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Inducible Expression of a Resistance-Nodulation-Division-Type Efflux Pump in *Staphylococcus aureus* Provides Resistance to Linoleic and Arachidonic Acids

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ABSTRACT

Although *Staphylococcus aureus* is exposed to antimicrobial fatty acids on the skin, in nasal secretions, and in abscesses, a specific mechanism of inducible resistance to this important facet of innate immunity has not been identified. Here, we have sequenced the genome of *S. aureus* USA300 variants selected for their ability to grow at an elevated concentration of linoleic acid. The fatty acid-resistant clone FAR7 had a single nucleotide polymorphism resulting in an $H_{121}Y$ substitution in an uncharacterized transcriptional regulator belonging to the AcrR family, which was divergently transcribed from a gene encoding a member of the resistance-nodulation-division superfamily of multidrug efflux pumps. We named these genes *farR* and *farE*, for regulator and effector of fatty acid resistance, respectively. Several lines of evidence indicated that FarE promotes efflux of antimicrobial fatty acids and is regulated by FarR. First, expression of *farE* was strongly induced by arachidonic and linoleic acids in an *farR*-dependent manner. Second, an $H_{121}Y$ substitution in FarR resulted in increased expression of *farE* and was alone sufficient to promote increased resistance of *S. aureus* to linoleic acid. Third, inactivation of *farE* resulted in a significant reduction in the inducible resistance of *S. aureus* to the bactericidal activity of 100 μM linoleic acid, increased accumulation of [^{14}C]linoleic acid by growing cells, and severely impaired growth in the presence of nonbactericidal concentrations of linoleic acid. Cumulatively, these findings represent the first description of a specific mechanism of inducible resistance to antimicrobial fatty acids in a Gram-positive pathogen.

IMPORTANCE

***Staphylococcus aureus* colonizes approximately 25% of humans and is a leading cause of human infectious morbidity and mortality. To persist on human hosts, *S. aureus* must have intrinsic defense mechanisms to cope with antimicrobial fatty acids, which comprise an important component of human innate defense mechanisms. We have identified a novel pair of genes, *farR* and *farE*, that constitute a dedicated regulator and effector of *S. aureus* resistance to linoleic and arachidonic acids, which are major fatty acids in human membrane phospholipid. Expression of *farE*, which encodes an efflux pump, is induced in an *farR*-dependent mechanism, in response to these antimicrobial fatty acids that would be encountered in a tissue abscess.**

Staphylococcus aureus has a dichotomous relation with human hosts, being able to establish an asymptomatic commensal relationship, but is also historically known as a leading cause of human infectious morbidity and mortality. Significantly, death attributed to *S. aureus* in the United States is now comparable to mortality rates for AIDS, tuberculosis, and viral hepatitis (1–3). Not surprisingly, therefore, *S. aureus* has been the subject of intensive research on mechanisms of pathogenesis and acquisition and transfer of antibiotic resistance and of efforts to identify potential vaccine antigens (4–6). Until the late 1990s, much of this was directed toward hospital-associated strains of methicillin-resistant *S. aureus* (HA-MRSA) to address the anticipated emergence of superbugs that would be resistant to all clinically useful antibiotics (7, 8). However, a new threat emerged in the late 1990s with community-acquired MRSA (CA-MRSA). Although these strains evolved in the community setting, one notorious strain known as USA300 has achieved pandemic status across North America and is now the leading cause of *S. aureus* infections, irrespective of community or hospital origin (9, 10). This has engendered greater attention toward identifying mechanisms of *S. aureus* persistence on human hosts and of host-to-host transmission.

Approximately 25% of humans are persistently colonized by *S. aureus*, where the preferred site of colonization is the anterior nares, and among colonized individuals, the bacterium is also frequently recovered from other body sites, including the axillae, perineum, hands, chest, and limbs (11). Accordingly, the bacteria's ability to persist on skin is an important mediator of trans-

Received 27 December 2014 **Accepted** 17 March 2015

Accepted manuscript posted online 23 March 2015

Citation Alnaseri H, Arsic B, Schneider JET, Kaiser JC, Scinocca ZC, Heinrichs DE, McGavin MJ. 2015. Inducible expression of a resistance-nodulation-division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids. J Bacteriol 197:1893–1905. doi:10.1128/JB.02607-14.

Editor: O. Schneewind

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.02607-14.

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doi:10.1128/JB.02607-14

mission, as underscored by the recent discovery that the hypertransmissible USA300 strain has overcome one of the innate defense barriers of the skin through horizontal gene transfer with *Staphylococcus epidermidis* to acquire resistance to toxic polyamines that restrict the growth of other *S. aureus* strains (12, 13). Other innate defense barriers of the skin include its acidic pH and antimicrobial fatty acids, foremost of which is sapienic acid that is released from triglycerides secreted by the sebaceous glands (14, 15). Nasal secretions also contain antimicrobial fatty acids, primarily linoleic, arachidonic, and palmitoleic acids or their corresponding cholesterol esters (16), and infected abscess tissue also contains abundant antimicrobial fatty acids (17, 18). Consequently, *S. aureus* is exposed to antimicrobial fatty acids not only during colonization but also during infection, and thus it is reasonable to hypothesize that *S. aureus* has evolved mechanisms of intrinsic resistance.

Among mechanisms that have been described are cell surface teichoic acids that can selectively restrict the access of palmitoleic acid to the cytoplasmic membrane (19) and a cell surface protein, IsdA, that is expressed in response to iron-limiting conditions and that also restricts the access of palmitoleic acid, or its isomer sapienic acid, to the cytoplasmic membrane (20). Other investigators have reported that *tet38*, encoding a major facilitator superfamily (MFS) efflux pump, promotes resistance to palmitoleic acid (21). Expression of *tet38* was induced by palmitoleic acid but not by linoleic acid, which suggested that there could be distinct mechanisms for coping with different antimicrobial fatty acids. Importantly, linoleic acid is an essential fatty acid for humans, which must be obtained from dietary sources, and is an essential precursor for synthesis of arachidonic acid. These two unsaturated fatty acids comprise a major proportion of unsaturated fatty acids in membrane phospholipid (22, 23). Therefore, the ability to sense and respond to linoleic acid could represent a specific sensory mechanism to signal colonization or infection of a human host, and yet specific mechanisms for regulating gene expression and intrinsic resistance in response to linoleic acid have not been reported.

To address this, we drew from our previous observation that exposure of *S. aureus* USA300 to a subinhibitory (25 μM) concentration of linoleic acid caused a robust induction of secreted protease expression, which led to proteolytic processing of a secreted glycerol ester hydrolase, Geh (24). We subsequently noted that when *S. aureus* cultures were supplemented with a trilinolein triglyceride substrate, Geh activity quickly liberated growth-inhibitory concentrations of linoleic acid (25). Moreover, 50 μM free linoleic acid imposed a 10- to 12-h growth delay in cultures of *S. aureus* USA300, which was then followed by unimpeded exponential growth; similar results were obtained with 50 μM trilinolein in wild-type *geh*-proficient *S. aureus* USA300, whereas growth of a *geh*-deficient mutant was unaffected by 50 μM trilinolein (24, 25). From these observations, we hypothesized that, in addition to the induction of expression of secreted proteases, there should also be an inducible mechanism for resistance of *S. aureus* to linoleic acid.

In related studies, selection of *S. aureus* strains that were able to grow at elevated concentrations of glycopeptides led to the identification of point mutations in the *vraS* sensor of antimicrobial glycopeptides (26, 27). Therefore, we adopted a similar strategy by conducting comparative genome sequencing of USA300 clones that were selected for their ability to initiate growth without a lag phase when they were inoculated into medium containing 50 μM linoleic acid. We now provide the first description of a novel gene pair, *farR-farE* (fatty acid resistance), constituting divergently transcribed genes that, respectively, encode a regulator and effector of *S. aureus* resistance to linoleic and arachidonic acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A list of bacterial strains and plasmids that were used or constructed for this study is provided in Table 1. *S. aureus* cultures were maintained as frozen stocks (−80°C) in 20% glycerol and streaked on tryptic soy broth (TSB) agar when required. TSB was supplemented, when required, with 10 μg/ml of erythromycin or chloramphenicol for propagation of strains bearing resistance markers. *Escherichia coli* strains were grown on LB agar or in LB broth containing 100 μg/ml ampicillin when required. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 180 rpm.

For experimental purposes, inoculum cultures of *S. aureus* were prepared by transferring cells from a single colony into 13-ml polypropylene tubes containing 3 ml of TSB supplemented with antibiotic, as required, followed by overnight incubation. After determination of the optical density at 600 nm (OD_{600}), aliquots of the overnight cultures were diluted into 25 ml of medium in 125-ml flasks to achieve an initial OD_{600} equivalent to 0.01. To supplement medium with different fatty acids, a 5 mM stock concentration was initially prepared in sterile TSB containing 1% dimethyl sulfoxide (DMSO) and then diluted into sterile TSB supplemented with 0.1% DMSO to achieve the desired concentration of fatty acids, ranging from 5 μM to 100 μM.

Selection and comparative genome sequencing of FAR clones. As reported previously, when an overnight culture of *S. aureus* USA300 was inoculated into fresh TSB containing 50 μM linoleic acid, there was a 10- to 12-h lag phase, followed by unimpeded exponential growth (24). Therefore, to promote the selection of fatty acid-resistant (FAR) clones, seven separate flasks of *S. aureus* USA300 were subjected to two consecutive cycles of growth to stationary phase in TSB–50 μM linoleic acid, after which samples of each culture were plated for isolation of single colonies. Colonies from each plate were screened to identify fatty acid-resistant clones that could initiate growth without a lag phase when inoculated into TSB–50 μM linoleic acid. A single FAR clone was then selected from each of the seven separate biologic replicates for comparative genome sequencing. For controls, two single colonies of USA300 were selected after two consecutive cycles of growth in TSB alone.

For comparative genome sequencing, genomic DNA was extracted from *S. aureus* using previously described protocols (28, 29). All samples for comparative genome sequencing were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada [http://www.lrgc.ca]) using an Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA) and 316 chips. Briefly, genomic DNA was quantified using a Qubit and Qubit double-stranded DNA (dsDNA) high-sensitivity assay (Life Technologies, Carlsbad, CA). Samples then underwent fragmentation and adapter and bar code ligation as per an Ion Xpress Fragment Library kit (catalog number 4469142, revision B) and size selection using a Pippin Prep system (Sage Science, Beverly, MA). The size of the final libraries was verified using an Agilent 2100 Bioanalyzer and a High Sensitivity DNA kit (Agilent Technologies Inc., Palo Alto, CA). Bar-coded libraries were pooled at equimolar concentrations, based on Qubit values, and the template dilution factor (TDF) for the final pooled library was calculated using molarity determined via quantitative PCR (qPCR) with an Ion Library Quantification kit (catalog number 4468802). Diluted libraries were processed as per the Ion OneTouch template kit (catalog number 4468007, revision B) for automated clonal amplification and sequenced using an Ion Express Template 200 kit (catalog number 4474280), Enrichment Station, and an Ion Sequencing 200 kit (catalog number 4471999, revision B). Sequence reads were mapped to the genome of *S. aureus* USA300 (FPR3757) (30) using CLC Genomics Workbench, version 7.0 (Boston, MA), and automated

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. aureus		
USA300 LAC	Community-associated MRSA, wild-type strain cured of resistance plasmids	24
RN4220	r_K^- m_K^+, capable of accepting foreign DNA	32
NE1393	Transposon insertion in SAUSA300_2490, Erm^r	34
NE2336	Transposon insertion in SAUSA300_2489, Erm^r	34
USA300 *farR*::ΦNE	USA300 LAC recipient of transposon from NE1393	This study
USA300 *farR*::ΦNE(pLI*farR*)	*farR*::ΦNE complemented with native *farR*, cloned in pLI50; Erm^r Cm^r	This study
USA300 *farR*::ΦNE(pCN*farR*)	*farR*::ΦNE complemented with pCN*farR* for cadmium-inducible expression, Erm^r Cm^r	This study
USA300 *farE*::ΦNE	USA300 LAC recipient of transposon from NE2336	This study
USA300 *farE*::ΦNE(pLI*farE*)	*farE*::ΦNE complemented with native *farE*, cloned in pLI50	This study
USA300 *farE*::ΦNE(pLI50)	*USA300 farE*::ΦNE with empty pLI50 vector, Cm^r	This study
USA300 Δ*tet38*	USA300 LAC with internal deletion of *tet38* (SAUSA300_0139)	This study
USA300 Δ*tet38-farE*::ΦNE	USA300 Δ*tet38* recipient of *farE*::ΦNE transposon insertion, Erm^r	This study
E. coli DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
Plasmids		
pLI50	*E. coli-S. aureus* shuttle vector	35
pLI*farR*	pLI50 with native *farR* gene	This study
pLI*farE*	pLI50 with native *farE* gene	This study
pGY*lux*	*E. coli-S. aureus* shuttle vector harboring promoterless *luxABCDE* operon	37
pCN51	*E. coli-S. aureus* shuttle vector with P_{cad} promoter for cadmium-inducible gene expression	36
pCN51c	pCN51 with *ermC* cassette replaced by *cat194* cassette from pRN7146	This study
pCN51*farR*	pCN51c with promoterless *farR* for cadmium-inducible expression of *farR*	This study
pKOR-1	*E. coli-S. aureus* shuttle vector; contains $P_{xyl\text{-}tetO}$, expresses antisense *secY* RNA	38
pKORΔ*tet38*	pKOR-1 containing upstream and downstream flanking sequences for deletion of *tet38*	This study

detection of single nucleotide polymorphisms (SNPs) was conducted using the neighborhood quality standard algorithm (31).

Strain and plasmid construction. Techniques for genetic manipulation of *S. aureus* were conducted according to established guidelines (32) and as described in our previous work (24, 25, 33). The University of Nebraska transposon mutant library (34) was used as a source of transposon insertions that inactivated SAUSA300_2490 (NE1393) and SAUS300_2489 (NE2336). These were transferred into plasmid-cured USA300 strain LAC, creating USA300 *farR*::ΦNE and USA300 *farE*::ΦNE, respectively (Table 1). All recombinant plasmids were first constructed as shuttle vectors in *E. coli* DH5α. The integrity of plasmids isolated from *E. coli* was confirmed by restriction enzyme digestion and nucleotide sequencing of the cloned DNA fragments prior to electroporation into *S. aureus* RN4220 as an intermediate host. From *S. aureus* RN4220, the individual plasmids were then introduced, via electroporation, into *S. aureus* USA300 or isogenic derivatives as required. Primers used for PCR amplification of gene segments that were required for plasmid construction are listed in Table S1 in the supplemental material.

Plasmid pLI50 (35) was used to complement mutations in SAUSA300_2490 (*farR*) and SAUSA300_2489 (*farE*). To complement *farE*, a 2.8-kb fragment was amplified by PCR of genomic DNA from *S. aureus* USA300 with forward and reverse primers farE_F1 and farE_R1. Similarly, a 1.2-kb product containing the native *farR* gene was amplified with primers farR_F1 and farR-R1. The PCR products were digested with KpnI and SacI and ligated into pLI50, which had been digested with the same enzymes. To construct pCN51*farR* in which expression of *farR* is dependent on the cadmium-inducible P_{cad} promoter, we first excised the *ermC* cassette from pCN51 by digestion with AvrII and XhoI and replaced it with a 1.0-kb AvrII-XhoI fragment containing the *cat194* cassette from pRN7146 (36). The resulting pCN51c plasmid was then digested with BamHI and AscI and ligated to a 605-nucleotide (nt) BamHI-AscI fragment containing the promoterless *farR* gene, which was generated by PCR with primers CNfarR_F and CNfarR_R. To construct pGY*farE*::*lux*, in which expression of the luciferase operon is driven from the *farE* promoter, a 396-bp fragment containing the intergenic segment between SAUSA300_2490 and SAUSA300_2489 (*farE*) was amplified with primers GYfarE_F and GYfarE_R and cloned into the BamHI and SalI sites of pGY*lux* (37).

A markerless in-frame deletion of *tet38* (SAUSA300_0139), encoding a major facilitator efflux pump, was constructed using pKOR-1 according to established protocols (25, 38). Briefly, sequences flanking the *tet38* locus were amplified by PCR using primers tet38-5′F and tet38-5′R to generate the upstream arm and primers tet38-3′F and tet38-3′R to generate the downstream arm. The upstream and downstream flanking arms were digested with SacII, ligated to one another, and then recombined into the temperature-sensitive pKOR-1 vector using *attB1* and *attB2* sites incorporated into the flanking sequences by the respective tet38-5′F and tet38-3′R primers. The resulting pKOR-1Δ*tet38* vector was first passaged through *S. aureus* RN4220 before being introduced into USA300 by electroporation. The correct deletion of codons 42 through 439 of the *tet38* gene was confirmed by PCR and DNA sequence analysis. The resulting USA300 Δ*tet38* strain was then used as a recipient for phage transduction, using USA300 *farE*::ΦNE as a donor (Table 1), to create USA300 Δ*tet38-farE*::ΦNE.

Assays of growth and bactericidal activity. For growth and bactericidal assays, inoculum cultures were supplemented with antibiotic where required, and these cultures were then inoculated into medium that lacked antibiotics to assess growth or bactericidal activity in the presence of antimicrobial fatty acids. For growth assays, flasks containing medium at a 1:5 ratio of medium volume to flask size and supplemented with the concentrations of fatty acid indicated in the figures or figure legends were inoculated to an initial OD_{600} of 0.01, and samples were withdrawn at hourly intervals for determination of the OD_{600}. All cultures were grown in triplicate or quadruplicate as specified in individual figure legends. For bactericidal assays, the overnight inoculum cultures were first subcultured into 25 ml of fresh TSB alone to prepare noninduced cells or in TSB containing 20 μM subinhibitory fatty acid to allow induction of intrinsic

resistance mechanisms. After growth to mid-exponential phase (OD_{600} of 0.5), these inoculum cultures were then inoculated into triplicate or quadruplicate flasks of fresh TSB (OD_{600} of 0.01; approximately 2×10^6 CFU/ml) containing a 100 μM bactericidal concentration of fatty acid. The cultures were then incubated with shaking at 37°C, and aliquots were withdrawn at hourly intervals for preparation of serial dilutions in sterile TSB. Subsequently, 10-μl aliquots from each dilution were spotted in quadruplicate onto TSB agar plates, and colonies were counted after 24 h of incubation. The mean of each quadruplicate technical replicate was entered as a single data point for each flask, from which the mean and standard deviation of the biologic replicate flasks were determined.

Assay for uptake of [^{14}C]linoleic acid. Assays for growth and uptake of [^{14}C]linoleic acid were conducted according to an established protocol (39), with modifications, to evaluate the influence of *farE* on accumulation of [^{14}C]linoleic acid in *S. aureus* cells. Briefly, quadruplicate cultures of *S. aureus* USA300 or the isogenic USA300 *farE*::ΦNE complemented with empty pLI50 vector or pLI*farE* were grown in TSB–20 μM linoleic acid to an OD_{600} of approximately 0.3 to allow induction of *farE*. The cultures were then supplemented with an additional 50 μM dose of linoleic acid and returned to the shaker. After 30 min of exposure to 50 μM linoleic acid, aliquots were withdrawn and supplemented with 0.2 μCi/ml of [^{14}C]linoleic acid. Aliquots of 200 μl were then removed at intervals of 1, 2, 5, and 10 min, and samples from each replicate were simultaneously filtered onto 0.45-μm-pore-size membrane filter discs using a vacuum manifold. The filters were then washed twice with 4 ml of 0.1 M phosphate buffer, pH 7.0, containing 1% Triton X-100 and, after a drying step, placed in scintillation vials containing 4 ml of Cytoscint scintillation cocktail (Fisher Scientific). Accumulated [^{14}C]linoleic acid was then quantified using a Beckman LS 6500 scintillation system. Data are expressed as picomoles of [^{14}C]linoleic acid accumulated per microgram of total cell lysate protein in each sample.

***farE*::*lux* reporter gene assays.** Inoculum cultures harboring pGY*farE*::*lux* or pGY*lux* control plasmid were subcultured into triplicate or quadruplicate flasks of TSB or TSB supplemented with different fatty acids to achieve an initial OD_{600} of 0.01. The cultures were incubated at 37°C with orbital shaking, and samples were withdrawn at hourly intervals for OD_{600} determinations. For quantification of luminescence, four 200-μl aliquots of each sample were added to 96-well white, opaque flat-bottom plates (Greiner Bio-one). After each well was supplemented with 20 μl of 0.1% (vol/vol) decanal in 40% ethanol, luminescence measurements were immediately taken on a BioTek Synergy H4 Hybrid Reader (BioTek, Winooski, VT) with 1 s of integration and a gain of 200. Data values were recorded as relative light units (RLU), corrected for background by subtraction of values recorded from cultures harboring the empty pGY*lux* vector. The data points were standardized for differences in growth by dividing RLU values by the recorded OD_{600} values of the cultures when samples were withdrawn.

Data analyses. Data points for growth, viability, and luciferase reporter gene assays were plotted and analyzed using Graph Pad Prism, version 6.0f. Significant differences at specific time points were determined by unpaired one-tailed Student's *t* tests.

RESULTS

Identification of single nucleotide polymorphisms in linoleic acid-resistant variants of *S. aureus*. The preferred site of *S. aureus* colonization of humans is the anterior nares, where concentrations of linoleic acid in nasal secretions can reach 40 to 50 μM (16). These values correlate with our previous work, where 50 μM linoleic acid caused a 10- to 12-h lag phase in growth of USA300, followed by unimpeded exponential growth (24). Following up on this, we observed that when stationary-phase cells from a primary culture grown in TSB–50 μM linoleic acid were reinoculated into the same medium, growth resumed without a lag phase (see Fig. S1 in the supplemental material). To determine if this was due to the selection of genetic variants with increased resistance to linoleic acid, stationary-phase cells from this second culture were plated on TSB agar for selection of single colonies. From these, we identified several that could initiate growth without a lag phase when they were inoculated into TSB–50 μM linoleic acid. Seven such fatty acid-resistant (FAR) clones were subjected to comparative genome sequencing, and two of these, designated FAR6 and FAR7, had an identical single nucleotide polymorphism (SNP): a C → T transition that alters the H_{121} codon (CAT) to Y (TAT) in a putative transcriptional regulator encoded by SAUSA300_2490 (30). FAR6 had a second SNP in a pyruvate oxidase encoded by *cidC*. Therefore, we focused on FAR7, which had just one SNP in SAUSA300_2490, and resequencing of this gene in USA300 and FAR7 confirmed the unique SNP in FAR7.

Description of the *farE-farR* locus. We hypothesize that SAUSA300_2490 and a divergently transcribed gene, SAUSA300_2489, respectively, comprise a regulator and effector gene pair that we have designated *farR* and *farE*, to denote predicted functions as a regulator and effector of fatty acid resistance. These assignments are supported by bioinformatics analyses. *farR* encodes a 182-amino-acid protein, with an N-terminal TetR family DNA binding domain (2.33e−4) and overall similarity to the AcrR cluster of orthologous groups of proteins (6.52e−9). In Gram-negative bacteria, AcrR regulators control expression of efflux pumps belonging to the AcrB family, which are often encoded by divergently transcribed genes, as with *acrR-acrABC* in *E. coli* (40) and the orthologous *mtrR-mtrCDE* arrangement in *Neisseria gonorrhoeae* (41). Similarly, *farE* is divergently transcribed from *farR* and encodes an 822-amino-acid protein that is annotated as a drug exporter of the resistance-nodulation-division (RND) superfamily (30), to which AcrB and orthologous efflux pumps are also assigned (42). Genome annotation also assigns FarE to the MMPL (mycobacterial membrane proteins, large) family of proteins, on the basis of homology to large membrane proteins of *Mycobacterium tuberculosis* that transport mycolic acids to the cell surface (43). Using protein structural modeling programs HHPRED and PHYRE2 (44, 45), FarR was predicted with greater than 99% confidence to resemble known AcrR family regulators, including PfmR and FadR of *Thermus thermophilus*, which control expression of genes involved in fatty acid synthesis and metabolism (46, 47), and MtrR, an efflux pump regulator of *Neisseria gonorrhoeae* (41, 48). Likewise, 80% of the FarE amino acid sequence was modeled with 100% confidence on the structure of AcrB from *E. coli* (49).

***farR* is required for inducible resistance to linoleic acid.** We hypothesized that *farR* should regulate expression of *farE* in response to antimicrobial fatty acids, which was addressed by constructing a *farE*::*lux* reporter, where expression of the *lux* operon is under transcriptional control of the *farE* promoter. When USA300(pGY*farE*::*lux*) was cultured in TSB, there was a modest peak of luciferase activity in early exponential growth, which quickly dissipated (Fig. 1). However, in TSB supplemented with 20 μM linoleic acid, luciferase activity was strongly induced in early exponential-phase cells and again dissipated as the cells progressed toward stationary phase. Importantly, no induction was observed in USA300 *farR*::ΦNE cells. Although USA300 *farR* appeared to exhibit superior growth to wild-type USA300 in TSB–20 μM linoleic acid (Fig. 1), our further analysis of this phenomenon uncovered that it reflects a growth penalty that is imposed on USA300 by forced expression of the *luxABCDE* genes. This was

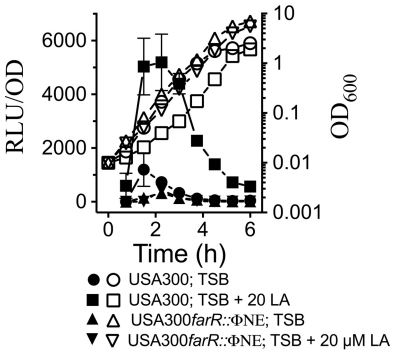

FIG 1 Linoleic acid induces expression of *farE*. Growth (OD_{600}; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300 *farR*::ΦNE, harboring the pGY*farE*::*lux* reporter vector, are charted. USA300 was grown in TSB or in TSB–20 μM linoleic acid (LA); USA300 *farR*::ΦNE was grown in TSB or in TSB–20 μM linoleic acid. Each value represents the mean and standard deviation of results of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point.

evident from a growth comparison of USA300 harboring either pGY*farE*::*lux* or empty pGY*lux* in TSB–20 μM linoleic acid, where cells carrying pGY*farE*::*lux* exhibited significantly slower growth than USA300 carrying the empty vector (see Fig. S2 in the supplemental material).

These assays suggested that USA300 should exhibit inducible resistance to the antimicrobial activity of linoleic acid. It was previously reported that exponential-phase cells of *S. aureus* were significantly more sensitive to the bactericidal activity of antimicrobial fatty acids than stationary-phase cells (17), which we confirmed in a preliminary experiment (see Fig. S3 in the supplemental material). Therefore, to assess inducible resistance, USA300 and USA300 *farR*::ΦNE were grown to mid-exponential phase in TSB (noninduced) or TSB–20 μM linoleic acid (induced) and then diluted to 10^6 CFU/ml in fresh TSB containing 100 μM linoleic acid. Noninduced USA300 suffered a >3-log loss of viability after 1 h of exposure to 100 μM linoleic acid (Fig. 2A), while the induced cells retained significantly greater viability at all time points such that there was only an approximate 40-fold loss of viability after 5 h. Furthermore, the induced USA300 *farR*::ΦNE cells exhibited a significantly greater loss of viability than induced USA300 after 2 h and onwards. Although the induced USA300 *farR*::ΦNE cells initially retained significantly greater viability than noninduced USA300 cells, they exhibited a progressive loss of viability such that after 4 h of exposure, the remaining viable cells did not significantly differ from noninduced USA300 cells.

To validate a role for *farR*, USA300 *farR*::ΦNE was complemented with empty pLI50 or pLI*farR* harboring *farR* and its native promoter to determine whether pLI*farR* could restore inducible resistance. Accordingly, when preinduced by growth in 20 μM linoleic acid, USA300 *farR*::ΦNE(pLI*farR*) retained significantly greater viability after 2 h of exposure to 100 μM linoleic acid than USA300 *farR*::ΦNE(pLI50) (Fig. 2B). Nevertheless, pLI*farR* did not appear to restore the level of inducible resistance to that of wild-type USA300, which retained approximately 10^5 CFU/ml viable cells after 5 h of exposure (Fig. 2A). We reasoned that this could be due to two variables: first, *farR* might be expressed at a high level from its native promoter on a multicopy plasmid; second, the FarR protein could engage nucleotide sequences on pLI*farR*, which contained the entire *farE-farR* intergenic segment, and these in *trans* interactions could limit the ability of FarR to regulate *farE* on the chromosome. To overcome these limitations, we expressed *farR* using the cadmium-inducible P_{cad} promoter and observed an approximate 100-fold difference in viability when USA300 *farR*::ΦNE(pCN*farR*) cells were exposed to 100 μM linoleic acid in the presence or absence of 10 μM cadmium (see Fig. S4 in the supplemental material). Cumulatively, these data support the contention that *farR* is required to manifest an inducible resistance phenotype in *S. aureus* USA300.

***farE* contributes to persistence and growth of *S. aureus* in the presence of linoleic acid.** We previously established that USA300 could grow in TSB containing 25 μM linoleic acid, whereas 50 μM linoleic acid imposed a 10- to 12-h lag phase. Our current reporter

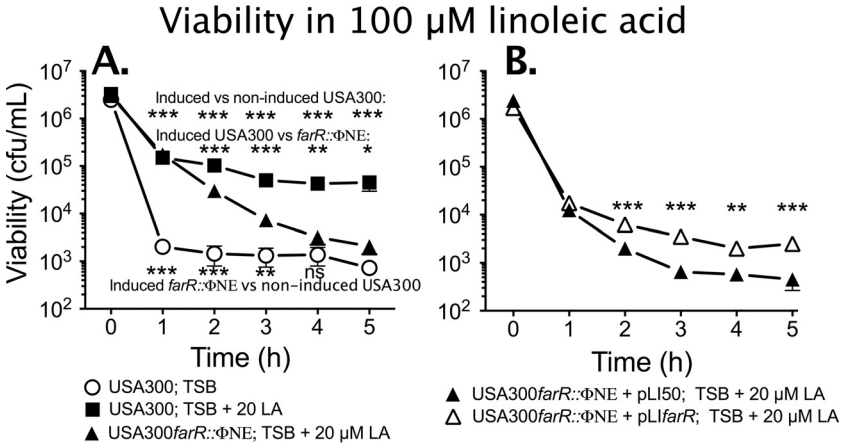

FIG 2 Sensitivity of USA300 and USA300 *farR*::ΦNE to the bactericidal activity of 100 μM linoleic acid (LA). (A) USA300 or USA300 *farR*::ΦNE challenge cells were grown to mid-exponential phase in TSB or in TSB–20 μM linoleic acid and then diluted to 10^6 CFU/ml in TSB containing 100 μM linoleic acid. Viability was monitored at hourly intervals. (B) USA300 *farR*::ΦNE was complemented with empty pLI50 vector or pLI*farR* and assayed for viability in 100 μM linoleic acid after initial growth in TSB–20 μM linoleic acid. All data points represent the means ± standard deviations of viability determinations from quadruplicate cultures. Significant differences in viability at each time point were determined by an unpaired one-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

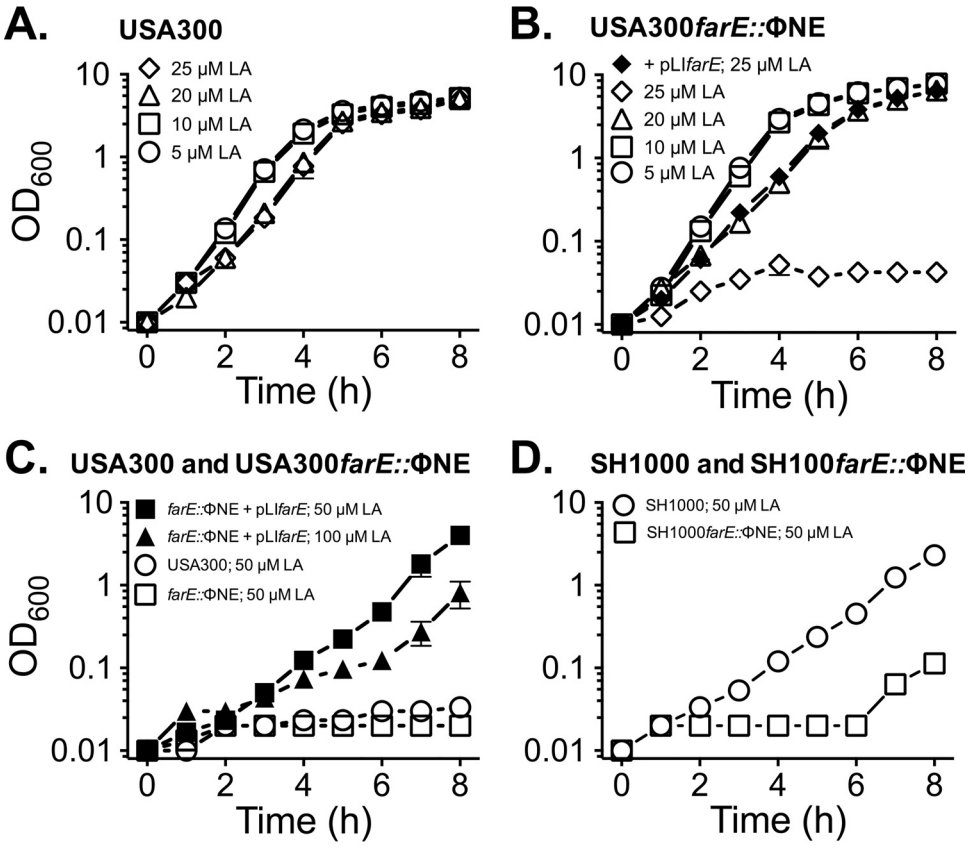

FIG 3 Mutation of *farE*::ΦNE enhances sensitivity of *S. aureus* to toxicity of linoleic acid. Growth of USA300 (A) or USA300 *farE*::ΦNE (B) in TSB supplemented with 5 μM, 10 μM, 20 μM, or 25 μM linoleic acid and that of USA300 *farE*::ΦNE(pLI*farE*) in TSB–25 μM linoleic acid were measured. (C) Growth of USA300 or USA300 *farE*::ΦNE in TSB–50 μM linoleic acid and growth of USA300 *farE*::ΦNE(pLI*farE*) in 50 μM or 100 μM linoleic acid. (D) Growth of *S. aureus* SH1000 or SH1000 *farE*::ΦNE in TSB–50 μM linoleic acid. Each data point represents the mean value of triplicate (A, C, and D) or quadruplicate (B) cultures.

gene assays also established that *farE* was induced by growth in TSB containing 20 μM linoleic acid. Therefore, we expected that *farE* would be required to support growth of *S. aureus* USA300 at an upper threshold of 25 μM linoleic acid and that induction of *farE* would confer protection against challenge of *S. aureus* with a 100 μM bactericidal concentration. To address the growth requirement, USA300 or USA300 *farE*::ΦNE was cultured in TSB containing 5, 10, 20, or 25 μM linoleic acid. USA300 was not adversely affected by 5 or 10 μM linoleic acid but exhibited slower growth in 20 or 25 μM linoleic acid (Fig. 3A). Comparatively, USA300 *farE*::ΦNE exhibited similar behavior at 5, 10, and 20 μM linoleic acid but was unable to initiate growth over 8 h of incubation in 25 μM linoleic acid (Fig. 3B). Furthermore, when USA300 *farE*::ΦNE was complemented with pLI*farE*, we observed growth restoration not only in 25 μM linoleic acid (Fig. 3B) but also in up to 100 μM linoleic acid (Fig. 3C). In contrast wild-type USA300 was unable to grow in 50 μM linoleic acid (Fig. 3C).

To ensure that the role of *farE* was not dependent on factors that are uniquely associated with the CA-MRSA strain USA300 genetic background, we transduced *farE*::ΦNE into *S. aureus* SH1000, which is a methicillin-susceptible laboratory strain that has the same multilocus sequence type (MLST) as USA300 (50). Although SH1000 exhibited somewhat greater intrinsic resistance to linoleic acid, as evident from its ability to grow in TSB–50 μM linoleic acid, SH1000 *farE*::ΦNE exhibited an extended lag phase, with no obvious growth over 6 h (Fig. 3D). Therefore, *farE* promotes growth of both MRSA and methicillin-susceptible *S. aureus* (MSSA) strains at elevated concentrations of linoleic acid.

To evaluate the role of *farE* in promoting inducible resistance, USA300 and USA300 *farE*::ΦNE were grown in TSB alone or in TSB containing 20 μM linoleic acid prior to subculture into 100 μM linoleic acid (Fig. 4). Consistent with *farE* not being appreciably expressed in noninduced cells, the noninduced USA300 and USA300 *farE*::ΦNE cultures both suffered a rapid loss of viability on exposure to 100 μM linoleic acid. However, when the cells were grown under inducing conditions prior to challenge with 100 μM linoleic acid, USA300 exhibited only a 10- to 40-fold loss of viability over 5 h and retained significantly greater viability at all time points than USA300 *farE*::ΦNE. Interestingly, the induced USA300 *farE*::ΦNE challenge cells still retained significantly greater viability than noninduced USA300, which suggests that factors in addition to *farE* may also promote inducible resistance. Cumulatively, these data confirm that *farE* contributes to the inducible resistance of *S. aureus* to the bactericidal activity of 100 μM linoleic acid and is also required to support growth in as low as 25 μM linoleic acid. It further appears that resistance is proportional to *farE* expression, as suggested by the ability of pLI*farE* to support growth of USA300 *farE*::ΦNE at concentrations of linoleic acid that could not be tolerated by USA300 (Fig. 3C).

The FAR7 clone exhibits increased expression of *farE*. FAR7

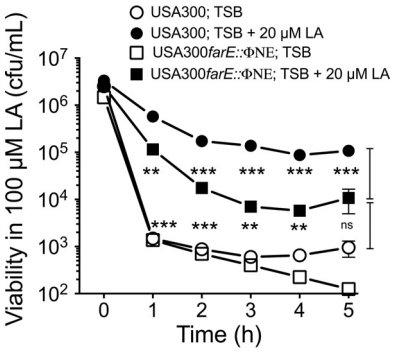

FIG 4 Sensitivity of USA300 and USA300 *farE*::ΦNE cells to the bactericidal activity of 100 μM linoleic acid. Cells of USA300 or USA300 *farE*::ΦNE were exposed to 100 μM linoleic acid after growth to mid-exponential phase in TSB or in TSB–20 μM linoleic acid. Each data point represents the mean value of quadruplicate cultures. *P* values are indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

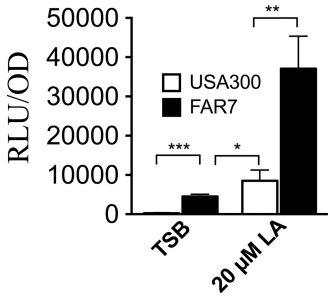

FIG 5 The FAR7 SNP causes enhanced induction of *farE* expression. The cultures were grown in TSB or TSB–20 μM linoleic acid (LA) as indicated. Data are expressed as relative luminosity units (RLU), standardized to one OD_{600} unit. Values represent the means of four replicates from each of four independent cultures. Measurements were taken from triplicate cultures when OD_{600} values reached approximately 0.5, and *P* values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

is distinguished from USA300 by an SNP in *farR* that changes H_{121} to Y in the gene product. This clone was selected for its ability to grow without a lag phase in TSB–50 μM linoleic acid, and our data suggest that this should be due to increased expression of *farE* as a consequence of the SNP in *farR*. This was confirmed by conducting *farE*::*lux* reporter gene assays in both USA300 and FAR7 (Fig. 5). When grown in TSB, FAR7 exhibited significantly greater luciferase activity than USA300, and during growth in TSB–20 μM linoleic acid, the luciferase activity in FAR7 significantly exceeded that of USA300. Therefore, the SNP that causes an H_{121}Y substitution in FarR results in a constitutive level of *farE* expression during growth in TSB and permits a significantly greater induced level of expression than could otherwise be achieved in USA300.

An H_{121}Y substitution in FarR is sufficient for increased resistance to linoleic acid. Since USA300 and FAR7 are differentiated on the basis of an SNP that causes an H_{121}Y substitution in FarR, we expected that this alone would be sufficient to promote increased resistance to linoleic acid. Accordingly, although FAR7 and USA300 exhibited no difference in growth when cultured in TSB, FAR7 was uniquely able to grow in TSB–100 μM linoleic acid (Fig. 6A). In bactericidal assays, noninduced USA300 and FAR7 suffered similar rapid losses of viability when exposed to 100 μM linoleic acid (Fig. 6B). Therefore, although there is some constitutive expression of *farE* during growth of FAR7 in TSB, this is not sufficient to promote resistance to 100 μM linoleic acid. However, when the assay was conducted with cells grown under inducing conditions, FAR7 did not exhibit any significant loss of viability over 5 h of exposure to 100 μM linoleic acid and exhibited significantly greater retention of viability from 3 to 5 h than USA300 (Fig. 6B). These observations are consistent with our *farE*::*lux* assays, where FAR7 exhibited a significantly higher induced level of *farE* expression than USA300, and support the contention that increased expression of *farE* correlates with increased resistance.

To further define the impact of the H_{121}Y substitution, USA300 *farR*::ΦNE was transformed with pLI50 harboring wild-type *farR* or the variant *farR7* allele derived from FAR7. With no complementation, USA300 *farR*::ΦNE exhibited no growth over 8 h of incubation in TSB–50 μM linoleic acid, and cells complemented with wild-type pLI*farR* were also unable to grow (Fig. 7). However, cells complemented with the variant *farR7* allele acquired the ability to grow in 50 μM linoleic acid and also, at a reduced rate, in 100 μM linoleic acid. Therefore, an SNP that introduces an H_{121}Y substitution in FarR is alone sufficient to confer increased resistance of *S. aureus* toward linoleic acid, presumably due to increased expression of *farE*.

Role of *farE* in resistance to other UFFA. Although *farE* is induced by and promotes resistance to linoleic acid, *S. aureus* would be exposed to a changing diversity and abundance of free fatty acids, dependent on the context within the human body. In a tissue abscess, pus contains high concentrations of unsaturated free fatty acids (UFFA), which could be derived from triglyceride (18, 51) or human cell membrane phospholipid, where each of the major unsaturated fatty acids, oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and arachidonic ($C_{20:4}$) acid, comprises approximately 13 to 15% of the total fatty acid content (22, 23). Conversely, although sapienic acid or its isomer palmitoleic acid ($C_{16:1}$) do not comprise a major proportion of the fatty acid profile of phospholipid, sapienic acid is the major unsaturated fatty acid in human sebum, both as free fatty acid and in sebum triglyceride (14, 52). Therefore, to better understand the biological role of *farE*, we evaluated the specificity of *farE* induction by these different fatty acids and the extent to which *farE* confers resistance to other fatty acids.

To evaluate the specificity of induction, USA300(pGY*farE*::*lux*) was grown to an OD_{600} of ~0.5 in TSB or in TSB supplemented with 20 μM fatty acid, followed by an assay of luciferase activity (Fig. 8A). There were significant differences in the abilities of different 18-carbon-chain-length fatty acids to induce *farE*::*lux* such that no induction was observed with saturated stearic acid ($C_{18:0}$) or oleic acid ($C_{18:1}$), while linoleic acid ($C_{18:2}$) was a strong inducer. Strikingly, arachidonic acid ($C_{20:4}$) promoted a significantly higher level of expression than linoleic acid, while linolenic acid ($C_{18:3}$) together with palmitoleic acid ($C_{16:1}$) and its isomer sapienic acid each facilitated an intermediate level of expression which was significantly greater than that of TSB alone but significantly less than the levels of linoleic and arachidonic acids.

Consistent with the modest induction by 20 μM palmitoleic acid, when a bactericidal assay was conducted with USA300 and USA300 *farE*::ΦNE cells that were preinduced by growth in 20 μM palmitoleic acid, there were no significant differences in retention of viability after exposure to 100 μM palmitoleic acid (Fig. 8B). However, when this assay was performed with arachidonic acid,

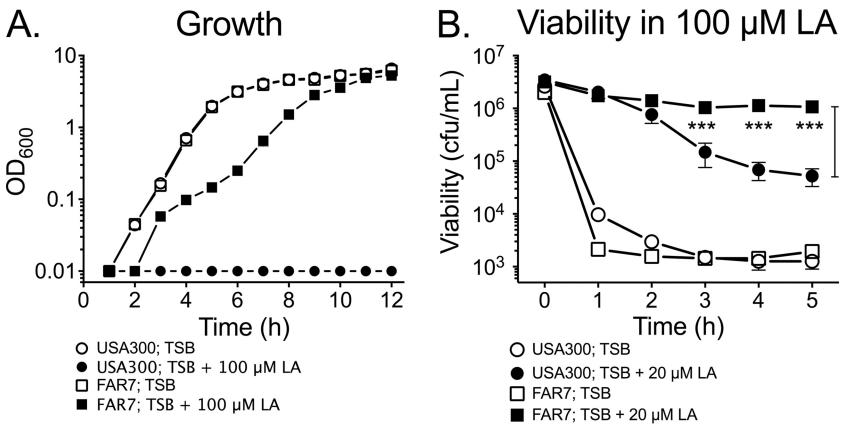

FIG 6 FAR7 is more resistant than USA300 to linoleic acid. (A) Growth analysis of USA300 and FAR7 cultured in TSB or in TSB–100 μM linoleic acid. (B) Bactericidal activity of 100 μM linoleic acid measured with USA300 or FAR7 challenge cells, prepared by growth to mid-exponential phase in TSB or in TSB–100 μM linoleic acid. Each data point represents the mean value of triplicate cultures. *P* values for comparison of induced USA300 and induced FAR7 cells are indicated by asterisks (***, $P < 0.001$).

USA300 retained significantly greater viability after 2 h of exposure than USA300 *farE*::ΦNE (Fig. 8B). Therefore, *farE* appears to have a primary role in mediating resistance to linoleic and arachidonic acids, which are the most effective inducers of *farE* expression.

Although *farE* did not promote resistance to palmitoleic acid, we nevertheless observed a significant induction of expression by 20 μM palmitoleic acid (Fig. 8A); moreover, FAR7 was able to grow in TSB containing 50 μM palmitoleic acid, whereas USA300 could not (see Fig. S5 in the supplemental material). This suggested that *farE* could still promote resistance to palmitoleic acid if it was expressed at a sufficiently high level. Furthermore, it was recently reported that *tet38*, which encodes a major facilitator superfamily efflux pump, was induced by palmitoleic acid and contributed to resistance (21). Therefore, we considered that one efflux pump might compensate for the loss of another, which could obfuscate the phenotype of USA300 *farE*::ΦNE when it was tested with palmitoleic acid. To address this, we constructed a markerless Δ*tet38* mutation in USA300, which was assayed for growth in TSB supplemented with 25 μM or 40 μM palmitoleic acid. The higher concentration imposed a lower growth rate, as evident from a time of 5 h being required for USA300 to achieve an OD_{600} of 0.5 compared to approximately 3 h in 25 μM palmitoleic acid (Fig. 9). Nevertheless, there were no discernible differences in growth between USA300 and the individual USA300 Δ*tet38* or USA300 *farE*::ΦNE mutants or the combined USA300 Δ*tet38-farE*::ΦNE double mutant. Therefore, neither *farE* nor *tet38* exerted a signif-

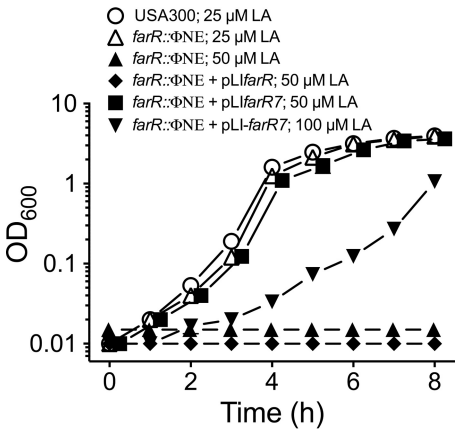

FIG 7 The variant *farR7* allele, but not wild-type *farR*, enables USA300 *farR*::ΦNE to grow at inhibitory concentrations of linoleic acid. USA300 was grown in TSB–25 μM linoleic acid, USA300 *farR*::ΦNE was grown in 25 μM or 50 μM LA, USA300 *farR*::ΦNE(pLI*farR*) was grown in 50 μM linoleic acid, and USA300 *farR*::ΦNE(pLI*farR7*) was grown in 50 μM or 100 μM linoleic acid. All data points represent the mean values of triplicate cultures.

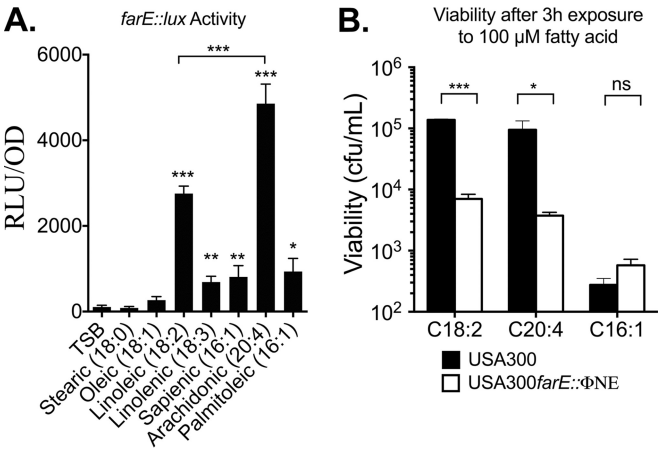

FIG 8 Influence of different antimicrobial fatty acids on induction of *farE* or viability of *S. aureus* USA300 and USA 300 *farE*::ΦNE. (A) Quantification of pGY*farE*::*lux*-dependent luciferase activity in *S. aureus* USA300 grown to an OD_{600} of 0.5 in TSB alone or in TSB supplemented with 20 μM fatty acid, as indicated. Each value represents the mean of quadruplicate measurements from each of four replicate cultures. *P* values indicate significant differences compared to growth in TSB alone or a significant difference between growth with linoleic and arachidonic acids. (B) Bactericidal activity of 100 μM linoleic acid ($C_{18:2}$), arachidonic acid ($C_{20:4}$), or palmitoleic acid ($C_{16:1}$) toward USA300 or USA300 *farE*::ΦNE cells. The inoculum cultures were grown to an OD_{600} of 0.5 in TSB supplemented with 20 μM concentrations of the respective fatty acids prior to challenge with a 100 μM bactericidal concentration. Asterisks indicate *P* values of significant differences between values for USA300 and USA300 *farE*::ΦNE. Each value represents the mean viability determination from quadruplicate cultures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant.

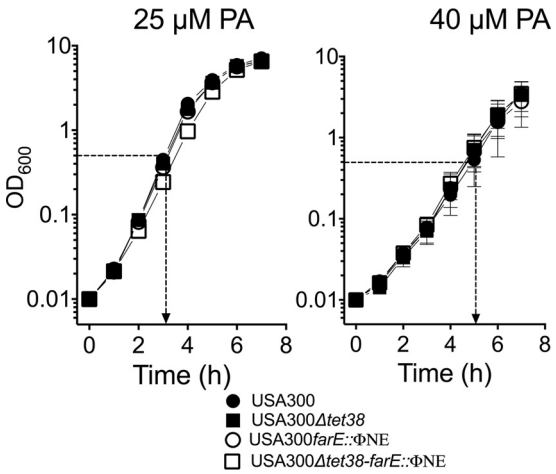

FIG 9 Effect of *farE*::ΦNE and Δ*tet38* mutations on growth of *S. aureus* in the presence of 25 μM or 40 μM palmitoleic acid (PA). USA300, USA300 *farE*::ΦNE, USA300 Δ*tet38*, and USA300 Δ*tet38-farE*::ΦNE were grown in TSB supplemented with 25 μM or 40 μM palmitoleic acid (PA), as indicated. The dotted line with the arrow depicts the time of growth at which the OD_{600} reached 0.5.

icant impact on resistance to palmitoleic acid under the conditions that we tested.

Inactivation of *farE* promotes increased uptake of [^{14}C]linoleic acid. Although many bacteria can derive energy from exogenous fatty acids through an inducible β-oxidation pathway (53), *S. aureus* lacks this ability, and its primary means of coping with exogenous fatty acids is through incorporation into phospholipid (19, 39, 54, 55). Since our data suggest that FarE promotes efflux of fatty acids, we expected that inactivation of *farE* would promote increased uptake of exogenous fatty acid. Prior to quantifying uptake of [^{14}C]linoleic acid, we first conducted a mock assay to evaluate the ability USA300 and USA300 *farE*::ΦNE to recover from exposure to an abrupt increase in the concentration of linoleic acid. Cultures were grown to an OD_{600} of 0.3 in TSB supplemented with subinhibitory 20 μM linoleic acid to allow induction of *farE* in USA300, and the cells were then challenged with a 50 μM dose of linoleic acid, followed by monitoring of the OD_{600} value. After 30 min, USA300, USA300 *farE*::ΦNE(pLI50), and USA300 *farE*::ΦNE(pLI*farE*) exhibited evidence of continued growth (Fig. 10A). However, beyond 30 min, growth of USA300 *farE*::ΦNE(pLI50) was severely impaired, whereas USA300 continued to grow, and USA300 *farE*::ΦNE(pLI*farE*) exhibited superior recovery. These data confirm that *farE* contributes to the ability of *S. aureus* USA300 to recover from an abrupt increase in the concentration of exogenous linoleic acid and that conditions of the assay were not bactericidal.

We next wished to address the question of whether FarE was responsible for actively extruding linoleic acid from the *S. aureus* cell. To do this, we performed uptake assays using [^{14}C]linoleic acid. We performed these assays on cells that were treated the same as for the growth experiments described in the legend of Fig. 10A, and cultures were supplemented with [^{14}C]linoleic acid 30 min after challenge with 50 μM linoleic acid. Strikingly, USA300 *farE*::ΦNE complemented with pLI*farE* exhibited the least accumulation of [^{14}C]linoleic acid, while USA300 *farE*::ΦNE harboring the empty pLI50 vector exhibited the greatest accumulation; wild-type USA300 exhibited intermediate accumulation (Fig. 10B). Importantly, this reflected an inverse correlation between recovery of growth after exposure to 50 μM linoleic acid and accumulation of [^{14}C]linoleic acid. Specifically, USA300 *farE*::ΦNE(pLI*farE*) exhibited the least accumulation of [^{14}C]linoleic acid, and its growth was not adversely affected; in contrast, USA300 *farE*::ΦNE(pLI50) exhibited the greatest accumulation, and its growth was severely impaired while wild-type USA300 exhibited intermediate growth and accumulation kinetics. These data support the contention that FarE-mediated efflux of unsaturated free fatty acids is required to support growth of *S. aureus* at elevated concentrations of antimicrobial fatty acid.

DISCUSSION

Through comparative genome sequencing of *S. aureus* USA300 variants that were selected for enhanced resistance to linoleic acid, we identified a regulator of fatty acid resistance, *farR*, and an effector of fatty acid resistance, *farE*, and this is, to our knowledge, the first description of a dedicated and inducible mechanism of *S. aureus* resistance to antimicrobial fatty acids. These genes bear similarity to the *acrR* and *acrB* paradigm in *E. coli*, where *acrR* and *acrB* were discovered through *in vitro* selection of acriflavine-resistant mutants, which mapped to the *acr* locus (40, 56, 57). The

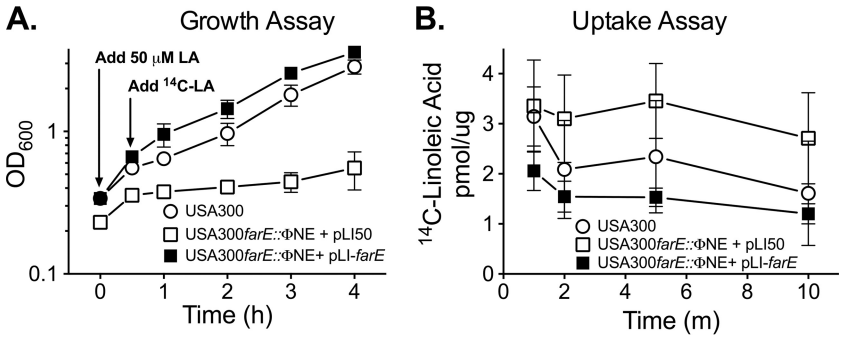

FIG 10 Growth (A) and uptake of [^{14}C]linoleic acid (B) following exposure of *S. aureus* USA300 and USA300 *farE*::ΦNE to an increase in concentration of linoleic acid. In panel A, quadruplicate cultures of USA300, USA300 *farE*::ΦNE(pLI50), or USA300 *farE*::ΦNE(pLI*farE*) were grown in TSB–20 μM linoleic acid to an OD_{600} of approximately 0.2 to 0.3. The cultures were then supplemented with an additional 50 μM dose of linoleic acid, and growth (OD_{600}) was measured after 30 min and then at hourly intervals. When this experiment was conducted for the purpose of quantifying uptake of [^{14}C]linoleic acid, the cultures were supplemented with 0.20 μCi/ml of [^{14}C]linoleic acid ([^{14}C]-LA) at the 30-min time point, and aliquots of culture were processed for quantification of [^{14}C]linoleic acid uptake at intervals of 1, 2, 5, and 10 min. Each data point represents the mean and standard deviation of values of quadruplicate samples.

emergence of antibiotic resistance in Gram-negative bacteria has also been attributed to the *in vivo* selection of mutations in the transcriptional repressor *acrR* which promote increased expression of the efflux pump encoded by *acrB* (58–60). Similarly, we discovered *farR* through *in vitro* selection of USA300 variants with increased resistance to linoleic acid. As with many proteins that possess an N-terminal TetR DNA binding domain, protein structural modeling and homology searches indicate that FarR belongs to the TetR/AcrR family of regulators, while FarE belongs to the RND family of multidrug efflux pumps, which include AcrB.

In addition to our own work, which supports a role for FarE as an efflux pump, other researchers using a different approach with *S. aureus* COL demonstrated that an amino acid substitution in FarE (SACOL2566) promotes resistance to a newly described oxadiazole family of antibiotics (61). In *E. coli*, polymorphisms that cause amino acid substitutions in AcrB can also accrue during *in vitro* selection of strains that are resistant to fluoroquinolone antibiotics (62), and in these examples, it is likely that resistance is due to amino acid substitutions that expand the substrate specificity of the efflux pump (61, 62). However, although AcrB family efflux pumps have been most extensively characterized as mediators of multidrug resistance, we contend that the primary function of FarE is to promote efflux of antimicrobial fatty acids that would be encountered during colonization or within a tissue abscess. This is consistent with the belief that members of the AcrB family, which are encoded by the core genome, evolved to promote efflux of host-derived toxic compounds, including bile salts and fatty acids (63–68).

It is especially significant that expression of *farE* was most strongly induced by linoleic and arachidonic acids. Since *S. aureus* cannot synthesize unsaturated fatty acids (69), our data suggest that *farE* is induced as part of a signaling pathway that is activated by host-specific unsaturated free fatty acids. In other work, bactericidal assays conducted with human nasal secretions established that cholesterol esters of linoleic and arachidonic acids were the principal components with bactericidal activity toward *Pseudomonas aeruginosa*, which does not colonize the nose, but that they did not affect viability of *S. aureus* (16); linoleic acid is also the principal antimicrobial fatty acid in homogenates of murine tissue abscesses (18, 51). Although arachidonic acid was not identified as a major fatty acid in abscess homogenates, it is a major unsaturated fatty acid in erythrocyte and leukocyte membrane phospholipid (23, 70), from which it is released by phospholipases at sites of infection and rapidly converted to inflammatory mediators (71). Therefore, the induction of *farE* in response to linoleic and arachidonic acids may represent an evolutionary feature that contributes to the success of *S. aureus* as a human pathogen.

Our observations are consistent with a requirement for FarE in maintaining membrane homeostasis when *S. aureus* is exposed to host-derived antimicrobial unsaturated free fatty acids. Since *S. aureus* cannot degrade exogenous fatty acids through β-oxidation, its primary means of coping with exogenous fatty acids is through incorporation into membrane phospholipid, which involves a novel fatty acid kinase pathway whereby phosphorylated fatty acid is directly incorporated into glycerol-3-phosphate (54, 55). This in itself may represent a primary means of detoxifying long-chain unsaturated free fatty acids, which promote loss of membrane integrity and cell death if allowed to accumulate in the cytoplasmic membrane (19). Importantly, *S. aureus* cannot synthesize unsaturated fatty acids and maintains membrane fluidity through synthesis of branched-chain fatty acids, primarily anteiso-C_{15} (69). From these considerations, we can envision two scenarios whereby FarE would be required under such conditions.

First, although some bacteria cease the *de novo* synthesis of fatty acids when provided with an exogenous supply of unsaturated fatty acids, this does not occur in *S. aureus*, which continues to synthesize fatty acids (72). However, under such conditions, there is reduced incorporation of endogenously synthesized anteiso-C_{15} into phospholipid, likely due to displacement or competition from the exogenous unsaturated fatty acid (72, 73). Consequently, it is likely that unutilized metabolites will accumulate, which could be dealt with through an efflux mechanism, and at least one study has proposed that the primary function of an RND family efflux pump is to promote efflux of fatty acids that are replaced as a result of membrane damage or phospholipid turnover (74). Second, although incorporation of unsaturated fatty acids into phospholipid may comprise an effective means of detoxification, it would also promote an increase in membrane fluidity which, if too severe, would compromise membrane function. In this context, we note from our analysis of uptake of [^{14}C]linoleic acid that USA300 *farE*::ΦNE cells exhibited significantly greater uptake of [^{14}C]linoleic acid than wild-type USA300 cells (Fig. 10B). Therefore, although growth of USA300 *farE*::ΦNE cells was impaired under these conditions (Fig. 10A), the cells continued to accumulate [^{14}C]linoleic acid, which suggests that there is sufficient metabolic capacity to incorporate unsaturated fatty acid into phospholipid at a level that is beyond the tolerance for proper membrane function. Consequently, FarE function could also be required under such conditions to ensure that incorporation of unsaturated fatty acid into phospholipid does not exceed a level of tolerance for membrane fluidity.

Although our data supported a role for *farE* in mediating resistance to linoleic and arachidonic acids, it did not confer resistance to palmitoleic acid, which is consistent with there being distinct mechanisms for resistance to unsaturated fatty acids of 16- and 18-carbon chain lengths. First, *S. aureus* exhibits a differential capacity to incorporate exogenous unsaturated 16- or 18-carbon fatty acids into membrane phospholipid. Oleic acid ($C_{18:1}$) is directly incorporated into phospholipid (72), but palmitoleic acid must first be extended by the *S. aureus* fatty acid biosynthesis machinery, in a rate-limiting step, to produce $C_{18:1}$, which is then incorporated into phospholipid (19). Perhaps due to the less efficient incorporation of $C_{16:1}$ fatty acids into phospholipid, *S. aureus* has evolved some capacity to exclude entry of palmitoleic and sapienic acids into the cytoplasm due to cell surface teichoic acids and the low iron-induced cell surface protein IsdA, which functions as a filtering mechanism to restrict penetration through the cell wall (19, 20). Other investigators also reported that a major facilitator superfamily efflux pump encoded by *tet38* promoted resistance to palmitoleic acid (21), and although we were not able to confirm this through construction of a USA300 Δ*tet38* deletion mutant, it may be that *tet38* functions in a strain-specific context.

It is further relevant to these considerations that expression of *tet38* was induced primarily by palmitoleic acid and much less effectively by linoleic acid, whereas we observed the opposite response for induction of *farE*. Importantly, with our identification of an SNP in *farR* that promotes increased expression of *farE*, we have provided the first mechanistic description of an efflux pump that is specifically induced in response to antimicrobial fatty acids in *S. aureus* and, at a broader level, in Gram-positive bacteria. FarR

belongs to the TetR/AcrR family of transcriptional regulators, which usually repress transcription of divergent genes by means of an N-terminal DNA binding domain that recognizes a specific operator site in the promoter segment of a target gene, and the affinity of this interaction is modulated by a C-terminal domain that binds a small inducing ligand (75, 76). In a relevant example, FadR of *Thermus thermophilus* represses expression of genes required to degrade fatty acids, which are derepressed upon binding of an acyl-coenzyme A (CoA) ligand to FadR (46). However, although *farE* is induced by antimicrobial fatty acids, we cannot yet conclude that *farR* is alone sufficient to regulate *farE*. If FarR functioned strictly as a repressor, then inactivation of *farR* should have caused derepression of *farE*. However, this was not observed, and *farR* was in fact needed for induction of *farE* (Fig. 1). Conversely, FAR7 exhibited a constitutive measure of *farE* expression, attributed to the $H_{121}Y$ substitution in FarR, which also conferred a significantly higher induced level of *farE* expression than could be achieved in wild-type USA300 (Fig. 8B).

As this substitution is not within the N-terminal DNA binding domain, which spans amino acids 28 to 61 of FarR, it should not directly affect the DNA binding function. However, in a potentially related example, FadR represses expression of genes required for β-oxidation of fatty acids, and the conformation of amino acids 106 to 119 in the C-terminal domain underwent a significant shift on binding of fatty acid, including R^{109}, which had an important role in maintaining the DNA-binding affinity even though it is not within the N-terminal DNA binding domain (77). Therefore, the $H_{121}Y$ substitution in FarR could still affect the function of the N-terminal DNA binding domain; alternatively, it may affect the ability of FarR to form functional oligomers, typically dimers or tetramers, which is another characteristic trait of the TetR family of regulators (75, 78).

Although most TetR regulators repress expression of divergently transcribed genes (75, 78), our observation that FarR is required for induction of *farE* is not unprecedented, and FarR may resemble a limited number of TetR regulators that trigger a broader cellular response to environmental insults (78–83). In one such example, the SczA metal ion-dependent transcriptional regulator of *Streptococcus pneumoniae* (82) binds to a specific operator site to repress transcription of a target gene in the absence of zinc, but when zinc is present, it binds to a different DNA segment upstream of the regulated gene to activate transcription. Alternatively, FarR may still function as repressor of *farE* in the absence of inducer, and then in the presence of exogenous fatty acid it may serve to promote expression of a positive-acting transcription factor that is needed to activate *farE*. This would partially conform to the AcrR-AcrB paradigm, where AcrR ensures that *acrB* is not expressed in the absence of an inducing stimulus, but other positive-acting factors are required to activate *acrB* (84–86). With these considerations in mind, work is in progress to determine the mechanism of FarR-dependent regulation of gene expression through analysis of its interaction with different fatty acids and target promoters and the scope of genes that are affected by this interaction.

ACKNOWLEDGMENTS

This work was supported by NSERC Discovery grants to M.J.M. and D.E.H. and by funding from the Schulich School of Medicine and Dentistry to M.J.M. J.E.T.S. is the recipient of an Ontario Graduate Scholarship award, and J.C.K. is the recipient of a Frederick Banting and Charles Best Canada Graduate Scholarship from the Canadian Institutes of Health Research.

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