Staphylococcus aureus Formyl-Methionyl Transferase Mutants Demonstrate Reduced Virulence Factor Production and Pathogenicity

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Inhibitors of peptide deformylase (PDF) represent a new class of antibacterial agents with a novel mechanism of action. Mutations that inactivate formyl methionyl transferase (FMT), the enzyme that formylates initiator methionyl-tRNA, lead to an alternative initiation of protein synthesis that does not require deformylation and are the predominant cause of resistance to PDF inhibitors in *Staphylococcus aureus*. Here, we report that loss-of-function mutations in FMT impart pleiotropic effects that include a reduced growth rate, a nonhemolytic phenotype, and a drastic reduction in production of multiple extracellular proteins, including key virulence factors, such as α -hemolysin and Panton-Valentine leukocidin (PVL), that have been associated with *S. aureus* pathogenicity. Consequently, *S. aureus* FMT mutants are greatly attenuated in neutropenic and nonneutropenic murine pyelonephritis infection models and show very high survival rates compared with wild-type *S. aureus*. These newly discovered effects on extracellular virulence factor production demonstrate that FMT-null mutants have a more severe fitness cost than previously anticipated, leading to a substantial loss of pathogenicity and a restricted ability to produce an invasive infection.

In bacteria, protein synthesis initiates with formyl-methionyl-tRNA_i, and therefore, all newly synthesized polypeptides contain an *N*-formyl-methionine terminal end that, in most cases, is not retained in mature proteins. As the polypeptides emerge from the ribosome, the *N*-formyl group is hydrolyzed by peptide deformylase (PDF), and subsequently, methionine aminopeptidase removes the *N*-terminal methionine. Deformylation plays a crucial role in protein maturation and has been shown to be essential for bacterial growth (1–4), as methionine aminopeptidase cannot hydrolyze *N*-blocked peptides (5). The ubiquitous nature of PDF, together with the fact that this function is not required for cytoplasmic protein synthesis in eukaryotes, has made this an attractive target for the development of new antibacterial agents. The successful purification and characterization of the native form of *Escherichia coli* PDF (6), as well as the discovery that the natural product actinonin is an inhibitor of PDF with weak antibacterial activity (7–9), triggered the search for additional PDF inhibitors. Since then, a large number of structurally diverse PDF inhibitors have been identified, including several compounds with demonstrated *in vivo* efficacy and good safety profiles (10). Three PDF inhibitors have progressed to clinical trials (11, 12), and one of them, GSK1322322, is in phase II clinical development for the treatment of respiratory tract and skin infections.

While mutations in *Fold* and *GlyA*, two enzymes involved in the synthesis of 10-formyl-tetrahydrofolate, have been described (13, 14), loss-of-function mutations in the gene encoding formyl-methionyl transferase (FMT), the enzyme that catalyzes the formylation of the initiator methionyl-tRNA, are the most common cause of resistance to PDF inhibitors in bacteria where FMT is not essential for viability, such as *Staphylococcus aureus* (3, 15), *Bacillus subtilis* (13), *Pseudomonas aeruginosa* (16), *Salmonella enterica* (14), and *E. coli* (15, 17, 18). In those organisms, protein synthesis can still initiate with unformylated methionyl-tRNA, bypassing the need for PDF function. However, FMT mutants show compromised growth *in vitro* (50% to 90% reduction in the growth rate compared to the wild type) (3, 14–18) and *in vivo* (3,

14). Whether these effects are simply due to slow translation initiation or additional factors was heretofore unknown.

In order to further understand the liability associated with mutations in FMT, a thorough comparative *in vitro* characterization of *S. aureus* FMT mutants versus the wild-type strain was undertaken, and their abilities to produce an infection in immunocompetent and immunocompromised animal models were investigated. These studies demonstrate that *S. aureus* FMT mutants show a substantial reduction in production of extracellular virulence factors and are significantly less pathogenic than their wild-type counterparts in animal models of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in these studies include *S. aureus* WCUH29 (hospital-acquired methicillin-resistant *S. aureus* [HA-MRSA]) (19), *S. aureus* PVL-2 (USA-300; Panton-Valentine leukocidin [PVL] positive; community-acquired MRSA [CA-MRSA]) (20), and their characterized derivatives (Table 1), as well as *S. aureus* MW2, 90265/97, and PK1. Bacteria were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates, in Mueller-Hinton (MH) broth, on CCY medium (21), or on agar-based MH plates, as indicated. To detect hemolysis, TSA plates supplemented with 5% sheep or rabbit blood were incubated at 37°C for 24 to 48 h and at 4°C for an additional 12 h when

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TABLE 1 Characterized *S. aureus* strains used in these studies

Strain	Genotype	Reference
WCUH29	WT; HA-MRSA	19
Δfnt	$\Delta fnt::tetM$	This study
FMT1	<i>fnt</i> + T550; frameshift	This study
FMT7	<i>fnt</i> C577T Q193stop	This study
FMT8	<i>fnt</i> – G434; frameshift	This study
FMT10	<i>fnt</i> C319T H107Y	This study
PDF18	<i>pdf</i> T176A V59D	This study
pYH4	Plasmid pYH4	25, 26
Δfnt (pYH4)	$\Delta fnt::tetM$; plasmid pYH4	This study
Δfnt (pYH4- <i>fnt</i>)	$\Delta fnt::tetM$; <i>fnt</i> cloned in pYH4	This study
PVL-2	WT; CA-MRSA; USA-300; PVL ⁺	20
Δfnt	$\Delta fnt::tetM$	This study

sheep blood was used. *S. aureus* WCUH29 strains used in the experimental animal models were grown on brain heart infusion (BHI) broth.

Antibacterial susceptibility testing. MICs of antibiotics were determined using the broth microdilution methodology recommended by the Clinical and Laboratory Standards Institute (22). The MIC was the lowest concentration of antibiotic that suppressed visible growth.

Construction of *fnt* deletion/insertion mutants. *S. aureus* WCUH29 *fnt* deletion mutants were constructed as described previously (23). A $\Delta fnt::tetA(M)$ cassette was first integrated into the *S. aureus* RN4220 chromosome and then moved into *S. aureus* WCUH29 by transduction with $\phi 11$ phage. To construct CA-MRSA-PVL-2 $\Delta fnt::tetA(M)$, a $\Delta fnt::tetA(M)$ cassette was cloned into pMAD and used as described previously (24), except that, after overnight growth at 42°C, the allelic-replacement mutants were directly selected in the presence of tetracycline and PDF inhibitor. All *fnt* allelic replacements were confirmed by PCR amplification. pYH4 and pYH4-*fnt*, from an open reading frame expression library of the *S. aureus* genome (25, 26), were used to transform *S. aureus* WCUH29 Δfnt .

Isolation and characterization of spontaneous resistant mutants. Overnight cultures of *S. aureus* strains were plated onto MH agar plates containing 4 times the MIC of the PDF inhibitor SB-734453, a member of the hydrazide family of PDF inhibitors [10; K. M. Aubart, A. B. Benowitz, S. B. Christensen, I. V. J. M. Karpinski, J. Lee, and D. J. Silva, 2003, preparation of *N*-hydroxy-*N*-(3-hydrazino-3-oxopropyl)formamide derivatives as peptide deformylase inhibitors with antibacterial activity, PCT international patent application WO 03 101442]. Resistant colonies were purified once on PDF inhibitor-containing plates and once on MH plates. *pdf* and *fnt* genes from mutants and their parent strains were amplified by PCR and sequenced to identify mutations.

Analysis of extracellular proteins by SDS-PAGE and Western immunoblotting. Culture supernatants of *S. aureus* strains WCUH29 and PVL-2, equivalent to overnight cultures with optical densities at 600 nm (OD_{600}) of 0.4, were concentrated by Speed-Vac (Savant) and analyzed by SDS-PAGE and Western immunoblotting, as described previously (28). *S. aureus* PVL-2 *lukF*-PV and *lukS*-PV genes were cloned onto a pET vector and overexpressed in *E. coli* BL21(DE3). His-tagged *LukF*-PV and *LukS*-PV were purified as previously described (29) and used to generate rabbit polyclonal antibodies. Alpha-toxin antibodies were provided by M. Burnham (30).

Measurement of cell clumping and coagulase activity. Cell clumping was qualitatively observed by mixing 20 μ l of a saline suspension of bacterial cells (2×10^9 CFU/ml), prepared from freshly grown colonies, with 50 μ l of serial 2-fold dilutions of a 2% (wt/vol) solution of commercial human fibrinogen in phosphate-buffered saline (PBS). Occurrence of clumping was noted after 3 min at room temperature. Coagulase activity was determined qualitatively by adding 0.5 ml of serial 2-fold dilutions of *S. aureus* culture supernatants in PBS to 0.5 ml of rabbit plasma diluted 1:3 in PBS. The mixtures were incubated at 37°C under static conditions and observed over a 24-h period.

Mice. Male CBA/J mice (19 to 24 g; Jackson Laboratory), used in the experimental models, were allowed food and water *ad libitum*. All procedures were performed in accordance with protocols approved by the GlaxoSmithKline Institutional Animal Care and Use Committee and met or exceeded the standards of the American Association for the Accreditation of Laboratory Animal Care, the United States Department of Health and Human Services, and all local and federal animal welfare laws.

Immunocompromised mice. Mice were dosed intraperitoneally (i.p.) once daily with 150 mg cyclophosphamide/kg of body weight (0.2 ml/mouse) on day 0 and with 100 mg/kg on days 3, 6, 8, 11, 13, and 15 of the study. On days 0, 4, 5, 7, 12, 14, 18, and 21, the mice were bled by cardiac puncture, and 200 to 300 μ l of blood was placed into EDTA-containing tubes and gently mixed. Samples were analyzed on an Advia 120 Hematology System (Siemens Diagnostics) to determine peripheral blood counts. Red blood cell counts remained relatively constant, with a slight decrease at days 18 and 21. White blood cell (WBC) and neutrophil counts decreased by day 4 to $\leq 1,000$ WBC/ μ l and ≤ 100 neutrophils/ μ l, respectively, and remained low until at least day 18. By day 21, 6 days after the last cyclophosphamide dose, white blood cell counts returned to baseline numbers and neutrophil counts increased to approximately four times baseline numbers. The mice maintained neutropenia (≤ 100 neutrophils/ μ l) from day 4 to day 18 of the study.

Murine hematogenous pyelonephritis infection model. *S. aureus* WCUH29 cultures, grown overnight in BHI broth, were centrifuged and washed three times in PBS. Bacterial suspensions ($OD_{600} \sim 0.2$) were diluted and plated onto TSA plates with 5% sheep blood to determine viable CFU. Five mice per group were inoculated with 0.1 ml of this suspension (containing approximately 10^7 CFU/mouse) by tail vein injection. The mice were monitored twice daily for signs of illness, and any that appeared moribund were euthanized prior to the end of the experiment. All remaining animals were euthanized by carbon dioxide overdose at days 3, 7, 14, 21, and 28 postinfection. Both kidneys were removed using an aseptic technique, placed in bags, and homogenized in PBS for 2 min in a Stomacher 80 Biomaster (Seward). Tenfold serial dilutions in sterile saline were plated on TSA supplemented with 5% sheep blood for enumeration of viable bacteria.

Infection in immunocompromised animals. Mice were treated i.p. with cyclophosphamide as described above—150 mg/kg on day –4 and 100 mg/kg on days –1, 2, 4, 7, 9 and 11—to create and maintain neutropenia for the study duration. Enumeration of the day for cyclophosphamide treatment has been adjusted to encompass the day infection started, day 0. The experimental model was performed as described above, except mice were inoculated with 10^3 CFU/mouse and the animals were euthanized at days 0, 2, 4, 7, 10, and 14 postinfection.

Survival studies. Infection was induced as described above. Ten immunocompetent animals per group, inoculated with approximately 10^7 CFU/mouse, or 20 neutropenic animals/group, inoculated with 10^3 CFU/mouse, were used in these studies. Mouse survival was followed for 14 days, but bacterial enumeration of kidneys was not performed.

RESULTS

In vitro characterization of *S. aureus* FMT mutants. Spontaneously occurring *S. aureus* WCUH29 mutants resistant to the PDF inhibitor SB-734453 (Aubart et al., PCT international patent application WO 03 101442) were isolated at a frequency of 5×10^{-7} , similar to that previously reported for different PDF inhibitors with other *S. aureus* strains (3, 15). From over 200 mutants, 37 small and 3 large colonies were selected for genetic characterization of the *fnt* and *pdf* genes. The strains with large-colony morphology were found to carry mutations in the *pdf* gene. This is the first time that mutations conferring resistance to PDF inhibitors in *S. aureus* have been identified in the target gene, although they occur at a much lower frequency (5×10^{-8} to 5×10^{-9}) and confer a lower level of resistance than *fnt* mutations. Two of the

FIG 1 Nonhemolytic phenotypes of different *S. aureus* WCUH29 FMT mutants in sheep blood agar plates. *S. aureus* WCUH29 and its derivative strains (Table 1) were streaked onto a TSA plate containing 5% sheep blood agar and screened for hemolytic activity by incubation at 37°C for 24 to 48 h and were then left at 4°C for 12 h. FMT-null mutants isolated from *S. aureus* strains PVL-2, MW2, 90265/97, and PK1 showed identical nonhemolytic phenotypes on sheep blood agar plates screened under these conditions.

three mutants had a V59D substitution in the PDF protein, which maps to the inhibitor-binding pocket in the PDF crystal structure (10). All strains with small-colony morphology carried mutations in the *fnt* gene that should lead to defective FMT proteins, including premature stop codons or frameshifts, or amino acid substitutions shown to disrupt the catalytic mechanism of the enzyme (31). As previously described (3, 15), FMT mutants, regardless of the type of mutation, showed a decrease in the growth rate with respect to the parent strain or the PDF mutants.

Further characterization revealed that *S. aureus* WCUH29 FMT mutants (Table 1), when screened on sheep blood agar plates, showed a nonhemolytic phenotype, while wild-type (WT) *S. aureus* and PDF mutant strains were hemolytic (Fig. 1), indicating that FMT mutants had impaired hemolysin production (32). The hemolytic phenotype could be restored in a genetically engineered *fnt* deletion mutant, *S. aureus* WCUH29 Δfnt , by the addition of the *fnt* gene in the pYH4 plasmid (25, 26) (Fig. 1), implying that loss of FMT function resulted in lack of hemolysis. This was a characteristic of all *S. aureus* FMT mutants, irrespective of their parent strain, as lack of hemolysis on sheep blood agar plates was also observed with FMT mutants from 4 other *S. aureus* strains, PVL-2, MW2, 90265/97, and PK1, encompassing both HA-MRSA and CA-MRSA with different antibiotic resistance phenotypes, including multidrug-resistant *S. aureus*. Moreover, the hemolytic activity of *S. aureus* 90265/97 was enhanced when incubating the plates at 4°C while its corresponding FMT mutants were still nonhemolytic, suggesting β -hemolysin production was affected (32).

Identical results were obtained when hemolysis was evaluated on rabbit blood agar plates. While PDF mutants had a wild-type hemolytic phenotype, all *S. aureus* WCUH29 FMT mutants analyzed were nonhemolytic, implying that α -hemolysin production was compromised (32). Complementation of *S. aureus* WCUH29 Δfnt with the *fnt* gene in *trans* again restored hemolysis on rabbit blood agar plates. Western immunoblotting analysis, performed using culture supernatants, showed >95% reduction in produc-

FIG 2 Reduction in alpha-toxin or PVL production in *S. aureus* FMT mutants. Culture supernatants from *S. aureus* WCUH29 strains grown in TSB or *S. aureus* PVL-2 strains grown in MH broth were fractionated by SDS-PAGE and subjected to Western immunoblotting with the indicated antisera. (A) Production of alpha-toxin in *S. aureus* WCUH29 strains. (B) Production of PVL subunits in *S. aureus* PVL-2 strains.

tion of α -hemolysin in the *S. aureus* FMT mutants, while the PDF mutant produced levels similar to those in the wild-type strain (Fig. 2A). Extracellular α -hemolysin production was restored to wild-type levels in *S. aureus* WCUH29 Δfnt (pYH4-fmt), where the *fnt* gene was supplied in *trans*. Production of α -hemolysin was similarly reduced in *S. aureus* FMT mutants from two additional strains tested, 90265/97 and PK1.

Lack of transformylase affected the production of additional virulence factors, with reductions in total extracellular proteins of at least 50% observed in supernatants of cultures from the different *S. aureus* WCUH29 FMT mutants. Separation of the proteins by SDS-PAGE showed a specific pattern, identical in all mutants, with substantial reductions seen in the production of at least six major exoproteins (Fig. 3A).

As CA-MRSA infections in the United States are predominantly caused by the USA300 clone, which secretes PVL toxin, an

FIG 3 Reduction of extracellular protein production in *S. aureus* FMT-null mutants. Culture supernatants from *S. aureus* WCUH29 strains grown in TSB or *S. aureus* PVL-2 strains grown in MH broth or CCY medium were fractionated by SDS-PAGE, followed by silver staining (A) or SimplyBlue Safestain (B) for visualization.

TABLE 2 *S. aureus* FMT-null mutations result in hypersensitivity to some 50S ribosomal inhibitors

Antibiotic compound	Class	MIC ^a (μg/ml) for <i>S. aureus</i> WCUH29 strain:			
		Wild type	Δfnt	FMT-8	FMT-10
Azithromycin	Macrolide	1	0.125	0.125	0.125
Erythromycin	Macrolide	0.25	0.016	0.016	0.016
Tylosin	Macrolide	1	0.063	0.063	0.063
Telithromycin	Ketolide	0.063	0.004	0.004	0.004
Lincomycin	Lincosamide	1	0.125	0.125	0.125
Virginiamycin	Streptogramin	2	0.125	0.25	0.25
Tiamulin	Pleuromutilin	0.25	0.03	0.03	0.03

^a No significant MIC changes (<2-fold variation) were observed against *S. aureus* FMT mutants with the following antibiotics: amoxicillin, bacitracin, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, kanamycin, linezolid, mupirocin, novobiocin, oxacillin, phosphomycin, piperacillin, rifampin, streptomycin, trimethoprim, and vancomycin.

fnt deletion mutant of a USA300 isolate was constructed. As expected, *S. aureus* PVL-2 Δfnt was nonhemolytic on sheep and rabbit blood agar plates, and its extracellular protein production was also severely impaired in different testing media (Fig. 3B). Specifically, production of the PVL subunits, LukS-PV and LukF-PV, could not be detected in *S. aureus* PVL-2 Δfnt when the strain was grown on CCY, a medium known to maximize extracellular production of PVL (33). This was further confirmed by Western blotting of PVL production in *S. aureus* PVL-2 Δfnt (Fig. 2B).

While diagnostic kits used clinically demonstrate that *S. aureus* WCUH29 FMT mutants are coagulase positive, like the parent strain, a qualitative evaluation of production of coagulase in culture supernatants and clumping factor in whole cells (the same strains indicated in Fig. 2A) showed that both activities were reduced in FMT mutants. Production could be restored to wild-type levels when the *fnt* gene was supplied in *trans*.

Finally, susceptibility testing of a representative set of *S. aureus* WCUH29 FMT mutants showed no cross-resistance with other marketed antibiotics. These mutants were 8- to 16-fold more susceptible to antibiotics that interact with 50S ribosomal subunits, such as macrolides, ketolides, lincosamides, streptogramins, and pleuromutilins, than the wild-type parent strain (Table 2). Interestingly, *S. aureus* FMT mutants could not be isolated when 1/4 MIC of erythromycin was added to a PDF inhibitor with an inoculum of 1.4×10^9 CFU of *S. aureus* WCUH29, indicating that subinhibitory concentrations of erythromycin can prevent spontaneous resistance development mediated by FMT mutation. This further suggests that all mutants with loss-of-function mutations in FMT are more susceptible to erythromycin regardless of their genotype.

Animal survival and culture studies comparing *S. aureus* wild-type and FMT mutant strains in a murine hematogenous pyelonephritis infection model. Given the reduction in virulence factor production observed in *S. aureus* FMT mutants, it was anticipated that their ability to cause an invasive infection would be impaired. In order to evaluate if *S. aureus* FMT mutants could produce a lethal infection, mice were inoculated intravenously with wild-type *S. aureus* WCUH29 (4.5×10^7 CFU/mouse) or representative FMT mutants (Table 1): Δfnt (1.6×10^7 to 2.1×10^7 CFU/mouse), FMT8 (2.1×10^7 to 2.3×10^7 CFU/mouse), and FMT10 (1.5×10^7 CFU/mouse). The animals (10/group) were

followed for 14 days in two separate experiments. All animals infected with wild-type organisms succumbed to infection by day 7, whereas those infected with *S. aureus* Δfnt , FMT8, or FMT10 showed 100%, 90%, and 95% survival rates, respectively (Fig. 4A).

In order to assess if the high survival rate observed with *S. aureus* FMT mutants was related to their inability to produce virulence factors or to impaired growth in the animals, the bacterial load present in the kidneys of mice infected with *S. aureus* WT or FMT mutant strains was enumerated at days 3, 7, 14, 21, and 28 postinfection (Fig. 4B). In this study, all mice infected with wild-type *S. aureus* died by day 7, while in the *S. aureus* FMT groups, only one animal, infected with *S. aureus* FMT10, died 10 days after infection. While similar bacterial counts were recovered from the kidneys of all animals infected with wild-type organisms, there were large variations in the bacterial loads recovered from the kidneys of animals infected with FMT mutant strains, with counts from animals in the same group differing by more than 10^3 CFU/kidney in many cases. Despite the variability, a very clear trend was observed in all groups, with mean kidney bacterial counts peaking between day 3 and day 7 and then decreasing over time. In fact, bacteria could not be detected in the kidneys of several animals infected with *S. aureus* Δfnt (2 animals each at days 21 and 28), FMT8 (3 animals at day 28), or FMT10 (1 animal at day 14).

Similar results were obtained in an additional experiment in which enumeration of bacteria from animals infected with these strains took place from day 28 to day 49 postinfection. In that study, all mice infected with wild-type *S. aureus* had succumbed to infection by day 8, while only one animal from the groups infected with *S. aureus* FMT mutants died by day 5, with all remaining animals surviving to day 49. In addition, kidney bacterial loads continued to decrease over time, and 60% of the mice had counts below the limit of detection at day 49.

While *S. aureus* FMT mutants were clearly capable of growing in the animals, albeit at a lower rate than wild-type organisms, they were substantially less pathogenic than the parent strain. In order to determine if the FMT isolates became more virulent after animal passage, an *S. aureus* Δfnt colony isolated from the 21-day-postinfection kidney homogenate was used to infect 10 animals, and survival was followed for 14 days. The survival rate was 100%.

Animal survival and culture studies comparing *S. aureus* wild-type and FMT mutant strains in a neutropenic murine hematogenous pyelonephritis infection model. As immunocompromised animals are more vulnerable to infection, a neutropenic murine hematogenous pyelonephritis infection model that mimicked the conditions of the immunocompetent model previously used was developed. In this model, neutropenia was maintained for the duration of the study. The inoculum of the wild-type strain was adjusted to produce a survival curve similar to that obtained in the nonneutropenic model, and therefore, studies were performed with an inoculum of 10^3 CFU/mouse.

An initial study involving 20 mice/group showed 0% survival of those animals infected with wild-type *S. aureus* WCUH29 by day 8 postinfection (Fig. 5A). However, mice infected with *S. aureus* Δfnt or FMT1 displayed 100% and 90% survival on day 14, respectively (Fig. 5A). These results are almost identical to those obtained in the nonneutropenic model.

The bacterial load present in the kidneys of these animals was determined in an additional study (5 mice/group) that included bacterial enumeration at days 0, 2, 4, 7, 10, and 14 postinfection.

FIG 4 *S. aureus* WCUH29 FMT mutants are attenuated in a murine pyelonephritis infection model. *S. aureus* WCUH29 wild type and FMT mutants, Δ fmt, FMT8, and FMT10, were used in these studies. (A) Survival study combining two separate experiments, both with 10 animals/group. (B) Bacterial counts from kidneys of infected mice (5 animals/group) at different days postinfection. Kidneys from animals infected with WT organisms could not be cultured after day 3 due to death. The means $\pm$ standard deviations are indicated by the black lines.

Similar to the survival study, infection with the wild-type strain caused death of all animals by day 7, whereas no deaths occurred in the *S. aureus* Δ fmt group and 2 animals died (days 6 and 14) in the *S. aureus* FMT1 group. Extremely large variations were observed in kidney bacterial counts of animals inoculated with FMT mutant strains (Fig. 5B). No bacteria were recovered from mice infected with *S. aureus* Δ fmt at day 2, a maximum bacterial load was obtained at day 4, and counts decreased at days 7 and 10, reaching undetectable levels at day 14. There were differences of approximately 10^5 CFU/kidney from mice within the same group, and in all cases, there were animals with bacterial levels below the limit of detection (2/5 mice at day 4 and 3/5 mice at days 7 and 10). A similar trend was observed in the animals infected with *S. aureus* FMT1. At days 7, 10, and 14, bacteria were undetectable in $\geq 50\%$ of the mice (4/5, 2/4, and 3/4 animals, respectively). As observed in the nonneutropenic-animal model, mice infected with wild-type *S. aureus* WCUH29 had similar bacterial counts in all groups enumerated.

DISCUSSION

Resistance is expected to occur with any new antibacterial agent that is used clinically. The frequency and level of resistance are

parameters that can be readily determined *in vitro* for any novel antibiotic. However, the rate at which resistance emerges once a compound is in the clinic will be determined by the controlled use of the antibiotic, the cost that the mutation inflicts on the fitness of the bacteria, and the ability of the bacteria to compensate for that cost while maintaining resistance.

As inactivation of FMT is the predominant cause of resistance to PDF inhibitors in certain pathogens, FMT mutants have been the focus of research by several groups assessing the validity of PDF as an antibacterial target (3, 4, 17). In our study, *S. aureus* FMT mutants were isolated at frequencies of 5×10^{-7} and showed, as previously described (3), a substantial reduction in *in vitro* growth rates, most likely due to the inefficient initiation of protein synthesis when unformylated methionyl-tRNAi is used (18). This slower initiation process may also account for the observation that these mutants are 8 to 16 times more susceptible than the wild-type strain to some protein synthesis inhibitors that bind to the 50S ribosomal subunit, such as macrolides, lincosamides, or pleuromutilins, while remaining equally susceptible to other antibacterial agents. Furthermore, we have discovered that loss-of-function mutations in *S. aureus* FMT have additional consequences, as FMT mutants are nonhemolytic and show a 50%

FIG 5 *S. aureus* WCUH29 FMT mutants are attenuated in a neutropenic murine pyelonephritis infection model. *S. aureus* WCUH29 wild type and FMT mutants, Δ fmt and FMT1, were used in these studies. (A) Survival study, with 20 animals/group. (B) Bacterial counts from kidneys of infected mice (5 animals/group) at different days postinfection. Bacterial counts from the kidneys of one dead animal (FMT1; day 14) are marked with a filled triangle. Kidneys from animals infected with WT organisms could not be cultured after day 4 due to death. The means $\pm$ standard deviations are indicated by the black lines.

reduction in total exoprotein production. Examination of some specific extracellular proteins revealed that key virulence factors associated with *S. aureus* pathogenicity were particularly impacted. In fact, Western immunoblotting analysis demonstrated a >95% reduction in alpha-toxin production in FMT mutants, as well as the elimination of PVL production in a CA-MRSA strain. These results are consistent with a slower translation process, as it has been demonstrated that protein synthesis inhibitors can reduce the synthesis of virulence factors even at concentrations that have no effect on bacterial growth (34, 35).

Although the molecular mechanisms that regulate the transition of *S. aureus* from a commensal bacterium to an invasive pathogen are not well understood, it is clear that production of a wide repertoire of virulence factors that allow *S. aureus* to attach to cells, degrade tissues, disseminate, and evade host defenses plays a critical role in its pathogenicity (36). In fact, alpha-toxin has been reported as a major cause of death in mice injected with *S. aureus* intraperitoneally (37) and in a murine model of *S. aureus* pneumonia (38, 39) and has also been described as a key virulence determinant in *S. aureus* brain abscess (40) and septic arthritis infections (41). In addition, deletion of *agrA*, a global regulatory gene responsible for the expression of most exoproteins, including all hemolysins and leukocidins (42), has an even more dramatic effect on the reduction of *S. aureus* pathogenicity (38, 43, 44). Due

to the pronounced decrease in exotoxin production observed in *S. aureus* FMT mutants, we investigated their ability to establish an infection in a murine hematogenous pyelonephritis model. All mice inoculated with the *S. aureus* wild-type strain died by day 7 postinfection, whereas those infected with *S. aureus* FMT mutants showed a dramatic reduction in lethality, with 90 to 100% survival rates 14 days postinfection. This extreme difference in survival rates is consistent with the impaired exoprotein/virulence factor production associated with FMT mutations.

Analysis of the bacterial load in the kidneys of infected mice revealed that *S. aureus* FMT mutants could initially grow *in vivo*, although not at the same rate as wild-type organisms, and that kidney bacterial counts decreased over time. In fact, 20% of the animals infected with *S. aureus* FMT mutants did not have any detectable bacteria in their kidneys at either 14, 21, or 28 days postinfection. This could be due to the inability of the mutants to establish an invasive infection or to the ability of the immune system to eradicate them. As neutropenic animals are more susceptible to bacterial infections, we established a pyelonephritis animal model where neutropenia was maintained for the duration of the study (14 days). As seen in the immunocompetent animals, $\geq 90\%$ of the mice infected with *S. aureus* FMT mutants survived while none of the animals infected with wild-type *S. aureus* lived beyond 8 days postinfection. In a culture study, *S. aureus* FMT mutants were capable of growing in some of the neutropenic animals but were clearly less pathogenic than the wild-type strain. Furthermore, bacteria could not be detected in the kidneys of two-thirds of the animals infected with FMT mutants 7 days after infection. The fact that similar results are observed in neutropenic and nonneutropenic animals suggests that the immune response is not the primary mechanism involved in the elimination of *S. aureus* FMT mutants.

The decreased pathogenicity demonstrated by *S. aureus* FMT mutants may be further accentuated in the presence of wild-type bacteria, since competition experiments performed in mice with *S. enterica* wild-type and FMT mutants showed that 2 days after injecting 10^5 CFU of both organisms, FMT mutants could be recovered at a frequency of only 5×10^{-5} to 4×10^{-6} (14). In addition, although mutations that partially compensated for the growth of *S. enterica* FMT mutants *in vitro* could be found after 50 to 150 serial passages (14, 45), these compensated mutants did not show improved growth rates in mice (14).

Our results agree with those of Margolis et al. (3), who reported the attenuation of *S. aureus* FMT mutants in a 4-day thigh abscess model of infection. In contrast, Zorz et al. (46) and Gjertsson et al. (47) did not observe differences in the growth of *S. aureus* FMT mutants with respect to the wild-type strain in their animal models. These discrepancies are likely explained by the different experimental designs. The study duration used by Zorz et al. (46) is too short, as we have demonstrated that periods longer than 3 days are necessary to observe differences between *S. aureus* WT and FMT mutant strains. Gjertsson et al. (47) used a wild-type *S. aureus* strain less virulent than WCUH29, which did not cause death after 16 days, making it difficult to compare the pathogenicities of *S. aureus* WT and FMT mutant strains.

Finally, over 50 *in vivo* efficacy studies have been performed in our laboratories, where *S. aureus* abscess groin infections (using an inoculum of 10^7 CFU/mouse) have been treated with efficacious doses of various PDF inhibitors twice daily for 4 days. At the end of every efficacy study, the antimicrobial susceptibilities of a

number of isolates from randomly selected animals were tested, and FMT mutants were never detected. This indicates that the eradication of wild-type *S. aureus* by treatment with PDF inhibitors does not promote growth of *S. aureus* FMT mutants in animal models. This is in contrast to the *in vitro* situation, where breakthrough growth of FMT mutants is always observed after 24 h in cell-killing studies with PDF inhibitors using an inoculum of $\sim 10^6$ CFU/ml. The different behaviors of these mutants *in vitro* versus *in vivo* further support the notion that lack of virulence, and not reduced growth, is primarily responsible for their compromised ability to cause productive infection in an animal.

In summary, this study shows that, although *S. aureus* FMT mutants can be isolated at high frequency *in vitro*, inactivation of FMT leads to a reduced growth rate both *in vitro* and in mice. More importantly, *S. aureus* FMT mutants show a significant decrease in production of virulence factors that translates into a substantial loss of pathogenicity in animal models. Therefore, it is anticipated that these mutants will have difficulty producing an invasive infection in humans.

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