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RESEARCH ARTICLE
Therapeutics and Prevention

Systematic Analysis of Efflux Pump-Mediated Antiseptic Resistance in *Staphylococcus aureus* Suggests a Need for Greater Antiseptic Stewardship

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ABSTRACT *Staphylococcus aureus*-associated infections can be difficult to treat due to multidrug resistance. Thus, infection prevention is critical. Cationic antiseptics, such as chlorhexidine (CHX) and benzalkonium chloride (BKC), are liberally used in health care and community settings to prevent infection. However, increased administration of antiseptics has selected for *S. aureus* strains that show reduced susceptibilities to cationic antiseptics. This increased resistance has been associated with carriage of specific efflux pumps (QacA, QacC, and NorA). Since prior published studies focused on different strains and on strains carrying only a single efflux gene, the relative importance of these various systems to antiseptic resistance is difficult to ascertain. To overcome this, we engineered a collection of isogenic *S. aureus* strains that harbored *norA*, *qacA*, and *qacC*, individually or in combination. MIC assays showed that *qacA* was associated with increased resistance to CHX, cetrimide (CT), and BKC, *qacC* was associated with resistance to CT and BKC, and *norA* was necessary for basal-level resistance to the majority of tested antiseptics. When all three pumps were present in a single strain, an additive effect was observed in the MIC for CT. Transcriptional analysis revealed that expression of *qacA* and *norA* was significantly induced following exposure to BKC. Alarmingly, in a strain carrying *qacA* and *norA*, preexposure to BKC increased CHX tolerance. Overall, our results reveal increased antiseptic resistance in strains carrying multiple efflux pumps and indicate that preexposure to BKC, which is found in numerous daily-use products, can increase CHX tolerance.

IMPORTANCE *S. aureus* remains a significant cause of disease within hospitals and communities. To reduce the burden of *S. aureus* infections, antiseptics are ubiquitously used in our daily lives. Furthermore, many antiseptic compounds are dual purpose and are found in household products. The increased abundance of antiseptic compounds has selected for *S. aureus* strains that carry efflux pumps that increase resistance to antiseptic compounds; however, the effect of carrying multiple pumps within *S. aureus* is unclear. We demonstrated that an isogenic strain carrying multiple efflux pumps had an additive resistance phenotype to cetrimide. Moreover, in a strain carrying *qacA* and *norA*, increased chlorhexidine tolerance was observed after the strain was preexposed to subinhibitory concentrations of a different common-use antiseptic. Taken together, our findings demonstrate cooperation between antiseptic resistance efflux pumps and suggest that their protective phenotype may be exacerbated by priming with subinhibitory concentrations of household antiseptics.

KEYWORDS *Staphylococcus aureus*, antimicrobial agents, benzalkonium chloride, chlorhexidine, efflux pumps

Citation LaBreck PT, Bochi-Layec AC, Stanbro J, Dabbah-Krancher G, Simons MP, Merrell DS. 2020. Systematic analysis of efflux pump-mediated antiseptic resistance in *Staphylococcus aureus* suggests a need for greater antiseptic stewardship. mSphere 5: e00959-19. https://doi.org/10.1128/mSphere.00959-19.

Editor Paul Dunman, University of Rochester

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Systematic analysis of efflux pump-mediated antiseptic resistance in Staphylococcus aureus suggests a need for greater antiseptic stewardship @scottymerrell1

Received 17 December 2019
Accepted 17 December 2019
Published 15 January 2020

Hospital- and community-acquired infections by *Staphylococcus aureus* are a significant cause of disease and economic burden in the United States. In particular, infections by methicillin-resistant *S. aureus* (MRSA) are difficult to prevent and treat because of the ability of the bacterium to persist on nosocomial surfaces, including within inpatient populations, and because of antibiotic resistance, respectively (1, 2). In spite of the treatment difficulties associated with hospital-acquired MRSA (HA-MRSA) infections, improved hygiene and disinfection strategies are decreasing the number of invasive infections (3). Indeed, the increased application of antiseptics has been able to reduce the number of hospital-acquired infections (HAI) in certain settings (4, 5). However, our dependency on antiseptics is not without consequence, as bacteria are evolving an increased tolerance to some common biocides (6, 7).

Two common antiseptic compounds, chlorhexidine (CHX) and benzalkonium chloride (BKC), have become standard biocides in our daily lives. CHX was developed in the 1950s but was not put into common usage until after 2000 (8, 9). As a presurgical scrub and skin prep, CHX has been shown to be effective at infection prevention and is well tolerated on patients. However, CHX use is not limited to the hospitals; CHX is widely available in over-the-counter soaps and is frequently used as a disinfectant (10). Prior to CHX, BKC was developed for antiseptic usage in 1935 and was broadly used in surgical preparations and hand soaps (11, 12). Today, BKC is found in numerous common-use products, including cosmetics, antiseptics/hand sanitizers, and pharmaceutical products. This overwhelming prevalence of BKC has prompted an FDA request for additional scientific data to support the safety and effectiveness of BKC in over-the-counter antiseptic rubs; this analysis is currently ongoing (13). Throughout the past 70 years, both CHX and BKC have demonstrated efficacy as antiseptics (14); nonetheless, over time their excessive use is selecting for bacterial strains that show increased resistance to both compounds (9, 15).

Efflux pumps provide one mechanism for increased antiseptic tolerance of bacteria to CHX and BKC. In *S. aureus*, the antiporter efflux pumps QacA, NorA, and QacC are known to offer increased resistance to a wide range of common antiseptics (16). Besides *norA*, which has recently been shown to be part of the core genome of *S. aureus* (17), *qacA* and *qacC* reside on a variety of conjugative and nonconjugative plasmids (18, 19). The majority of *qac* prevalence studies demonstrate that while *qacA* and *qacC* do not commonly coexist within *S. aureus*, the presence of all three efflux pumps within *S. aureus* isolates does occur (20–22); however, the functional consequences of harboring all three pumps are unknown. For *S. aureus* strains that carry either *qacA* or *qacC*, the prevalence varies greatly and depends on the geographical location and sample population being studied. Typically, *qacA* and *qacC* prevalence is higher in isolates from Asia and Europe than in those from North America (23). Overall, there has been an increasing trend in *qacA* prevalence in Asian countries (24), and new data from the United States suggest a similar trend as well (22, 25). The identification of *qacA* and *qacC* carried on conjugative plasmids suggests the presence of selective pressures that drive the mobilization of these genes across *S. aureus* strains. Furthermore, this observation is further supported by the expansion of *qacA* to other pathogenic bacteria (26, 27).

As the prototypical antiseptic resistance pump in *S. aureus*, QacA has been well characterized and shown to impart increased tolerance to bisbiguanides, quaternary ammonium compounds (QACs), diamides, and aromatic dyes (28). QacA is composed of 14 transmembrane segments and is grouped in the major facilitator superfamily of transporters (29). Expression of *qacA* is controlled by the QacR repressor, which is coded for by the adjacently encoded *qacR* (30). To induce *qacA* expression, substrates of QacA directly bind to QacR and elicit a structural change that releases QacR from the *qacA* promoter (31, 32). Different compounds have been shown to stimulate *qacA* expression to various degrees (30, 33).

As another plasmid-encoded efflux pump, *qacC*, which is also known as *smr*, has been shown to impart increased resistance to QACs and to aromatic dyes (28). In addition to large low-copy-number multiresistance plasmids, *qacC* is found on small

rolling-circle replicating (RCR) plasmids (34). *qacC* is largely conserved among different plasmids; however, the promoter of *qacC* is known to differ between low-copy-number and RCR plasmids (34). The small (107-amino-acid [aa]) QacC efflux pump is member of the small multidrug resistance (SMR) family, has 4 transmembrane segments, and likely functions as a homodimer (35, 36).

As mentioned above, *norA* was recently described as part of the core genome of *S. aureus*, but was shown to exist as multiple possible alleles (17). *norA* is known to impart resistance to a variety of structurally diverse compounds, and previous work suggests that NorA may be responsible for the majority of basal antiseptic resistance in *S. aureus* (37, 38). In terms of expression, many different *norA* promoter mutations are documented that cause increased expression of the gene. These mutations result in increased tolerance to hydrophilic fluoroquinolones and to antiseptics (37–39). The importance of *norA* in antiseptic tolerance is understated, and it is plausible that different *norA* alleles and/or expression changes account for the large portion of *qacA*-negative strains that show increased CHX tolerance (9).

Collectively, *qacA*, *qacC*, and *norA* have been characterized individually. Nonetheless, antiseptic resistance phenotypes are not well documented for strains that contain various combinations of these pumps. We engineered isogenic *S. aureus* strains that contain various combinations of all three pumps and then examined their MICs and minimal bactericidal concentrations (MBCs) to various substrates. Additionally, we assessed the expression of the different efflux pumps after exposure to various biocides. From these data we developed, tested, and confirmed the hypothesis that preexposure to one biocide could subsequently increase the resistance to a different antiseptic. Given the widespread prevalence of these antiseptics in common-use items, this finding presents a potential cause for concern.

RESULTS

Creation and characterization of isogenic strains. To examine the phenotypic effect of carrying *norA*, *qacA*, and *qacC* in different combinations, we constructed 13 isogenic strains in the *S. aureus* RN4220 strain background (Table 1). For the *norA* isogenic strains, mutations/changes were made in the chromosome via allelic exchange. Previous work indicates that *norA* is important for basal-level resistance to a wide variety of compounds (37, 38). To test this phenotype, we deleted *norA* in RN4220 to create RN4220 Δ*norA* and then complemented the deletion using pCN51-*norA*. In addition to the phenotypic changes associated with the presence or absence of *norA*, a number of studies demonstrate that increased expression of *norA* is associated with higher levels of antiseptic and antibiotic resistance (38, 40). Therefore, we created two strains bearing naturally occurring distinct promoter mutations in *norA* that have been demonstrated to increase *norA* expression (38). The first mutation, a polymorphism in the −10 promoter region where 5′TACAAT3′ is mutated to 5′TATAAT3′, likely increases the binding affinity of RNA polymerase to the promoter. The second mutation is also known as the *flq* mutation and represents a polymorphism in the 5′ untranslated region (UTR) of *norA* whereby a polynucleotide tract is mutated from 5′TTTTT3′ to 5′TTCTT3′; this mutation is known to increase the half-life of the mRNA transcript (39, 41). The strains containing these promoter mutations were named RN4220 N1 and RN4220 N2, respectively. Both of these mutations increased the relative expression of *norA* (Fig. 1A).

To enable direct comparison between plasmid-carried efflux genes, we cloned *qacA* and *qacC* variants into pCN38 with their native promoters (30, 34). For the *qacA* isogenic strain, *qacA* and *qacR*, which encodes *qacA*'s regulator, QacR, were cloned into pCN38 to create RN4220 (pCN38-*qacA qacR*). As for the *qacC* isogenic strains, previous reports have shown *qacC* to have two different promoter sequences; these sequences vary based on the plasmid backbone that *qacC* is carried on. To determine if these promoter differences result in different phenotypic effects, we synthesized *qacC* and its promoter from pSK41 (a large conjugative plasmid) (42) and *qacC* plus 133 bp upstream of *qacC* from pSK89; this promoter sequence is still undefined (43). Both *qacC* variants were cloned into pCN38 to create RN4220 (pCN38-*qacC* [pSK41]) and RN4220 (pCN38-*qacC*

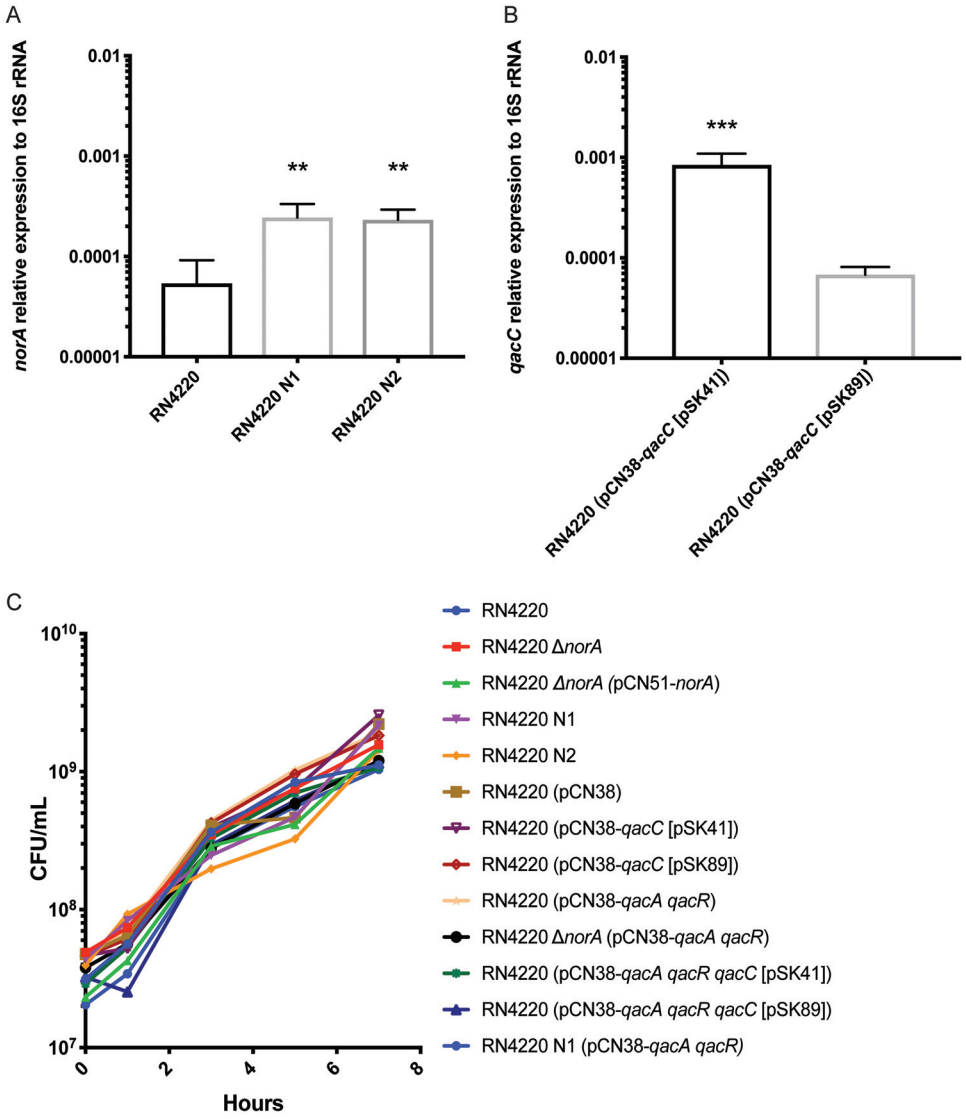

FIG 1 Isogenic strain characterization. (A) The expression of *norA* relative to that of the 16S rRNA gene was examined in the isogenic strains RN4220, RN4220 N1, and RN4220 N2. Bar graphs indicate the geometric means from 3 biologically independent replicates, and error bars represent the geometric SD. A statistically significant difference from RN4220 ($P < 0.01$) is indicated by double asterisks. Statistical analysis was conducted by one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons. (B) The expression of *qacC* relative to that of the 16S rRNA gene was examined in the isogenic strains RN4220 (pCN38-*qacC* [pSK41]) and RN4220 (pCN38-*qacC* [pSK89]). A statistically significant difference between strains ($P < 0.001$) is indicated by triple asterisks. Statistical analysis was conducted using a Student *t* test. Bar graphs indicate the geometric means from 3 biologically independent replicates, and error bars represent the geometric SD. (C) Growth curve of isogenic strains. Overnight cultures were subcultured and strains were grown for 7 h. CFU were determined at 0, 1, 3, 5, and 7 h. The geometric means from at least 2 biologically independent replicates are plotted.

[pSK89]). Expression of *qacC* in logarithmically grown cells was different between the two promoters; RN4220 (pCN38-*qacC* [pSK41]) showed a 10-fold increase in expression compared to that of RN4220 (pCN38-*qacC* [pSK89]) (Fig. 1B). Finally, to characterize the phenotype that results from harboring multiple pumps in a single strain, we also created strains that carried various combinations of the efflux pumps described above (Table 1). In the isogenic strains that were designed to carry multiple *qac* genes, *qacA*, *qacR*, and *qacC* were cloned into the same plasmid.

To ensure that genetic manipulation of RN4220 did not result in any obvious fitness defects of the constructed strains, levels of growth of the strains were compared in BBL Mueller-Hinton II (cation-adjusted) broth (MHB) across a standard growth curve; no

TABLE 1 Strain and plasmid descriptions

Strain or plasmid	Lab strain designation	Description[a]	*Gene* (*norA*, *qacC*, and/or *qacA*) present or other description[b]	Reference
S. aureus strains				
RN4220	DSM1935	Wild type	*norA*	62
RN4220 Δ*norA*	DSM1936	*S. aureus* RN4220 with a clean deletion of *norA*	None	This study
RN4220 N1	DSM1937	RN4220 with a *norA* promoter mutation in the −10 element (TACAAT to TATAAT)	*norA*$^+$	This study
RN4220 N2	DSM1938	RN4220 with a *norA* promoter mutation in the 5′ untranslated region (TTTTT to TTCTT)	*norA*$^+$	This study
RN4220 (pCN38)	DSM1715	RN4220 carrying cloning vector pCN38	*norA*	47
RN4220 (pCN38-*qacC* [pSK41])	DSM1939	RN4220 carrying pCN38 containing *qacC* from pSK41 (accession number AF051917.1)	*norA*, *qacC*$^+$	This study
RN4220 (pCN38-*qacC* [pSK89])	DSM1940	RN4220 carrying pCN38 containing *qacC* from pSK89 (accession number M37889.1)	*norA*, *qacC*	This study
RN4220 (pCN38-*qacA qacR*)	DSM1941	RN4220 carrying pCN38 containing *qacA* and *qacR* from pC02 (accession number CP012121.2)	*norA*, *qacA*	This study
RN4220 (pCN38-*qacA qacR qacC* [pSK41])	DSM1942	RN4220 carrying pCN38 containing *qacA*, *qacR*, and *qacC* from pSK41	*norA*, q*acA*, *qacC*$^+$	This study
RN4220 (pCN38-*qacA qacR qacC* [pSK89])	DSM1943	RN4220 carrying pCN38 containing *qacA*, *qacR*, and *qacC* from pSK89	*norA*, *qacA*, *qacC*	This study
RN4220 Δ*norA* (pCN38-*qacA qacR*)	DSM1944	RN4220 Δ*norA* carrying pCN38 containing *qacA* and *qacR*	*qacA*	This study
RN4220 N1 (pCN38-*qacA qacR*)	DSM1945	RN4220 N1 carrying pCN38 containing *qacA* and *qacR*	*norA*$^+$, *qacA*	This study
RN4220 (pCN51)	DSM1946	RN4220 carrying pCN51	*norA*	This study
RN4220 Δ*norA* (pCN51)	DSM1947	RN4220 Δ*norA* carrying pCN51	None	This study
RN4220 Δ*norA* (pCN51-*norA*)	DSM1948	RN4220 Δ*norA* carrying pCN51 containing *norA* from *S. aureus* RN4220	*norA*	This study
RN4220 (pCN51-*qacC*)	DSM1950	RN4220 carrying pCN51 containing *qacC* from pSK41 (accession number AF051917.1)	*norA*, *qacC*	This study
RN4220 (pCN51-*qacA*)	DSM1949	RN4220 carrying pCN51 containing *qacA* from pC02 (accession number CP012121.2)	*norA*, q*acA*	This study
2014.C02 (also known as C02)	DSM1418	Clinical isolate carrying *qacA*-positive pC02 (accession number CP012121.2)	*norA*, q*acA*	18
1969.N	DSM1504	Clinical isolate carrying *qacA*-positive p1969.Nb (accession number CP016860.2)	*norA*, *qacA*	18
2148.C01	DSM1499	Clinical isolate carrying *qacA*-positive p2148.C01b (accession number CP017095.2)	*norA*, *qacA*	18
5118.N	DSM1491	Clinical isolate carrying *qacA*-positive p5118.Nb (accession number CP016854.2)	*norA*, *qacA*	18
E. coli strains				
DH5 α (pCN38)	DSM1595	DH5 α carrying pCN38		47
TOP10 (pCN38-*qacC* [pSK41])	DSM1972	TOP10 carrying pCN38 containing *qacC* from pSK41 (accession number AF051917.1)		This study
TOP10 (pCN38-*qacC* [pSK89])	DSM1973	TOP10 carrying pCN38 containing *qacC* from pSK89 (accession number M37889.1)		This study
TOP10 (pCN38-*qacA qacR*)	DSM1974	TOP10 carrying pCN38 containing *qacA* and *qacR* from pC02 (accession number CP012121.2)		This study
TOP10 (pCN38-*qacA qacR qacC* [pSK41])	DSM1975	TOP10 carrying pCN38 containing *qacA*, *qacR*, and *qacC* from pSK41		This study
TOP10 (pCN38-*qacA qacR qacC* [pSK89])	DSM1976	TOP10 carrying pCN38 containing *qacA*, *qacR*, and *qacC* from pSK89		This study
DH5 α (pCN51)	DSM1933	DH5 α carrying pCN51		This study
TOP10 (pCN51-*norA*)	DSM1977	TOP10 carrying pCN51 containing *norA* from *S. aureus* RN4220		This study
TOP10 (pCN51-*qacC*)	DSM1978	TOP10 carrying pCN51 containing *qacC* from pSK41 (accession number AF051917.1)		This study
TOP10 (pCN51-*qacA*)	DSM1979	TOP10 carrying pCN51 containing *qacA* from pC02 (accession number CP012121.2)		This study
TOP10 (pKOR1-Δ*norA*)	DSM1930	TOP10 carrying pKOR1 containing a fused upstream and downstream region of *norA*		This study
TOP10 (pKOR1-N1)	DSM1931	TOP10 carrying pKOR1 containing *norA* promoter with the N1 mutation		This study
TOP10 (pKOR1-N2)	DSM1932	TOP10 carrying pKOR1 containing *norA* promoter with the N2 mutation		This Study
Plasmids				
pCN38		Shuttle vector for *E. coli* and *S. aureus*	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	67
pCN38-*qacC* [pSK41]		pCN38 containing *qacC* from pSK41	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	This study

(Continued on next page)

TABLE 1 (Continued)

Strain or plasmid	Lab strain designation	Description[a]	*Gene* (*norA*, *qacC*, and/or *qacA*) present or other description[b]	Reference
pCN38-*qacC* [pSK89]		pCN38 containing *qacC* from pSK89	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	This study
pCN38-*qacA qacR*		pCN38 containing *qacA* and *qacR* from *S. aureus* 2014.C02	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	This study
pCN38-*qacA qacR qacC* [pSK41]		pCN38 containing *qacA* and *qacR* from *S. aureus* 2014.C02 and *qacC* from pSK41	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	This study
pCN38-*qacA qacR qacC* [pSK89]		pCN38 containing *qacA* and *qacR* from *S. aureus* 2014.C02 and *qacC* from pSK89	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*	This study
pCN51		Shuttle vector for *E. coli* and *S. aureus*. Plasmid multicloning site is located downstream of a cadmium-inducible promoter.	Encodes ampicillin resistance in *E. coli* and erythromycin resistance in *S. aureus*	67
pCN51-*norA*		pCN51 containing *norA* from *S. aureus* RN4220	Encodes ampicillin resistance in *E. coli* and erythromycin resistance in *S. aureus*	This study
pCN51-*qacA*		pCN51 containing *qacA* from *S. aureus* 2014.C02	Encodes ampicillin resistance in *E. coli* and erythromycin resistance in *S. aureus*	This study
pCN51-*qacC*		pCN51 containing *qacC* from pSK41	Encodes ampicillin resistance in *E. coli* and erythromycin resistance in *S. aureus*	This study
pKOR1-Δ*norA*		pKOR1 containing the Δ*norA* construct	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*. Plasmid used to create *S. aureus* RN4220 Δ*norA*.	This study
pKOR1-N1		pKOR1 containing the N1 *norA* promoter construct	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*. Plasmid used to create *S. aureus* RN4220 N1.	This study
pKOR1-N2		pKOR1 containing the N2 *norA* promoter construct	Encodes ampicillin resistance in *E. coli* and chloramphenicol resistance in *S. aureus*. Plasmid used to create *S. aureus* RN4220 N2.	This study

[a]Accession numbers are from GenBank.
[b]"+" indicates increased expression.

TABLE 2 Isogenic strain MICs

Strain	MIC (μg/ml)[a]											
	Chlorhexidine		Chlorhexidine digluconate		Benzalkonium chloride		Cetrimide		Pentamidine isethionate		Ethidium bromide	
	Median	Max	Median	Max	Median	Max	Median	Max	Median	Max	Median	Max
RN4220	1.6	1.6	2	4	4	4	2	2	25	25	8	8
RN4220 Δ*norA*	*0.8*	*0.8*	*1*	*2*	*1*	*2*	*0.5*	*0.5*	*1.56*	*1.56*	*2*	*2*
RN4220 Δ*norA* (pCN51-*norA*)	**3.2**	**3.2**	**4**	4	**8**	**8**	**8**	**8**	**50**	**50**	**16**	**16**
RN4220 N1	1.6	1.6	**4**	4	**8**	**8**	**4**	**4**	25	**50**	**16**	**16**
RN4220 N2	**3.2**	**3.2**	**4**	4	**8**	**8**	**4**	**4**	**50**	**50**	**32**	**32**
RN4220 (pCN38)	1.6	1.6	2	*2*	4	4	2	2	*12.5*	*12.5*	8	8
RN4220 (pCN38-*qacC* [pSK89])	1.6	1.6	2	*2*	4	4	2	2	*12.5*	*12.5*	8	8
RN4220 (pCN38-*qacC* [pSK41])	1.6	1.6	**3**	4	**16**	**16**	**8**	**8**	*12.5*	25	**128**	**128**
RN4220 (pCN38-*qacA qacR*)	**3.2**	**3.2**	**4**	4	**16**	**16**	**4**	**4**	**400**	**400**	**128**	**128**
RN4220 Δ*norA* (pCN38-*qacA qacR*)	**3.2**	**3.2**	**4**	4	**16**	**16**	**4**	**8**	**300**	**400**	**128**	**128**
RN4220 (pCN38-*qacA qacR qacC* [pSK41])	**3.2**	**3.2**	**4**	4	**16**	**16**	**16**	**16**	**200**	**400**	**128**	**128**
RN4220 (pCN38-*qacA qacR qacC* [pSK89])	**3.2**	**3.2**	**4**	4	**8**	**16**	**8**	**8**	**200**	**400**	**128**	**128**
RN4220 N1 (pCN38-*qacA qacR*)	**3.2**	**3.2**	**4**	4	**16**	**16**	**8**	**8**	**400**	**400**	**128**	**256**
S. aureus 2014.C02	**3.2**	**3.2**	**4**	**8**	**16**	**16**	**8**	**8**	**400**	**400**	**256**	**256**

[a]Values are from ≥3 independent replicates. Values in italics indicate a MIC less than that of the wild type. Bold values indicate MIC greater than that of the wild type. Max, maximum.

significant differences in growth were observed (Fig. 1C). In addition, to determine if the various efflux systems altered antibiotic resistance of the strains, all of the isogenic strains were tested for antibiotic resistance using the BD Phoenix system. Resistance profiles for ampicillin-sulbactam, cefazolin, cefoxitin, clindamycin, daptomycin, erythromycin, gentamicin, levofloxacin, linezolid, minocycline, moxifloxacin, nitrofurantoin, oxacillin, penicillin G, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were defined, and no significant changes in antibiotic resistance were observed between the isogenic strains (data not shown). Taken together, the basic characterizations indicate that the isogenic strains are comparable to wild-type RN4220 in terms of growth and antibiotic resistance.

Isogenic strain MICs and MBCs to various antiseptic compounds. To determine the relative contribution of the various efflux pumps to antiseptic resistance, we next determined the MIC and MBC to CHX, CHX digluconate, BKC, cetrimide, pentamidine isethionate, and ethidium bromide for each of the isogenic strains. Two forms of CHX were tested since literature has reported MICs using both pure CHX and the more soluble CHX digluconate (37, 44). BKC and cetrimide are QACs that represent commonly used antiseptics, and pentamidine isethionate and ethidium bromide are known substrates for NorA, QacA, and QacC (28, 37). To facilitate MIC and MBC comparisons to an additional strain background, we also included the recent *qacA*-positive isolate *S. aureus* 2014.C02 (C02) (44).

The MICs of the individual compounds against the various strains are presented in Table 2. In terms of NorA, deleting *norA* resulted in increased susceptibility to all of the tested compounds. This strongly suggests that *norA* is important for basal resistance to all of the tested compounds. Furthermore, strains that expressed higher levels of *norA* showed higher levels of resistance to the majority of antiseptics (Table 2). Of note, after several failed attempts to clone *norA* and its native promoter into pCN38, the functional complementation of *norA* was accomplished by fusing *norA* to the cadmium-inducible promoter carried in pCN51. The leaky expression of this promoter provided functional complementation and resulted in a phenotype similar to that of the isogenic strains that overexpress *norA* (Table 2). Thus, in terms of NorA, the results demonstrated increased susceptibility to antiseptics in RN4220 Δ*norA* juxtaposed with increased antiseptic resistance in strains overexpressing *norA* (Table 2). Taken together, the data indicate that NorA is a key efflux pump for antiseptic resistance.

Two distinct phenotypes were observed with the two *qacC* variants (Table 2). The strains harboring *qacC* [pSK41] showed increased resistance to BKC, cetrimide, and

TABLE 3 Isogenic strain MBCs

Strain	MBC (μg/ml)[a]											
	Chlorhexidine		Chlorhexidine digluconate		Benzalkonium chloride		Cetrimide		Pentamidine isethionate		Ethidium bromide	
	Median	Max	Median	Max	Median	Max	Median	Max	Median	Max	Median	Max
RN4220	3.2	3.2	2	8	8	8	4	4	25	50	16	32
RN4220 Δ*norA*	3.2	3.2	**4**	*4*	8	8	*2*	*2*	*3.1*	*3.1*	*2*	*8*
RN4220 Δ*norA* (pCN51-*norA*)	3.2	3.2	**4**	*4*	8	8	**8**	**8**	**50**	50	16	*16*
RN4220 N1	3.2	3.2	**4**	*4*	8	8	4	**8**	**50**	50	**32**	32
RN4220 N2	3.2	3.2	**8**	**16**	**16**	**16**	**8**	**16**	**100**	**100**	**32**	**64**
RN4220 (pCN38)	3.2	3.2	**4**	*4*	*4*	8	*2*	4	25	*25*	16	*16*
RN4220 (pCN38-*qacC* [pSK89])	3.2	3.2	2	*4*	8	8	4	4	25	*25*	**32**	32
RN4220 (pCN38-*qacC* [pSK41])	3.2	3.2	**4**	8	**32**	**32**	**8**	**16**	*25*	*25*	**256**	**256**
RN4220 (pCN38-*qacA qacR*)	3.2	3.2	**4**	8	**16**	**16**	**8**	**16**	**400**	**800**	**256**	**256**
RN4220 Δ*norA* (pCN38-*qacA qacR*)	3.2	3.2	**8**	**16**	**16**	**32**	4	**8**	**400**	**400**	**128**	**128**
RN4220 (pCN38-*qacA qacR qacC* [pSK41])	3.2	**6.4**	**8**	8	**16**	**16**	**16**	**16**	**200**	**200**	**256**	**256**
RN4220 (pCN38-*qacA qacR qacC* [pSK89])	3.2	3.2	**8**	8	**32**	**64**	**16**	**16**	**200**	**200**	**128**	**128**
RN4220 N1 (pCN38-*qacA qacR*)	**6.4**	**6.4**	**4**	8	**16**	**16**	**16**	**16**	**400**	**400**	**256**	**256**
S. aureus 2014.C02	3.2	3.2	**8**	8	**16**	**16**	**8**	**16**	**400**	**800**	**256**	**512**

[a]Values are from 3 independent replicates. Values in italics indicate an MBC less than that of the wild type. Bold values indicate an MBC greater than that of the wild type.

ethidium bromide. In comparison, strains carrying *qacC* [pSK89] exhibited no increases in antiseptic resistance compared to RN4220.

Similar to the case with *norA*, carriage of *qacA* was associated with increased resistance to the majority of tested antiseptics (Table 2). Strains containing *qacA* displayed increased resistance to CHX, BKC, and the highest observed resistance to pentamidine isethionate and ethidium bromide. Unexpectedly, in RN4220 Δ*norA* (pCN38-*qacA qacR*), the absence of *norA* appeared inconsequential; carriage of *qacA* provided levels of resistance similar to those of the strain that carried both *qacA* and *norA* [RN4220 (pCN38-*qacA qacR*)]. Similarly, even compared to those of the isogenic strains overexpressing *norA*, the isogenic *qacA*-containing strains demonstrated enhanced resistances to several of the compounds. Of note, the isogenic RN4220 *qacA*-containing strains were phenotypically similar to the previously characterized naturally isolated *qacA*-positive strain C02.

Among the strains that carried multiple efflux pumps, RN4220 N1 (pCN38-*qacA qacR*) demonstrated a resistance phenotype similar to that of strain C02, which naturally harbors both *norA* and *qacA* (Table 2). The functional compatibility of *qacA*, *qacC*, and *norA* in a single strain was most evident in RN4220 (pCN38-*qacA qacR qacC* [pSK41]); this strain displayed the highest resistance to cetrimide (Table 2). Thus, strains that carry multiple antiseptic efflux pumps may show increased resistance to some antiseptics.

The MBC data (Table 3) showed a trend similar to that of the MIC data (Table 2). However, similar to prior reports (37, 45), there was increased variability in the MBC data. For example, RN4220 N1 (pCN38-*qacA qacR*) displayed the highest resistance to CHX; however, this observation was not maintained with CHX gluconate. Overall, when combined with the MIC data, these results demonstrated that *qacA* provided the highest level of resistance; however, strains with promoter mutations in *norA* also showed enhanced resistance compared to that of wild-type RN4220. Additionally, the data suggest that strains carrying all three efflux pumps may show increased resistance to some antiseptics.

Fitness cost of efflux gene overexpression. As described above, we were unsuccessful in our initial attempts to complement the RN4220 Δ*norA* strain by cloning *norA* with its native promoter into pCN38. On the few occasions where pCN38-*norA*-containing *S. aureus* colonies were obtained, sequencing of *norA* revealed nonsynonymous mutations in the coding sequence (data not shown). This difficulty led us to hypothesize that *norA* expression is limited in *S. aureus* by a fitness cost associated with

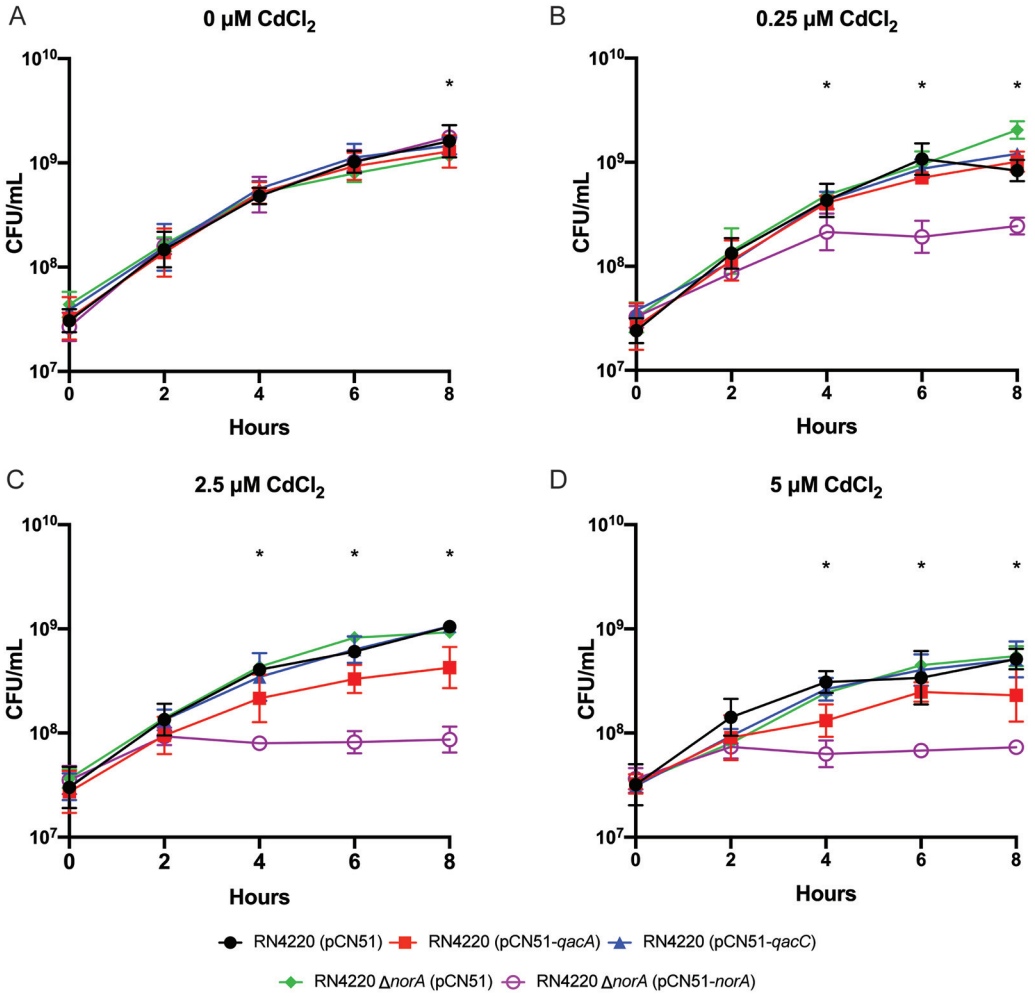

FIG 2 Overexpression of *norA*, *qacA*, and *qacC*. Promotorless *norA*, *qacA*, and *qacC* were transcriptionally fused to a cadmium-inducible promoter in pCN51. Overnight cultures were diluted to an OD_{600} of 0.05, and strains were grown for 8 h in increasing concentrations of cadmium chloride. CFU were determined every 2 h for 8 h. Each panel indicates a growth curve in the presence of the indicated concentration of cadmium chloride. The geometric mean and geometric SD are plotted from ≥3 biologically independent replicates. *, $P < 0.05$ between RN4220 Δ*norA* (pCN51) and RN4220 Δ*norA* (pCN51-*norA*). In panel A, the observed significance between RN4220 Δ*norA* (pCN51) and RN4220 Δ*norA* (pCN51-*norA*) was due to the nearly identical CFU counts between replicates and likely not biologically relevant. Significant differences were determined by two-way ANOVA with Tukey's correction for multiple comparisons.

norA overexpression. This led us to wonder if overexpression of *qacA* and *qacC* could also affect the fitness of *S. aureus*. To test the effects of efflux gene overexpression, we generated isogenic strains that carried *norA*, *qacA*, and *qacC* under the control of the cadmium-inducible promoter found in pCN51; empty-pCN51-containing control strains were also constructed [RN4220 (pCN51) and RN4220 Δ*norA* (pCN51)] and analyzed. Even low concentrations of cadmium chloride resulted in a significant decrease in growth of RN4220 (pCN51-*norA*) (Fig. 2). For the strain overexpressing *qacA*, a slight, but statistically insignificant, growth defect was evident at 2.5 μM and higher concentrations of cadmium chloride. Of note, we did not observe any growth inhibition associated with overexpression of *qacC* at any concentration of cadmium chloride. Taken together, these data indicated that overexpression of *norA* resulted in a pronounced fitness cost, overexpression of *qacA* resulted in a mild defect, and overexpression of *qacC* showed no growth phenotype. Thus, overall levels of expression of *norA* and *qacA* in *S. aureus* may be limited by a cost to host fitness.

Efflux pump expression after antiseptic exposure. Although a few studies have looked at efflux gene expression in response to antiseptic exposure (30, 33, 46), there

is currently a poor overall understanding of how antiseptic exposure may affect transcription of *norA*, *qacA*, and *qacC*. To address this gap, the isogenic strains were exposed to subinhibitory concentrations of CHX and BKC and expression of the various genes was monitored every 15 min for 1 h. After exposure to CHX, *qacC* [pSK41] showed no change in expression. In comparison, *qacA*, *norA*, and *qacC* [pSK89] showed a transient 2-fold increase in expression at 15 min, but expression levels returned to basal levels by 30 min postexposure (Fig. 3A). In comparison, exposure to BKC resulted in a sustained 10-fold increase in *qacA* expression (Fig. 3B). Similarly, *norA* showed a sustained 2-fold increase in expression (Fig. 3B). Expression was unchanged for *qacC* [pSK41] and *qacC* [pSK89]. Taken together, these observations support previous reports that indicate that CHX is not a strong inducer of efflux gene expression compared to BKC, which is known to induce both *qacA* and *norA* expression (33).

Though isogenic strains are a valuable tool that can mitigate strain background issues and allow one to compare various strains with a greater understanding of exact strain differences, the genetic content found in various clinical isolates likely affects expression of genes of interest. Furthermore, little is known about *qacA* expression when the gene is carried on native plasmids; the majority of transcriptional studies have involved cloning of *qacA* and *qacR* into shuttle vectors (30, 46). Given these facts and the apparent importance of QacA in antiseptic resistance (Tables 2 and 3), we next assessed *qacA* expression changes in response to CHX and BKC exposure in a small collection of previously described *S. aureus* USA300 isolates (18). Unlike the constructed RN4220 strain, no *qacA* expression changes were observed in the USA300 strains after the addition of CHX (Fig. 3C). In contrast, a 3- to 7-fold increase in *qacA* expression was clearly evident after BKC induction (Fig. 3D). Given the differences in the transcriptional changes observed in the various strains, we reasoned that the basal levels of *qacA* expression might also differ across the various strains. Indeed, compared to that of the RN4220 isogenic strain, the USA300 isolates all showed increased basal levels of *qacA* expression (Fig. 3E). Though not completely clear, these differences are likely affected by the basic plasmid characteristics of the plasmids on which *qacA* is carried. For example, pCN38 and pC02 show differences in copy number (47) and replication initiation genes (47). Furthermore, gene copy number likely affects the basal level of expression and ultimate potential for induction of *qacA* within a strain. Taken together, the results indicate that though there are strain differences, early transcriptional activation of efflux genes can occur with different compounds but is most robust and sustained upon exposure to BKC.

Overexpression of *qacA* increases CHX resistance. The expression differences observed with BKC and CHX suggested that *qacA*-mediated CHX resistance may be limited by overall weak CHX-mediated induction of *qacA*. Thus, we sought to determine if increased production of *qacA* could further enhance CHX resistance using the isogenic strain carrying *qacA* and *qacR* [RN4220 (pCN38-*qacA qacR*)] compared to an isogenic strain carrying *qacA* under the control of a cadmium-inducible promoter [RN4220 (pCN51-*qacA*)]. A CHX broth macrodilution assay was performed with these strains in increasing concentrations of CHX with or without the addition of exogenous cadmium chloride; appropriate vector controls were included. In the absence of cadmium chloride, RN4220 (pCN38-*qacA qacR*) showed the highest MIC to CHX (~0.8 μg/ml), followed by RN4220 (pCN51-*qacA*), which had a MIC of ~0.4 μg/ml (Fig. 4A). When 2.5 μM cadmium chloride was added to the medium, the MIC of RN4220 (pCN38-*qacA qacR*) was unchanged (Fig. 4B). However, RN4220 (pCN51-*qacA*) showed a MIC that increased to 1.2 μg/ml. Thus, overexpression of *qacA* in RN4220 (pCN51-*qacA*) significantly increased CHX resistance compared to *qacA* expressed from its native promoter. This increase suggests that *qacA*-mediated resistance to CHX may be limited by the overall induction potential of CHX and that a potential mechanism for increased CHX resistance in *qacA* harboring isolates would be to alter regulation of *qacA* expression.

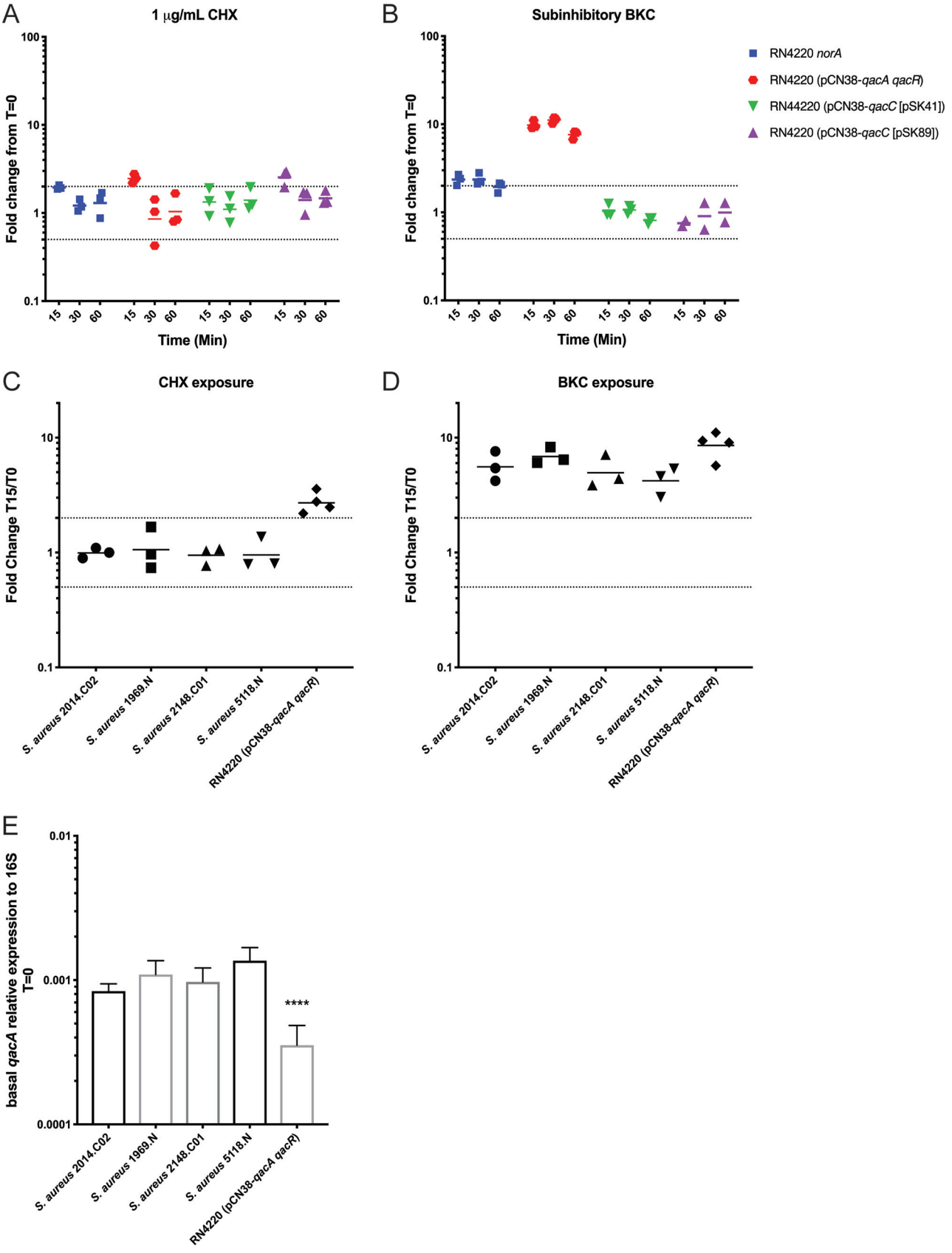

FIG 3 Efflux pump gene expression before or after antiseptic exposure. (A and B) The expression of *norA*, *qacA*, and the two *qacC* variants after exposure to 1 μg/ml of CHX or subinhibitory concentrations of BKC {2.5 μg/ml for RN4220 and RN4220 (pCN38-*qacC* [pSK89]) or 5 μg/ml for RN4220 (pCN38-*qacA*

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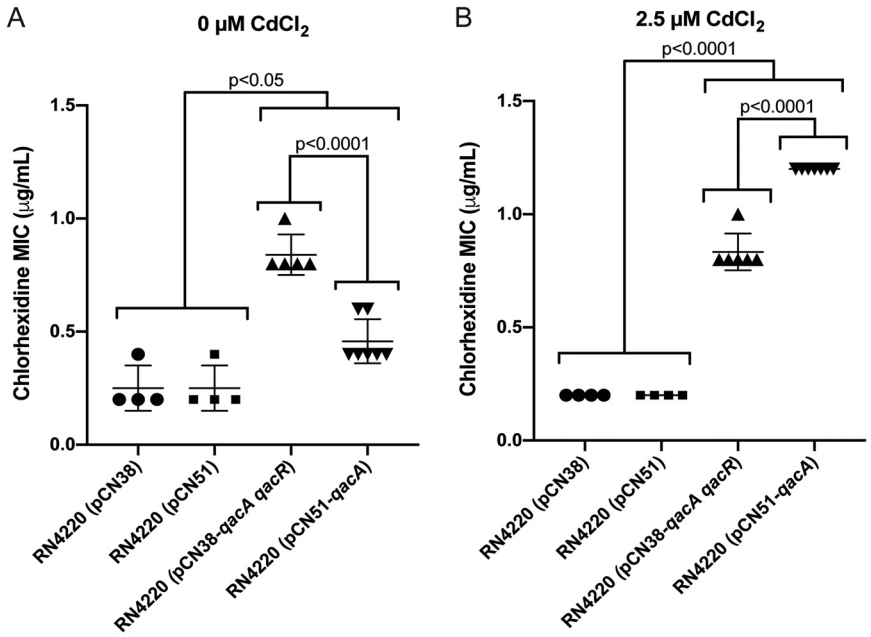

FIG 4 Overexpression of *qacA* increases CHX resistance. The MIC to CHX was determined for RN4220 (pCN38-*qacA qacR*), RN4220 (pCN51-*qacA*), and the indicated RN4220 vector controls in the presence of 0 μM or 2.5 μM cadmium chloride. The data from at least 4 biologically independent replicates are plotted with the mean and standard deviation indicated by error bars. The error bars represent the SD. The *P* values above the graphs indicate statistically significant differences between the indicated groups as determined by one-way ANOVA with Tukey's correction for multiple comparisons.

Preexposure to BKC increases CHX tolerance. The sustained increases in *norA* and *qacA* induced by BKC (Fig. 3B and D) combined with the abundance of BKC and other QACs found in personal-care and household products present a possible source for selection for increased CHX tolerance in *S. aureus* strains that harbor both genes. Indeed, we reasoned that even transient low-level exposure to these ubiquitous compounds may decrease the killing potential of other antiseptics. To investigate this possibility, we next assessed if preexposure to BKC would increase CHX tolerance. To this end, RN4220 (pCN38-*qacA qacR*) cells were exposed to water (vehicle control) or a subinhibitory concentration of BKC for 60 min. Cells were then washed and inoculated into inhibitory concentrations of CHX and the percent survival was determined at different time points after CHX exposure. Though no dramatic differences between treatment groups were seen at 5 μg/ml of CHX, differences in survival were apparent at 10 μg/ml of CHX; the tendency of BKC-treated cells to be more tolerant to CHX exposure was evident at 60 s (Fig. 5A) and statistically significant at 120 s (Fig. 5B).

We observed the same enhanced tolerance in C02, a clinical MRSA isolate, albeit with a higher concentration and longer exposure times (Fig. 5C and D). C02 exposed to BKC displayed enhanced survival in 15 μg/ml of CHX (Fig. 5D). Indeed, the increase in survival seen with BKC-exposed cells was approximately 15-fold the survival seen with

FIG 3 Legend (Continued)
qacR) and RN4220 (pCN38-*qacC* [pSK41])} was examined. Fold differences in gene expression compared to expression prior to the addition of antiseptic were calculated at 15, 30, and 60 min. The geometric means from ≥3 biologically independent replicates are plotted (except for panel B; two replicates were completed for RN4220 (pCN38-*qacC* [pSK89]), as no change was observed). The dotted lines indicate a 2-fold increase or decrease in expression. (C and D) The expression of *qacA* in *S. aureus* USA300 strains was examined after exposure to 1 μg/ml of CHX or 5 μg/ml of BKC. For comparison, expression results from RN4220 (pCN38-*qacA qacR*) were included. Fold differences in gene expression compared to expression prior to the addition of antiseptic were calculated at 15, 30, and 60 min. The geometric means from ≥3 biologically independent replicates are plotted. (E) The basal level relative expression of *qacA* compared to the 16S gene was examined in the indicated strains. The geometric mean is plotted, and the error bars indicate the geometric SD. ****, $P < 0.0001$ compared to the values for all other strains. Significant differences were determined by one-way ANOVA with Tukey's correction for multiple comparisons.

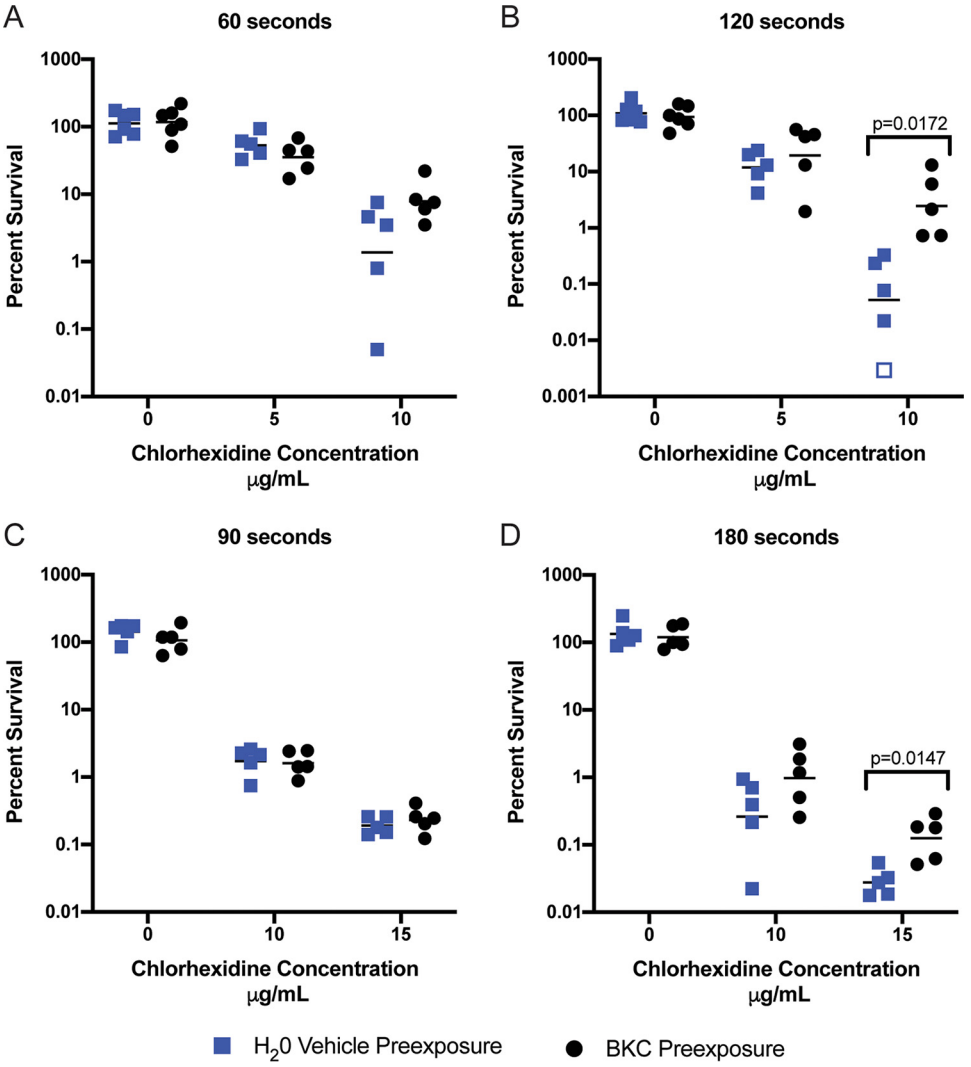

FIG 5 Preexposure to BKC increases CHX resistance. (A and B) RN4220 (pCN38-*qacA qacR*) was grown to log phase and exposed to either H_2O (vehicle control) or BKC for 60 min. Cells were then washed and inoculated into the indicated concentration of CHX. CFU were determined immediately following inoculation and at 60 and 120 s. (C and D) C02 was grown to log phase and exposed to either H_2O (vehicle control) or BKC for 60 min. Cells were then washed and inoculated into the indicated concentration of CHX. CFU were determined immediately following inoculation and at 90 and 180 s. Percent survival was calculated as (CFU at *N* seconds/CFU of input) × 100, where *N* equals indicated chlorhexidine exposure times. Graphed values for percent survival are from ≥5 independent replicates, and the bars indicate the geometric means. The open square represents a replicate for which no bacteria were recovered and is graphed at the limit of detection. The *P* values above the graphs indicate statistically significant differences as determined by Student *t* test with a correction for multiple comparisons using the Holm-Sidak method.

control cells. Thus, preexposure to a subinhibitory concentration of BKC can increase tolerance to CHX in *S. aureus* strains that contain *norA* and *qacA*.

DISCUSSION

Given the skyrocketing rates of antibiotic resistance, infection prevention has become even more important than in the decades before widespread resistance. Among prevention approaches, antiseptics are crucial in the battle against hospital-acquired infections and pan-drug-resistant bacteria. Consequently, a number of these compounds have come into common use in the hospital as well as community setting. For example, CHX is a well-tolerated antiseptic that has been shown to be effective as a presurgical skin preparation. Though prior thought suggested that antiseptic resistance was unlikely (7), the ubiquitous use of CHX in hospitals and the community has led to

the appearance of strains of bacteria, including *S. aureus*, that are more resistant to CHX (9, 48). Furthermore, the increasing presence of QACs such as BKC in everyday personal-care products appears to have provided an additional selection for efflux-mediated antiseptic resistance in bacteria. In *S. aureus*, the functional redundancy of efflux pumps associated with antiseptic resistance highlights the importance of these pumps within this pathogen. Moreover, the presence of multiple pumps provides multiple pathways for resistance evolution. Using a panel of isogenic *S. aureus* strains, we investigated the contribution of *norA*, *qacA*, and *qacC* to antiseptic resistance. Though *qacA* imparted the highest level of resistance of an individual pump, all three genes could individually alter antiseptic resistance. Moreover, strains that carried multiple efflux pumps showed the highest level of achieved resistance for some antiseptics. Overexpression of *qacA* or *norA* further increased CHX resistance. Alarmingly, preexposure of two different *norA*- and *qacA*-positive strains to subinhibitory concentrations of BKC subsequently increased their resistance to CHX. Given the widespread prevalence of these antiseptics in common-use items, this finding presents a potential cause for concern.

In terms of evolution of antiseptic resistance, it is important to consider that while *qacA* and *qacC* are typically horizontally acquired factors, *norA* exists as part of the core genome of *S. aureus*; *qacA* and *qacC* typically reside on a variety of conjugative and nonconjugative plasmids (18, 42, 49, 50). Importantly, naturally occurring *S. aureus* isolates that concomitantly carry *norA*, *qacA*, and *qacC* have been documented (20–22, 51). Unfortunately, the genome sequences of these strains are not available, so it is impossible to know whether genomic localization of the three genes follows the expected paradigm. Interestingly, no available plasmid sequence in the NCBI GenBank database contains both *qacA* and *qacC*. However, there are several examples of *Staphylococcus haemolyticus* isolates (GenBank accession numbers CP011116.1, CP025396.1, and CP024809.1) that harbor *qacA* and an *smr* family efflux pump within the chromosome. In all three of these genomes, the two genes are separated by >200 kb, suggesting that separate integration events are responsible for dual carriage. While the majority of *S. aureus* literature indicates that *qacA* typically resides on a plasmid (23), a recent publication from Australia described stable chromosomal integration of a *qacA*-positive plasmid in *S. aureus* clinical isolates (48). The integration event was linked to increased CHX tolerance and was hypothesized to result in a change in *qacA* expression. Thus, genomic location likely affects *qacA* expression, which, in turn, affects downstream antiseptic resistance. Indeed, our data that show that basal-level *qacA* expression differs depending on the plasmid on which the gene is carried (Fig. 3E) appear to support this hypothesis.

Given the complete conservation of the *qacA* promoter sequence in the strains that we examined, it is not completely clear at a mechanistic level why the basal levels of *qacA* expression differ across the examined strains. One possibility could be plasmid copy number. However, the four clinical isolates that showed the highest levels of basal *qacA* expression all harbor RepA_N replication initiation proteins, which are believed to mediate theta plasmid replication (52) and to be carried at lower copy numbers than RCR plasmids like pCN38-*qacA qacR*. Indeed, we previously showed that pC02 has a copy number of 3 or 4, while pCN38 has a copy number of 9 to 18 (47). Thus, counterintuitively, the higher levels of basal *qacA* expression were seen in the strains with a lower plasmid copy number and thus lower *qacA* copy number. This finding likely suggests that the difference in *qacA* expression is intricately tied to the amount of the QacR regulator that is produced in the various strain backgrounds. Prior work indicates that *qacR* is carried adjacent to *qacA* and is either regulated by an unknown mechanism or constitutively expressed (53, 54). Furthermore, QacR represses *qacA* by binding to the *qacA* promoter as a pair of dimers (31). Thus, if expression of *qacR* is directly proportional to its copy number in the genome, additional copies of *qacR* would increase the number of QacR proteins available to form dimers. Thus, a strain with a higher plasmid copy number would produce more QacR, which could then function to turn off *qacA* expression. Conversely, a strain with a lower plasmid copy number would produce less QacR, and thus *qacA* expression could be higher due to a

decrease in the amount of the repressor. Intriguingly, this regulatory model also explains the increased resistance seen upon *qacA* and *qacR* integration into the chromosome (48); in a single copy, smaller amounts of functional QacR dimers would lead to increased expression of *qacA* and thus increased CHX resistance. Future targeted mechanistic studies will seek to investigate the proposed regulatory model.

The importance of evolutionary changes in gene expression as a means to increase antiseptic resistance is clearly supported by data from Hardy et al. (9), who proposed that the temporal evolving increase in *S. aureus* CHX MBC may be associated with mutations in *norA* or in *norB*, which is another broad-substrate efflux pump. In this as well is in other studies (37, 38), increased expression of *norA* was associated with enhanced resistance (Tables 2 and 3). Indeed, prior reports have identified numerous *norA* promoter mutations that are associated with increased expression (38–41). However, our data strongly suggest that the extent of *norA* expression within *S. aureus* is limited by a previously unrecognized fitness cost (Fig. 2). Indeed, we were unable to move a plasmid containing *norA* under the control of its own promoter into *S. aureus*. Conversely, we were able to clone *norA* under the control of the cadmium-inducible promoter in pCN51, but we then observed a clear and significant dose-dependent growth defect of RN4220 (pCN51-*norA*) upon addition of cadmium chloride. This finding suggests a delicate balance in the tolerable expression levels of *norA*, which, in turn, places a theoretical limit to the degree of *norA* overexpression-mediated resistance that can occur in a strain. Beyond *norA* expression, it is worth mentioning that a recent report defined three possible *norA* alleles (17). It is intriguing to speculate that these various alleles may have different functional consequences in terms of *norA*-mediated antiseptic resistance; however, this topic remains to be explored.

Beyond *S. aureus*, antiseptic resistance has been observed in other bacterial species. For example, *in vitro* evolution experiments with vancomycin-resistant *Enterococcus faecium* and *Pseudomonas stutzeri* demonstrated that stable CHX resistance development was associated with efflux pump mutations and/or alterations in cellular membrane lipid composition (55, 56). Furthermore, it is known that Gram-negative cells are inherently more resistant to CHX than Gram-positive cells (6); this resistance is likely attributable to the additional lipid membrane. Nonetheless, increased resistance to CHX relative to wild-type susceptibilities has been demonstrated for numerous Gram-negative species (6). For example, a multidrug-resistant isolate of *Klebsiella pneumoniae* that was obtained from a CHX soap dispenser was able to grow in 1% CHX soap (57). Clearly, increased CHX tolerance is evident in clinically relevant bacterial species and is likely being selected for by QACs that have found their ways into many common-use items and thus into the environment.

Little is currently known about the possible effects of exposure to subinhibitory concentrations of antiseptics on bacterial cellular processes. However, a recent study demonstrated that subinhibitory CHX exposure can increase vancomycin and daptomycin resistance gene expression in *Enterococcus faecium* (58). Furthermore, induction of biofilm formation by subinhibitory QAC exposure provides another potential mechanism for increased antibiotic and antiseptic resistance (59). To date, multiple studies have shown that increased expression of *qacA* and *norA* can be induced by subinhibitory exposure to various antiseptic QACs (33, 46). These prior findings led us to the discovery that preexposure to BKC could reduce CHX-mediated killing of *S. aureus* (Fig. 5). Given this finding, one has to wonder if other QACs can promote a similar phenotype in *S. aureus* or other bacterial pathogens. Since they are important cationic surfactants, QACs are incorporated into numerous personal-care products, such as makeup, soaps, shampoos, conditioners, and lotions. Given this, it is not difficult to imagine that a patient using several of these products prior to a surgical intervention might "prime" bacterial cells to exhibit enhanced tolerance to a CHX presurgical skin preparation. This could then increase the chance of a subsequent surgical site infection. Indeed, future studies should be directed to examine the phenotypic effect of cosmetic QACs on efflux pump expression in *S. aureus* and other bacteria. Given that it is imperative that antiseptics remain highly effective at preventing infections in this age

of increased antibiotic resistance, it is likely time to consider greater antiseptic stewardship.

MATERIALS AND METHODS

Bacterial growth conditions and information. Strains were cultured in BBL Mueller-Hinton II (cation-adjusted) broth (MHB), 0.1× concentrated MHB, tryptic soy broth (TSB; Becton, Dickinson), or lysogeny broth (LB; Becton, Dickinson) as indicated for the individual experiments. Bacteria were grown at 37°C stationary or shaking at 190 rpm as indicated. When solid medium was used, broth was supplemented with 1.7% agar (Alfa Aesar). Antiseptic neutralizing broth was made as previously described (60) and contained 3% asolectin (Sigma), 10% Tween 80 (Sigma), and 1% peptone (Fisher). Neutralizing agar was modified from reference 60 and contained 1.5% TSB, 0.3% azolectin, 1% Tween 80, and 1.7% agar. All isogenic strains were constructed in the *S. aureus* RN4220 background (61, 62). Primer sequences are listed in Table 4.

Construction of *S. aureus* RN4220 Δ*norA*. *norA*, which is located in the chromosome of *S. aureus*, was deleted by allelic exchange using pKOR1 (63). To construct the pKOR1-Δ*norA* vector, splicing by overlap extension (SOE) PCR was used to generate the vector insert. First, the region upstream of *norA* (1,153 bp) was amplified from *S. aureus* RN4220 genomic DNA using primers Nup-For and Nup-Rev, and the region downstream of *norA* (1,024 bp) was amplified using primers Ndn-For and Ndn-Rev. The primers included a 26-nucleotide overlapping region between the upstream and downstream regions of *norA*. Following amplification of the regions, the upstream fragment was fused to the downstream fragment by SOE PCR using Nup-For and Ndn-Rev. The resulting fragment was cloned into pKOR1 via Gateway cloning (Thermo Fisher Scientific). The resulting plasmid, pKOR1-Δ*norA*, was transformed into chemically competent *Escherichia coli* TOP10 cells, and transformants were selected on LB agar supplemented with 100 μg/ml of ampicillin (Thermo Fisher Scientific). Colonies were selected and the insert was verified by PCR using the primers Nup-For and Ndn-Rev. The resulting pKOR1-Δ*norA* plasmid was used to transform *S. aureus* RN4220, and the subsequent deletion of *norA* was completed by following the described protocol (63) to create *S. aureus* RN4220 Δ*norA*. The deletion of *norA* was verified by PCR using primers Ncon-For and Ncon-Rev.

Construction of *S. aureus* RN4220 N1. To construct *S. aureus* RN4220 N1, site-directed mutagenesis by allelic exchange was used to create a point mutation in the −10 region of the *norA* promoter. First, the region upstream of the N1 mutation was amplified with primers Nup-For and N1up-Rev using *S. aureus* RN4220 DNA as a template. The sequence of primer N1up-Rev contained a C-to-T transition mutation in the −10 region of *norA* at nucleotide 24793 of GenBank sequence NZ_AFGU01000065.1. The downstream region of N1 was amplified with primers N1dn-For and N12dn-Rev. Similarly, the sequence of primer N1dn-For contained a C-to-T transition mutation in the −10 region of *norA*. The two amplified regions contained a 30-bp overlap and were joined by SOE PCR using primers Nup-For and N12dn-Rev. The resulting fragment was cloned into pKOR1 via Gateway cloning. The constructed plasmid, pKOR1-N1, was used to create the point mutation in the promoter region of *norA* in *S. aureus* RN4220 following the protocol described above. To verify the mutation, the DNA was PCR amplified with primers Nup-For and Nup-Rev and sequenced with primers Nseq-For and Nseq-Rev.

Construction of *S. aureus* RN4220 N2. To construct the *S. aureus* RN4220 N2 strain, site-directed mutagenesis by allelic exchange was used to create a point mutation in the 5′ UTR of the *norA* promoter. First, the region upstream of *norA* was amplified with primers Nup-For and N2up_Rev using *S. aureus* RN4220 DNA as a template. The sequence of primer N2up-Rev contained a T-to-C transition mutation in the 5′ UTR of *norA* at nucleotide 24779 of GenBank sequence NZ_AFGU01000065.1. The downstream region of N2 was amplified with primers N2dn-For and N12dn-Rev. Similarly, the sequence of primer N2dn-For contained the transition mutation in the 5′ UTR of *norA*. The two amplified regions contained a 35-bp overlap and were joined by SOE PCR using primers Nup-For and N12dn-Rev. The resulting fragment was cloned into pKOR1 via Gateway cloning. The constructed plasmid, pKOR1-N2, was used to create the point mutation in the promoter region of *norA* in *S. aureus* RN4220 by following the protocol described above. To verify the mutation, the DNA was PCR amplified with primers Nup-For and Nup-Rev and sequenced with primers Nseq-For and Nseq-Rev.

Construction of isogenic *S. aureus* RN4220 strains carrying *qacA* and *qacC*. pCN38 was selected as the backbone to carry the plasmid-located efflux genes. *qacA* and its native regulator along with *qacR* and its native promoter were PCR amplified using primers 38*qacA*-For and 38*qacA*-Rev. The resulting product was digested with BamHI and SalI enzymes and ligated into a similarly digested pCN38. pCN38-*qacA qacR* was transformed into *E. coli*. Transformants were plated on LB agar supplemented with 100 μg/ml of ampicillin. Single colonies were picked and were grown overnight in LB supplemented with ampicillin. Plasmids were isolated from the overnight cultures with the QIAprep spin miniprep kit, and the insert was verified by amplification with 38*qacA*-For and 38*qacA*-Rev. The resulting plasmid was electroporated into *S. aureus* RN4220 to create *S. aureus* RN4220 (pCN38-*qacA qacR*). The sequence of pCN38-*qacA qacR* was confirmed by sequencing with primers *qacA*F, *qacA*seq-Rev, pCN38-For, and pCN38-Rev.

Gblocks (Integrated DNA Technologies) were synthesized to create sequences for the two *qacC* variants: *qacC* from pSK41 (GenBank accession number AF051917.1) and *qacC* from pSK89 (GenBank accession number M37889.1). The synthesized products were digested with SmaI and SacI enzymes and individually ligated into the similarly digested pCN38 vector. The *qacC*-containing plasmids were individually transformed into *E. coli* and plasmids were obtained with the QIAprep spin miniprep kit. To verify the insert, recovered plasmids were amplified with pCN38-For and pCN38-Rev. The resulting *qacC*-containing plasmids were electroporated into *S. aureus* RN4220 to create strains RN4220 (pCN38-

TABLE 4 Primer and Gblock sequences

Primer	Primer sequence (5′–3′)[a]	Reference
38qacA-For	AAAGGATCCAGATCTTCTCACAGCGTCCTAAA	This study
38qacA-Rev	AATTGTCGACTTTCAAACCTAGTCATCCTTGCC	This study
qacAF	GCTGCATTTATGACAATGTTTG	51
qacAseq-Rev	GATAAAATTGTAGAAGGAATATCCC	This study
51qacA-For	AATAGTCGACTATAGACCGATCGCACGGT	This study
51qacA-Rev	AATAGGATCCCTACAATATCTAAAAATATATGTTTAGTAC	This study
51-seqFor	GCAGATAATGATGATCGCCCTAG	This study
51-seqRev	TCTGTTAACTTATTAACTCTTTCCGC	This study
51norA-For	AATAGTCGACGGAGAAAAAAGAGGTGAGCAT	This study
51norA-Rev	AATAGGATCCACACCAAAATACTTATGCTACATATT	This study
51norA-seqFor	ATGATACGACCAGCCATTAC	This study
51norA-seqRev	CTAGTAATCCTAAATCACTACCAG	This study
Smr-F	ATAAGTACTGAAGTTATTGGAAGT	51
Smr-R	TTCCGAAAATGTTTAACGAAACTA	51
pCN38-For	GCTCACATGTTCTTTCCTGC	47
pCN38-Rev	ATTAAGTTGGGTAACGCCAG	47
qRT 16S rRNA FWD	GTGGAGGGTCATTGGAAACT	68
qRT 16S rRNA REV	CACTGGTGTTCCTCCATATCTC	68
qRTqacA-For	GTTGCATCTGCTCTAATAATG	69
qRTqacA-Rev	GGCTACCAAGTACTGCTA	69
qRTqacC-For	GCCATAAGTACTGAAGTTATTGG	This study
qRTqacC-Rev	TAGTGGTAGGTGTTGCATTG	This study
qRTnorA-For	GGTGGTATGAGTGCTGGTATGG	44
qRTnorA-Rev	GCATACGATGTGAAACTTCTGCC	44
Nup-For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCTAAGAAGACACCAAGC	This study
Nup-Rev	CAATATACACCAAAATACTTATGCTAATGCTCACCTCTTTTTTCTCCATGTC	This study
Ndn-For	GACATGGAGAAAAAAGAGGTGAGCATTAGCATAAGTATTTTGGTGTATATTG	This study
Ndn-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGACCAGATAAATGTTCTTCTGGTAC	This study
Ncon-For	GCCAACGGTGAGAAGTCAATG	This study
Ncon-Rev	CATTTGCTTGTATGAGTTCTGCGAC	This study
N1up-Rev	GTTTCTATATTATATTACAACATTGCTAC	This study
N1dn-For	GTAGCAATGTTGTAATATAATATAGAAAC	This study
N12dn-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCAATATACACCAAAATACTTATGCTA	This study
N2up-Rev	CGTAAGAAGTTTCTATATTGTATTACAACATTGC	This study
N2dn-For	GCAATGTTGTAATACAATATAGAAACTTCTTACG	This study
Nseq-For	GGTCATCTGCAAAGGTTGTTATAC	This study
Nseq-Rev	CTCCATGTCATGCTTAAAGCTG	This study
pCN51-qacC gBlock	AATAGTCGACCGAAAATTAAAAGGAGTTAAAAATGCCTTATATTTATTTAATAATAGCCATAAGTACTGAAGTTATTGGA AGTGCATTTCTTAAATCTTCAGAAGGCTTTTCAAAATTTATACCATCCTTAGGAACAATAATTTCATTTGGAATTTGTTTCTAT TTTTTAAGTAAAACAATGCAACACCTACCACTAAATATAACTTATGCAACTTGGGCGGGACTAGGTTTAGTCTTAACAACCG TAGTCTCAATAATTATTTTCAAAGAACAAATAAATCTAATAACTATAGTATCTATAGTTTTAATCATAGTCGGCGTAGTTTCG TTAAACATTTTCGGAACATCGCATTAAGGATCCAATA	This study
qacC(pSK41) gBlock	AAAAGAGCTCATAAATTTTCTCGGCATAAATGCATGTTTACTGTAAAATTGATACTGATACAAATAAAAAATAAAAGGATAGT TGCAAATGAAAAATACTTAGAATAAAATTAAATAAAATACGAAAATTAAAAGGAGTTAAAAATGCCTTATATTTATTTAAT AATAGCCATAAGTACTGAAGTTATTGGAAGTGCATTTCTTAAATCTTCAGAAGGCTTTTCAAAATTTATACCATCCTTAGGAAC AATAATTTCATTTGGAATTTGTTTCTATTTTTTAAGTAAAACAATGCAACACCTACCACTAAATATAACTTATGCAACTTG GGCGGGACTAGGTTTAGTCTTAACAACCGTAGTCTCAATAATTATTTTCAAAGAACAAATAAATCTAATAACTATAGTATCTATA GTTTTAATCATAGTCGGCGTAGTTTCGTTAAACATTTTCGGAACATCGCATTAATTGCTTTATTCCAATTGCT TTCCCGGGAAAA	This study
qacC(pSK89) gBlock	AAAAGAGCTCACTCGTTTCAAAAACCTTTCAAAAACCATCAATCCACAAAAATACCACGCGAATGACACTCAAAATACAA GACTACAATTAAAAAATACTTAGAATAAAATTAAATAAAATACGAAAATTAAAAGGAGTTAAAAATGCCTTATATTTATTTA ATAATAGCCATAAGTACTGAAGTTATTGGAAGTGCATTTCTTAAATCTTCAGAAGGCTTTTCAAAATTTATACCATCCTTAGGA ACAATAATTTCATTTGGAATTTGTTTCTATTTTTTAAGTAAAACAATGCAACACCTACCACTAAATATAACTTATGCAAC TTGGGCGGGACTAGGTTTAGTCTTAACAACCGTAGTCTCAATAATTATTTTCAAAGAACAAATAAATCTAATAACTATAGTATC TATAGTTTTAATCATAGTCGGCGTAGTTTCGTTAAACATTTTCGGAACATCGCATTAATTGCTTTATTCCAATTGCTTT CCCGGGAAAA	This study

[a]Underlining indicates restriction enzyme sites for cloning and additional nucleotides for increased restriction digestion efficiencies.

qacC [pSK41]) and RN4220 (pCN38-*qacC* [pSK89]). The correct sequences of pCN38-*qacC* [pSK41] and pCN38-*qacC* [pSK89] were confirmed by sequencing with primers pCN38-For and pCN38-Rev.

To create the strains carrying *qacA* and *qacC*, pCN38-*qacA qacR* was isolated from *S. aureus* RN4220 (pCN38-*qacA qacR*) and was digested with SmaI and SacI. The synthesized Gblocks *qacC* [pSK41] and *qacC* [pSK89] were similarly digested and independently ligated into the digested pCN38-*qacA qacR* plasmid. The pCN38-*qacA qacR qacC* [pSK41] and pCN38-*qacA qacR qacC* [pSK89] plasmids were processed through *E. coli* and *S. aureus* as described above to generate strains RN4220-pCN38 (*qacA qacR qacC* [pSK41]) and RN4220-pCN38 (*qacA qacR qacC* [pSK89]).

pCN38-*qacA qacR* was isolated from *S. aureus* RN4220 (pCN38-*qacA qacR*) and was electroporated into electrocompetent *S. aureus* RN4220 N1 and *S. aureus* RN4220 Δ*norA* to generate *S. aureus* RN4220 N1 (pCN38-*qacA qacR*) and *S. aureus* RN4220 Δ*norA* (pCN38-*qacA qacR*).

Construction of *S. aureus* RN4220 strains with controllable expression of *qacA*, *qacC*, and *norA*. pCN51 was used as the vector to carry promoterless *qacA*, *qacC*, and *norA*. The individual efflux genes

were cloned downstream of a cadmium-inducible promoter in the multicloning site of pCN51. *qacA* was PCR amplified from pC02 (GenBank accession number CP012121.2) template DNA using primers 51qacA-For and 51qacA-Rev. *norA* was PCR amplified from RN4220 template DNA using primers 51norA-For and 51norA-Rev. A Gblock for *qacC* was synthesized using the pSK41 sequence (GenBank accession number AF051917.1) *qacC* sequence. All efflux gene products were digested with SalI and BamHI and were ligated into a similarly digested pCN51. The pCN51 constructs were transformed into *E. coli* and correct inserts were confirmed as follows: in the pCN51-*qacA* plasmid using the 51qacA-For and 51qacA-Rev primers, in the pCN51-*norA* plasmids using the 51norA-For and 51norA-Rev primers, and in the pCN51-*qacC* plasmids using the Smr-F and Smr-R primers. pCN51-*qacA* and pCN51-*qacC* were electroporated into *S. aureus* RN4220 to create *S. aureus* RN4220 (pCN51-*qacA*) and *S. aureus* RN4220 (pCN51-*qacC*). pCN51-*norA* was electroporated into *S. aureus* RN4220 Δ*norA* to create RN4220 Δ*norA* (pCN51-*norA*). The sequence of pCN51-*qacA* was confirmed by sequencing with primers 51-seqFor, 51-seqRev, and qacAseq-Rev. The sequence of pCN51-*norA* was confirmed by sequencing with primers 51-SeqFor, 51seqRev, 51norA-seqFor, and 51norA-seqRev. The sequence of pCN51-*qacC* was confirmed by sequencing with primers 51-seqFor and 51-seqRev.

Isogenic strain growth curves. Cultures of the isogenic strains were grown overnight in MHB with the appropriate antibiotic selection added for plasmid maintenance. The following day, overnight cultures were subcultured to an optical density at 600 nm (OD_{600}) of 0.05 in 2 ml of MHB without antibiotics and strains were grown for 7 h with shaking at 37°C. After inoculation and at hours 1, 3, 5, and 7 of the growth curves, 10 μl was removed from the cultures and was serially diluted and plated to enumerate CFU. A similar method was used to determine the growth kinetics of strains that harbored pCN51 containing *qacA*, *qacC*, or *norA*. Isogenic strains were grown overnight in MHB with added erythromycin for plasmid maintenance. The following day, overnight cultures were subcultured to an OD_{600} of 0.05 in 2 ml of MHB without antibiotics and containing 0, 0.25, 2.5, or 5 μM cadmium chloride. Strains were grown for 8 h with shaking at 37°C and CFU were determined at time points 0, 2, 4, 6, and 8.

Phoenix antibiotic susceptibility testing. Strains were inoculated from freezer stocks onto Mueller-Hinton agar (MHA) or MHA plus antibiotics for plasmid maintenance, and individual colonies were chosen for susceptibility testing using the Becton, Dickinson Phoenix automated identification and susceptibility system (Franklin Lakes, NJ), following the manufacturer protocol and reagents. Briefly, pure colonies from overnight culture plates were suspended in the Phoenix ID broth and vortexed to generate a uniform suspension to a density of 0.5 McFarland as determined using the BD PhoenixSpec nephelometer. Twenty-five microliters of the 0.5 McFarland suspension was added to the antibiotic susceptibility testing (AST) broth and mixed gently by inversion to avoid generating bubbles. The ID and AST broths were added by pouring into the respective inoculation port of the PMIC/ID-107 panel (number 448607). Panels were placed into the instrument and run to completion (18 to 24 h). Inocula were plated on TSA overnight to ensure culture purity prior to interpretation of results. MICs to ampicillin-sulbactam, cefazolin, cefoxitin, clindamycin, daptomycin, erythromycin, gentamicin, levofloxacin, linezolid, minocycline, moxifloxacin, nitrofurantoin, oxacillin, penicillin G, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were calculated by the BD Phoenix instrument software, with interpretations determined according to Clinical and Laboratory Standards Institute (CLSI) M100 guidelines based on the bacterial identification (64).

Microbroth antiseptic MIC dilutions. Antiseptic MICs were determined by following the CLSI broth microdilution protocol, with minor modifications (65). Cultures of *S. aureus* were grown overnight in MHB with the appropriate antibiotic selection added for plasmid maintenance. Overnight cultures were diluted to an OD_{600} of 0.063 in MHB without antibiotics (which is equivalent to a McFarland dilution of 0.5). A subsequent 1/20 dilution was performed in MHB without antibiotics, and from that, 10 μl was inoculated into microtiter wells containing 2-fold dilutions of chlorhexidine (0.2 μg/ml to 6.4 μg/ml; Sigma), chlorhexidine digluconate solution (20%; 0.5 μg/ml to 64 μg/ml; Sigma), benzalkonium chloride (0.5 μg/ml to 64 μg/ml; Sigma), alkyltrimethylammonium bromide (cetrimide; 0.5 μg/ml to 32 μg/ml; Sigma), pentamidine isethionate salt (two separate dilution series: 1.156 to 100 μg/ml and 500 μg/ml to 1,600 μg/ml; Sigma), or ethidium bromide (2 μg/ml to 1,024 μg/ml; VWR). The inoculum was $\sim 5 \times 10^5$ CFU/ml. MICs were determined after 16 to 20 h of incubation at 37°C without shaking.

To determine MBCs, 10 μl was collected from wells that did not show any turbidity changes and plated on MHA to check for killing. The MBC was calculated as described previously (37).

CHX macrobroth dilutions. CHX macrobroth dilutions were performed as previously described (18, 44), with minor modifications; the 1-ml broth macrodilution increased chlorhexidine concentrations by intervals of 0.2 μg/ml (0.2 μg/ml to 1.4 μg/ml) with or without the addition of 2.5 μM cadmium chloride and an inoculum of $\sim 5 \times 10^4$ CFU/ml.

RNA expression analysis. Bacterial strains were grown overnight in MHB supplemented with antibiotics for plasmid maintenance. The next day, cells were subcultured 1/100 in 10 ml of MHB without antibiotics and grown to OD_{600} of $\sim$0.500. One milliliter of the preinduced culture was collected at the initial time point (time zero). The remaining cells were exposed to a subinhibitory concentration of antiseptic CHX (1 μg/ml) or BKC {2.5 μg/ml for RN4220 and RN4220 (pCN38-*qacC* [pSK89]) or 5 μg/ml for all other strains}, and 1-ml samples were collected at 15, 30, and 60 min after exposure. For collection, the samples were pelleted by centrifugation at 13,000 $\times$ *g* for 2 min, supernatant was removed, and the cells were immediately frozen in liquid nitrogen. Frozen cells were stored at −80°C until RNA extraction.

To isolate RNA, pelleted cells were resuspended in 200 μl of phosphate-buffered saline (PBS). Cells were mixed with 200 μl of TRIzol (Thermo Fisher Scientific) and lysed by bead beating for 4 min on setting 10 with 0.1-mm glass beads in the Bullet Blender 5 (Next Advance). After bead beating, 700 μl of

TRIzol was added to the lysed cells and the solution was briefly vortexed. Subsequently, 600 μl of chloroform was added to the lysed cells and the solution was briefly vortexed. Samples were spun at 2,844 $\times$ *g* for 20 min at 4°C. The aqueous phase was removed and mixed with equal parts of isopropanol alcohol, and RNA was processed as directed by the EasyRNA kit (Qiagen). RNA quality was examined on a 2% agarose gel.

cDNA synthesis and quantitative reverse transcription-PCR (qRT-PCR) were performed as described previously (66). The qRT-PCRs included a 16S rRNA reference gene and the efflux gene(s) of interest (*norA*, *qacA*, or *qacC*). Primers are listed in Table 4 and identified by the "qRT" name. The following cycling conditions were used for all qRT-PCRs: 95°C for 10 min (1 cycle) and 40 cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 20 s. Gene expression relative to the 16S rRNA reference gene was calculated by the $2^{-\Delta CT}$ method; fold change in gene expression over time was calculated by the $2^{-\Delta\Delta CT}$ method (70).

Antiseptic preexposure test. RN4220 (pCN38-*qacA*) was grown overnight in MHB supplemented with chloramphenicol for plasmid maintenance. The strain was subcultured 1/50 in 25 ml of MHB without antibiotics and grown to an OD_{600} of ~0.500. At that time, the culture was split into two 10-ml cultures and exposed to 5 μg/ml of BKC or to water as a vehicle control (50 μl). The cultures were grown in the presence of the inducer for 60 min. Following induction, cells were centrifuged at 2,630 $\times$ *g* for 5 min. Supernatant was removed and cells were resuspended in 10 ml of 0.1$\times$ concentrated MHB. Cells were then centrifuged at 2,630 $\times$ *g* for 5 min, and supernatant was removed from the pelleted cells. The washed cells were resuspended in 5 ml of 0.1$\times$ MHB and standardized to an OD_{600} of 0.400 in 0.1$\times$ MHB. A total of 250 μl of the standardized culture was aliquoted into 1.750 ml of 0.1$\times$ MHB supplemented with 0, 5, or 10 μg/ml of CHX. After inoculation, 10 μl was immediately taken from the CHX-treated cells to enumerate starting CFU (input). Subsequent 10-μl samples were removed at 60 and 120 s during CHX exposure (output). Cells were diluted in neutralizing broth and plated on neutralizing TSA. Plates were incubated at 37°C overnight and CFU were enumerated the following day.

A similar method was used for *S. aureus* C02, with the following modifications; C02 was grown overnight in MHB without antibiotics, and after standardization to an OD_{600} of 0.400 in 0.1$\times$ MHB, the cells were aliquoted into 1.750 ml of 0.1$\times$ MHB supplemented with 0, 10, or 15 μg/ml of CHX. After inoculation, 10 μl was immediately taken from the CHX-treated cells to enumerate starting CFU (input). Subsequent 10-μl samples were removed at 90 and 180 s after CHX exposure (output).

Percent survival was calculated using the following equation: (CFU at *N* seconds/CFU of input) $\times$ 100, where *N* equals indicated chlorhexidine exposure times.

ACKNOWLEDGMENTS

These studies were supported by a U.S. Department of Defense Program project grant (HT9404-12-1-0019) and a Military Infectious Diseases Research Program award (HU0001-15-2-0031).

The views expressed in this article reflect the results of research conducted by the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, The Uniformed Services University, the Henry M. Jackson Foundation, or the U.S. government. Some of the authors are either a military service member (M.P.S.) or federal/contracted employee of the United States government (J.S.). This work was prepared as part of their official duties. Title 17 U.S.C. 105 provides that "copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. 101 defines a U.S. government work as work prepared by a military service member or employee of the U.S. government as part of that person's official duties.

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