

# *Staphylococcus aureus* NorD, a Putative Efflux Pump Coregulated with the Opp1 Oligopeptide Permease, Contributes Selectively to Fitness *In Vivo*

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*Staphylococcus aureus* readily infects humans, causing infections from mild superficial skin infections to lethal bacteremia and endocarditis. Transporters produced by *S. aureus* allow the pathogen to adapt to a variety of settings, including survival at sites of infection and in the presence of antibiotics. The native functions of many transporters are unknown, but their potential dual contribution to fitness and antimicrobial resistance highlights their importance in staphylococcal infections. Here, we show that *S. aureus* NorD, a newly recognized efflux pump of the major facilitator superfamily, contributes to fitness in a murine subcutaneous abscess model. In community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain MW2, *norD* was selectively upregulated 36-fold at the infection site relative to growth *in vitro*, and the *norD* mutant demonstrated significant fitness impairment in abscesses, with fitness 20- to 40-fold lower than that of the parent MW2 strain. Plasmid-encoded NorD could complement the fitness defect of the MW2 *norD* mutant. Chromosomal *norD* expression is polycistronic with the upstream oligopeptide permease genes (*opp1ABCDF*), which encode an ABC oligopeptide transporter. Both *norD* and *opp1* were upregulated in abscesses and iron-restricted culture medium and negatively regulated by Fur, but only NorD contributed to fitness in the murine abscess model.

*Staphylococcus aureus* is a versatile pathogen that causes a variety of diseases from food poisoning, skin infections, and abscesses to lethal bacteremia and endocarditis (21). Its adaptability is facilitated by a range of virulence factors and by factors that contribute to its survival and fitness in multiple environments within the host (2). Among the contributors to *S. aureus* fitness are a range of transmembrane transporters that can mediate uptake of nutrients or export of metabolites and toxic substances, including in some cases export of antimicrobial agents causing antimicrobial resistance (5, 9, 15, 16, 26, 28).

We found previously that several staphylococcal efflux pumps were selectively overexpressed within the milieu of experimental subcutaneous abscesses and that these pumps not only contributed to antimicrobial resistance when overexpressed *in vitro* but also contributed to bacterial survival within the abscess in the absence of antimicrobial agents (12). Both NorB and Tet38, members of the major facilitator superfamily (MFS) of secondary transporters encoded on the *S. aureus* chromosome, contributed selectively to fitness in the abscess environment and conferred low-level resistance to fluoroquinolones and tetracycline, respectively.

In order to identify other transporters that might also be important for bacterial survival in this environment, we evaluated the MFS transporter NorD. Like *norB* and *tet38*, *norD* expression was selectively upregulated in abscesses. Herein, we present data indicating that NorD contributes substantially to *S. aureus* fitness in an abscess infection model, and we identified a low-iron environment as a potential physiologic trigger for its upregulation *in vivo*. The selective fitness defect of a *norD* mutant is substantially greater than that reported for *norB* (12). Interestingly, expression of *norD* appears to be polycistronic with the upstream oligopeptide permease ABC family transporter *opp1ABCDF*, together

forming the *opp1-norD* operon. Although *opp1* alone appears to contribute little to *S. aureus* fitness in the abscess environment, all genes in the operon are coregulated in a low-iron medium *in vitro* and during infection. Intact *fur* (encoding the ferric uptake repressor) but not *mgrA* was necessary for *norD* induction under iron-limiting conditions.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All *S. aureus* strains, with the exception of RN4220 (*r*<sup>-</sup>) and RN6390, were derivatives of methicillin-resistant *S. aureus* (MRSA) strain MW2 (1, 2). *S. aureus* strains were cultivated in Trypticase soy broth (TSB) (Difco, Sparks, MD), and *Escherichia coli* strains were grown in Luria-Bertani broth (LB) (Difco) at 37°C, unless stated otherwise. The following antibiotics were obtained from Fisher (Sewanee, GA) or Sigma (St. Louis, MO) and used at the concentrations indicated: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin, 50 µg/ml; tetracycline, 3 µg/ml; and anhydrotetracycline, 100 µg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma) was used at 60 µg/ml in LB agar. To measure the induction of NorD in iron-restricted medium, strain MW2 was cultivated for 30 min in TSB supplemented with 400 µM dipyriddy (Sigma) with or without the addition of 50 µM ferrous sulfate (Sigma) (30).

**Construction of *norD*, *opp1*, *mgrA*, and *fur* mutants.** We constructed an in-frame deletion, omitting all but the first 6 and the last 5 codons of *norD* (MW2386) in *S. aureus* MW2 (Fig. 1). The in-frame deletion frag-

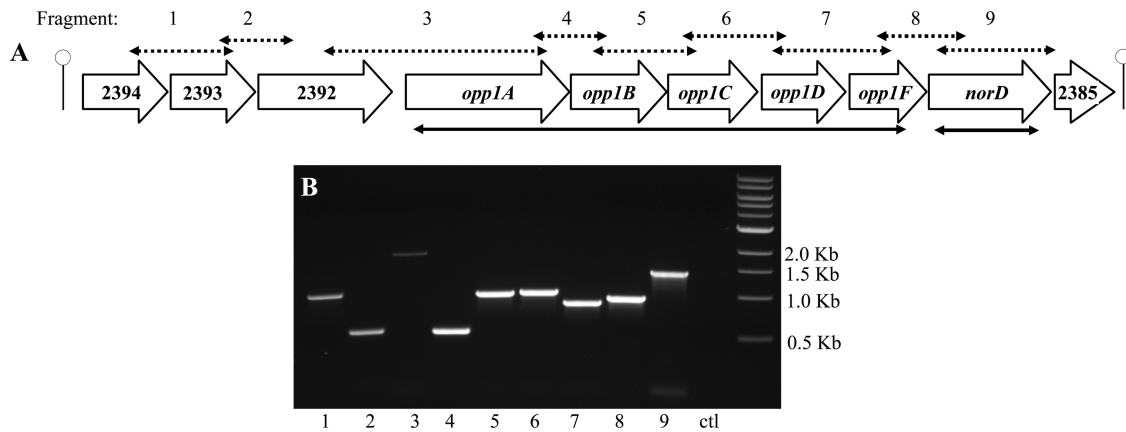
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**FIG 1** Schematic depiction of the *S. aureus* strain MW2 *opp1-norD* chromosomal operon from MW2394 to MW2385. (A) Stemmed loops indicate the putative Rho-independent terminators. The extent of deletions in the *opp1ABCDF* and *norD* genes are indicated by the solid black double-headed arrows below the schematic map. Double-headed dashed arrows indicate cotranscription of genes demonstrated by RT-PCR of cDNA synthesized using a reverse primer to MW2385. (B) PCR products of the predicted sizes arrayed by agarose gel electrophoresis. The control lane (ctl) containing the RNA sample as the PCR template demonstrates no residual DNA after DNase I digestion.

ment was generated using splicing by overlap extension PCR (11, 17, 19) with the primers *norD-UpF* with *norD-UpR* and *norD-DnF* with *norD-DnR* (Table 1). The insert was first cloned in pCR2.1, a TA cloning vector, in *E. coli* TOP10 (Invitrogen) and was PCR amplified using primers *attB1-norD-F* and *attB2-norD-R* to add *attB1* and *attB2* sites on either end of the amplicon. These two *attB* sites facilitated Gateway cloning (BP clonase II enzyme mix; Invitrogen) of the PCR products into shuttle vector pKOR1 (3) to generate p*ΔnorD*. The correct construct was confirmed by DNA sequencing, and p*ΔnorD* was electroporated into *S. aureus* RN4220, isolated, and then electroporated into *S. aureus* MW2. Allelic exchange using plasmid p*ΔnorD* was performed as previously described (3). The extent of the in-frame deletion of 386 amino acids resulted in a *ΔnorD* mutant, which retained only 11 intact codons. The MW2 *norD* mutant was confirmed by sequence analysis of its PCR product. *opp1* and *mgrA* in-frame deletions were also constructed in strain MW2 using the same strategy. Specifically, out of 1,657 codons in *opp1*, there are 32 codons retained in the MW2 *opp1* strain, and *norD* is intact in this strain. The MW2 *mgrA* deletion mutant retains 20 of 148 *mgrA* codons. The PCR primers used for constructing the deletion mutants are listed in Table 1. The MW2 *fur* mutant was constructed by bacterial phage transduction. Briefly, an *S. aureus* phage 85 lysate was prepared from Newman *fur::tet* (kindly provided by Dominique Missiakas and Chuan He). A single MW2 transductant isolated on LB plates containing 5  $\mu$ g/ml tetracycline was isolated and phenotypically characterized; the *fur* open reading frame (ORF) disruption was confirmed by PCR.

**Cloning of *norD* and construction of the *norD* complementation vector.** The entire 1,194 bp of the *norD* coding sequence was amplified from *S. aureus* MW2 chromosomal DNA using primer pairs *NdeI-PnorD-F* and *BamHI-PnorD-R*. The amplicon was cloned into *NdeI* and *BamHI* sites of shuttle vector pOS1-*P<sub>lgt</sub>* to generate p*NorD*, in which *norD* transcription is under the control of the lipoprotein diacylglycerol transferase (*lgt*) promoter (37). These plasmids were electroporated into *S. aureus* RN4220 and then into MW2 *norD* and maintained by addition of 10  $\mu$ g/ml chloramphenicol to the culture medium, unless otherwise stated.

**Mouse subcutaneous abscess model.** Swiss Webster male mice aged 4 to 6 weeks were used for the subcutaneous abscess model, as previously described (6, 13). Briefly, exponential-phase *S. aureus* cultures were prepared by diluting overnight cultures 1:100 into TSB and incubating at 37°C with rotation until the culture medium reached an optical density at 600 nm ( $OD_{600}$ ) of 0.8. Cells of a single strain or a 1:1 mixture of mutant and parental strains were washed with and diluted 1:20 in phosphate-buffered saline (PBS). The cell suspension was then mixed with an equal

volume of autoclaved Cytodex-1 beads (131 to 220  $\mu$ m; Sigma) in PBS to generate the inoculum mentioned in the legend to Fig. 5. The suspension of cells and beads (0.2 ml) was injected subcutaneously in each shaved flank of a mouse anesthetized with ketamine and xylazine. After 48 h, the abscesses were excised and homogenized, and the bacterial burden was determined by enumerating CFU on LB agar plates. For *in vitro* experiments, 10  $\mu$ l of the bacterial suspension was inoculated into 4 ml of TSB and grown for ~24 h at 37°C with rotation. For fitness competition experiments, to distinguish the two strains, we used a streptomycin-resistant derivative of *S. aureus* MW2 designated MW2<sup>SM</sup>, which grows in the presence of 100  $\mu$ g streptomycin/ml. MW2<sup>SM</sup> is a spontaneous mutant selected for its resistance to 500  $\mu$ g streptomycin/ml, and its growth rate is the same as the parental strain MW2 (not shown). Their metabolic profiles are also identical as tested by API assays (bioMérieux) (J. Lee, unpublished data). The total CFU recovered from abscesses (or culture) of the two competing bacterial strains were evaluated using the competitive index (CI), which is defined as the output/input ratio. The ratio was calculated as follows: number of CFU of the competing strain/number of CFU of the MW2<sup>SM</sup> strain. For *in vivo* comparisons, a CI near one indicates similar numbers of CFU of the two competing strains recovered from abscesses, suggesting comparable fitness of the two strains. A CI of <1 indicates the fitness advantage of the parental strain MW2<sup>SM</sup> over the competitor. The difference between paired strains was analyzed using Wilcoxon signed rank test by comparing their CIs to one, a theoretical median. For comparison of two strains injected separately, the medians of recovered CFU were analyzed using the nonparametric Wilcoxon rank sum test. Each *P* value was estimated with two tails.

**Preparation of bacterial RNA and real-time RT-PCR.** Total *S. aureus* RNA was isolated using the RNeasy minikit (Qiagen, Valencia, CA). For exponential-phase bacterial RNA, cells were collected at an  $OD_{600}$  of 0.3 to 0.5, and the cells were lysed with lysostaphin (Sigma). For stationary-phase bacteria, RNA was extracted from overnight cultures (24 h) in TSB. Bacterial RNA expressed *in vivo* was isolated from abscesses as previously described (12). All RNA samples were digested with DNase I (Turbo DNA-free; Ambion, Austin, TX) to ensure the absence of detectable DNA by PCR. Real-time reverse transcription-PCR (RT-PCR) was performed in two steps. cDNA was synthesized using the Verso cDNA kit (ABgene, Epsom, Surrey, United Kingdom) with a gene-specific reverse primer. Quantitative PCR (qPCR) amplifications were conducted using SYBR green master mix (ABgene), as previously described (12). The primers used for reverse transcription and qPCR are listed in Table 1. Gene expression was estimated by the  $\Delta\Delta CT$  method relative to the expression of the endogenous reference gene *gmk* (36). The difference of relative expres-

**TABLE 1** Sequences of primers used in this study

Use and primer	Sequence
Construction of mutants	
mgrA-UpF	5'-GTTTAAGATCGCAACAAACACA
mgrA-UpR	5'-ATAAGCGATCAGACATTAAGTTCTCCTCC
mgrA-DnF	5'-GTCTGATCGCTTATTAGGTAAAGTCATTC ATGC
mgrA-DnR	5'-CTGCATCAACATGGATTTAGA
attB1-mgrA-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT GTTTAAGATCGCAACAAACACA
attB2-mgrA-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT CTGCATCAACATGGATTTAGA
norD-UpF	5'-TGTTGGAAGAAGTCGGTCTATC
norD-UpR	5'-ACTACTCGCTCAAGCCATTGCACCTTTCAT
norD-DnF	5'-GCAATGGCTTGAGCGAGTAGTCTTTAATG AAAA
norD-DnR	5'-TAATACATGGTGGGAATTCATTCC
attB1-norD-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT TGTTGGAAGAAGTCGGTCTATC
attB2-norD-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT TAATACATGGTGGGAATTCATTCC
opp1-UpF	5'-CTGAAACGCGAAACA
opp1-UpR	5'-TCTATTTTCTCATTGCTTTTCTCTT
opp1-DnF	5'-GCAAATGAGAAAAATAGAAGAACAATT CCGACAAGC
opp1-DnR	5'-AATACCACTGTTCAAC
attB1-opp1-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGC TCTGAAACGCGAAACA
attB2-opp1-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGG TAATACCACTGTTCAAC
Real-time RT-PCR	
opp1A-RTf	5'-ACGATCCACAAAGTACTATTGC
opp1A-RTr	5'-TAAATGCGTCATCAATGCTGTT
opp1B-RTf	5'-AATCCAGCTGTGACAATTTTAC
opp1B-RTr	5'-GCATCGCTTCAAGTAACCAATTTT
opp1C-RTf	5'-ATGTCTTTTTAGGATTAGCAGC
opp1C-RTr	5'-GTCAGTACCTAGTAGATGTTGA
opp1D-RTf	5'-TGGTAGCGGTAATCAATCACT
opp1D-RTr	5'-GCGATTGAGATAATGACAACATTGA
opp1F-RTf	5'-GAAAGGTGTGTCATTGAGTGT
opp1F-RTr	5'-CCGGTTCTCAATACCTAATAT
mw2385-RTf	5'-TTTCGCAAAATTTATTCGTCGTCC
mw2385-RTr	5'-GCTCTACAAAGTTATATTGGCAGTA
mw2392-RTf	5'-TTCGAAATCTTGCCAGATAT
mw2392-RTr	5'-GCTTAAATGGTACAGCTGAAAA
mw2392-r	5'-ATGCGCCTCGTTTTGTATT
mw2393-RTf	5'-TGATATTGATCCACAAGCCG
mw2393-RTr	5'-ACCTTTTGATCCGTAATTGT
mw2394-RTf	5'-TGAAGTTCAAATGCCACAAG
mw2394-RTr	5'-ACTTGTTTAACTGGAATCAC
norD-RTf	5'-ATGAAAGGTGCAATGGCTTG
norD-RTr	5'-GCTATGGCATTGATGATCAAAA
gmk-RTf	5'-TATCAGGACCATCTGGAGTAGG
gmk-RTr	5'-CATCAACTCACCTTCACGC
Construction of <i>norD</i> complementary plasmid	
NdeI-PnorD-F	5'-TGAACATATGAAAGGTGCAATGGCTTG
BamHI-PnorD-R	5'-GTTTGATCCTCATTAAGACTACTCGCT GGGC

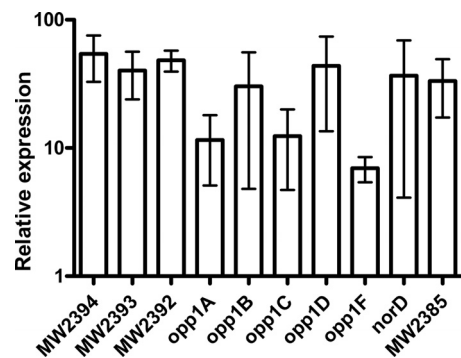
sion measured by RT-PCR was analyzed by Mann-Whitney U tests. Each *P* value was estimated with two tails.

## RESULTS

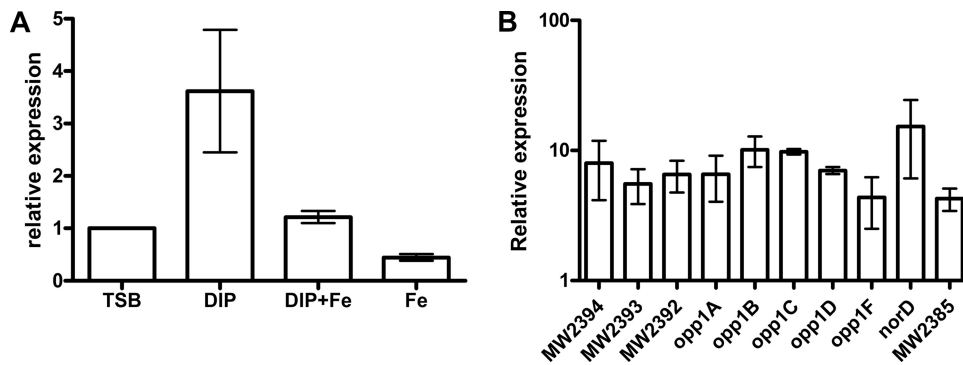
***norD*, which encodes a highly conserved efflux pump among *S. aureus* strains, is upregulated during infection.** MW2386, a chromosomal gene of *S. aureus* MW2, encodes a protein with 397 amino acids. Sequence analysis predicts it to be an integral membrane protein with an N-terminal signal sequence and 12 membrane-spanning helices; it is a member of the MFS, as are the multidrug resistance transporters (NorA, NorB, and NorC) in *S. aureus*. Thus, we designated this gene *norD*. In addition to MW2, all of the 33 other *S. aureus* strains with available genome sequences (NCBI, 1 August 2012), harbor a chromosomal copy of *norD*, and BLAST results showed that the amino acid sequence is highly conserved (identity > 96%).

The similarity of the amino acid sequence and molecular structure of NorD to other efflux pumps suggests that NorD plays a role similar to that of other efflux pumps. We previously reported that efflux pump NorB in *S. aureus* MW2 confers fitness advantages at the site of infection in the murine subcutaneous abscess model (12). Thus, we infected mice with strain MW2 and assessed the relative expression of *norD* in total bacterial RNA recovered from an abscess versus *in vitro* cultures using qRT-PCR. Compared to its expression *in vitro* at stationary phase (24 h) in TSB, *norD* was upregulated about 36-fold in abscesses (Fig. 2). The effect was even greater compared to expression in exponential phase *in vitro* (90-fold [data not shown]). This result is similar to that for *norB*, which is also selectively upregulated (171-fold) in the mouse subcutaneous abscess model (12).

***norD* is linked to the *opp1* operon, which is also selectively overexpressed in abscesses.** In strain MW2, *norD* is 11 bp downstream of *opp1F*, which encodes a subunit of the ABC type transporter Opp1, a putative oligopeptide permease (Fig. 1). Typically, five subunits constitute an Opp permease (15), including a substrate-binding protein (OppA), two permease proteins (OppB and OppC), and two ATP-binding proteins (OppD and OppF). The genome of *S. aureus* contains four *opp* loci, the constituent genes of which are expressed as an operon, including *opp1ABCDF* upstream of *norD*. Previous studies indicated that Opp1 does not transport nitrogen in *S. aureus* (5, 15), and its substrates for transport are unknown. Instead, disrupted *opp1* has been reported to



**FIG 2** Expression of the *opp1-norD* operon in *S. aureus* MW2 recovered from murine subcutaneous abscesses relative to *in vitro* gene expression. Relative expression was determined by real-time RT-PCR using specific primers for each gene to synthesize cDNA. Both the medians and the ranges of relative expression from at least two independent experiments are shown.



**FIG 3** The expression of genes in the *opp1-norD* operon is upregulated under low free iron conditions. Dipyrindyl (DIP) and  $\text{FeSO}_4$  (Fe) were added to final concentrations of 400  $\mu\text{M}$  and 50  $\mu\text{M}$ , respectively, when the  $\text{OD}_{600}$  of the *S. aureus* MW2 culture reached  $\sim 0.4$ . Cultures were incubated at 37°C for 30 min before the total bacterial RNA was extracted. (A) Expression of *norD* by strain MW2 under conditions of rich and low free iron. (B) Expression of genes in *opp1-norD* operon in MW2 under low free iron conditions. Both the medians and the ranges of relative expression from at least two independent experiments are shown.

attenuate *S. aureus* in murine infection models (9, 26). Thus, we examined whether *opp1* was also selectively upregulated in the subcutaneous abscess environment. The amount of transcripts of all *opp1* genes, by means of qRT-PCR, increased from 7- to 54-fold in abscesses compared to their expression *in vitro* (Fig. 2).

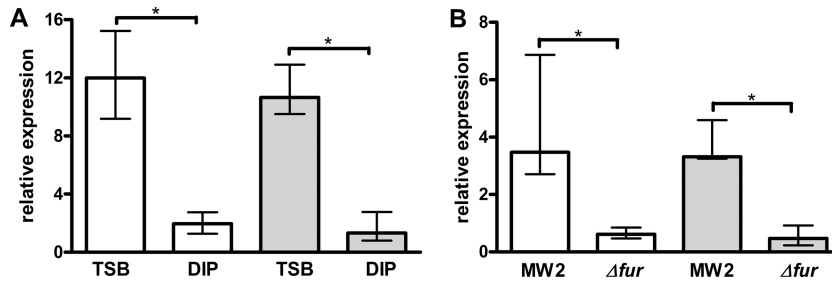
***opp1* and *norD* are regulated by environmental iron.** Upregulated expression of *norD* during infection implies that certain environmental signals that exist *in vivo* trigger changes in gene expression. A variety of such signals mimicking the *in vivo* settings, such as starvation (saline), oxidative stress ( $\text{H}_2\text{O}_2$ ), and acid stress (pH 5.5), were tested *in vitro* for effects on *norD* expression. None of these signals induced upregulation of *norD* transcription (data not shown). In contrast, a shift to an acidic environment (pH 5.5) caused a 3-fold decrease in *norD* expression (data not shown). However, free iron restriction, which occurs at many sites of infection (7, 30), upregulated *norD* expression. We compared *norD* expression in strain MW2 grown in TSB supplemented with 400  $\mu\text{M}$  dipyrindyl (a ferrous iron chelator) with and without the addition of 50  $\mu\text{M}$  ferrous sulfate. The presence of dipyrindyl alone increased *norD* expression by 3.6-fold compared to that in TSB (Fig. 3A). This induction did not occur (1.2-fold) when ferrous sulfate was added together with dipyrindyl. Furthermore, the addition of ferrous sulfate to TSB without dipyrindyl resulted in a 2.1-fold decrease in *norD* expression. All of the genes in the *opp1-norD* operon were upregulated when dipyrindyl was added to the culture medium, with increases ranging from 3.5- to 15.3-fold (Fig. 3B). These data suggest that free iron concentrations are an environmental signal for *opp1-norD* expression. Genes for other *S. aureus* efflux pumps (*norA*, *norB*, *norC*, and *tet38*) were not inducible under limiting iron conditions (data not shown).

***opp1* and *norD* are components of the same operon.** Because of genetic arrangement and coregulation of *opp1* and *norD* in abscesses and *in vitro* under iron-limiting conditions, we determined whether the *opp1ABCDEF* genes and *norD* were cotranscribed as components of an operon. Using an online tool for identifying Rho-independent terminator (<http://rna.igmors.u-psud.fr/toolbox/arnold/index.php>), we identified two putative Rho-independent terminators in the transcription orientation of *norD*; one was upstream of MW2394, and the other was downstream of MW2385 (Fig. 1), suggesting an operon extending from MW2394 to MW2385. We analyzed the transcripts of these genes in *S. au-*

*reus* MW2 grown in culture by synthesizing cDNA using a specific reverse primer to MW2385 and then PCR amplification using primer pairs spanning two adjacent ORFs (Fig. 1). All of the PCRs had products of the appropriate size, suggesting cotranscription of genes from MW2394 to MW2385, and so we named it the *opp1-norD* operon. A putative promoter element is located in the intergenic region upstream of MW2394. In addition to *opp1ABCDEF* and *norD*, there are four ORFs (MW2394-MW2392 [upstream of *opp1*] and MW2385 [downstream of *norD*]), which encode hypothetical proteins of unknown function (Fig. 1). The *opp1-norD* operon is highly conserved in other *S. aureus* strains. All data presented herein indicate coregulation of these genes under different conditions, supporting their organization in a single operon.

**Regulation of *opp1-norD* expression involves *fur* but not *mgrA*.** *Fur* is an iron-binding bacterial protein that regulates many iron-responsive genes (4, 7, 18). Because expression of *S. aureus opp1-norD* was responsive to environmental iron availability, *Fur* is a likely regulator for the operon. To test this hypothesis, we constructed a *fur* mutant of *S. aureus* MW2 and monitored the expression of *opp1-norD* under low free iron conditions (Fig. 4). In *S. aureus* MW2, dipyrindyl increased expression of *norD* (3.5-fold) and MW2394 (3.3-fold), but induction was significantly reduced in the MW2 *fur* mutant, 0.6- and 0.5-fold, respectively (both  $P = 0.0286$ , Fig. 4B). Furthermore, basal levels of *norD* and MW2394 were 12.0-fold and 10.7-fold higher, respectively, in the MW2 *fur* mutant than in the parental strain under rich free iron conditions. However, when cultivated under low free iron conditions, the difference of the basal levels was significantly reduced to 2.0- and 1.3-fold (both  $P = 0.0286$ , Fig. 4A). These results indicate that *Fur* functions either directly or indirectly as a repressor of *norD* and *opp1*.

*MgrA* is a global transcriptional regulator that negatively modulates the expression of *norB*, *norC*, and *tet38* (33, 35). Thus, we determined whether *mgrA* also affects the expression of *norD*. The levels of expression of *norD* did not differ, however, between *S. aureus* MW2 *mgrA* mutant and MW2. The expression profiles of the genes in *opp1* and *norD* mutants (see below) also revealed no autofeedback regulation of the *opp1-norD* operon (MW2392 [1.8-fold] and *norD* [1.9-fold] in MW2 *opp1* mutant and *opp1F* [1.1-fold] in MW2 *norD* mutant relative to that in MW2, respectively).

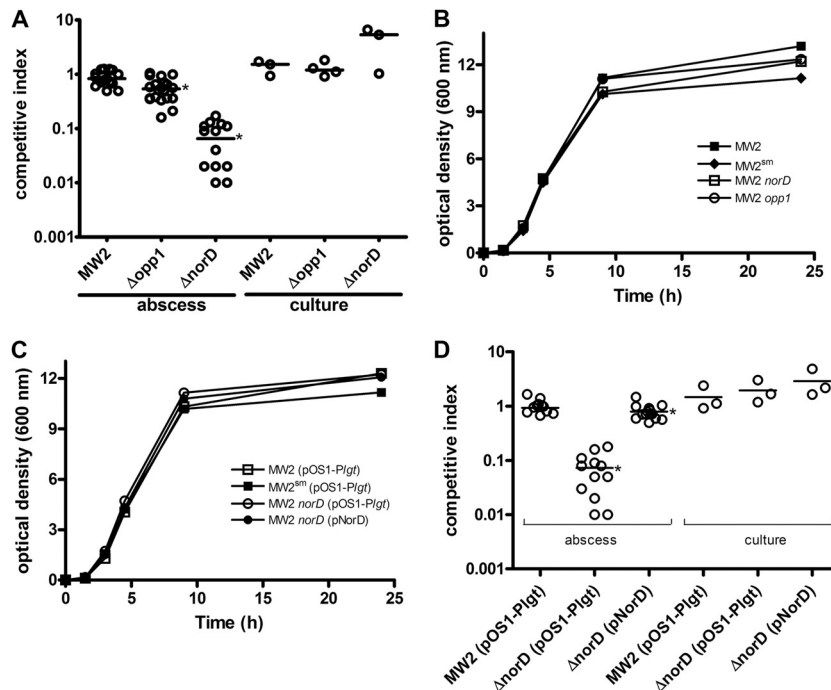


**FIG 4** Relative expression of *norD* and MW2394 in *S. aureus* MW2 and an MW2 *fur* mutant under low free iron conditions. (A) Expression of *norD* and MW2394 in the MW2 *fur* mutant relative to that in MW2. (B) Expression of *norD* and MW2394 in MW2 and in the MW2 *fur* mutant under low free iron conditions relative to that under rich free iron conditions. TSB culture was supplemented with 400  $\mu$ M dipyrindyl (DIP) when the culture OD<sub>600</sub> reached about 0.4 and incubated for 30 min. Median values that were significantly different are indicated by a bar and asterisk. Both the medians and the ranges of relative expression from four independent experiments are shown.

***norD* mutants, but not *opp1* mutants, exhibit a fitness defect in subcutaneous abscesses.** Selective increases in expression of transporters *norD* and *opp1* in subcutaneous abscesses suggested that their functions may be important for bacterial fitness in the abscess milieu. To address this possibility, we constructed individual in-frame deletion mutants of *norD* and *opp1* in *S. aureus* MW2 and evaluated their fitness relative to that of MW2 using competition assays in the subcutaneous abscess infection model. In order to score the survival of mutant versus parental strains, we used as a comparator a streptomycin-resistant variant of MW2 (MW2<sup>SM</sup>), which dem-

onstrated no fitness defect in competition assays with MW2 when the two strains were injected in roughly equal numbers in mouse flanks and harvested at 48 h (CI = 0.83) or grown *in vitro* up to 24 h (CI = 1.52) (Fig. 5A). Thus, MW2<sup>SM</sup> is comparable to MW2 in its growth *in vivo* and *in vitro*.

In direct competition experiments with *S. aureus* MW2<sup>SM</sup> and MW2 *norD*, the *norD* mutant exhibited a substantial fitness defect with a median CI of 0.065 ( $P < 0.0001$ ) at 48 h (Fig. 5A). Moreover, in 5 out of 14 abscesses, MW2 *norD* colonies were not detected among 96 colonies patched on LB plates containing 100



**FIG 5** The *S. aureus* MW2 *norD* mutant exhibits a fitness defect in the murine subcutaneous abscesses. (A) Fitness competition between *S. aureus* MW2 and MW2 *opp1*, MW2 *norD*, and MW2<sup>SM</sup> mutants in the murine subcutaneous abscess model. Exponential-phase bacteria, together with Cytodex beads, were injected subcutaneously in both flanks of each mouse at a typical input ratio for each bacterial strain of 1:1. The inoculum was  $1 \times 10^6$  to  $5 \times 10^6$  CFU in 0.2 ml for each lesion. After 48 h, the bacterial load of each abscess was estimated by quantitative plate cultures. For *in vitro* TSB cultures, the numbers of bacteria were determined after 24 h. Competitor strains were distinguished by resistance to streptomycin in strain MW2<sup>SM</sup>. A competitive index (CI) was calculated as the output ratio of two strains divided by the input ratio. Each circle represents the CI for one abscess, and the horizontal bars represent medians for the group. (B and C) Growth curves of strains cultivated in TSB broth at 37°C. Representative results of repeated experiments are shown. (D) The fitness defect of the MW2 *norD* mutant in murine subcutaneous abscesses was complemented by plasmid-encoded NorD. All strains competed with MW2<sup>SM</sup>(pOS1-Plgt) as described above. The data shown in panels A and D are the results compiled from three independent experiments. An asterisk indicates that the median CI is significantly different from one.

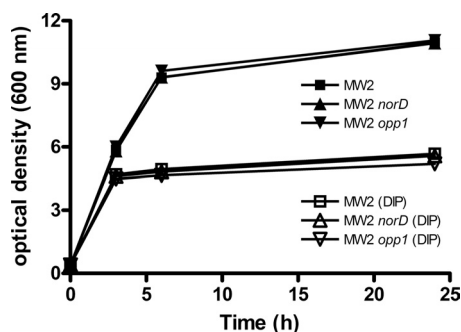


FIG 6 Growth of *S. aureus* MW2 and *opp1* and *norD* mutants in TSB broth at 37°C after the addition of 0.4 mM DIP when the OD<sub>600</sub> reached 0.4. Representative results of repeated experiments are shown.

μg/ml streptomycin. In contrast, the MW2 *norD* mutant exhibited a small growth advantage over MW2<sup>SM</sup> *in vitro* (CI = 5.3). We also performed a set of single-strain assays to investigate the fitness difference between MW2 and MW2 *norD*, in which each of two strains with similar inocula was singly injected on opposite flanks of mice. At 48 h after injection, the median numbers of viable bacteria recovered from each abscess were  $7.3 \times 10^7$  CFU for MW2 and  $0.3 \times 10^7$  CFU for the MW2 *norD* mutant, a 24-fold difference ( $P < 0.0001$ ). In contrast, the numbers of CFU from *in vitro* cultures (24 h) were similar for the two strains ( $1.5 \times 10^{10}$  CFU ml<sup>-1</sup> for MW2 and  $1.8 \times 10^{10}$  CFU ml<sup>-1</sup> for the MW2 *norD* mutant). The growth curves of these strains *in vitro* were similar (Fig. 5B), indicating that the fitness defect of the *norD* mutant is specific for the abscess environment. Thus, the *norD* mutant exhibits a selective *in vivo* growth defect in both competition and single-strain assays.

To complement the fitness defect of *S. aureus* MW2 *norD* mutant *in vivo*, we constructed pNorD carrying the entire *norD* ORF from strain MW2 cloned into the NdeI and BamHI sites of pOS1-Plgt (27, 37). *norD* was strongly expressed from pNorD as determined by qRT-PCR, and pNorD was stable in MW2 and its derivatives when grown in TSB for up to 72 h in the absence of chloramphenicol and during infection for up to 48 h (data not shown). The *in vitro* growth rate of MW2(pNorD) was similar to that of the wild-type strain for up to 24 h (Fig. 5C). In competition assays (Fig. 5D), MW2(pNorD) showed only a modest decrease in fitness compared to MW2 (CI of 0.73;  $P = 0.02$ ). As noted above, the CI of the MW2 *norD* mutant was 0.065, and thus, the *in vivo* fitness defect of the *norD* mutant was substantially complemented by pNorD. As noted above, there is no autofeedback regulation related to *norD* as suggested by the comparable transcript level of *opp1F* (1.1-fold) in MW2(pNorD) relative to that in MW2.

In contrast to the striking fitness defect of the *norD* mutant *in vivo* (CI of 0.065), an in-frame *opp1* deletion mutant exhibited only a slight defect (CI of 0.53;  $P = 0.0002$ ) (Fig. 5A). Compared to strain MW2, neither the *opp1* nor the *norD* mutant showed any difference in their growth *in vitro* in rich or low free iron medium (Fig. 6).

## DISCUSSION

In this study, we have identified and characterized the *opp1-norD* operon of *S. aureus* based on its selective overexpression in a murine subcutaneous abscess model. The operon spans from MW2394 to MW2385, and it includes an *opp1* locus and

*norD*, a newly recognized gene encoding an efflux pump. *Opp1* is a member of the ABC transporter family that is energized by ATP. NorD is a member of the MFS transporter family that is energized by hydrogen and other ion gradients across the bacterial cytoplasmic membrane. In strain RN6390, the transcription of these genes was confirmed to be linked, extending from upstream SAOUHSC\_02770 to SAOUHSC\_02762 and corresponding to MW2394 to *norD* in strain MW2 (15). We have further characterized the arrangement of these two transporters and determined their relative contributions to fitness in a mouse infection model.

NorD is related to other MFS efflux transporters, such as NorA, NorB, NorC, and Tet38. Like *norD*, *norB* and *tet38* are also selectively upregulated in the abscess environment, and *norB* and *tet38* mutants showed selective fitness defects in murine abscesses (12). Overexpression of *norB* confers increased resistance to quinolones, such as ciprofloxacin and moxifloxacin, and overexpression of *tet38* confers increased resistance to tetracycline (33). In contrast, overexpression of *norD* in *S. aureus* MW2 produced no detectable change in susceptibility to quinolones (ciprofloxacin, norfloxacin, moxifloxacin, delafloxacin, and levofloxacin), tetracyclines, polymyxin B, daptomycin, nalidixic acid, trimethoprim, ethidium bromide, or triclosan (data not shown). Thus, the substrates of NorD are not known, and it is likely that the natural substrates of NorB and Tet38 that contribute to their ability to confer fitness in an abscess environment are also not directly related to their antibiotic resistance properties but reflect instead the broad substrate profiles of MFS transporters. MFS and other efflux transporters may function to remove toxic metabolites from the cytoplasm or environmental toxins from the bacterial cytoplasm or cell membrane; for example, AcrAB-TolC of *E. coli* is induced by and provides resistance to bile salts, likely contributing to the ability of *E. coli* to colonize the intestinal tract (29). It is, thus, plausible that NorD removes an as yet unidentified toxin present in abscesses, thereby enhancing *S. aureus* survival in the neutrophil-rich environment of the abscess.

Four polycistronic *opp* operons (including *opp1*) are present in the *S. aureus* genome, along with one monocistronic *opp5A* gene. Although these operons are annotated as oligopeptide permeases, only *opp3* has been shown to have a role in the uptake of oligopeptides. Furthermore, *opp3* alone appears to be sufficient for this function (15). *opp2* and *opp5A* function in nickel transport and are attenuated in a staphylococcal urinary tract infection model (16). None of the *opp* operons other than *opp1* has a downstream coregulated transporter gene like *norD*. Thus, the function of *opp1* remains unknown. *opp1*, although coregulated with *norD*, appears not to be a major contributor to fitness in the abscess model, unlike *norD*. A previous study demonstrated that *opp1* transposon mutants were attenuated in abscess, wound, and systemic infection models (9). Given our findings that an *opp1* in-frame deletion mutant exhibited little attenuation in an abscess infection model, it is likely that *opp1* attenuation seen in the prior study reflected the polar effects of the *opp1* transposon insertion on *norD* expression. The lack of a fitness defect in the *opp1* mutant could reflect redundancy of function, consistent with multiple *opp* operons present in the staphylococcal genome.

A condition known to be present in the abscess environment that triggered the increased expression of *opp1* and *norD* *in vitro* was low free iron. Other conditions found in abscesses, such as oxidative stress and low pH, did not affect *in vitro* expression of

*opp1* or *norD*, but low pH has been shown to increase the expression of *norB* (31). Because of their upregulation in response to iron, *opp1* and *norD* could be directly involved in iron acquisition or metabolism. However, since neither mutant exhibited a growth defect *in vitro* under iron-limiting conditions, it is more likely that low iron may be an *in vivo* signal for *S. aureus*, allowing increased gene expression.

Bacteria sense iron-restricted environments in part through the Fur protein (4, 7, 14, 18), which binds ferrous ions, dimerizes, and binds to Fur box DNA sequences (18, 25, 30, 38) upstream of iron-regulated genes. In *S. aureus*, it is estimated that the Fur regulon comprises ~20 operons involving ~50 genes. A recent study revealed that the *S. aureus* efflux pump NorA is a member of the Fur regulon and may function to export a siderophore for acquisition of environmental ferric ions (10). Fur positively modulates *norA* expression by interacting directly with a Fur box in the *norA* promoter region. By regulating *norA* expression, Fur thus contributes to quinolone resistance in *S. aureus*. In the MW2 *fur* mutant, *opp1-norD* gene upregulation diminished under iron-restricted conditions, suggesting that Fur regulates expression of the *opp1-norD* genes. Our data and a previous study suggest that the promoter of the *opp1-norD* operon is located in the intergenic region upstream of MW2395 (15). However, we could not identify the Fur binding site in the region by gel shift assays with purified Fur protein (data not shown), suggesting the possibility of indirect regulation of *opp1-norD* by a separate Fur-regulated gene product.

MgrA regulates expression of genes encoding other Nor efflux pumps and virulence factors (8, 20, 22–24, 32–34), but it does not affect regulation of the *opp1-norD* operon. Our data show that *opp1-norD* expression was comparable in *S. aureus* MW2 and MW2 *mgrA* mutant in both rich medium and under low free iron conditions (data not shown), suggesting that regulation of *opp1-norD* is independent of MgrA. In preliminary studies, an *mgrA* mutant showed only a 2-fold defect in fitness *in vivo*, confirming that it does not regulate *opp1-norD* *in vivo*.

In summary, we have identified in *S. aureus* MW2 a putative efflux pump designated NorD. Its expression is polycistronic with upstream *opp1ABCDF*, thus forming the *opp1-norD* operon. The operon is ubiquitous among staphylococci, and the genes in the operon are coregulated. *opp1-norD* expression is selectively increased within subcutaneous abscesses relative to growth *in vitro*. *fur*-dependent upregulation of *opp1* and *norD* was observed in *S. aureus* MW2 grown in a low-iron environment *in vitro*, a condition that is characteristic of infection sites within mammalian hosts. The efflux pump NorD, but not *Opp1*, significantly contributed to staphylococcal fitness in subcutaneous abscesses, a common form of *S. aureus* infection.

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## REFERENCES

1. Anonimus. 1999. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999. *JAMA* 282:1123–1125.
2. Baba T, et al. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 359:1819–1827.
3. Bae T, Schneewind O. 2006. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 55:58–63.
4. Baichoo N, Helmann JD. 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. *J. Bacteriol.* 184:5826–5832.
5. Borezee-Durant E, Hiron A, Piard JC, Juillard V. 2009. Dual role of the oligopeptide permease Opp3 during growth of *Staphylococcus aureus* in milk. *Appl. Environ. Microbiol.* 75:3355–3357.
6. Bunce C, Wheeler L, Reed G, Musser J, Barg N. 1992. Murine model of cutaneous infection with Gram-positive cocci. *Infect. Immun.* 60:2636–2640.
7. Carpenter BM, Whitmire JM, Merrell DS. 2009. This is not your mother's repressor: the complex role of Fur in pathogenesis. *Infect. Immun.* 77:2590–2601.
8. Chen PR, et al. 2006. An oxidation-sensing mechanism is used by the global regulator MgrA in *Staphylococcus aureus*. *Nat. Chem. Biol.* 2:591–595.
9. Coulter SN, et al. 1998. *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Mol. Microbiol.* 30:393–404.
10. Deng X, et al. 2012. Expression of multidrug resistance efflux pump gene *norA* is iron responsive in *Staphylococcus aureus*. *J. Bacteriol.* 194:1753–1762.
11. Ding Y, Davis BM, Waldor MK. 2004. Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. *Mol. Microbiol.* 53:345–354.
12. Ding Y, Onodera Y, Lee JC, Hooper DC. 2008. NorB, an efflux pump in *Staphylococcus aureus* MW2, contributes to bacterial fitness in abscesses. *J. Bacteriol.* 190:7123–7129.
13. Ford CW, Hamel JC, Stapert D, Yancey RJ. 1989. Establishment of an experimental model of a *Staphylococcus aureus* abscess in mice by use of dextran and gelatin microcarriers. *J. Med. Microbiol.* 28:259–266.
14. Hantke K. 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol. Gen. Genet.* 182:288–292.
15. Hiron A, Borezee-Durant E, Piard JC, Juillard V. 2007. Only one of four oligopeptide transport systems mediates nitrogen nutrition in *Staphylococcus aureus*. *J. Bacteriol.* 189:5119–5129.
16. Hiron A, et al. 2010. A nickel ABC-transporter of *Staphylococcus aureus* is involved in urinary tract infection. *Mol. Microbiol.* 77:1246–1260.
17. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
18. Horsburgh MJ, Ingham E, Foster SJ. 2001. In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.* 183:468–475.
19. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77:61–68.
20. Kaatz GW, Thyagarajan RV, Seo SM. 2005. Effect of promoter region mutations and *mgrA* overexpression on transcription of *norA*, which encodes a *Staphylococcus aureus* multidrug efflux transporter. *Antimicrob. Agents Chemother.* 49:161–169.
21. Lowy FD. 1998. *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339:520–532.
22. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY. 2006. Transcription profiling of the *mgrA* regulon in *Staphylococcus aureus*. *J. Bacteriol.* 188:1899–1910.
23. Luong TT, Lee CY. 2006. The *arl* locus positively regulates *Staphylococcus aureus* type 5 capsule via an *mgrA*-dependent pathway. *Microbiology* 152:3123–3131.
24. Manna AC, Cheung AL. 2006. Expression of SarX, a negative regulator of *agr* and exoprotein synthesis, is activated by MgrA in *Staphylococcus aureus*. *J. Bacteriol.* 188:4288–4299.
25. Mazmanian SK, Ton-That H, Su K, Schneewind O. 2002. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 99:2293–2298.
26. Mei JM, Nourbakhsh F, Ford CW, Holden DW. 1997. Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol. Microbiol.* 26:399–407.
27. Schneewind O, Mihaylova-Petkov D, Model P. 1993. Cell wall sorting

- signals in surface proteins of Gram-positive bacteria. *EMBO J.* 12:4803–4811.
28. Speziali CD, Dale SE, Henderson JA, Vines ED, Heinrichs DE. 2006. Requirement of *Staphylococcus aureus* ATP-binding cassette-ATPase FhuC for iron-restricted growth and evidence that it functions with more than one iron transporter. *J. Bacteriol.* 188:2048–2055.
  29. Thanassi DG, Cheng LW, Nikaido H. 1997. Active efflux of bile salts by *Escherichia coli*. *J. Bacteriol.* 179:2512–2518.
  30. Torres VJ, et al. 2010. *Staphylococcus aureus fur* regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. *Infect. Immun.* 78:1618–1628.
  31. Truong-Bolduc QC, et al. 2011. Implication of the NorB efflux pump in the adaptation of *Staphylococcus aureus* to growth at acid pH and in resistance to moxifloxacin. *Antimicrob. Agents Chemother.* 55:3214–3219.
  32. Truong-Bolduc QC, Ding Y, Hooper DC. 2008. Posttranslational modification influences the effects of MgrA on *norA* expression in *Staphylococcus aureus*. *J. Bacteriol.* 190:7375–7381.
  33. Truong-Bolduc QC, Dunman PM, Strahilevitz J, Projan SJ, Hooper DC. 2005. MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. *J. Bacteriol.* 187:2395–2405.
  34. Truong-Bolduc QC, Hooper DC. 2007. Transcriptional regulators NorG and MgrA modulate resistance to both quinolones and  $\beta$ -lactams in *Staphylococcus aureus*. *J. Bacteriol.* 189:2996–3005.
  35. Truong-Bolduc QC, Strahilevitz J, Hooper DC. 2006. NorC, a new efflux pump regulated by MgrA of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 50:1104–1107.
  36. Vandecasteele SJ, Peetermans WE, Merckx R, Van Eldere J. 2001. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. *J. Bacteriol.* 183:7094–7101.
  37. Wardenburg JB, Williams WA, Missiakas D. 2006. Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 103:13831–13836.
  38. Xiong AM, Singh VK, Cabrera G, Jayaswal RK. 2000. Molecular characterization of the ferric-uptake regulator, Fur, from *Staphylococcus aureus*. *Microbiology* 146:659–668.