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MECHANISMS OF RESISTANCE

Daptomycin Resistance and Tolerance Due to Loss of Function in *Staphylococcus aureus dsp1* and *asp23*

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ABSTRACT Lipopeptide daptomycin is a last-line cell-membrane-targeting antibiotic to treat multidrug-resistant *Staphylococcus aureus*. Alarmingly, daptomycin-resistant *S. aureus* isolates have emerged. The mechanisms underlying daptomycin resistance are diverse and share similarities with resistances to cationic antimicrobial peptides and other lipopeptides, but they remain to be fully elucidated. We selected mutants with increased resistance to daptomycin from a library of transposon insertions in sequent type 8 (ST8) *S. aureus* HG003. Insertions conferring increased daptomycin resistance were localized to two genes, one coding for a hypothetical lipoprotein (SAOUHSC_00362, Dsp1), and the other for an alkaline shock protein (SAOUHSC_02441, Asp23). Markerless loss-of-function mutants were then generated for comparison. All transposon mutants and knockout strains exhibited increased daptomycin resistance compared to those of wild-type and complemented strains. Null and transposon insertion mutants also exhibited increased resistance to cationic antimicrobial peptides. Interestingly, the Δ*dsp1* mutant also showed increased resistance to vancomycin, a cell-wall-targeting drug with a different mode of action. Null mutations in both *dsp1* and *asp23* resulted in increased tolerance as reflected by reduced killing to both daptomycin and vancomycin, as well as an increased tolerance to surfactant (Triton X-100). Neither mutant exhibited increased resistance to lysostaphin, a cell-wall-targeting endopeptidase. These findings identified two genes core to the *S. aureus* species that make previously uncharacterized contributions to antimicrobial resistance and tolerance in *S. aureus*.

KEYWORDS *Staphylococcus aureus*, antibiotic resistance, daptomycin, vancomycin

Staphylococcus aureus is a leading cause of life-threatening infections, both in the hospital and in the community (1, 2). Compounding the problem, effective treatment has become extremely challenging with the emergence of multidrug-resistant lineages, in particular, methicillin-resistant *S. aureus* (MRSA) (1). Two last-line antibiotics remain generally active against MRSA infections: cell-wall-targeting vancomycin (VAN; glycopeptide family) and daptomycin (DAP; lipopeptide family) (3). Acquired high-level resistance to vancomycin has been described in clinical isolates of *S. aureus*, but so far, those remain sporadic and rare occurrences. Nevertheless, there is a disconcerting erosion in the efficacy of vancomycin for the treatment of *S. aureus* associated with MIC creep in MRSA strains (1, 4). In contrast, the cyclic lipopeptide daptomycin shows efficient bactericidal activity against MRSA (5, 6), but the emergence of *S. aureus* DAP-resistant mutant strains (DAP-R) during the course of protracted treatment is a common finding (7, 8).

Daptomycin shares structural similarities with cationic antimicrobial peptides (CAMPs) and a similar mode of action involving membrane disruption (9). Daptomycin

Citation Barros EM, Martin MJ, Selleck EM, Lebreton F, Sampaio JLM, Gilmore MS. 2019. Daptomycin resistance and tolerance due to loss of function in *Staphylococcus aureus dsp1* and *asp23*. Antimicrob Agents Chemother 63:e01542-18. https://doi.org/10.1128/AAC.01542-18.

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Received 23 July 2018
Returned for modification 21 August 2018
Accepted 23 October 2018
Accepted manuscript posted online 5 November 2018
Published 21 December 2018

is thought to insert in bacterial membranes of Gram-positive bacteria, in a phosphatidylglycerol-dependent fashion, and induce the formation of pores (10). The formation of such pores, which have high permeability to Na^+, K^+, and alkali metal ions, leads to the disruption of proton motive force, alterations in cell envelope homeostasis, and ultimately cell death (7). The emergence of resistant isolates in the clinic is generally due to spontaneous mutations in genes and pathways involved in bacterial cell membrane (CM) and cell wall biosynthesis and modification (11). The most common resistance mechanism involves the alteration of the cell surface charge leading to the repulsion of the anionic daptomycin molecules (12). This primarily occurs via gain-of-function nonsynonymous mutations in the phosphatidylglycerol lysyltransferase MprF, producing positively charged lysyl-phosphatidylglycerol, a major component of the bacterial membrane (13, 14). Similarly, overexpression of the *dlt* operon, encoding proteins required for the incorporation of D-alanine in teichoic acids and resulting in a net increase in positive charge, has been described for daptomycin-resistant *S. aureus* (15–17). A second major resistance mechanism involves changes in the membrane composition via the alteration of phospholipid membrane metabolism. As such, mutations in phosphatidyltransferase PgsA and in cardiolipin synthase Cls2 provoke a decrease in the amount of phosphatidylglycerol in the membrane, impacting fluidity and ultimately reducing daptomycin binding and efficacy (18, 19). A third known resistance mechanism involves alteration of complex transcriptional regulatory networks governing the cell envelope stress response and membrane homeostasis (20). Decreased or increased transcription of the *walKR* or *vraSR* two-component regulatory system, respectively, has been linked to increased daptomycin resistance in *S. aureus* (12, 21–23). Additionally, polymorphisms in RNA polymerase subunits RpoB and RpoC (19, 24) have been associated with a daptomycin resistance phenotype, but those seem to indirectly affect the expression of the *dlt* operon or other cell wall biosynthesis pathways, thus explaining the characteristic increased cell wall thickness (25, 26).

To understand better the cell wall and physiologic dynamic in *S. aureus* that defines susceptibility and resistance to daptomycin, we screened a saturating bank of transposon insertions in *S. aureus* HG003 for increased resistance likely resulting from a loss-of-function mutation. We isolated, identified, validated, and recapitulated the loss-of-function mutations in two genes that, when mutated, resulted in increased daptomycin resistance. One codes for a hypothetical lipoprotein (SAOUHSC_00362) and the second codes for an alkaline shock protein (SAOUHSC_02441, Asp23). These genes were further characterized for their roles in the resistance and tolerance of *S. aureus* to daptomycin and other antimicrobials.

RESULTS

Identification of *S. aureus* transposon mutants with increased MIC to daptomycin. We first determined the MIC of daptomycin for the *S. aureus* HG003 transposon mutant library pool (which we described previously to consist of 71,700 unique insertions) (27) and for a control subpool composed of 10 independent transposon mutants selected randomly. The growth of the mutants in the control pool, consisting of insertions not expected to be involved in daptomycin resistance, was inhibited by 0.25 μg/ml of daptomycin (Fig. 1). To determine whether mutants were present in the complete pool that exhibited the increased daptomycin resistance, it was grown in the presence of 0.25 μg/ml of drug (Fig. 1). We further enriched for the desired mutants by subculturing the outgrowth of the MIC assay in fresh medium supplemented with daptomycin (1 μg/ml). After plating and overnight growth on brain heart infusion (BHI) agar plates, 29 *S. aureus* colonies were confirmed to possess increased daptomycin MICs. The genomes of these strains were sequenced. Most possessed insertions in one of two genes, either SAOUHSC_00362 ($n = 20$), encoding a hypothetical lipoprotein (named here Dsp1 for daptomycin susceptibility protein 1), or SAOUHSC_02441 ($n = 6$), encoding an alkaline shock protein, Asp23. Additional transposon insertions were also detected in the gene SAOUHSC_01975 ($n = 1$), encoding a hypothetical protein, and in an intergenic region ($n = 2$).

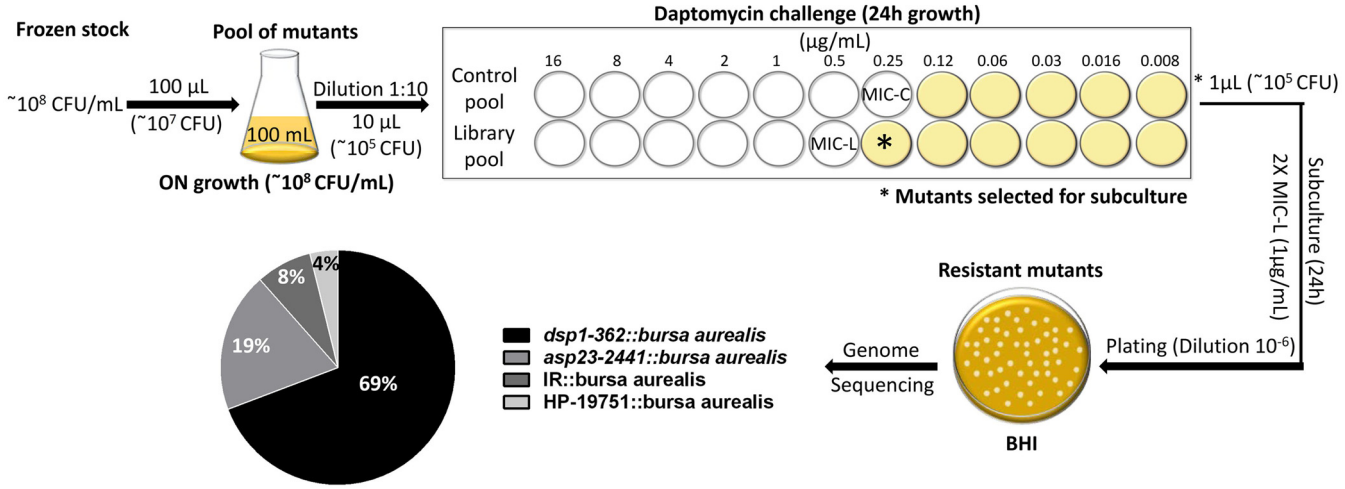

FIG 1 Transposon library antibiotic treatment and transposon mutant identification. A 10-μl aliquot of an overnight culture of the transposon library (library pool) and a pool generated from 10 independent mutants (control pool), each containing 10^6 CFU, were inoculated into a final volume of 200 μl Muller-Hinton broth in a 96-well plate broth microdilution format. DAP MIC was determined by the growth of the control pool (MIC-C). The contents of the library pool growing at 0.25 μg/ml DAP (1× MIC-C) were subcultured in 1 μg/ml (2× MIC-L). This culture was diluted and plated on BHI plates to recover isolated resistant mutants. Genomic DNA was harvested from individual colonies and subjected to whole-genome sequencing. Interrupted genes by transposon insertion were identified after alignment with the NCTC8325 whole genome.

Inactivation of either *dsp1* or *asp23* results in increased resistance to daptomycin and other antimicrobials. An analysis of all *S. aureus* genomes available in the GenBank database suggested that *dsp1* and *asp23* are genes in this species and are present in all genomes. For both genes, we constructed markerless null mutants in *S. aureus* RN4220 for follow up analysis. In this background, increased daptomycin resistance was also observed, indicating that the resistance initially observed was unlikely to result from polar effects of transposon insertion. The Δ*dsp1* and Δ*asp23* null mutants exhibited 2-fold increases in the daptomycin MIC compared to that of the parental RN4220 (Table 1), and neither gene deletion was associated with a discernible growth defect in the absence of antibiotic selection (Fig. 2).

Interestingly, these mutants also showed increased resistance to various CAMPs (i.e., bacitracin, colistin, nisin, and polymyxin). Unexpectedly, the Δ*dsp1* mutant (but not the *asp23* mutant) also showed 2-fold increased resistance to vancomycin (Table 1, Fig. 2D).

Overexpression of *dsp1* or *asp23* causes increased susceptibility to daptomycin. To unambiguously associate Δ*dsp1* and Δ*asp23* with daptomycin resistance, we complemented the deletions with wild-type genes cloned into the xylose-inducible expression vector pEPSA5. Growth curves of the Δ*dsp1* and Δ*asp23* strains, with an empty pEPSA5, showed that the carriage of this shuttle vector has no effect on the ability of these strains to replicate in the presence of 1 μg/ml of daptomycin, even in the presence of xylose (Fig. 3A and C). When complemented with their respective wild-type alleles cloned into pEPSA5 (Δ*dsp1_comp* and Δ*asp23_comp*), both strains retained the ability to grow in 1 μg/ml of daptomycin in the absence of xylose

TABLE 1 Antibiotic susceptibilities of the mutant strains

	MIC (μg/ml)[a]												
Strain	DAP	VAN	BAC	CST	NIS	PMB	CIP	CHL	ERY	GEN	LZD	OXA	TET
RN4220	1	1	32	256	128	32	1	16	0.5	1	4	0.5	2
Δ*dsp1*	**2**	**2**	64	512	256	64	1	16	0.5	1	4	0.5	2
Δ*asp23*	**2**	1	64	512	256	64	1	16	0.5	1	4	0.5	2

[a]DAP, daptomycin; VAN, vancomycin; BAC, bacitracin; CST, colistin; NIS, nisin; PMB, polymyxin B; CIP, ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; OXA, oxacillin; TET, tetracycline.

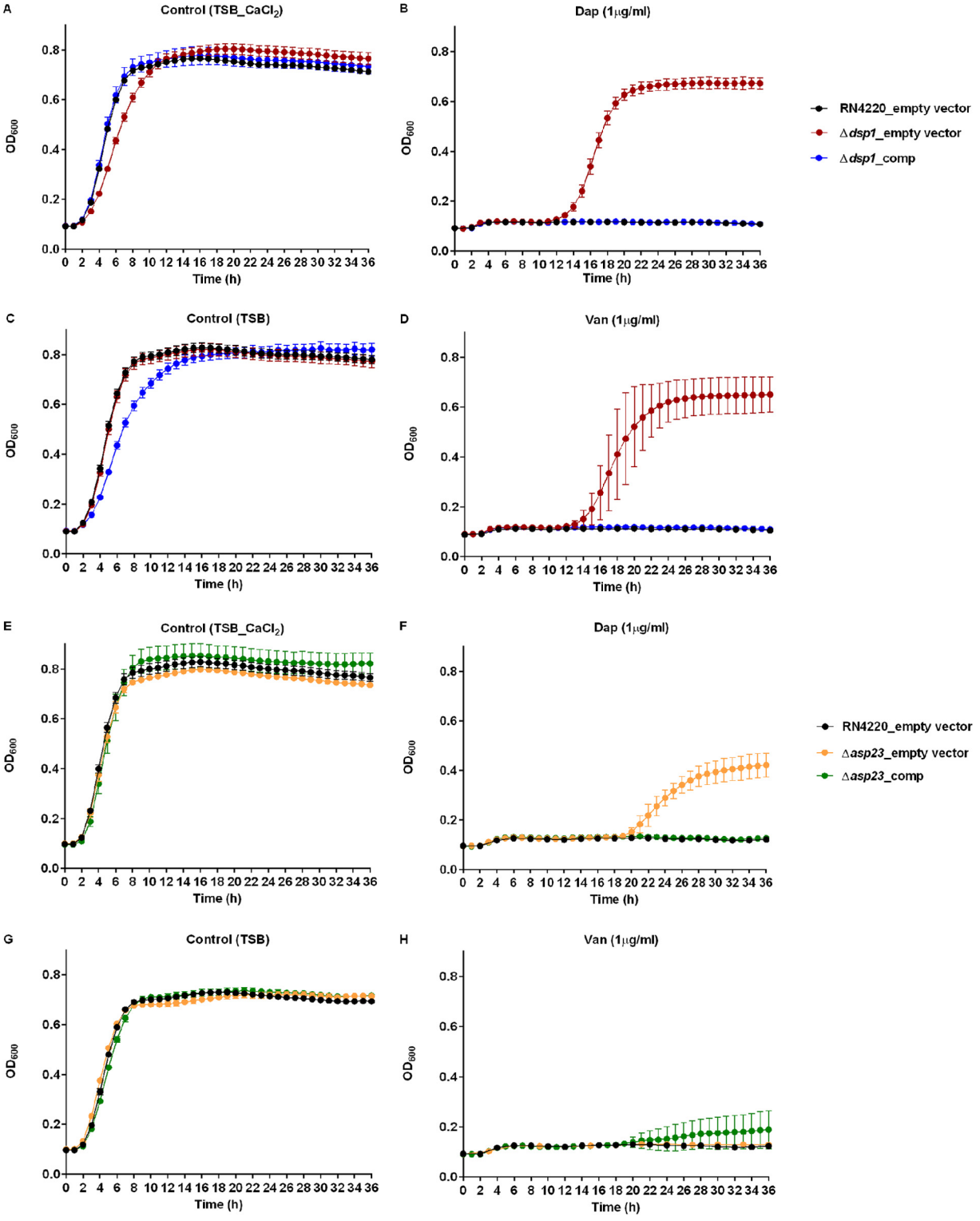

FIG 2 Growth kinetics of Δ*dsp1* (A to D), Δ*asp23* (E to H), and complemented strains upon challenge with 1 μg/ml DAP (B and F) or 1 μg/ml VAN (D and H). TSB $CaCl_2$ was used as a control for DAP challenge (A and E), and plain TSB was used as a control for VAN challenge (C and G). The data are median values from three experiments for each condition, and error bars represent the standard deviations.

induction. However, in the presence of 0.25% or greater xylose, both the Δ*dsp1_comp* and Δ*asp23_comp* strains were inhibited by 1 μg/ml of daptomycin.

Inactivation of *dsp1* or *asp23* results in reduced killing by vancomycin and daptomycin. We performed killing assays for both knockout mutants in the presence

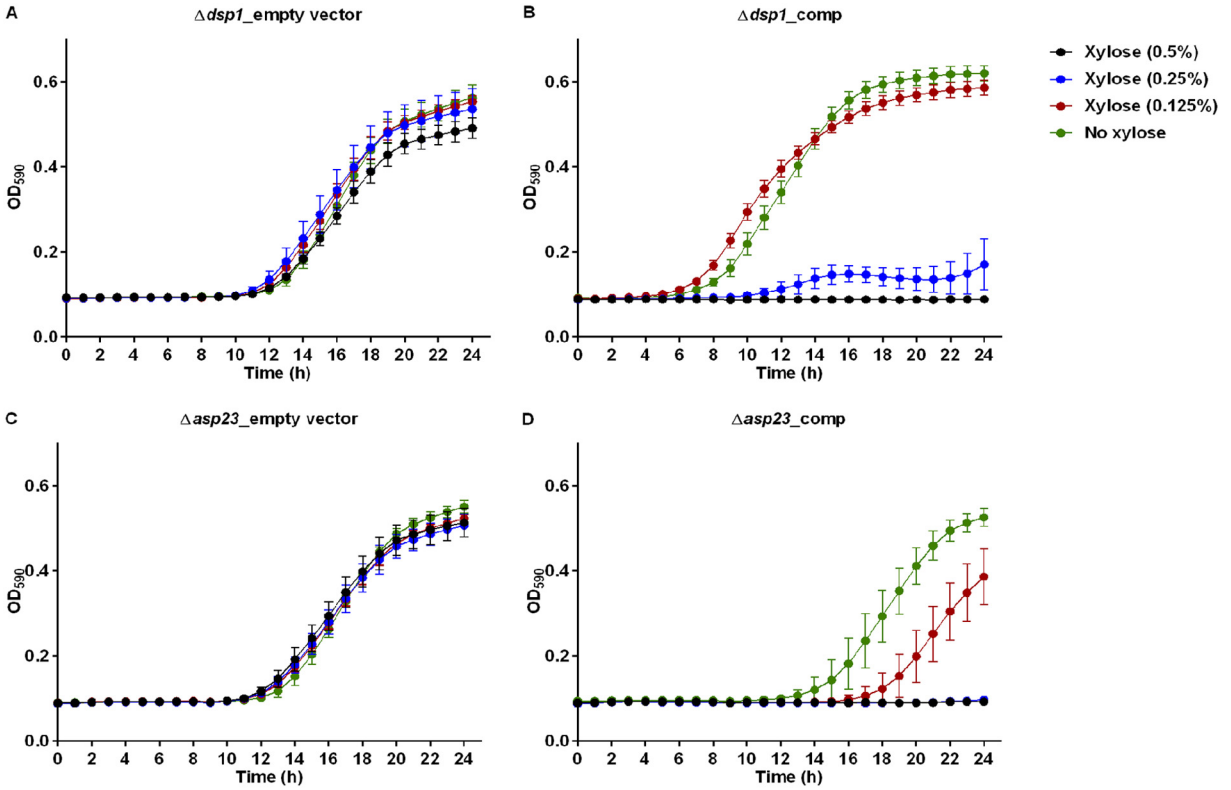

FIG 3 Overexpression of genes *dsp1* (B) and *asp23* (D) in the complemented strains in the presence of DAP (1 μg/ml). Mutant strains with empty vector pEPSA5 were used as a control (A and C). The data are median values from three experiments for each condition, and error bars represent the standard deviations.

of 5 μg/ml daptomycin (Fig. 4A). Starting from a 10^8 CFU/ml inoculum, the survival of the parental RN4200 after 2 h was below our technical limit of detection (1,000 CFU/ml). Strikingly, under the same conditions, $>10^5$ CFU/ml was recovered for both mutants. Even after 24 h, the Δ*dsp1* and Δ*asp23* mutants both retained readily detectable viable cells ($>10^4$ CFU/ml). Interestingly, the increased tolerance of *S. aureus* Δ*dsp1* and Δ*asp23* mutants was found not to be specific to the lipopeptide daptomycin, as a similar phenotype was observed when the cells were challenged with 5 μg/ml of vancomycin (Fig. 4B). For gene *dsp1*, an increased tolerance to DAP and VAN was

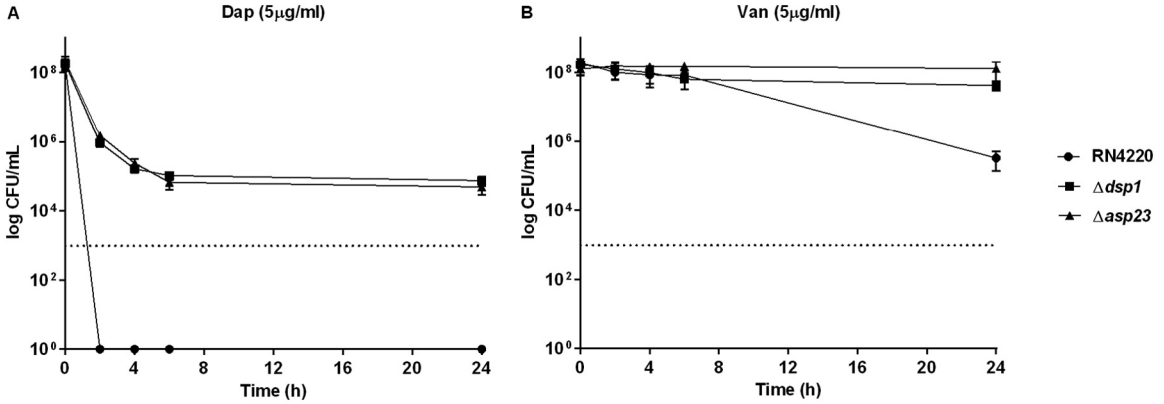

FIG 4 Increased survival of Δ*dsp1* and Δ*asp23* mutants in DAP (A) and VAN (B) exposure assays. Unpaired *t* tests were used to determine statistical significance after 24-h treatments. For daptomycin: Δ*dsp1*, $P = 0.0197$ and Δasp23, $P = 0.0171$; for vancomycin: Δ*dsp1*, $P = 0.0243$ and Δ*asp23*, $P = 0.0193$. The experiment was performed in three independent replicates; means and standard deviations are indicated. Detection limit, 1,000 CFU/ml.

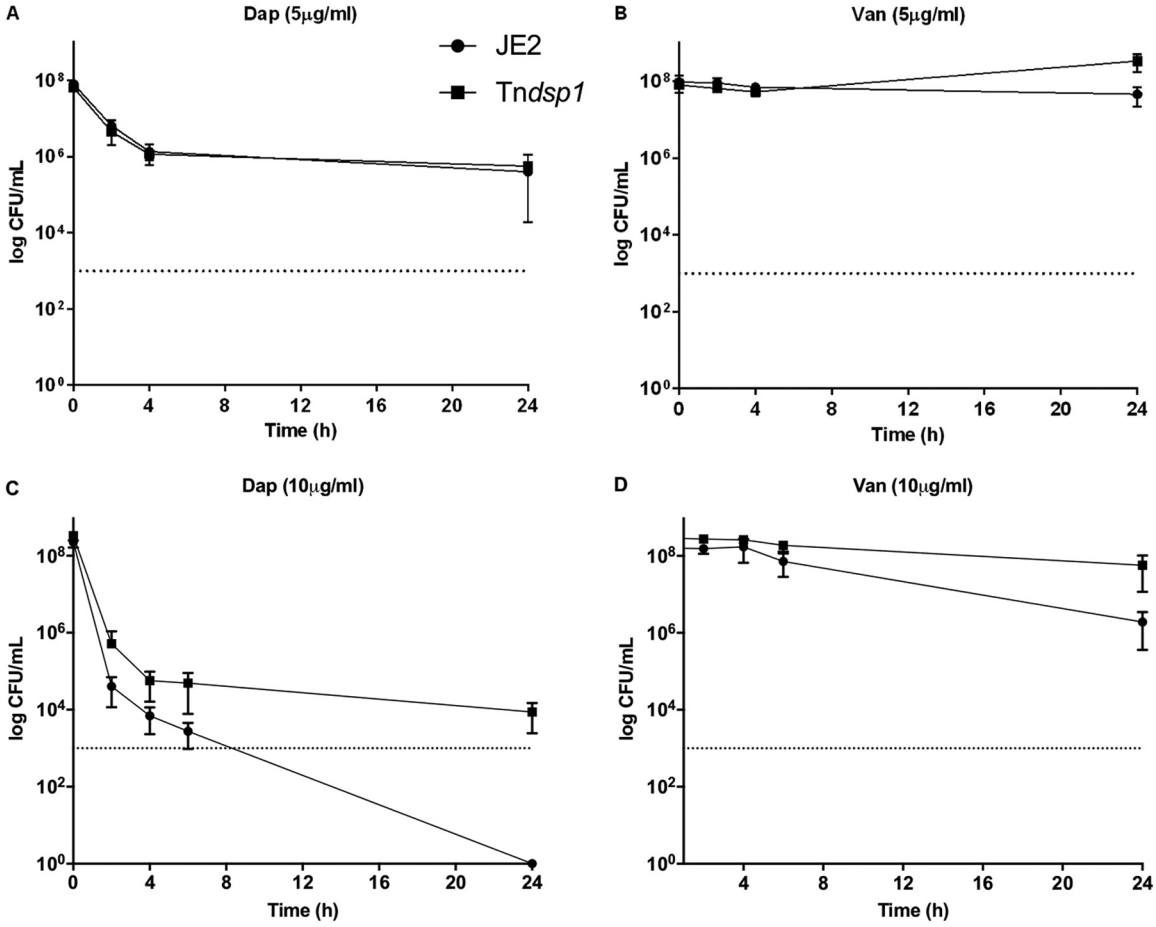

FIG 5 Survival of USA300 wild-type JE2 and mutant Tn*dsp1* strains in 5 μg/ml and 10 μg/ml DAP (A and C) and 5 μg/ml and 10 μg/ml VAN (B and D) exposure assays. Both JE2 and Tn*dsp1* strains show similar survival when challenged with 5 μg/ml of antibiotics, while the Tn*dsp1* strain showed significantly increased tolerance to 10 μg/ml of VAN or DAP. Unpaired *t* tests were used to determine statistical significance after 24-h treatments. For 10 μg/ml daptomycin: Tn*dsp1*, $P = 0.0213$; for 10 μg/ml vancomycin: Tn*dsp1*, $P = 0.0444$. The experiment was performed in three independent replicates; means and standard deviations are indicated. Detection limit, 1,000 CFU/ml.

observed in a transposon mutant (Tn*dsp1*) obtained from the Nebraska Transposon Mutant Library (NTML) (28) compared to that of wild-type JE2 (Fig. 5), a methicillin-resistant strain derived from the highly characterized community-associated *S. aureus* USA300 LAC (28). This was independent from their resistance phenotype, as both Tn*dsp1* and JE2 showed no change in the MIC for DAP (1 μg/ml) or VAN (2 μg/ml). This suggests that gene *dsp1*, core to *S. aureus*, is generally involved in vancomycin and daptomycin tolerance in this species. This is likely true for gene *asp23* but was not tested experimentally, as no transposon mutants were available in the NTML.

Mutant strains show reduced autolytic activity, similar susceptibility to lysostaphin, and no alteration in cell surface charge. Induced autolysis assays were performed to identify the basis for the observed increased resistance to DAP of the mutant strains. The susceptibility to cell lysis was characterized by monitoring the kinetics of Triton X-100-induced autolysis over time. Wild-type RN4220 showed ~30% cell survival at 3 h in the presence of 0.05% Triton X-100. In contrast, more than 50% of the *dsp1* and *asp23* mutants survived after 3 h of treatment (Fig. 6A), suggesting enhanced DAP tolerance may result from alterations to the cell envelope. Alternatively, for lysostaphin-induced autolysis, the mutant strains exhibited similar susceptibility to the parental strain, with ~40% survival after 3 h (Fig. 6B). These results suggest that the Δ*dsp1* and Δ*asp23* mutants possess membrane modifications or other cell wall changes that result in DAP resistance and that peptidoglycan itself does not appear to be

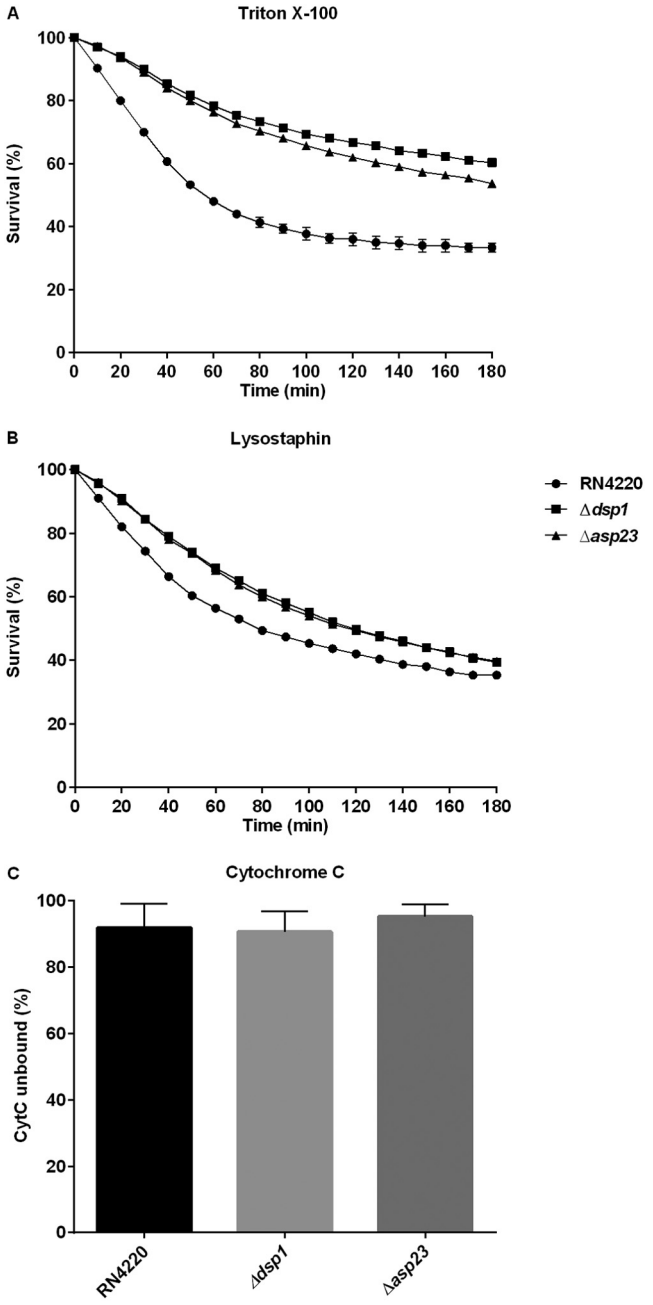

FIG 6 Ability of Δ*dsp1* and Δ*asp23* mutants to induced autolysis in the presence of lysostaphin (A) or Triton X-100 (B) and to repulse cationic cytochrome *c* (C). Unpaired *t* tests were used to determine statistical significance after 24-h treatment with Triton X-100. Δ*dsp1*, $P < 0.0001$ and Δ*asp23*, $P < 0.0001$. The data are mean values from three experiments for each condition, and error bars represent the standard deviations.

involved. Therefore, the possibility that the alteration of cell surface charge was be responsible for repelling DAP was evaluated by quantifying the association of the highly cationic molecule, cytochrome *c*, with the staphylococcal surface (Fig. 6C). However, no difference in cytochrome *c* binding was observed.

DISCUSSION

Prior use of VAN during treatment of VAN-intermediate strains of *S. aureus* (VISA) has been linked to an increased resistance to DAP (11, 29), suggesting that some alterations of the cell wall have the ability to affect the susceptibility to both drugs. DAP is a drug

of last resort for treating multidrug-resistant *S. aureus*. Therefore, the development of resistance during therapy is a serious threat, as it leaves clinicians without effective options for treatment. Despite numerous studies examining DAP resistance evolution, both *in vitro* and *in vivo*, the mechanisms by which bacteria become resistant to DAP are not fully understood (12). Mutations in several genes have been described to trigger DAP resistance, although none of these mutations are sufficient to confer high-level resistance *per se* (30).

Here, two genes not previously associated with daptomycin resistance were identified in a screen of transposon insertion mutants in *S. aureus* HG003. A sequence analysis showed that one gene encodes a hypothetical lipoprotein (SAOUHSC_00362) and the other (*asp23*) an alkaline-shock protein (SAOUHSC_02441). The staphylococcal alkaline shock protein 23 (Asp23) is one of the most abundant proteins expressed by *S. aureus*. It is a 23-kDa protein that accumulates in the soluble protein fraction of *S. aureus* cells following alkaline shock (31). The stress response gene *asp23* is the last gene in a four-gene operon transcribed from three different σ^B-dependent promoters (31, 32). The deletion of *asp23* has been linked to increased transcription of cell wall stress-associated genes, and most of the upregulated genes were previously shown to also be induced in the presence of VAN (33), including the two-component system VraSR and peptidylprolyl isomerase PrsA (34). Recently, a point mutation in *asp23*, among other mutations, was reported in a DAP-R strain that resulted from serially passaging *S. aureus* in increasing DAP concentrations. A C-to-T transition (Glu47Lys) was observed in *asp23*, but not directly associated with the DAP-R phenotype (30). We show that *asp23* loss of function increases DAP resistance and also confers increased tolerance to both DAP and VAN. On the basis of the known function of Asp23 in stress response, we hypothesize that a deletion of *asp23* in the context of DAP treatment results in transcriptional changes that indirectly increase DAP resistance.

Beyond DAP and VAN, the Δ*asp23* and Δ*dsp1* mutants also exhibited higher tolerance to all CAMPs tested. CAMPs are widely distributed in nature and constitute key effectors of innate immune responses to infection in organisms ranging from mammals to plants. In some respects, DAP resembles CAMPs because of its peptide content, charge, and mode of action targeting membrane function (35). Mishra and coworkers (36) provided evidence that prior exposure to endovascular host cationic peptides could conceivably drive the selection of DAP-R strains. Cross-resistance between DAP and other CAMPs that target the bacterial cell membrane has also been reported, especially in association with mutations that appear to lead to a gain of function of *mprF*. MprF modifies anionic phospholipids and thereby introduces positive charges into the membrane surface, which reduce the affinity for DAP and CAMPs (14). The overexpression of the *dtl* operon causes increases in net positive charges in the cell membrane and has also been described in DAP-R strains (15–17).

As most adaptations in DAP-R strains have been associated with cell membrane and cell wall changes, induced autolysis assays were conducted using Triton X-100 or lysostaphin challenge. Bacterial cell autolysis is regulated by various mechanisms, including the modification of the cell wall peptidoglycan and the regulation of the expression of genes associated with cell wall hydrolase activities (37, 38). The ability of the nonionic surfactant Triton X-100 to induce cellular lysis is associated with the disruption of the plasma membrane, triggering the autolysis of the peptidoglycan layer by cellular autolysins (39). Lysostaphin has a direct effect in the cell wall peptidoglycan by cleaving the cross-linking pentaglycine bridges (40). Our results showed that both *dsp1* and *asp23* deletions are associated with decreased bacterial autolysis induced by the nonionic detergent, Triton X-100, but no effect was observed in controlling lysostaphin-induced lysis. These data support the hypothesis that DAP resistance in the mutant strains is likely caused by changes in the cell membrane, perhaps by modulating the abundance of membrane-associated proteins and thus affecting membrane stability or fluidity. Here, we described two genes that upon inactivation lead to an increased tolerance to both DAP and VAN. Studies are under way to further elucidate

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
Escherichia coli DH5α	Cloning host	Invitrogen
Staphylococcus aureus		
HG003	Wild type; NCTC8325 derivative; *rsbU* and *tcaR* repaired	49
RN4220	Wild type; NCTC8325 derivative; restriction deficient, prophage cured	50
RN4220 Δ*dsp1*	Locus SAOUHSC_00362 deletion mutant	This study
RN4220 Δ*asp23*	*asp23* deletion mutant	This study
RN4220 Δ*dsp1*_Comp	Δ*dsp1* complemented strain	This study
RN4220 Δ*asp23*_Comp	Δ*asp23* complemented strain	This study
JE2	Wild type; derived from *S. aureus* USA300 LAC	NTML (28)
Tn*dsp1*	Transposon insertion at position 34 of *dsp1* gene in JE2 background	NTML (28)
Plasmids		
pKOR1	*E. coli/S. aureus* shuttle vector; thermosensitive origin of replication; ATc induced counterselection; Amp^r, Chl^r	42
pEPSA5	*E. coli/S. aureus* shuttle vector; expression vector; Amp^r, Chl^r	45

[a]Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant.

the mechanisms by which *dsp1* and *asp23* contribute to the susceptibility of *S. aureus* to these antimicrobials.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids are listed in Table 2. The transposon library constructed in *S. aureus* HG003 using the *Bursa aurealis* system has been previously described (27). *S. aureus* and *Escherichia coli* strains were grown in tryptic soy broth (TSB; BD Diagnostics Systems) and Luria broth (LB; BD Diagnostics Systems) medium, respectively, at 37°C under continuous shaking, unless indicated otherwise. *E. coli* DH5α (Invitrogen) was used as the cloning host for pEPSA5 and pKOR1 plasmids that were subsequently transferred to *S. aureus* RN4220. Antibiotic markers were selected when appropriate with 50 μg/ml ampicillin (Amp; Sigma-Aldrich) and 10 μg/ml chloramphenicol (Chl; Sigma-Aldrich). Medium broth was supplemented with $CaCl_2$ (50 μg/ml; Sigma-Aldrich) when DAP was tested. Anhydrotetracycline (ATc; Sigma-Aldrich) at a final concentration of 1 μg/ml was used to select mutant strains. Genes cloned into pEPSA5 had their expression induced with xylose (0.5%, Sigma-Aldrich).

Transposon mutant library screen. The *mariner*-based *Bursa aurealis* transposon insertion system was previously used to construct a mutagenized library in HG003. This library has been described to consist of 71,700 unique insertions in the HG003 chromosome, with 57,191 insertions occurred within coding regions (27), and it was used in this work as described. Frozen aliquots of the transposon mutant library pool and a "little pool" generated from 10 independent transposon mutants selected at random were thawed and diluted 1:1,000 into 100 ml of Mueller-Hinton (MH; Moltox) broth and grown overnight at 37°C. The culture was diluted 1:10 into 10 ml of MH broth supplemented with $CaCl_2$. The cultures were further diluted (1:20) into 200 μl fresh MH $CaCl_2$ broth with 2-fold dilutions of DAP (16 to 0.008 μg/ml; Sigma-Aldrich) in 96-well plates at 37°C for 24 h. Two rounds of selection in DAP were performed under the same conditions. The MIC of the little pool was determined, and wells above this MIC from the full mutant library were diluted 1:100 and subcultured twice in a higher concentration (2$\times$ the MIC) of DAP to increase the sensitivity of the detection of mutants showing decreased susceptibility to DAP. The mutants were serially diluted 1:10^8 into phosphate-buffered saline (PBS) and then plated on tryptic Soy agar (TSA) plates to recover isolated mutant strains. Two independent experiments were performed.

Molecular cloning and DNA isolation. The primers used in this study were synthesized by Integrated DNA Technologies (Table 3). Qiagen kits were used for the isolation of bacterial genomic DNA and plasmids. For PCR product cleanup, ExoSAP-IT (Affymetrix; USB) was used. Phusion high-fidelity DNA polymerase, PCR reagents, restriction enzymes, calf-intestinal alkaline phosphatase (CIP), and T4 DNA ligase from New England BioLabs and Gateway BP Clonase II enzyme mix from Invitrogen were used according to the manufacturer's protocols.

Whole-genome sequencing for identification of mutated genes. Genomic DNA was isolated with the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Sequencing libraries were prepared using an Illumina Nextera XT Library Prep kit and subjected to 250-bp paired-end sequencing on the MiSeq platform at the Massachusetts Eye and Ear Infirmary Ocular Genomics Institute (Boston, MA, USA). Sequence reads were assembled *de novo* (CLC Genomics Workbench 8.0.3; Cambridge, MA, USA) and aligned to the transposon sequence using Geneious R8 (41).

Construction of mutant strains and genetic complementation. Gene deletions from the genome of *S. aureus* RN4220 were made by homologous recombination with plasmid pKOR1 according Bae and Schneewind (42) with minor modifications. Briefly, two DNA segments of ~1kb in length up- and downstream of the candidate genes were PCR-amplified from the chromosomal DNA of *S. aureus* HG003. Overlap regions were introduced in the primer sequence next to the gene to be deleted, and *att* recombination sites were introduced at the distal ends, as listed in Table 3. The two segments flanking

TABLE 3 Oligonucleotides used in this study

Primer name	Sequence	Reference or source
Knockout construction		
362-51-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAAGAGCAATTGATTAATAAAGG	This study
362-31-OL	AATGATGGCTTTCAGAATGTAATATAACTCCTTAGTTTATCT	This study
362-32-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAAGATGAAATCAGTGCGAATCC	This study
362-52-OL	GAGTTATATTACATTCTGAAAGCCATCATTGCGAAA	This study
2441-51-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGTTTTATTTACAAAAACTAGGG	This study
2441-31-OL	TTTTTATCGAATAACAATAGATTCTCCTTTTACTTG	This study
2441-32-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGAACGAAGATAGTACTCAAATTAG	This study
2441-52-OL	AAAGGAGAATCTATTGTTATTCGATAAAAAGGGCTT	This study
Complementation construction		
362-Fw-EcoRI	GCGAGAATTCAGGAGGTACATTATGAAAAAGAGATTACTAC	This study
362-Rev-BamHI	GCGAGGATCCTTATTTATCGATAACATCACTCTTG	This study
2441-Fw-EcoRI	GCGAGAATTCAGGAGGTCTATTATGACTGTAGATAACAATAAAG	This study
2441-Rev-BamHI	GCGAGGATCCTTATTGTAAACCTTGTCTTTCTTGG	This study

each target gene were recombined by SOEing PCR according to Horton et al. (43). The PCR products were cloned individually into pKOR1 by recombination reactions (Gateway BP Clonase II enzyme mix). The resulting plasmids were transformed into *E. coli* DH5α chemically competent cells according to the manufacturer's recommendations and subsequently into *S. aureus* RN4220 competent cells via electroporation (44). Further allelic replacement was achieved as described previously (42) and verified by Sanger sequence analysis.

To complement the mutant strains, the target genes were PCR amplified using the primers listed in Table 3 and cloned into the pEPSA5 shuttle vector (45) using restriction endonucleases EcoRI and BamHI.

MIC determination. MICs were determined using the CLSI broth microdilution assay guidelines (46, 47) for different antimicrobials against the parent strain *S. aureus* RN4220 and isogenic mutants. All MIC assays were performed in triplicates.

Killing curve. Killing curve assays were performed in the presence of DAP and VAN. Briefly, an overnight culture was diluted 1:100 into MH or MH $CaCl_2$ broth and grown at 37°C to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.3). Cultures were exposed to 5 μg/ml DAP or VAN, 5× the MIC of parent strain, and incubated at 37°C for 24 h. CFU after 0, 2, 4, 6, and 24 h were quantified by the track dilution method using six 10-fold dilutions and 10 μl aliquot for plating.

Growth curve. Growth curves were performed with *S. aureus* RN4220, its mutants, and complemented strains. RN4220 transformed with empty pEPSA5 vector was also included in the analysis. Overnight cultures were diluted and adjusted to an OD_{600} of 0.08 to 0.1 (~10^8 CFU/ml). The cultures were inoculated (1:20) in 200 μl fresh TSB or TSB $CaCl_2$ broth and challenged with 2-fold dilutions of DAP or VAN (4 to 0.125 μg/ml) in 96-well plates at 37°C for 36 h. The OD_{600} values were monitored every hour using a BioTek plate reader. Chloramphenicol (10 μg/ml) and xylose (0.5%) were included to maintain pEPSA5 plasmid vector and to induce the expression of cloned genes, respectively. Growth curves were generated by plotting the OD_{600} values over time. The data are reported as mean values from four independent experiments.

Induced autolysis assay. For Triton X-100-induced autolysis, cells were grown in TSB to an OD_{600} of 1.0 and chilled on ice before harvesting. After washing the cells with ice-cold water, the bacterial pellets were resuspended in the same volume of 50 mM Tris-HCl (pH 7.5) containing 0.05% Triton X-100 (J. T. Baker Inc.). Induced autolysis was measured spectrophotometrically during incubation at 37°C as a decrease in OD_{600} over time. For lysostaphin (BioVendor)-induced autolysis assays, strains were also grown to an OD_{600} of 1.0 and harvested by centrifugation. Cells were washed with water and resuspended in PBS supplemented with 200 ng/ml lysostaphin. Cell autolysis was determined as described above. The results were normalized to the OD_{600} at time zero (OD0) and the percent survival at indicated times was determined.

Cytochrome *c* binding assay. The relative positive surface charge of *S. aureus* strains was determined by quantifying the association of the positively charged molecule cytochrome *c* (Sigma) to the staphylococcal surface as previously described (48). Briefly, after overnight growth in TSB, cultures were diluted 1:100 in fresh medium and allowed to regrow to logarithmic phase (OD_{600} of ~0.3). After centrifugation, the cells were washed twice with MOPS (morpholinepropanesulfonic acid) buffer (20 mM, pH 7.0, the bacterial suspension was adjusted to an OD_{600} of 1.0, and 1 ml aliquots were harvested. The cell pellets were resuspended in 200 μl MOPS buffer and 50 μl of cytochrome *c* solution was added (equine heart, 2.5 mg/ml in MOPS buffer; Sigma). After 10 min at room temperature, the samples were harvested at 12,000 × *g*, supernatants were recovered, and the OD_{530} was measured spectrophotometrically. The experiment was repeated in triplicates.

ACKNOWLEDGMENTS

This work was supported by CNPq and has been funded in part by DHHS/NIH/NIAID grants AI072360 and AI083214 (Harvard-wide Program on Antibiotic Resistance) (M.S.G.).

We declare no competing financial interests.

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