF32 and MWF33 had the same MIC for FASII drugs that targeted the condensing enzymes of FASII. These included cerulenin, 31.3 μg/ml; thiolactomysin, 25 μg/ml; platenacin, 0.8 μg/ml; and platencin, 0.8 μg/ml. Thus, the selected fabI mutations conferred resistance only to AFN-1252 and not to drugs that targeted another pathway enzyme.

Repeated attempts at generating mutants resistant to higher concentrations of AFN-1252 by selection on plates containing 1 μg/ml AFN-1252 using either the wild-type strain RN4220, or strains MWF32 or MWF33 yielded no colonies. Thus, we were unable to directly select for more resistant clones or to evolve a more resistant clone starting with either of the two resistant clones we obtained in the first round of selection. These data indicated that these two mutants were the only missense mutations in fabI that led to a significantly increased MIC for AFN-1252. We investigated this hypothesis by generating all possible missense mutations at position 99 and used heterologous expression in E. coli to rank the resistance phenotypes of each mutant based on the MIC (Table 1). FabI(M99T) was the most resistant of the single missense mutations that could occur at position 99. Also, amino acid changes at position-99 that would not be expected from the selection method because they involved either 2 or 3 base pair changes also were not more resistant than FabI(M99T) (Table 1).
Phenotypes of the AFN-1252-Resistant Strains—The growth rates of the two fabI mutant strains were compared to wild-type (Fig. 2A). Strain MWF32 expressing the Fabl(M99T) protein had a doubling time in LB media that was indistinguishable from the parental strain RN4220 (27-30 min doubling time). In contrast, strain MWF33 (Fabl(Y147H)) had a significantly impaired growth rate (120 min doubling time) (Fig. 2A). If the slow growth rate of strain MWF33 was due to the Fabl(Y147H) protein being catalytically defective, then supplementation with exogenous anteiso 15:0/17:0 fatty acids and lipoate should cure the deficiency in FASII activity and accelerate the growth rate. Supplementation with exogenous fatty acids did restore the doubling time of strain MWF33 to a wild-type rate (35 min) (Fig. 2A). Supplementing strains RN4220 or MWF32 with exogenous fatty acids did not increase their growth rate. The transcription of the fab genes in S. aureus is controlled by the FapR repressor (34). This repressor is released from its DNA binding sites by malonyl-CoA, which accumulates when FASII activity is reduced (35). Thus, a hallmark of FASII inhibition in S. aureus is an increase in the transcription of the fab genes (26). The mRNA levels of pslX, in the fapR-pslX-fabD-fabG operon, fabH in the fabH-fabF operon, and fabl were measured by qRT-PCR to determine if the mutations compromised Fabl function in vivo, which would be reflected by a compensatory upregulation of gene expression in the mutant strains. All three genes exhibited increased expression in both mutant strains, indicating that both fabl mutations resulted in catalytically compromised enzymes and triggered the upregulation of the FapR regulon (Fig. 2B). These data suggested that deficiencies in the activity of the mutant fabl alleles led to the compensatory upregulation of pathway enzymes to maintain the rate of FASII. In the case of strain MWF33, these compensatory changes were not sufficient to restore the normal growth of the strain (Fig. 2A). The elevated fab gene expression in strain MWF32 suggested that Fabl(M99T) was also catalytically deficient in vivo, but that the compensatory upregulation of the fab genes (including fabl) was sufficient to prevent a deficit in fatty acid production resulting in a normal growth phenotype. Methionine is the only residue found at position-99 in S. aureus Fabls, and the gene expression data indicated that Fabl(M99T) was catalytically compromised in vivo. These data provided insight into why we were not able to select for a Fabl(M99T, Y147H) double mutant using higher concentrations of AFN-1252 starting with either strain MWF32 or MWF33. The elimination of these two clearly important drug-protein interactions should give rise to a more resistant enzyme, and indeed when we synthesized the Fabl(M99T, Y147H) double mutant and tested it in the E. coli system, the MIC for AFN-1252 was shifted more than either of the single mutants alone (Table 1). Thus, the Fabl(M99T, Y147H) was more resistant to AFN-1252 than either Fabl(M99T) or Fabl(Y147H), but was also predicted to be more catalytically impaired in vivo than the single mutants. We tested this idea by cloning the two single mutants and the double mutant into the pCL15 expression plasmid and transforming strain RN4220. We then compared the growth rates of the strains in the presence of 4 ng/ml AFN-1252 to chemically knockout the chromosomally-encoded wild-type Fabl activity. The strain expressing Fabl(M99T) grew with a doubling time of 72 ± 1 min and the Fabl(Y147H) expressing strain had a doubling time of 101 ± 6 min. The Fabl(M99T, Y147H) expressing strain had a doubling time of 321 ± 25 min illustrating the catalytic deficiency of the double mutant in the context of the S. aureus FASII enzymes. Thus, we concluded that repeated attempts to select for the double mutant failed because it was too impaired to support the growth of S. aureus.

Although the strains MWF32 and MWF33 were more resistant to AFN-1252, there was still a MIC for the drug. Previous work concluded that Fabl was the only AFN-1252 target in S. aureus (26), therefore we determined if FASII was still inhibited in the mutant strains (Fig. 2C). AFN-1252 most potently inhibited [14C]acetate incorporation in strain RN4220, was less effective in strain MWF32, and strain MWF33 was the most resistant. The ACP pools of AFN-1252-treated wild-type and mutant strains were compared to the ACP pool of the untreated cells (Fig. 2D). In untreated cells, non-esterified ACP was the only clearly discernible protein form. In AFN-1252-treated cells, a series of short-chain acyl-ACP accumulated. Treatment of the two mutant strains with higher concentrations of AFN-1252 resulted in the same pattern of acyl-ACP accumulation as noted in the wild-type strain. Thus, strains MWF32 and MWF33 both exhibited increased resistance to AFN-1252, but the MIC for AFN-1252 in these strains was still attributed to on-target inhibition of the mutant Fabl enzymes.

Enzymology of Fabl Mutant Proteins—The wild-type and the mutant Fabl enzymes were expressed and purified. Fabl(Y147H) was not stable to short-term storage and precipitated from the
storage buffers making this enzyme difficult to characterize. Freshly isolated FabI(Y147H) exhibited a marked catalytic defect (Fig. 3A). The calculated FabI(Y147H) kcat was 2.3 ± 0.8 min⁻¹ compared to the FabI kcat of 30.8 ± 5.5 min⁻¹. This catalytic deficiency of FabI(Y147H) was consistent with the fatty acid-dependent growth phenotype of strain MWF33 (Fig. 2A). The roles played by Tyr157 and Lys164 located in the conserved YX₆K motif in NADP(H) binding and hydride transfer to carbon-3 of the substrate are an established characteristic of this protein family (36). Whether the Tyr147 hydroxyl was important for activity was tested by constructing the FabI(Y147F) mutant. The E. coli MIC analysis system confirmed that the FabI(Y147F)-expressing cells did not have increased resistance to AFN-1252 compared to FabI (Table 1), suggesting that FabI(Y147F) bound AFN-1252 with approximately the same affinity as FabI. Like FabI(Y147H), purified FabI(Y147F) was not stable and had a similar low kcat (3.9 ± 0.3 min⁻¹). The conserved Tyr147 may form a hydrogen bond with the thioester carbonyl of enoyl-ACP to align the substrate in the active site and promote the reaction. This interpretation for the role for Tyr147 in catalysis was also arrived at based on molecular dynamics simulations of enoyl-ACP binding to E. coli FabI (37).

Whether AFN-1252 bound to the free enzyme or one of the nucleotide-bound forms was determined by incubating [¹⁴C]AFN-1252 with FabI, FabI plus NADP⁺, or FabI plus NADPH. The samples were then fractionated with a size exclusion column, and the location of FabI was determined by UV detection and [¹⁴C]AFN-1252 was determined by scintillation counting. [¹⁴C]AFN-1252 co-eluted with the protein only in presence of NADPH, illustrating that AFN-1252 only bound to the FabI-NADPH complex (Fig. 3B).

The high-affinity binding of AFN-1252 presented a challenge in measuring its binding to FabI or its mutant derivatives. The usual FabI assay uses enoyl-N-acetylcysteamines or enoyl-CoAs as substrate analogs (11,38). However, the low affinity of FabI for enoyl-CoA meant that this assay required low μM FabI concentrations to observe detectable rates. These high protein concentrations cannot be used to kinetically determine the affinity of nanomolar inhibitors with any accuracy. Therefore, our assays used crotonyl-ACP which allowed us to measure initial rates using 20-50 nM FabI. Nonetheless, AFN-1252 binding was still high enough that the formation of the FabI-NADPH-AFN-1252 complex affected the free concentration of the inhibitor necessitating the use of Morrison’s quadratic equation (31) to take this effect into account in calculating binding constants (See “Experimental Procedures”). The affinity of AFN-1252 for FabI and FabI(M99T) was measured kinetically with saturating concentrations of NADPH and crotonyl-ACP. As expected, the FabI(M99T) mutant enzyme was more resistant against AFN-1252, with a Kᵢ of 69 nM versus 4 nM for FabI under saturating substrate concentrations, accounting for the increased resistance against AFN-1252 at the cellular level (Fig. 3C). The low activity of FabI(Y147H) (Fig. 3A) meant that μM concentrations of enzyme were needed in the assays precluding an accurate measurement of AFN-1252 binding to this mutant enzyme. However, all of the biological data indicated that FabI(Y147H) was approximately 2-fold more resistant to AFN-1252 than FabI(M99T) (Fig. 1B; Table 1).

The FabI(M99T) enzyme had turnover rates as fast as the wild type enzyme (Fig. 3A). The kinetic parameters of the FabI(M99T) mutant were compared to wild type enzyme, and no significant differences were observed. The FabI(M99T) mutant had a Kᵢₚ of 42.1 ± 8.6 μM for NADPH and 5.5 ± 0.8 μM for crotonyl-ACP compared to 55 ± 7.5 μM for NADPH and 14.4 ± 5.5 μM for crotonyl-ACP for FabI (Fig. 3D). FabI exhibited substrate inhibition by crotonyl-ACP (Kᵢₚ = 20.1 ± 8.4 μM) while the M99T mutant did not, although the observed substrate inhibition was likely not physiologically relevant because enoyl-ACPs were not normally detected in vivo (Fig. 2D), and thus their concentrations were likely below the FabI Kᵢₚ.

Triclosan binding in the M99T mutant—The increased susceptibility of strain MWF32 to triclosan was explored by examining the time dependent nature of triclosan binding to FabI. Triclosan binding to E. coli FabI exhibited slow-binding kinetics with the full effect of drug occurring 50-80 seconds after the reaction was initiated (Fig. 4A). Triclosan binding to S. aureus FabI was significantly faster, reaching equilibrium within 20 seconds (Fig. 4B). However, triclosan binding to FabI(M99T) was a slower process taking close to 100 seconds from the onset for the full effect of triclosan inhibition to be manifested (Fig. 4C). To compare affinities for triclosan to the FabI and FabI(M99T) enzymes, both enzymes were pre-incubated with triclosan and NADP⁺ for 10 min to allow inhibitor to bind before the start of the enzymatic reaction. These experiments showed that FabI(M99T) had a higher affinity for triclosan with a
K<sub>i</sub><sup>app</sup> = 7.9 nM compared to K<sub>i</sub><sup>app</sup> = 13.1 nM for FabI (Fig. 4D). The FabI(M99T) enzyme was added to assay mixtures containing different concentrations of triclosan, and the progress curves were measured (Fig. 4C). The k<sub>obs</sub> term, which measures the rate of the onset of inhibition, was determined from the progress curve for each concentration of triclosan, and plotted against the concentration of triclosan (Fig. 4C, inset). The k<sub>obs</sub> vs [triclosan] plot fit best to a straight line indicating reversible, slowing binding of triclosan to FabI(M99T), with an apparent on rate (k<sub>i</sub>) of 1.7×10<sup>4</sup> M<sup>-1</sup>sec<sup>-1</sup>. This “slow-binding” behavior of triclosan is characteristic of its interactions with many FabIs (7,12,39), and the importance of slow off rates to the in vivo drug efficacy has recently become clear (12,39). However, the basis for the lower MIC in our closed system was attributed to the higher affinity of triclosan for the FabI(M99T)-NAD<sup>+</sup> complex.

**DISCUSSION**

This work identifies the M99T mutation as the most common mechanism for the acquisition of increased resistance to AFN-1252 in *S. aureus*. Met99 resides on a conformationally flexible loop that covers the FabI active site following the binding of NADPH and substrate (11). The helical segment containing Met99 in the FabI-NADPH-AFN-1252 ternary complex is not a well-conserved structural element in bacterial FabIs. Residues 95-117 are disordered in the FabI crystal structure to allow the entry of substrates into the active site, and form a structured lid over the active site in the FabI-NADP(H), FabI-NADP(H)-triclosan and FabI-NADP(H)-AFN-1252 complexes (11,40). Met99 forms a close interaction with the oxotetrahydrooxazine portion of AFN-1252 that contributes a significant hydrophobic interaction that is clearly required for high-affinity AFN-1252 binding (Fig. 5). An analysis of FabI protein sequences deposited in the NCBI Microbial Genomes database shows that Staphylococcal species uniformly have Met99, whereas other FabIs have more hydrophilic and smaller residues in this position. This fact suggests that the interaction between Met99 and AFN-1252 accounts for the selective anti-Staphylococcal activity of the drug (22). Although FabI(M99T) did not have a pronounced catalytic defect using crotonyl-ACP as substrate, the actual in vivo substrates are an elongating series of branched-chain enoyl-ACPs. This blind spot in our analysis may be relevant because the upregulation of the lipid biosynthetic genes in strain MWF32 compared to strain RN4220 illustrates that the FapR genetic regulatory switch is increasing protein expression to compensate for a deficiency in the pathway. Although the nature of this inferred defect is not clear, the cells expressing the mutant protein are able to compensate and do not have an observable growth phenotype in the laboratory. Further experiments will be required to determine if strains with the FabI(M99T) defect are attenuated for growth in animal models.

FabI(Y147H) was the only other AFN-1252 mutant found in our study (1 in 50). In contrast to Met99, Tyr147 is a conserved residue in eubacterial FabIs. The 3-methylbenzofuran rings of AFN-1252 bind in the catalytic pocket making strong hydrophobic interactions with Tyr147 (Fig. 5). The absence of this interaction in FabI[Y147H] accounts for the lower affinity of AFN-1252. The low catalytic activity of FabI[Y147H] prevented the calculation of a binding constant from kinetic experiments. However, the collective in vivo data suggests that the AFN-1252 K<sub>i</sub> for FabI[Y147H] is 2-fold higher than for FabI[M99T]. In bacterial FabIs, this invariant tyrosine residue is flanked by a serine/threonine and a large hydrophobic residue such as leucine or phenylalanine. Structurally, the tyrosine residue is at a spatially conserved position forming a part of the active site of the FabI enzyme in the crystal structures of FabI from *S. aureus* (11,40), *E. coli* (41), *Helicobacter pylori* (42), *Bacillus subtilis* (43), and *Bacillus cereus* (43). The analysis of these structures, our site-directed mutagenesis and molecular dynamics simulations (37) all point to Tyr147 promoting the reaction by hydrogen bonding to the substrate thioester carbonyl. This hypothesis explains the compromised catalytic activity of FabI[Y147H] and the marked growth defect of strain MWF33.

Understanding the number and nature of AFN-1252 resistance mutations in FabI has important implications to the potential therapeutic use of AFN-1252. The low number of AFN-1252-resistant mutations is attributed to many of the key interactions that occur between AFN-1252 and the backbone amide of Ala97, and the hydrogen bond network and stacking interactions that bridge the drug, protein and NADPH cofactor (25). It is difficult to envision missense mutations that would selectively break these key interactions and still preserve cofactor binding and catalysis. Both mutant strains are more resistant to AFN-1252, but because AFN-1252 is such a high-affinity inhibitor, they remained sensitive to 0.25-0.5 µg/ml of the drug. This suggests that animals infected with the resistant strains could be treated by administering...
higher concentrations of AFN-1252 because the MIC for AFN-1252 in the resistant strains is still lower than most antibiotics, such as linezolid (1-4 µg/ml), a front-line drug used to treat *S. aureus* (44). Animal models testing the efficacy of AFN-1252 deliver doses of the drug that generate serum levels over 1.5 µg/ml (45). The idea that AFN-1252 resistant mutants could be successfully treated by simply delivering the same or higher doses of drug will be important to test in animal infection models. It is also significant that we were unable to obtain *S. aureus* mutants that were resistant to higher AFN-1252 concentrations than FabI(M99T) and FabI(Y147H) even when starting with strains MWF32 and MWF33. We reasoned that the FabI(M99T,Y147H) double mutant would be more resistant to AFN-1252 due to the elimination of two key interactions, and confirmed this hypothesis by constructing and analyzing the double mutant enzyme. However, the in vivo gene expression data indicate that both FabI(M99T) and FabI(Y147H) are functionally compromised leading us to conclude that the FabI(M99T,Y147H) double mutant is even more deficient. This idea was corroborated by finding that a strain possessing a plasmid expressing FabI(M99T,Y147H) grew very poorly on low concentrations of AFN-1252 that knocked out the endogenous wild-type FabI. These data suggest that the double mutant does not arise in our selection schemes because a single copy of this defective enzyme cannot support the growth of *S. aureus*.

Our study also provides insight into cross resistance against drugs targeting the same enzyme. While AFN-1252 binds to the FabI-NADPH complex (Fig. 5) and triclosan binds to the FabI-NADP+ complex (3), the two inhibitors occupy the same substrate/product binding pocket. Of the two AFN-1252 resistant mutants, FabI(Y147H) has increased resistance against triclosan while FabI(M99T) is actually more sensitive to triclosan. Future infection models will be required to determine if the poor growth of the strain MWF33 expressing the FabI(Y147H) protein compromises the virulence of *S. aureus* or whether the strain can obtain sufficient fatty acids from the host to support colonization. CG400462, a FabI inhibitor developed by CrystalGenomics, gave rise to the same two resistant mutants as we found with AFN-1252 (14) suggesting that it binds in a similar manner to the FabI-NADPH complex. Other FabI targeted compounds including CG400549 (13), MUT056399 from Mutabilis (15), and triclosan analogs from Xu et al. (9) give rise to resistant mutants (F204C/L/S/F and A95V) more typically found for triclosan (46,47) that were not detected in our study. Thus, the concern that spread of triclosan resistance would invalidate the use of FabI-targeted drugs (48) is limited to the FabI(Y147H) mutant in the AFN-1252 case.

**Acknowledgements**—[14C]AFN-1252 was a generous gift from Nachum Kaplan of Affinium Pharmaceuticals. We thank Matt Frank, Pam Jackson and Chitra Subramanian for their expert technical assistance, and the Protein Production Shared Resource for protein expression and purification.

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ACS Chem. Biol. 4, 221-231


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TABLE 1
Complementation and AFN-1252 sensitivity for missense mutations affecting positions 99 and 147 of S. aureus FabI
Complementation was scored as growth (Y) or no growth (N) at 42°C when plasmids expression of the indicated mutants were transformed into E. coli strain JP1111 (fabI(Ts)). The sensitivity to AFN-1252 was determined after transforming the plasmids into E. coli strain ANS1 and the MICs were determined as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Met99 Substitution</th>
<th>Complementation (Y/N)</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
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<td>0.1</td>
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<tr>
<td>1 Base Pair Change</td>
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</tr>
<tr>
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<tr>
<td>M99V</td>
<td>Y</td>
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</tr>
<tr>
<td>M99L</td>
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<td>0.1</td>
</tr>
<tr>
<td>M99I</td>
<td>Y</td>
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</tr>
<tr>
<td>M99R</td>
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<td>0.2</td>
</tr>
<tr>
<td>M99K</td>
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<td>0.1</td>
</tr>
<tr>
<td>2 Base Pair Change</td>
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</tr>
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<td>Y</td>
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</table>
the kobs calculated from each progress curve against the concentration of triclosan of that progress curve.

\( \mu \)

independent experiments. (gyrA was set to 1 and the relative levels of expression in the other two strains compared using MWF33 compared to the wild-type parent, strain RN4220. The level of gene expression in strain RN4220 \( \mu \) mutant supplemented with 133 \( \mu \)M anteiso15:0 and 66% anteiso17:0 in 0.1% Brij-58 to permit growth of this fatty acid auxotroph \( \square \). C, MICs for AFN-1252 against S. aureus strain RN4220 (○), MWF32 (FabI(M99T)) (■), MWF33 (FabI(Y147H)) (□), and PS01 (ΔaccD) supplemented with 133 \( \mu \)M anteiso15:0 and 66 \( \mu \)M anteiso17:0 in 0.1% Brij-58 (■). Cell growth was monitored by OD600 and the samples with no drug present were set at 100% growth.

**FIGURE 1.** Susceptibility of AFN1252-resistant S. aureus strains to FabI-directed inhibitors. A, Structures of triclosan and AFN-1252. The * indicates the carbon in AFN-1252 that was radiolabeled. B, MIC of AFN-1252 in LB media against S. aureus strains RN4220 (●), MWF32 (FabI(M99T)) (○), MWF33 (FabI(Y147H)) (■), and PS01 (ΔaccD) supplemented with 133 \( \mu \)M anteiso15:0 and 66% anteiso17:0 in 0.1% Brij-58 to permit growth of this fatty acid auxotroph (□). C, MICs for AFN-1252 against S. aureus strain RN4220 (○), MWF32 (FabI(M99T)) (■), MWF33 (FabI(Y147H)) (□), and PS01 (ΔaccD) supplemented with 133 \( \mu \)M anteiso15:0 and 66 \( \mu \)M anteiso17:0 in 0.1% Brij-58 (■). Cell growth was monitored by OD600 and the samples with no drug present were set at 100% growth.

**FIGURE 2.** The effect of fabI mutations on the growth rates of the resistant strains. A, The growth rate of strain MWF32(FabI(M99T)) supplemented with 133 \( \mu \)M anteiso15:0, 66 \( \mu \)M anteiso17:0 and 10 \( \mu \)M lipoate in 0.1% Brij-58 (FA) (●) or with only 0.1% Brij-58 (○). The growth rate of strain MWF33 (FabIY147H) mutant supplemented with 133 \( \mu \)M anteiso15:0, 66 \( \mu \)M anteiso17:0 and 10 \( \mu \)M lipoate in 0.1% Brij-58 (FA) (■) or with only 0.1% Brij-58 (□). The growth experiments shown are representative of triplicate, independent experiments. B, Increased expression of the pLSX, fabH, and fabI genes in strains MWF32 and MWF33 compared to the wild-type parent, strain RN4220. The level of gene expression in strain RN4220 was set to 1 and the relative levels of expression in the other two strains compared using gyrA as the calibrator gene. Data were calculated from triplicates. C, AFN-1252 inhibition of \( ^{14} \)C-acetate incorporation into the lipids of strains RN4220 (wild-type) (●), MWF32 (FabI(M99T)) (○) and MWF33 (FabI(Y147H)) (■). Data were calculated from triplicate experiments. D, ACP pool composition in wild-type and resistant strains treated with AFN-1252. ACP species were separated by urea gel electrophoresis and visualized by a western blot using anti-ACP antibodies. Although higher levels of AFN-1252 were required to inhibit the growth of the two resistant strains, the pattern of acyl-ACP accumulation following treatment was the same in all cases. The gel shown was representative of three experiments.

**FIGURE 3.** Biochemical properties of FabI and its AFN-1252-resistant mutants. A, Enzyme velocity versus concentration plots were performed in triplicate for FabI (●), FabI(M99T) (○), and FabI(Y147H) (■) under saturating substrate concentrations. B, The elution profile of \( ^{14} \)C-AFN-1252 from a Superdex G200 gel filtration column when incubated with FabI (●), FabI plus NADPH (○), and FabI plus NADP' (■). The chromatograms shown are representative of three gel filtration experiments. C, Velocity of FabI (●) and FabI(M99T) (○) at different concentrations of AFN-1252. Duplicate data sets were fit via the Morrison equation for tight binding inhibitors to determine the \( K_{app} \). D, Initial velocities of FabI (●) and FabI(M99T) (○) were determined in triplicate as a function of the crotonyl-ACP concentration. The apparent \( K_m \) values were similar, but FabI exhibited substrate inhibition at the higher concentrations.

**FIGURE 4.** Inhibition of FabI and its AFN-1252-resistant mutants by triclosan. A, Enzymatic progress curves of the E. coli FabI reaction with and without triclosan. The traces shown are representative of triplicate experiments. B, Enzymatic progress curves of the S. aureus FabI reaction with and without triclosan. The traces shown are representative of triplicate experiments. C, Enzymatic progress curve of the FabI(M99T) enzyme at 0 \( \mu \)M (●), 0.25 \( \mu \)M (○), 0.5 \( \mu \)M (■), 0.75 \( \mu \)M (□), 1 \( \mu \)M (▲), 1.25 \( \mu \)M (△), 1.5 \( \mu \)M (▼), or 2 \( \mu \)M triclosan (▽). The traces shown are representative of duplicate experiments. Inset plots the kobs calculated from each progress curve against the concentration of triclosan of that progress curve. D, Velocity of FabI (●) and FabI(M99T) (○) at different concentrations of triclosan. Duplicate data sets were fit via the Morrison equation for tight binding inhibitors to determine the \( K_i \) app.

**FIGURE 5.** View of the FabI-NADPH-AFN-1252 ternary complex. The structure was rendered in Pymol (PDBID: 4FS3) (25). The FabI active site is depicted as a green ribbon diagram, NADP(H) carbons are pink, AFN-1251 carbons are yellow, and Met99 and Tyr147 side chain carbons are in cyan. Met99 interacts with the oxotetrahydronaphthyridine part of AFN-1252, and on the other side of the active site Tyr147 interacts with the 3-methylbenzofuran portion. The loss of these important van der Waals interactions explained the
increased resistance of Fabl[M99T] and Fabl[Y147H] to AFN-1252. Other key interactions between and the Fabl-NADP(H) complex are hydrogen bond interactions between the carbonyl of the cis-amide of AFN-1252 and the 2'-hydroxyl of NADPH and the hydroxyl of Tyr157, and between the pyridyl nitrogen and the N-acyl hydrogen of the oxotetrahydrodronaphthyride part of AFN-1252 and the backbone amide of Ala95. The cis-amide also π-stacks with the nicotinamide ring of NADPH.
Figure 2.
Figure 3.
Figure 4.
Resistance to AFN-1252 arises from missense mutations in *Staphylococcus aureus* Enoyl-Acyl carrier protein reductase (FabI)
Jiangwei Yao, John B. Maxwell and Charles O. Rock

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