

Perturbation of *Staphylococcus aureus* Gene Expression by the Enoyl-Acyl Carrier Protein Reductase Inhibitor AFN-1252

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This study examines the alteration in *Staphylococcus aureus* gene expression following treatment with the type 2 fatty acid synthesis inhibitor AFN-1252. An Affymetrix array study showed that AFN-1252 rapidly increased the expression of fatty acid synthetic genes and repressed the expression of virulence genes controlled by the SaeRS 2-component regulator in exponentially growing cells. AFN-1252 did not alter virulence mRNA levels in a *saeR* deletion strain or in strain Newman expressing a constitutively active SaeS kinase. AFN-1252 caused a more pronounced increase in *fabH* mRNA levels in cells entering stationary phase, whereas the depression of virulence factor transcription was attenuated. The effect of AFN-1252 on gene expression *in vivo* was determined using a mouse subcutaneous granuloma infection model. AFN-1252 was therapeutically effective, and the exposure (area under the concentration-time curve from 0 to 48 h [AUC_{0–48}]) of AFN-1252 in the pouch fluid was comparable to the plasma levels in orally dosed animals. The inhibition of fatty acid biosynthesis by AFN-1252 in the infected pouches was signified by the substantial and sustained increase in *fabH* mRNA levels in pouch-associated bacteria, whereas depression of virulence factor mRNA levels in the AFN-1252-treated pouch bacteria was not as evident as it was in exponentially growing cells *in vitro*. The trends in *fabH* and virulence factor gene expression in the animal were similar to those in slower-growing bacteria *in vitro*. These data indicate that the effects of AFN-1252 on virulence factor gene expression depend on the physiological state of the bacteria.

Methicillin-resistant *Staphylococcus aureus* is a leading cause of skin and soft tissue infections in the United States (1), and its prevalence has reached epidemic proportions in the world (2). The spread of multidrug-resistant organisms has spurred the development of new drugs to attack this infectious agent. One such compound is AFN-1252, a potent small-molecule inhibitor of staphylococcal type 2 fatty acid synthesis (FASII) that targets the enoyl-acyl carrier protein (ACP) reductase step (FabI) (3–5). The suitability of targeting bacterial fatty acid synthesis to combat Gram-positive bacteria was questioned by Brinster et al. (6), who concluded that the ability of these bacteria to incorporate exogenous fatty acids into membrane phospholipids would render inhibition of the pathway ineffective in the context of a host where such fatty acids are readily available. However, the molecular basis for the differential susceptibility of Gram-positive pathogens to FASII inhibitors has been uncovered, providing an understanding of why FASII inhibitors are effective against *S. aureus* even in the presence of an extracellular source of fatty acids (7). These data resolved the debate concerning the validity of using fatty acid synthesis inhibitors to treat *S. aureus* infections (8, 9) and are consistent with the examples of fatty acid synthesis inhibitors that show efficacy in *S. aureus*-infected animal models (5, 8, 10–14).

The production of extracellular mediators (virulence factors) plays an important role in the pathogenicity of *S. aureus* (15). The *sae* regulatory system is a central downstream effector in the regulatory network that controls the expression of major virulence genes (16). SaeRS is a 2-component regulator where a membrane-bound sensor (SaeS) activates a transcriptional regulator (SaeR) that influences virulence gene expression by direct interaction with target promoters (17–19). *S. aureus* strains with deletions in *saeRS* are less virulent than their wild-type counterparts, illustrating that SaeRS-regulated virulence factor expression is critical for

pathogenesis in animal infection models (19–21). A number of antibiotic classes are known to modulate the expression of staphylococcal virulence factors under laboratory conditions (22–30). For example, the increased expression of virulence factors by the β -lactams is mediated by SaeRS in *S. aureus* (31). The impact of fatty acid synthesis inhibitors on virulence factor gene expression has not been studied in detail, although a report in 1984 that cerulenin treatment blunted alpha-toxin secretion (32) suggests that such inhibitors may also influence virulence factor production. Whether the laboratory studies correlating virulence gene expression and antibiotic treatment are recapitulated *in vivo* remains an open question.

There are also pathway-specific gene expression changes that are associated with specific antibiotic classes, and these expression profiles generate a characteristic signature for the specific branch of metabolism inhibited by the antibiotic (33, 34). With antibiotics targeting bacterial fatty acid synthesis, the upregulation of *fab* genes is a transcriptional signature that is not shared by other antibiotic classes (33, 34). In *S. aureus*, the expression of the *fab* gene set is controlled by the transcriptional regulator FapR (35), and the upregulation of *fab* gene expression following the inhibi-

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tion of fatty acid synthesis is reflected by increased levels of mRNA encoding the Fab enzymes (36). Despite these correlations between patterns of virulence gene expression and specific pathway inhibitors in the laboratory, there is little understanding of whether these experiments translate *in vivo*. The goals of this study were to evaluate the efficacy and pharmacokinetic parameters of AFN-1252 in a mouse subcutaneous (s.c.) granuloma infection model, to determine if virulence and pathway-specific gene expression are altered by AFN-1252 treatment *in vitro*, and to test whether these effects of AFN-1252 are recapitulated *in vivo*. The mouse subcutaneous granuloma *S. aureus* infection model was selected to facilitate acquiring samples at specific treatment intervals for transcriptional analysis, pharmacokinetics, and efficacy.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strain RN4220 was obtained from the American Type Culture Collection. Strain PDJ22 ($\Delta saeR$) was constructed by the insertion of a group II intron into the *saeR* gene of strain RN4220 at 42 bp after the initiation codon (37) using the primer design software provided by Sigma-Aldrich (Targetron system). The insertion was verified by PCR using primers outside the intron insertion site. The USA300 and Wood46 strains were obtained through the Network of Antimicrobial Resistance in *S. aureus* (NARSA) program supported under NIAID/NIH contract no. HHSN272200700055C, and the Newman strain was kindly provided by Mark Hart (NCTR, Jefferson, AR). For *in vitro* studies, *S. aureus* strains were grown in tryptone broth (TB) to mid-log phase (optical density at 600 nm [OD₆₀₀], 0.45) or stationary phase (OD₆₀₀, 2.2) and then split into 2 aliquots for treatment with solvent control (dimethyl sulfoxide [DMSO]) or treatment with AFN-1252 (0.05 $\mu\text{g/ml}$), cerulenin (500 $\mu\text{g/ml}$), or clarithromycin (1.5 $\mu\text{g/ml}$). For *in vivo* studies, *S. aureus* Wood46 was cultured overnight on tryptic soy agar (TSA), and the cells were suspended in tryptic soy broth and counted to generate an infecting inoculum of 8.0 log₁₀ CFU/ml.

Affymetrix array analysis. The effect of AFN-1252 on global abundance of gene transcripts was analyzed using the *S. aureus* Affymetrix array technology. Strain RN4220 was grown to an OD₆₀₀ of 0.45 in tryptone broth. The cultures were then split into 2 aliquots and treated with 50 ng/ml AFN-1252 or an equal volume of DMSO (0.1% final concentration) for 15 min. RNA was extracted from the cells using the Ambion RNAqueous kit (Applied Biosystems) according to the manufacturer's instructions with the modification of using lysostaphin for 15 min at room temperature to lyse the cells. The quality of the RNA was checked with Agilent Technologies 2100 Bioanalyzer Laboratory-on-a-chip before proceeding with the microarray. Affymetrix protocols were used to synthesize, fragment, and terminally label the cDNA. Hybridization, washing, and scanning of cDNA to GeneChip *S. aureus* Genome Array were performed according to the manufacturer's instructions (Affymetrix). The arrays were analyzed with GeneChip operating software, and global scaling was used to normalize the data from different arrays. Spotfire DecisionSite 9.1.1 was used to analyze the data. Changes were reported if they were greater than ± 1.4 -fold with a *P* value of < 0.05 using the Student *t* test to compare values. There were 6 data sets used for the analysis (3 treated with DMSO and 3 treated with AFN-1252).

Measurement of mRNA levels. Quantitative reverse transcription-PCR (qRT-PCR) was used to determine the levels of transcript mRNAs. Bacterial strains were grown in tryptone broth at 37°C to mid-log phase (OD₆₀₀, 0.45) for log-phase experiments or at the transition to stationary phase (OD₆₀₀, 2.2). The cultures were treated with selected antibiotics or DMSO solvent as the control. Laboratory-grown cells were harvested by centrifugation and washed with 1 ml of RNAlater solution (Ambion). Total RNA was then immediately isolated from the bacterial cells using the RNAqueous kit (Ambion) per the manufacturer's instructions, except that treatment with lysostaphin was included to lyse the cells and LiCl was used to precipitate the RNA. The pelleted RNA was resuspended in nu-

lease-free water, and a 10- μg aliquot was treated with the Turbo DNA-free kit (Ambion) to remove any remaining genomic DNA. First-strand cDNA was generated using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System, and samples were run in triplicate. Negative controls (distilled water) and RNA samples without the reverse transcription step were run in duplicate. Dissociation curves were generated after each real-time PCR run to check for nonspecific amplification. PCR mixtures were composed of SYBR green PCR Master Mix (Applied Biosystems), 150 nM (each) primer, and cDNA synthesized from either 10 ng or 15 ng of total RNA for *in vitro* and *in vivo* samples, respectively. The expression levels of various housekeeping genes were analyzed (38) under the different treatment conditions and compared to 16S RNA, and *gmk* was the least altered and was used as the calibrator to compare mRNA levels between strains and treatments. Template curves consisting of at least 7 points ranging from 50 ng to 50 pg of total RNA input were run for each primer set to verify that they produced linear responses and had the same relative efficiency in the PCR as in the *gmk* calibrator. The primers used are listed in Table S1 in the supplemental material. The values were compared using the threshold cycle (C_T) method (39), and the amount of cDNA present ($2^{-\Delta C_T}$) was reported relative to the *gmk* calibrator.

Mouse granuloma infection model. Subcutaneous (s.c.) air pockets were aseptically formed on the dorsal aspect of anesthetized female, CD-1 mice (6 to 8 weeks of age) 5 days prior to infection. Air pockets were immediately injected with 0.4% croton oil (irritant), and 1 ml of sterile intravenous (i.v.) saline solution was injected into pouches 3 days later. Five-day-old pouches were infected with 7.1 to 7.5 log₁₀ CFU of *S. aureus* Wood46, and animals were orally (p.o.) dosed with 100 mg AFN-1252/kg of body weight at 2, 26, and/or 50 h after infection. AFN-1252 was formulated as a 1% poloxamer suspension (Pluronic F-127; Sigma-Aldrich). Control mice were not treated. Pouch fluid was collected from animals at defined time points postdosing and transferred into tubes for RNA extraction, CFU enumeration, or determination of AFN-1252 concentration by liquid chromatography-mass spectrometry (LC-MS) analysis. Additionally, heart blood was collected at the same time points and processed for plasma separation and collection. Pouch fluid samples used for CFU counting were immediately 10-fold serially diluted in sterile 1 \times phosphate-buffered saline (PBS), plated onto tryptic soy agar (TSA) containing 5% activated charcoal, and incubated overnight at 37°C. Pouch fluid and plasma samples were stored at -80°C if they were not immediately analyzed or processed for analysis.

Bacterial RNA extraction from pouch fluid exudates. Pouch fluid was transferred into 2-ml screw-cap tubes loaded with 1 ml of TRIzol (Invitrogen) and one-half volume of 0.1-mm zirconia-silica beads (sterile). Tubes were placed into the bead beater and processed for 1 min at maximum speed. Ice-cold chloroform was added to each tube and then cold centrifuged (4°C) for 15 min at 12,000 $\times g$. A second treatment with TRIzol was done for each upper phase, followed by another chloroform extraction and centrifugation step. RNA was precipitated by adding ice-cold isopropanol to upper phases, and RNA was pelleted in each sample by cold centrifugation for 10 min at 15,000 $\times g$. After RNA pellets were resuspended in 75% ethanol, they were cold centrifuged for 5 min at 7,500 $\times g$ and dried for 10 min at room temperature. RNase-free water was added to each RNA sample and then treated with Turbo DNase (Ambion) for 30 min at 37°C. Turbo DNase inactivating reagent was added to the samples, and they were centrifuged for 2 min at 10,000 $\times g$. Upper phases were transferred into RNase-free tubes and stored at -80°C until analyzed by qRT-PCR as described above.

Quantification and pharmacokinetic analysis of AFN-1252 in plasma and pouch fluid. Plasma and pouch fluid samples taken from study animals were placed into the wells of a V-bottomed 96-well plate and then mixed with the internal standard solution of deuterated AFN-1252 and methanol. The mixtures were vacuum filtered through an Isolute SLE+ extraction plate (Biotage), ethyl acetate was added to each well,

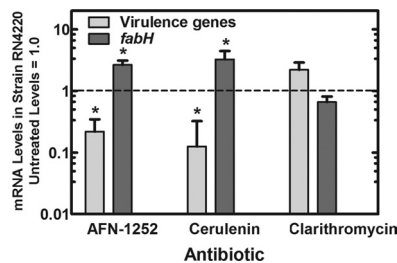


FIG 1 Depression of virulence factor mRNA levels in *S. aureus* treated with fatty acid synthesis inhibitors. Strain RN4220 was grown to mid-log phase in tryptone broth and treated with either AFN-1252 (50 ng/ml), cerulenin (500 μ g/ml), or clarithromycin (1.5 μ g/ml) for 15 min to arrest either lipid or protein synthesis. The MICs for these drugs in strain RN4220 determined in tryptone broth as described previously (7) were as follows: AFN-1252, 4 ng/ml; cerulenin, 62 μ g/ml; and clarithromycin, 310 ng/ml. RNA was extracted, and the levels of four randomly selected virulence factor mRNAs (*saeP*, *ehp*, *efb*, and *hlgC*) were calculated and averaged, in addition to *fabH*, by quantitative RT-PCR. Significance between treated and untreated samples was calculated using the Student *t* test, and standard deviations were obtained from triplicate determinations derived from triplicate experiments. *fabH* mRNA was significantly increased in response to AFN-1252 and cerulenin (*, $P < 0.05$) but not significantly altered by clarithromycin. The average levels of virulence gene mRNAs were significantly decreased in response to AFN-1252 and cerulenin (*, $P < 0.05$). Clarithromycin-treated cells had a modest increase in virulence gene expression, but it was not significant.

and dissolved compound was eluted from the Isolute SLE+ plate under low vacuum for 5 min. Ethyl acetate was evaporated, and samples were reconstituted in a methanol-water-formic acid (500:500:1) solution. Samples were loaded into the autosampler of a Thermo Finnigan Surveyor high-pressure liquid chromatograph (HPLC) coupled to an LCQ Deca mass spectrometer, and three separate injections of each sample were used to calculate the concentration of AFN-1252 against the peak response ratio of the calibration curve. The AFN-1252 concentration for each time point was averaged between studies and entered into PK Solutions 2.0 software (Summit Research Services, Montrose, CO) to calculate the pharmacokinetic parameters for both pouch and plasma samples.

Microarray data accession number. The complete, triplicate data set can be viewed and accessed under accession number [GSE19400](https://www.ncbi.nlm.nih.gov/geo/) in the NCBI GEO database (www.ncbi.nlm.nih.gov/geo/).

RESULTS

Gene expression changes associated with FASII inhibition in *S. aureus*. An Affymetrix array experiment was used to obtain a global view of the effect of the abrupt cessation of fatty acid synthesis at the enoyl-ACP reductase step (FabI) by AFN-1252 (7) on the pattern of cellular gene expression. The complete list of genes with significant expression changes ($P < 0.05$) is found in Table S2 in the supplemental material, and the complete, triplicate data set can be viewed and accessed under accession number [GSE19400](https://www.ncbi.nlm.nih.gov/geo/) in the NCBI GEO database (www.ncbi.nlm.nih.gov/geo/). Previous work established the mechanism for the alteration in *fab* gene expression in response to FASII inhibitors. In *Bacillus subtilis* and *S. aureus*, the activation of FASII gene expression that occurs following the interruption of fatty acid synthesis is controlled by the FapR transcriptional regulator (35, 40–42). FapR is a repressor that binds to the *fab* gene promoters and represses transcription. FapR is released from its binding sites by either malonyl coenzyme A (malonyl-CoA) or malonyl-ACP (43). The inhibition of FASII leads to the accumulation of malonyl-CoA due to the continued production of this intermediate by acetyl-CoA carboxylase coupled with its impaired utilization due to FASII inhibition (41).

Therefore, an increased expression of FASII genes that constitute the FapR regulon in *S. aureus* was anticipated and observed following AFN-1252 treatment (see Table S2). There may be an unknown stress response that contributes to the regulation of *fabH*, but stresses induced by pH (44), alcohol (45), or antibiotics that target other pathways (33, 34) do not alter *fabH* expression. The upregulation of the FapR-regulated genes in AFN-1252-treated cells was verified by the quantitative measurement of *fabH* (and *plsX* [data not shown]) gene expression following AFN-1252 treatment (Fig. 1). The *fabH* mRNA abundance increased approximately 3-fold within 15 min of AFN-1252 treatment. The same level of *fabH* transcriptional activation occurred after blocking FASII at the β -ketoacyl-ACP synthase step using cerulenin, but not with clarithromycin, a protein synthesis inhibitor (Fig. 1). Among the FASII genes controlled by this mechanism, *fabH* was the most highly activated by FASII inhibition in *S. aureus* and was selected as the marker for the genetic response of cells to FASII inhibition.

A second result was the significant repression of a group of transcripts associated with *S. aureus* virulence. Specifically, the mRNA levels for α -, β -, and γ -hemolysins and two fibrinogen binding proteins (Ehp and Efb) decreased over 3-fold within 15 min of AFN-1252 treatment in the Affymetrix array experiment (Table 1). A unifying feature of the virulence gene expression pattern was that the virulence genes are positively regulated by the SaeRS two-component regulator (46–48). The SaeRS system also autoregulates its own expression (*saeR* and *saeS* genes) along with the *saeP* and *saeQ* genes, which are located adjacent to the *saeRS* operon (49). Therefore, the involvement of the SaeRS two-component system in the response to AFN-1252 was also directly indicated by the reduced expression of all four *sae* genes (Table 1). A set of four virulence factor genes that exhibited significant levels of downregulation by AFN-1252 treatment and represented the

TABLE 1 Virulence-related genes downregulated by AFN-1252

Locus tag	Gene	Fold change ^a	<i>P</i> value	Function
SA0309	<i>geh</i>	1.54	0.002	Lipase
SA0660	<i>saeS</i>	1.77	0.009	Two-component sensor
SA0661	<i>saeR</i>	2.45	0.003	Two-component regulator
SA0662	<i>saeQ</i>	4.94	0.000	Hypothetical
SA0663	<i>saeP</i>	4.40	0.000	Hypothetical
SA1000	<i>ehp</i>	3.63	0.001	Fibrinogen binding protein
SA1003	<i>efb</i>	3.49	0.000	Fibrinogen binding protein
SA1007		1.70	0.009	Alpha-toxin
SA1750		2.05	0.002	Map-w
SA1752		8.18	0.000	β -Hemolysin
SA1811	<i>hlyB</i>	3.63	0.000	β -Hemolysin
SA1812		2.72	0.005	γ -Hemolysin
SA2206	<i>sbi</i>	3.72	0.001	IgG binding protein
SA2207	<i>hlyA</i>	4.21	0.000	γ -Hemolysin
SA2208	<i>hlyB</i>	4.99	0.001	γ -Hemolysin
SA2209	<i>hlyC</i>	4.78	0.000	γ -Hemolysin
SA2461	<i>icaD</i>	1.54	0.007	Intracellular adhesion
SA2462	<i>icaC</i>	1.61	0.003	Intracellular adhesion

^a Average fold decrease in mRNA abundance derived from the Affymetrix array experiment. Triplicate control and experimental samples were analyzed. Shown are *S. aureus* genes with significantly ($P < 0.05$) different expression levels (± 1.4 -fold) following a 15-min treatment with 50 ng/ml AFN-1252. See Table S2 in the supplemental material for a summary of all significantly altered genes and accession number [GSE19400](https://www.ncbi.nlm.nih.gov/geo/) in the NCBI GEO database for the complete data set.

overall trend in virulence factor expression were randomly selected for quantitative analysis of SaeRS-regulated gene expression (*hlgC*, *ehp*, *efb*, and *saeP*) (Table 1). The inhibition of virulence gene expression was validated by combining the average levels of four transcripts (*hlgC*, *ehp*, *efb*, and *saeP*) that were coordinately downregulated following AFN-1252 treatment (Fig. 1). Similar reductions in virulence factor expression were associated with cerulenin treatment (Fig. 1). However, the average expression levels for these four virulence markers were elevated 2-fold in cells treated with the protein synthesis inhibitor clarithromycin. These data indicated that the attenuation of virulence factor gene expression was linked to the inhibition of fatty acid biosynthesis.

AFN-1252 effect on virulence factor mRNA levels was mediated by SaeRS. The idea that the effect of AFN-1252 was mediated by the *sae* system was tested by the construction of deletion strain PDJ22 (Δ *saeR*) from strain RN4220, which would inactivate signaling through SaeRS. Experiments with this strain were corroborated by the analysis of additional *S. aureus* strains with known lesions in the SaeRS pathway (Fig. 2A). The average expression level of the virulence factors was lowest in strain PDJ22 (Δ *saeR*), which lacked an intact SaeRS signaling pathway. The levels of virulence factor expression in a panel of strains were compared by normalizing their levels of expression to those in strain PDJ22, set to 1 (Fig. 2A). The USA300 isolate is wild type with respect to the regulatory system involving SaeRS and did not express high levels of virulence factors *in vitro*. This was in agreement with previous findings indicating that external stimuli, like serum or antimicrobial peptides, were required for virulence factor production in other isolates of USA300 (50, 51). Strains RN4220 (52) and Wood46 (53) expressed higher levels of SaeRS-directed virulence factor mRNA due to inactivating mutations in *rsbU* and *sigB*, respectively, which are negative regulators of SaeRS (54). Strain Newman has a point mutation in the sensory domain of SaeS that produces constitutively active signaling through SaeRS (16, 55), and this strain had the highest average levels of virulence factor transcripts, >10-fold higher than those in strain PDJ22 (Fig. 2A).

The ability of AFN-1252 to impact virulence factor mRNA levels in this strain collection correlated with the activity of the SaeRS system (Fig. 2B). AFN-1252 did not alter virulence factor mRNA levels in strain PDJ22 (Δ *saeR*), and there was also a minimal effect in the USA300 isolate, in which the intact SaeRS pathway appeared not to be actively inducing virulence factor expression *in vitro*. In contrast, AFN-1252 treatment significantly reduced virulence factor mRNA levels in strains RN4220 and Wood46. Importantly, the high levels of virulence factor mRNA in strain Newman were not impacted by AFN-1252 (Fig. 2B), which is presumably due to the constitutive and unregulated signaling through the SaeRS system. AFN-1252 treatment significantly upregulated the expression of *fabH* in all of the strains, showing that the pathway-specific action of the drug was a universal occurrence. These data suggested a link between the effects of AFN-1252 treatment on virulence factor transcription and SaeRS regulatory activity in *S. aureus*.

Therapeutic effect of AFN-1252 on *S. aureus* in the mouse granuloma model. *S. aureus* Wood46, a clinical isolate known to produce virulence factors (53) (Fig. 2A), was selected for use in the mouse granuloma infection model. Selection of this strain was based on its prior use in animal models, as well as the *in vitro* effects of AFN-1252 on Wood46 gene transcription. The first experiments involved administering a single oral dose of AFN-1252

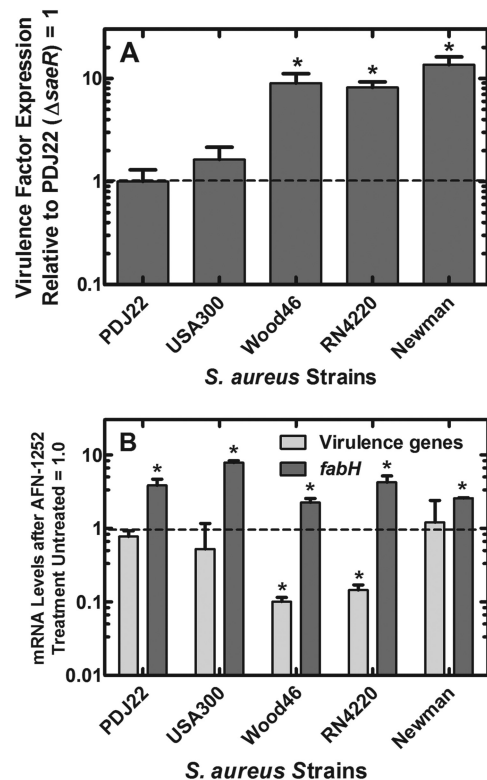


FIG 2 Relative virulence factor production by *S. aureus* strains. (A) Basal level of virulence factor mRNA levels in a panel of *S. aureus* strains. The expression level in strain PDJ22 (Δ *saeR*) was then set to 1, and the expression levels in the indicated strains were compared to the levels in the *saeR*-knockout strain. The differences between strains PDJ22 and USA300 were not significant. Strains Wood46, RN4220, and Newman exhibited significantly higher levels of virulence factor mRNA than did strains PDJ22 and USA300. *, $P < 0.05$. (B) Levels of virulence factor and *fabH* mRNA in strains treated with AFN-1252. In both experiments, the mRNA levels of four virulence-related genes (*ehp*, *efb*, *hlgC*, and *saeP*) and *fabH* were measured by qRT-PCR using *gmk* as the internal control, and triplicate determinations for each gene were averaged. Expression of *fabH* was significantly increased in all cases (*, $P < 0.05$). Virulence gene expression was only significantly decreased by 8- to 10-fold in strains Wood46 and RN4220 (*, $P < 0.05$). All strains had the same MIC for AFN-1252, 4 ng/ml (7). Significance between treated and untreated samples was calculated using the Student *t* test, and error bars indicate standard deviations.

(100 mg/kg) 2 h after infecting the granuloma pouches and examining the effect of AFN-1252 on pouch-associated CFU within 48 h of dosing (Fig. 3A). Little change in the bacterial load was detected within the first 12 h of AFN-1252 treatment, but between 24 and 48 h, there was a 3.0- \log_{10} reduction in the mean CFU/ml of pouch fluid collected from AFN-1252-treated animals compared to untreated animals (Fig. 3A). Wood46 counts in untreated pouch fluid increased to 7.5 \log_{10} CFU/ml at 24 h, while bacterial counts in AFN-1252-treated pouches dropped to 4.4 \log_{10} CFU/ml in 24 h. The pouch-associated CFU counts did not change between 24 and 48 h in the AFN-1252-treated animals. This response to a single dose of AFN-1252 was similar to the transient effect of a single oral dose of linezolid in the rat granuloma model, which initially showed a decrease in *S. aureus* CFU counts within 48 h of dosing that was followed by a rebound in bacterial growth (56). When 100 mg/kg of AFN-1252 was orally administered once a day for 3 days (+2, +26, and +50 h), AFN-1252 reduced 72-hour *S. aureus* CFU counts in the pouches of

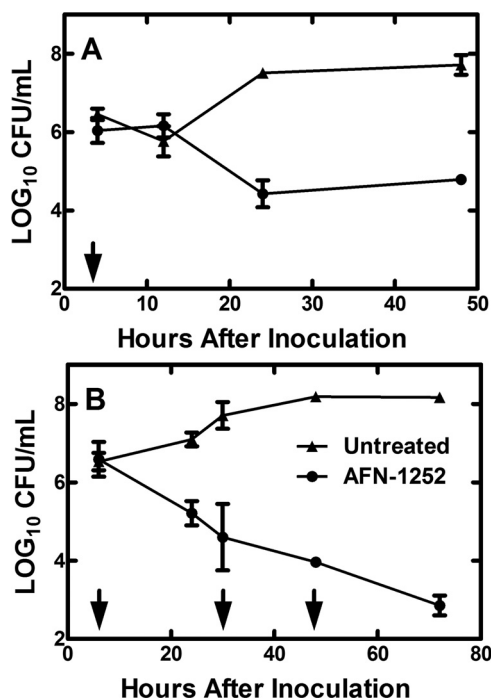


FIG 3 Effect of AFN-1252 treatment on *S. aureus* in the mouse granuloma model. Subcutaneous mouse granuloma pouches were infected with 3.2×10^7 CFU of strain Wood46. (A) An oral dose of 100 mg/kg of AFN-1252 was administered at 2 h postinfection (arrow). (B) Oral doses of 100 mg/kg of AFN-1252 were administered at 2, 26, and 50 h postinfection (arrows). At the indicated times in both panels, the pouch fluid was recovered and bacterial CFU/ml of pouch fluid were determined in triplicate. CFU in pouches from animals treated with AFN-1252 (●) were compared to that in untreated controls (▲). Error bars indicate standard deviations.

treated animals by 5 orders of magnitude compared to untreated controls, which was near the detection limit for this model (Fig. 3B). These data illustrate that orally administered AFN-1252 can reach therapeutic levels in the mouse granuloma pouch and effectively resolve the *S. aureus* infection associated with the pouch fluid.

Pharmacokinetics of AFN-1252 in the mouse granuloma model infected with *S. aureus*. AFN-1252 levels were measured in the plasma and pouch fluid of mice with *S. aureus* Wood46-infected granulomas over a 48-hour time course following a single oral dose of AFN-1252 at 100 mg/kg (Fig. 4). The maximum concentration of drug in serum (C_{max}) of AFN-1252 was higher in the plasma (3.5 $\mu\text{g}/\text{ml}$) of infected animals than in pouch fluid (2.1 $\mu\text{g}/\text{ml}$), and the time to maximum concentration of drug in serum (T_{max}) for each compartment was estimated to be approximately 1 h after treatment. Additionally, the elimination half-lives for AFN-1252 were similar for the pouch fluid (4.9 h) and plasma (4.4 h). The calculated exposure (area under the concentration-time curve from 0 to 48 h [AUC_{0-48}]) of AFN-1252 in plasma was 22.5 $\mu\text{g} \cdot \text{h}/\text{ml}$, while the AUC_{0-48} of AFN-1252 in granuloma pouch fluid was 19.9 $\mu\text{g} \cdot \text{h}/\text{ml}$. Therefore, orally dosed AFN-1252 quickly (T_{max} of 1.0 h) penetrated infected pouch fluid at an exposure level comparable to that for plasma (85% of the plasma AUC) and remained detectable in pouch fluid for 24 h (Fig. 4). The pharmacokinetic data supported the efficacy results of AFN-1252 in the model and validated the use of this animal model to

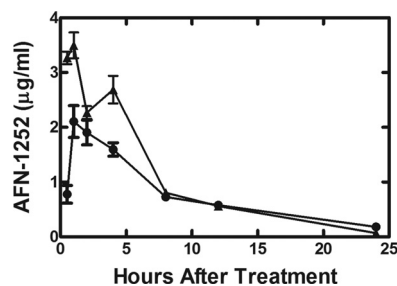


FIG 4 Levels of AFN-1252 in serum and pouch fluid following a single oral dose. *S. aureus* Wood46-infected animals received a single oral dose of AFN-1252 (100 mg/kg) 2 h after infection (time zero). At the indicated times, blood plasma and pouch fluid were sampled and AFN-1252 levels were determined in plasma (▲) and in the pouch exudate (●) by LC-MS. Mean values represent the plasma and pouch fluid levels of 3 to 9 samples harvested at the indicated times after AFN-1252 dosing. A total of 3 replicates were analyzed for each sample by LC-MS, and the limit of detection for the assay was 0.01 $\mu\text{g}/\text{ml}$. Error bars indicate standard deviations. AFN-1252 levels at 48 h were below detection.

measure the *in vivo* effects of AFN-1252 on *S. aureus* gene expression.

***S. aureus* Wood46 virulence factor and *fabH* expression in AFN-1252-treated pouches.** We were able to examine *S. aureus* mRNA expression in the mouse granuloma model for up to 12 hours after treatment with AFN-1252, but the substantial decrease in the number of recovered bacteria in the treated pouches after 12 h (>99% reduction by 24 h) (Fig. 3A) precluded obtaining a sufficient sample for RNA analysis at later time points. Pouch samples used in mRNA expression experiments were taken over the 12 h that immediately followed the single dose of AFN-1252 (100 mg/kg), when there was little change in the bacterial load in both treated and untreated animals (Fig. 5A). During the first 12 h following oral administration of AFN-1252, there was a substantial and persistent 3.5- to 6.5-fold increase in the levels of *fabH* mRNA harvested from pouch-associated Wood46 cells at all time points (Fig. 5B), which was higher than the increase in *fabH* levels in the *in vitro* cultures of Wood46 after being exposed to AFN-1252 (~2-fold). The highest levels of *fabH* mRNA were present in the 2-hour samples (6.5-fold), and elevation in *fabH* level persisted up to 12 h (~3.5-fold). These data indicated the presence of AFN-1252 at levels in the pouch fluid that inhibit the FASII cycle for the entire 12 h following AFN-1252 dosing.

The impact of AFN-1252 on *S. aureus* Wood46 virulence factor mRNA levels was not as strong in the mouse granuloma model as was observed on the laboratory bench (Fig. 5C). Two hours after AFN-1252 dosing, there was an average ~2-fold reduction in virulence factor mRNA levels and mRNA levels continued to be suppressed for up to 12 h (1.6-fold). In comparison, there was a 3- to 4-fold decrease in average virulence factor mRNA levels following AFN-1252 treatment of *S. aureus* Wood46 cultures in the laboratory (Fig. 2B). One important difference in the two experiments was that the laboratory data were obtained in rapidly growing cells (Table 1; Fig. 1), whereas the CFU of strain Wood46 did not increase during the course of the experiment in the pouch model (Fig. 5A). The unchanged level of pouch-associated CFU during the first 12 h may reflect removal of *S. aureus* cells by the immune system coupled with their replacement by cell division, which has been previously shown with *Escherichia coli* in this model (57). However, it stands to reason that pouch-associated bacteria may

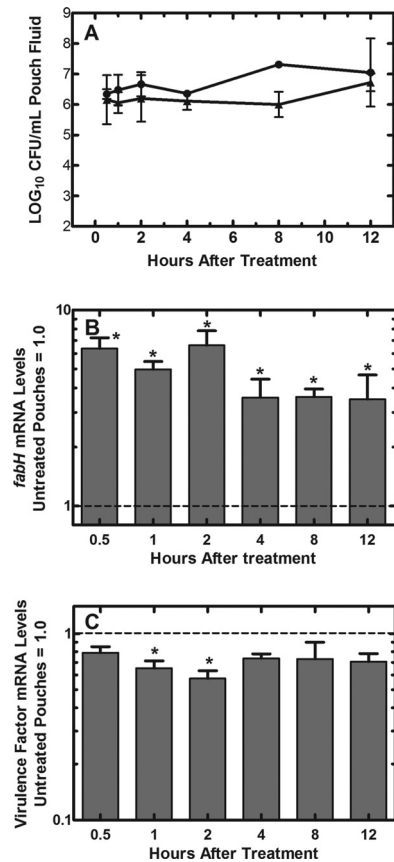


FIG 5 Virulence factor and *fabH* expression levels following treatment of infected pouches with AFN-1252. (A) Bacterial load in the pouches of AFN-1252-treated (▲) and untreated (●) pouches. (B) The levels of *fabH* mRNA in AFN-1252-treated samples relative to *fabH* expression levels in untreated samples. At all time points, *fabH* transcript levels were significantly elevated over controls ($P < 0.05$). (C) The average levels of 4 virulence factor mRNAs expressed in the AFN-1252-treated pouches relative to the expression levels in untreated controls. qRT-PCR was performed using *gmk* as the internal standard. The significance of differences between samples was determined by the Student *t* test. The modest reduction in virulence gene expression levels was significant only at 1 and 2 h ($*, P < 0.05$). Error bars indicate standard deviations. The exception is the 8-h untreated-pouch time point in panel A, which consisted of only a single determination.

be growing at a lower rate than in the log-phase laboratory model explored in Fig. 1. Therefore, we examined if the growth rate of *S. aureus* Wood46 influenced the AFN-1252 effect on gene expression in laboratory cultures (Fig. 6). The same gene panel used *in vivo* was also used to compare the effect of AFN-1252 on gene expression in the logarithmic growth phase to the effect in cells whose growth rate had decreased by 80% as they entered early stationary phase. Whereas AFN-1252 robustly suppressed virulence factor gene expression in log-phase Wood46 (OD_{600} , 0.45), there was little alteration in the levels of virulence factor mRNA in Wood46 by AFN-1252 when the cells were harvested at an OD_{600} of 2.2 (Fig. 6). A comparison of the basal levels of virulence factor expression in the slower-growing cells to that in the log-phase cells showed that there was a 2-fold increase in the average levels of virulence factor mRNA in the denser cell cultures over those in the log-phase cells. The effect of AFN-1252 on *fabH* mRNA levels was higher in the slower-growing cultures than in the log-phase cells

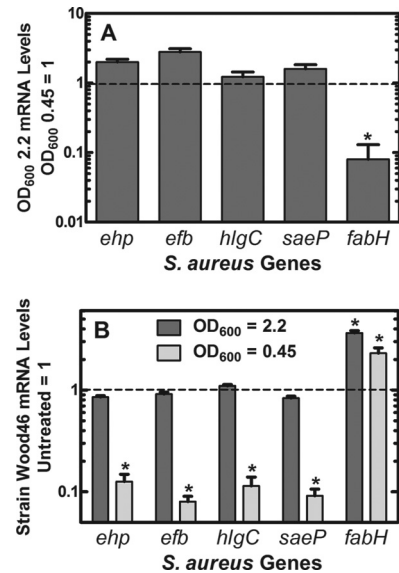


FIG 6 Growth rate dependence of AFN-1252 on virulence factor and *fabH* mRNA levels. Strain Wood46 was subjected to treatment with AFN-1252 (50 ng/ml) for 15 min either during the logarithmic phase of growth (OD_{600} , 0.45) or after growth slowed as the cells began to enter stationary phase (OD_{600} , 2.2). Transcript abundance in triplicate samples was measured in triplicate by qRT-PCR using *gmk* as the calibrator. (A) mRNA levels detected at an OD_{600} of 0.45 were set to 1.0 and compared to the same measurements at an OD_{600} of 2.2. *fabH* mRNA was significantly decreased in the samples at the OD_{600} of 2.2 ($*, P < 0.05$). mRNA levels for the 4 virulence factors were either unchanged (*hlgC*) or modestly elevated (~ 2 -fold) with *ehp*, *efb*, and *saeP*. (B) mRNA levels before AFN-1252 treatment were normalized to 1, and the changes in virulence factor and *fabH* mRNAs triggered by AFN-1252 are plotted. There was no significant difference between virulence gene expression in cells treated with AFN-1252 at an OD_{600} of 2.2, but all 4 virulence factors were decreased by about an order of magnitude in cells treated with AFN-1252 at an OD_{600} of 0.45 ($*, P < 0.05$). *fabH* mRNAs were significantly increased in response to AFN-1252 under both experimental conditions ($*, P < 0.05$). Significance between treated and untreated samples was calculated using the Student *t* test, and error bars indicate standard deviations.

(Fig. 6). This finding reflected the higher average increase in *fabH* mRNA in the pouch model (Fig. 5B) over that in logarithmically growing cells (Fig. 6). However, the total cellular levels of *fabH* mRNA were lower in slower-growing stationary cells than in cells in log phase. Thus, the higher fold induction following AFN-1252 treatment was due to the lower basal levels of *fabH* mRNA in the slower-growing cell population. These experiments illustrated that the inverse relationships of *fabH* and virulence gene expression levels in exponential-phase lab cultures of *S. aureus* Wood46 after exposure to AFN-1252 were not reproduced in Wood46 cells harvested from infected granuloma pouches, which may be explained by the different physiological states of the cells in each environment. This divergence in genetic responses to AFN-1252 treatment may not only be the result of *in vitro* versus *in vivo* differences (immune response) but could also be the result of the growth rate and/or density of Wood46 in the pouch fluid. In the future, it may be of interest to evaluate additional strains in the animal model to further understand the interaction between AFN-1252 and the SaeRS virulence regulation pathway in *S. aureus*.

DISCUSSION

Our data link the repression of virulence gene expression in *S. aureus* by AFN-1252 to the function of the SaeRS 2-component

system. The suite of virulence factors downregulated by AFN-1252 is coordinately regulated by SaeRS, and virulence factor attenuation does not occur in cells lacking a functional SaeRS system or in cells with constitutive SaeR signaling. Most 2-component regulators have sensor components with a single transmembrane helix connecting the external ligand binding domain with an internal kinase domain (58). SaeRS belongs to a small subset of these regulators with the external ligand binding domain replaced by a series of membrane-spanning helices (59). These regulators are thought to sense perturbations in the membrane environment (18). One well-characterized member of this family is DesK from *Bacillus subtilis* (60). This regulator utilizes the transmembrane segment to sense the fluidity of the membrane and adjusts the transcription of an acyl desaturase to adjust the biophysical properties of the phospholipid bilayer. Similarly, SaeRS is thought to sense perturbations in the intramembrane environment, and a point mutation in the first transmembrane segment of SaeS leads to constitutive signaling through SaeRS (48). Membrane perturbations by antibacterial host factors (51), by biocides (55), or by altering membrane phospholipid composition (61) all affect SaeRS-dependent virulence factor production. Similarly, we propose that the abrupt cessation of phospholipid synthesis in AFN-1252-treated cells alters the membrane properties and signaling through SaeRS in *S. aureus*. This effect is most pronounced in rapidly growing cells that are actively forming new membrane phospholipid but is attenuated in slower-growing cultures where the rate of new phospholipid formation is reduced.

The importance of virulence factors in *S. aureus* pathogenesis has focused laboratory research on how antibiotics affect the expression of virulence genes. A survey of 31 antibiotics showed a strong induction of virulence factor expression with subinhibitory concentrations of β -lactams and an almost complete inhibition of expression by clindamycin (25). Linezolid is another protein synthesis inhibitor that reduces exoprotein production in *S. aureus* at subinhibitory concentrations (23, 30). As anticipated from the mechanism of action of linezolid, the primary effect of this functional class of antibiotics on virulence factor expression is mediated through translation rather than transcription (24). Our results extend this body of work to identify fatty acid synthesis inhibitors as a class of antibiotics that attenuate virulence factor transcription via their effect on the SaeRS system. However, the laboratory results do not exactly match the findings in the animal model. The pathway-specific transcriptional effect of AFN-1252 on FASII gene expression was preserved in the mouse granuloma model infected with strain Wood46, but the reduction in virulence gene transcription was attenuated. One potential reason for this difference is that the cells in the pouch are growing slower than are cells in laboratory culture. Although the pouch CFU did not change during the 12 h of our experiments, this level of viable bacteria may reflect the elimination of the bacteria by the host immune system coupled with their replacement by dividing bacteria (57). In support of the idea that slower-growing cells impact the effect of AFN-1252 on virulence factor expression, a comparison between rapidly and slow-growing cells in the laboratory recapitulated the reduced impact of AFN-1252 on virulence factor expression in slower-growing cells. However, this may not be the only factor that influences the expression of virulence genes in the pouch. The fact that the level of virulence factor expression is known to depend on the type of medium used for cell growth in

laboratory experiments (62) suggests that host environment plays an important role. Factors are present in the animal that are absent in the laboratory that may activate the SaeRS system to counteract the effect of AFN-1252 (50, 51), or RNA turnover may be reduced by *S. aureus* stress response pathways that are activated in response to the host (63). Our evaluation of virulence factor mRNA levels in the mouse granuloma model illustrates that the extension of laboratory findings on virulence gene expression to *in vivo* infection models needs further research.

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