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MECHANISMS OF RESISTANCE

A Novel, Widespread *qacA* Allele Results in Reduced Chlorhexidine Susceptibility in *Staphylococcus epidermidis*

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ABSTRACT Chlorhexidine gluconate (CHG) is a topical antiseptic widely used in health care settings. In *Staphylococcus* spp., the pump QacA effluxes CHG, while the closely related QacB cannot due to a single amino acid substitution. We characterized 1,050 cutaneous *Staphylococcus* isolates obtained from 173 pediatric oncology patients enrolled in a multicenter CHG bathing trial. CHG susceptibility testing revealed that 63 (6%) of these isolates had elevated CHG MICs (≥4 μg/ml). Screening of all 1,050 isolates for the *qacA/B* gene (the same *qac* gene with A or B allele) by restriction fragment length polymorphism (RFLP) yielded 56 isolates with a novel *qacA/B* RFLP pattern, *qacA/B*$_{273}$. The CHG MIC was significantly higher for *qacA/B*$_{273}$-positive isolates (MIC$_{50}$, 4 μg/ml; MIC range, 0.5 to 4 μg/ml) than for other *qac* groups: *qacA*-positive isolates ($n = 559$; MIC$_{50}$, 1 μg/ml; MIC range, 0.5 to 4 μg/ml), *qacB*-positive isolates ($n = 17$; MIC$_{50}$, 1 μg/ml; MIC range, 0.25 to 2 μg/ml), and *qacA/B*-negative isolates ($n = 418$, MIC$_{50}$, 1 μg/ml; MIC range, 0.125 to 2 μg/ml) ($P = 0.001$). A high proportion of the *qacA/B*$_{273}$-positive isolates also displayed methicillin resistance (96.4%) compared to the other *qac* groups (24.9 to 61.7%) ($P = 0.001$). Whole-genome sequencing revealed that *qacA/B*$_{273}$-positive isolates encoded a variant of QacA with 2 amino acid substitutions. This new allele, named *qacA4*, was carried on the novel plasmid pAQZ1. The *qacA4*-carrying isolates belonged to the highly resistant *Staphylococcus epidermidis* sequence type 2 clone. By searching available sequence data sets, we identified 39 additional *qacA4*-carrying *S. epidermidis* strains from 5 countries. Curing an isolate of *qacA4* resulted in a 4-fold decrease in the CHG MIC, confirming the role of *qacA4* in the elevated CHG MIC. Our results highlight the importance of further studying *qacA4* and its functional role in clinical staphylococci.

KEYWORDS CHG, *Staphylococcus*, *Staphylococcus epidermidis*, chlorhexidine, chlorhexidine gluconate, coagulase-negative staphylococci, *qacA*, *qacB*, *qacA/B*

Staphylococcus epidermidis is a typical resident of the skin flora and an important cause of device-associated infections, especially central line-associated bloodstream infections (1). The success of *S. epidermidis* as an opportunistic pathogen derives from its ability to bind indwelling devices through the formation of a biofilm (2–4) and the high rate of antimicrobial resistance within the population (5, 6).

With a favorable safety profile and broad-spectrum and residual activity (7), chlorhexidine gluconate (CHG) is a promising option for skin cleansing and antisepsis for the prevention of device-associated infections. Bathing with CHG has been demonstrated to reduce the rates of central line-associated bloodstream infections (8, 9), acquisition of multidrug-resistant organisms (10), and blood culture contamination, which is frequently caused by *S. epidermidis* (11, 12). Furthermore, topical applications

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of CHG have been demonstrated to significantly reduce the cutaneous microbial burden (13, 14). However, increasing usage of CHG may select for organisms with decreased susceptibility to CHG and increased resistance to commonly prescribed antimicrobials (13, 15–17).

In *Staphylococcus* spp., *qacA* encodes a 514-amino-acid, 14-transmembrane-segment pump with the capacity to efflux CHG (18–20). The pump encoded by *qacB* (a *qacA* allelic variant) differs from that encoded by *qacA* by only 7 to 9 nucleotides but does not have the ability to efflux CHG (18, 21). A single nucleotide variant (SNV) (968C>A) resulting in a substitution, Ala323Asp, in transmembrane segment 10 accounts for the different substrate specificities of QacA and QacB (18). Currently, three alleles of *qacA* have been described; however, no functional differences between the pumps encoded by these three alleles have been reported (21).

Beyond its capacity to efflux CHG, QacA is responsible for the efflux of a broad range of mono- and divalent cations, including dyes and quaternary ammonium compounds (20). In *S. epidermidis*, *qacA* is most frequently carried by the plasmid pSK105, which also carries an *aacA/aphD* bifunctional aminoglycoside resistance gene (22). Other plasmids carrying *qacA* may contain the trimethoprim resistance gene *dfrA*, the *blaZ* β-lactamase, or genes encoding heavy metal efflux pumps (22).

In addition to QacA, the 107-amino-acid, 4-transmembrane-segment efflux pump encoded by *smr*, also known as *qacC*, has been implicated in the efflux of CHG (23–25). While it is unrelated to QacA and QacB, Smr demonstrates the capacity to efflux a similar, yet narrower range of monovalent cations (24, 25).

In our study, cutaneous *Staphylococcus* isolates were obtained from pediatric oncology patients enrolled in a multicenter randomized controlled CHG bathing trial. We identified a subpopulation of isolates with an elevated CHG MIC, which we defined as an MIC of ≥4 μg/ml. To investigate the genetic basis of the elevated CHG MIC, we screened the isolates for *qacA/B* (the same *qac* gene with A or B allele) via PCR and restriction fragment length polymorphism (RFLP). From this screening, we identified a previously undescribed RFLP pattern, termed *qacA/B*$_{273}$, in a subset of isolates. We then determined whether the *qacA/B*$_{273}$ RFLP pattern was associated with a significantly higher CHG MIC compared to that for the *qacA*-positive, *qacB*-positive, and *qacA/B*-negative isolates. We also describe the sequence of the novel *qacA* allele, referred to as *qacA4*, producing the novel *qacA/B*$_{273}$ RFLP pattern and characterized the isolates carrying *qacA4*. Furthermore, through curing experiments, we investigated the role of *qacA4* in causing elevated CHG MICs in *S. epidermidis*.

RESULTS

Overview of study population. In total, 1,050 cutaneous *Staphylococcus* isolates were obtained from 173 patients. The study isolates primarily consisted of coagulase-negative *Staphylococcus* species, with *S. epidermidis* being the most frequently recovered species (53.1%), while *S. aureus* accounted for just 2.9% of the study population (Table 1). In addition to *S. epidermidis*, 17 other coagulase-negative *Staphylococcus* species were identified in the study population. Of note, the species of four coagulase-negative *Staphylococcus* isolates could not be determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

A subset of *Staphylococcus* isolates has an elevated CHG MIC. Measuring the CHG MICs across all 1,050 isolates yielded 63 isolates with elevated CHG MICs, defined as an MIC of ≥4 μg/ml (Fig. 1a). All of these isolates were identified as *S. epidermidis*.

Identification of a novel *qacA/B* RFLP pattern. Isolates were screened for the *qacA/B* genes to explore the genetic basis of the elevated CHG MICs. PCR amplification of the *qacA/B* genes resulted in an 864-bp product. Digestion of the *qacA/B* PCR product with AluI resulted in the presence of a characteristic 198-bp fragment for *qacA*-positive isolates and a characteristic 165-bp fragment for *qacB*-positive isolates. A third subpopulation of isolates was distinguished by the appearance of a 273-bp fragment (Fig. 2), and this subpopulation is hereafter referred to as *qacA/B*$_{273}$-positive isolates.

TABLE 1 Overview of the cutaneous *Staphylococcus* isolates included in this study[a]

Species	Total no. of isolates	% of isolates
S. aureus	30	2.9
Coagulase-negative staphylococci	1,020	97.1
S. epidermidis	558	53.1
S. hominis	267	25.4
S. capitis	62	5.9
S. warneri	47	4.5
S. haemolyticus	22	2.1
S. pasteuri	16	1.5
S. saprophyticus	9	0.9
S. lugdunensis	8	0.8
S. cohnii	4	0.4
S. caprae	4	0.4
S. pettenkoferi	4	0.4
S. condiment	3	0.3
S. schleiferi	3	0.3
S. simulans	3	0.3
S. auricularis	2	0.2
S. equorum	2	0.2
S. sciuri	1	0.1
S. xylosus	1	0.1
Staphylococcus spp.	4	0.4

[a]Data are for 1,050 cutaneous *Staphylococcus* isolates identified by MALDI-TOF MS.

Of the 1,050 isolates, 632 contained *qacA/B*, as identified by PCR. Based on the results of the RFLP analysis, 559 were classified as *qacA* positive, 17 as *qacB* positive, and 56 as *qacA/B*$_{273}$ positive (Table 2). The *qacA/B* genes were detected in 8 different coagulase-negative *Staphylococcus* species. When screened for the carriage of *smr*, 279 of the 1,050 isolates were classified as *smr* positive (Table 2). In total, 12 unique coagulase-negative *Staphylococcus* species carried *smr*. Notably, the *qacA/B* genes and *smr* were not detected in any of the *S. aureus* isolates.

The *qacA/B*$_{273}$ RFLP pattern is associated with an elevated CHG MIC. Next, the relationship between elevated CHG MICs and detection of the *qacA*, *qacB*, and *smr* genes was examined. *qacA/B* was detected in each of the 63 isolates with an elevated CHG MIC: 54 were classified as *qacA/B*$_{273}$ positive and 9 were classified as *qacA* positive (Fig. 1b). None of the isolates with an elevated CHG were classified as *qacB* positive. Furthermore, 51 of the 63 isolates with an elevated CHG MIC were categorized as *smr* positive, and the remaining 12 were classified as *smr* negative (Fig. 1c).

To further investigate if the *qacA/B*$_{273}$ RFLP pattern was associated with an elevated CHG MIC, differences in the CHG MIC distributions of the *qacA/B*-containing isolates were assessed. The CHG MIC was significantly higher for the *qacA/B*$_{273}$-positive isolates than for the *qacA*-positive, *qacB*-positive, and *qacA/B*-negative isolates ($P = 0.001$); the results did not change when restricting the analyses to one randomly chosen isolate per patient per *qacA/B* group (Table 2). In addition, the CHG MIC distributions of the *smr*-positive and *smr*-negative isolates were compared. The CHG MIC was significantly higher for the *smr*-positive isolates than for the *smr*-negative isolates ($P = 0.02$); however, this comparison was no longer significant when the analyses were restricted to one randomly chosen isolate per patient per *smr* group, as one individual accounted for 20% of the *smr*-positive isolates with elevated MICs ($P = 0.11$) (Table 2).

Additionally, the CHG MIC distributions associated with the *qacA/B* and *smr* resistance gene combinations among all isolates were assessed to determine if a particular resistance gene combination was associated with elevated CHG MICs. This comparison revealed that *qacA/B*$_{273}$ rather than a particular resistance gene combination was associated with elevated CHG MICs ($P = 0.001$); the results did not change when restricting the analyses to one randomly chosen isolate per patient per gene combination (Table 3).

The *qacA/B*$_{273}$-positive isolates exhibited higher rates of resistance to methicillin (96.4%) and other commonly prescribed antimicrobials, including erythromycin (ERY;

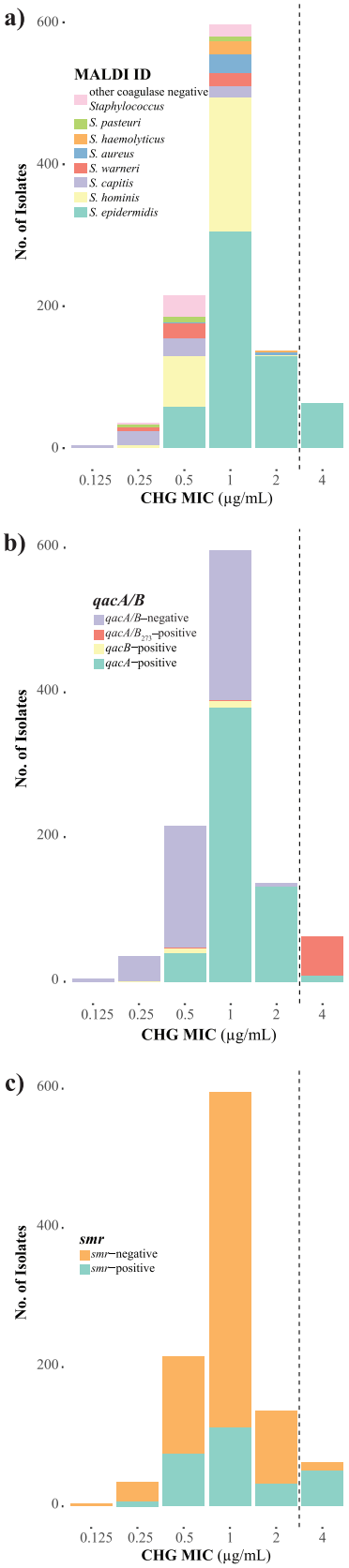

FIG 1 CHG MIC distribution of the 1,050 cutaneous *Staphylococcus* isolates included in our study, grouped by species (a), *qacA/B* PCR and RFLP patterns (b), and *smr* PCR results (c). The dashed line indicates the concentration that we defined as an elevated CHG MIC (≥4 μg/ml). MALDI ID, species identified by MALDI-TOF MS.

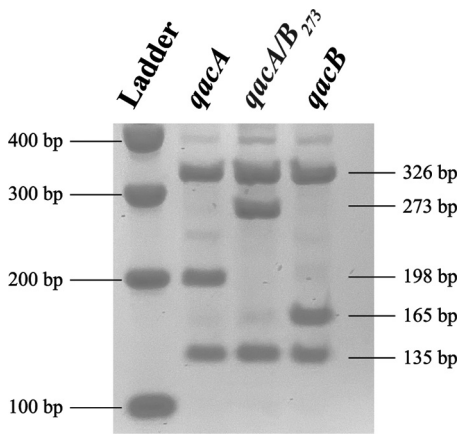

FIG 2 RFLP patterns observed from the AluI restriction digest of the *qacA/B* PCR amplicon. Isolates were classified as *qacA* positive, *qacA/B*$_{273}$ positive, or *qacB* positive based on the presence of a 198-bp, 273-bp, or 165-bp fragment, respectively (21). Ladder, 100-bp markers (Promega).

92.9%), ciprofloxacin (CIP; 96.4%), gentamicin (GEN; 89.3%), and sulfamethoxazole-trimethoprim (SXT; 98.2%), than the *qacA*-positive, *qacB*-positive, and *qacA/B*-negative isolates ($P < 0.001$ for all comparisons); the results did not change when restricting the analyses to one randomly chosen isolate per patient per *qacA/B* group (Table 4). All *qacA/B* genotypes exhibited rates of resistance of <1% to linezolid (LZD), rifampin (RIF), and vancomycin (VAN).

Whole-genome sequencing of *qacA/B*-positive isolates yields novel *qacA* alleles. To further investigate the *qacA/B* genes in the *qacA/B*$_{273}$-positive isolates, the genomes of 9 *qacA/B*$_{273}$-positive *S. epidermidis* isolates were compared to the genomes of 10 *qacA*-positive and 4 *qacB*-positive *S. epidermidis* isolates (see Table S1 in the supplemental material). All 9 of the *qacA/B*$_{273}$-positive isolates had elevated CHG MICs, while none of the 10 *qacA*-positive and 4 *qacB*-positive isolates had elevated CHG MICs.

The sequences of the *qacA/B* genes were highly conserved in the 9 *qacA/B*$_{273}$-positive isolates with elevated CHG MICs. As the *qacA/B* genes of the *qacA/B*$_{273}$-positive isolates contained the distinguishing *qacA* nucleotide 968A, the gene was classified as a novel allele of *qacA*. As shown in Fig. 3a, this allele contained three SNVs (470C>G, 819G>A, and 1133C>T) compared to the sequence of the *qacA* gene (GenBank accession number AB566410) of a reference *S. epidermidis* sequence type 2 (ST2) strain (GenBank accession number GCA_900086615.1) (26) and is henceforth referred to as *qacA4* (GenBank accession number MK040360). The SNV at position 1133 in *qacA4* resulted in the loss of an AluI digestion site, explaining the novel RFLP pattern observed in Fig. 2. Two of the SNVs resulted in amino acid substitutions Ala157Gly and Ala378Val in transmembrane segments 5 and 12, respectively (Fig. 3b).

Compared to the three previously characterized alleles of *qacA* (*qacA1* [GenBank accession number GU565967], *qacA2* [21], and *qacA3* [GenBank accession number MK040360]) and the *qacA* alleles of the 10 *qacA*-positive *S. epidermidis* isolates, *qacA4*

TABLE 2 Comparison of CHG MIC distributions of the *qacA*-positive, *qacB*-positive, *qacA/B*$_{273}$-positive, and *qacA/B*-negative isolates and CHG MIC distributions of *smr*-positive and *smr*-negative isolates

Genotype	Total no. of isolates	CHG MIC (μg/ml)		
		50%	90%	Range
qacA positive	559	1	2	0.5–4
qacB positive	17	1	1	0.25–2
qacA/B$_{273}$ positive	56	4	4	0.5–4
qacA/B negative	418	1	1	0.125–2
smr positive	279	1	2	0.25–4
smr negative	771	1	1	0.125–4

TABLE 3 Comparison of the CHG MIC distributions associated with the eight CHG resistance gene combinations detected in our isolates

Genotype	Total no. of isolates	CHG MIC (μg/ml)		
		50%	90%	Range
qacA positive and *smr* positive	101	1	2	0.5–4
qacA positive and *smr* negative	458	1	2	0.5–4
qacB positive and *smr* positive	5	1	1.6	0.5–2
qacB positive and *smr* negative	12	0.75	1	0.25–1
qacA/B$_{273}$ positive and *smr* positive	50	4	4	4–4
qacA/B$_{273}$ positive and *smr* negative	6	4	4	0.5–4
qacA/B negative, *smr* positive	123	0.5	1	0.25–1
qacA/B negative and *smr* negative	295	1	1	0.125–2

differed from these sequences by at least three SNVs, including all three that distinguished *qacA4* from the reference *qacA* sequence (Fig. S1). Notably, from the 10 *qacA*-positive isolates that we sequenced, we identified 5 additional novel *qacA* alleles: *qacA7* (GenBank accession number MK040363), *qacA8* (GenBank accession number MK040364), *qacA9* (GenBank accession number MK040365), *qacA10* (GenBank accession number MK040366), and *qacA11* (GenBank accession number MK040367) (Table S2; Fig. S1). Similarly, comparing the sequence of *qacA4* to the other sequences of *qacA* deposited in the NCBI GenBank database further confirmed that the SNVs at the three positions described above were unique to *qacA4*.

We also sequenced the genomes of the two *qacA/B*$_{273}$-positive isolates that did not have elevated CHG MICs (MIC < 4 μg/ml) to investigate their discordant genotypic-phenotypic relationship (Table S1). Neither of the *qacA/B* genes in these two isolates contained the three identifying *qacA4* mutations, and their sequences differed from the reference *qacA* sequence by six SNVs (Fig. 4). These SNVs resulted in six and seven amino acid substitutions compared to the reference *qacA* and *qacA4* sequences, respectively (Fig. 4). The *qacA/B* genes in these isolates were classified as two additional alleles of *qacA*, referred to as *qacA5* (GenBank accession number MK040361) and *qacA6* (GenBank accession number MK040362). Due to an SNV at position 1132, which resulted in the loss of an AluI digestion site, *qacA5* and *qacA6* displayed digestion patterns identical to the pattern for *qacA4*.

We next sequenced the genomes from the nine *qacA*-positive isolates that had elevated CHG MICs.

From these isolates, we identified 4 *qacA* alleles: *qacA10*, *qacA12*, *qacA13*, and *qacA14* (Table S2; Fig. S2). The sequences of the *qacA* genes in these isolates differed from the reference *qacA* sequence by 1 to 5 amino acid substitutions and from the *qacA4* sequence by 2 to 8 amino acid substitutions (Fig. S2). The allele *qacA14*, identified in isolate 96.5, contained one of the distinguishing coding changes of *qacA4*,

TABLE 4 Comparison of proportion of *qacA*-positive, *qacB*-positive, *qacA/B*$_{273}$-positive, and *qacA/B*-negative isolates resistant to commonly prescribed antimicrobials

Antimicrobial[a]	% of isolates:			
	qacA positive	*qacB* positive	*qacA/B*$_{273}$ positive	*qacA/B* negative
FOX[b]	61.7	52.9	96.4	22.7
ERY[b]	76.0	88.2	92.9	30.1
CIP[b]	25.0	5.9	96.4	8.4
GEN[b]	15.0	0	89.3	2.6
SXT[b]	60.1	35.3	98.2	22.7
LZD	0	0	0	0
RIF	0.9	0	0	0
VAN	0	0	0	0

[a]CIP, ciprofloxacin; SXT, sulfamethoxazole-trimethoprim; FOX, cefoxitin; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; RIF, rifampin;VAN, vancomycin.

[b]$P < 0.001$ for all comparisons. Results were generated using all isolates; the results did not change when restricting the analyses to one randomly chosen isolate per patient per *qacA/B* group.

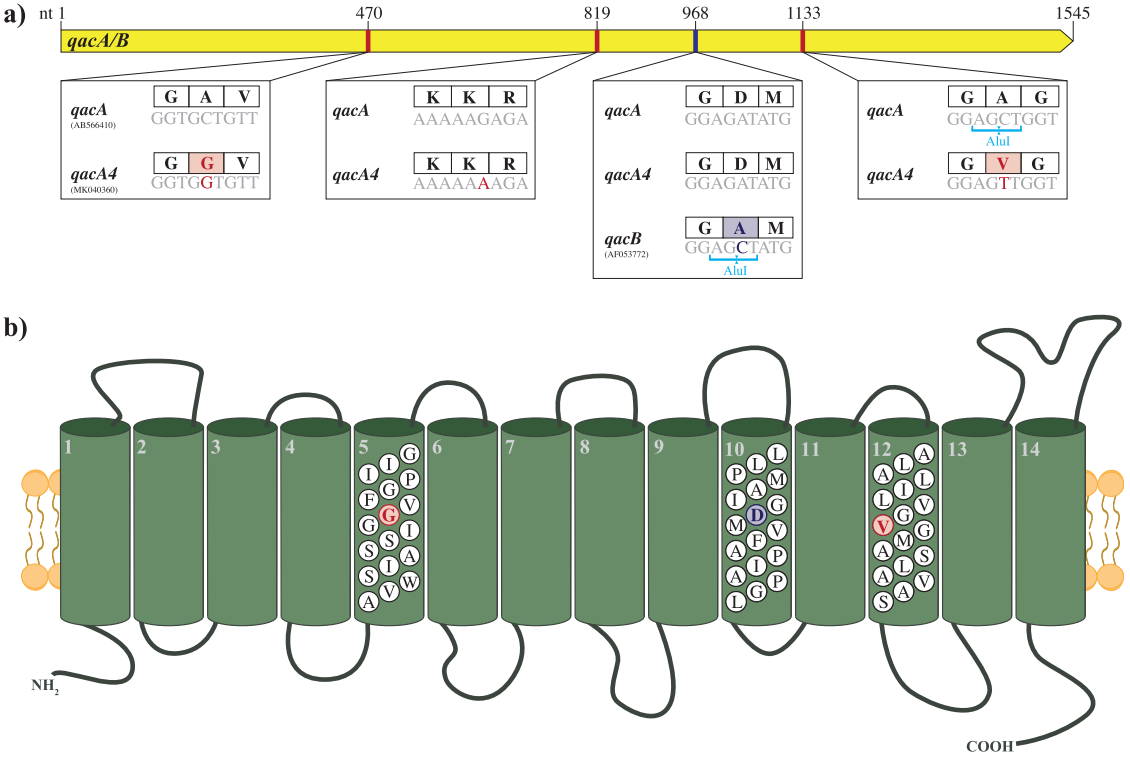

FIG 3 (a) Comparison of the sequences of *qacA4* (GenBank accession number MK040360), a reference *qacA* sequence (GenBank accession number AB566410), and a reference *qacB* sequence (GenBank accession number AF053772). The associated AluI restriction sites are shown below the nucleotide (nt) sequences, and the corresponding amino acid sequences are displayed in the boxes above the nucleotide sequences. (b) Structure of the predicted efflux pump encoded by *qacA4* (adapted from reference 20 with permission). The residues which distinguish QacA4 from the reference QacA sequence are highlighted in red. Those which distinguish QacA from QacB are displayed in blue.

Ala157Gly, but not the other coding change. This allele encoded a unique amino acid substitution Pro328Leu, which distinguished the allele from the reference *qacA* and *qacA4* sequences. Another isolate, 86.4, with an elevated CHG MIC carried the same *qacA10* allele as isolate 110.3, which did not have an elevated CHG MIC. The amino acid substitutions in these novel *qacA* alleles occurred in transmembrane segments 5, 6, 9, 10, 12, and 13 and in the extracellular loop between transmembrane segments 5 and 6.

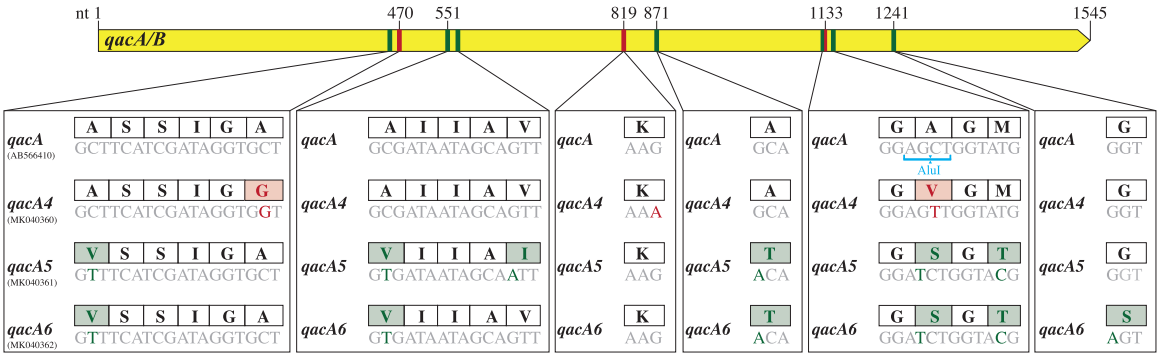

FIG 4 Comparison of the sequences of *qacA4* (GenBank accession number MK040360), *qacA5* (GenBank accession number MK040361), and *qacA6* (GenBank accession number MK040362) and the reference *qacA* sequence (GenBank accession number AB566410). The associated AluI restriction sites are shown below the nucleotide sequences, and the corresponding amino acid sequences are displayed in the boxes above the nucleotide sequences. The nucleotides which distinguish the *qacA4* sequence from the reference *qacA*, *qacA5*, and *qacA6* sequences are highlighted in red. Those which distinguish the *qacA5* and/or *qacA6* sequence from the *qacA4* and the reference *qacA* sequences are displayed in green.

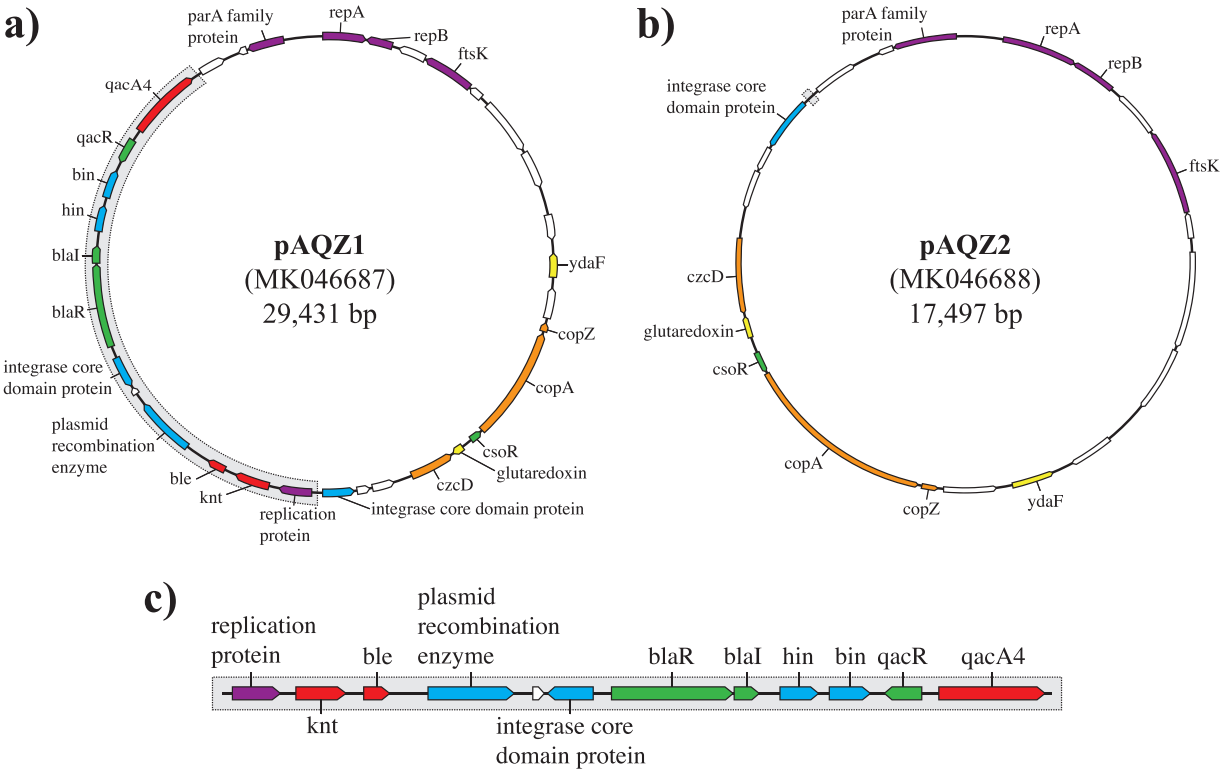

FIG 5 Schematic representations of plasmid pAQZ1 containing *qacA4* obtained from the *de novo* assemblies of isolates 91.2 and 107.2 (a), plasmid pAQZ2 retained in isolate 107.2_{cured} (b), and the 11,934-bp segment of pAQZ1 eliminated through the curing experiments (c). Open reading frames (ORFs) shown in red depict resistance genes, ORFs in orange describe heavy metal efflux genes, ORFs in green represent transcriptional regulator genes, ORFs in blue depict recombinase genes, ORFs in purple describe replication genes, ORFs in white represent hypothetical proteins, and ORFs in yellow depict genes with other functions.

Identification of the novel resistance plasmid pAQZ1 containing the *qacA4* allele. The genomic context of the *qacA4* allele in *de novo* assemblies of two separate *qacA/B*$_{273}$-positive isolates with high coverage, isolates 91.2 and 107.2, was examined to understand whether *qacA4* was encoded on the chromosome or on a plasmid. Both isolates carried *qacA4* on a 29,431-bp circular contig with coverage that was 2.8 times higher than the average chromosomal coverage, consistent with it being carried by a plasmid (Fig. 5a). The circular nature of the contig was verified by conducting PCR across the predicted junction site (data not shown). The plasmid, henceforth designated pAQZ1 (GenBank accession number MK046687), carrying *qacA4* contained the RepA replication initiation protein with a RepA_N domain (pfam06970). Similar to other RepA_N family plasmids (27, 28), the origin of replication of pAQZ1 is likely contained within *repA*. The plasmid also carried several genes involved in heavy metal efflux, including *copZ*, *copA*, *czcD*, the *knt* kanamycin resistance gene, the *ble* bleomycin resistance gene, and an incomplete β-lactamase operon.

When the sequence of pAQZ1 was compared to plasmid sequences deposited in GenBank, several regions of pAQZ1 showed high sequence similarity (>99%) with previously characterized *S. aureus* and coagulase-negative *Staphylococcus* plasmids (GenBank accession numbers CP017465 and CP023967). The complete sequence of pAQZ1, however, did not fully align with any single, previously characterized *S. aureus* or coagulase-negative *Staphylococcus* plasmid. When queried against the sequences in the NCBI WGS database, the sequence of pAQZ1 showed high sequence similarity and a query coverage of 68% and 86%, respectively, to two previously sequenced contigs from two coagulase-negative *Staphylococcus* isolates (GenBank accession numbers JZUM01000030.1 and QSTD01000014.1).

Curing analysis *in vitro* confirms that *qacA4* is responsible for the elevated CHG MICs. Transformation of the pan-susceptible type strain *S. epidermidis* TÜ1457 with

TABLE 5 Comparison of CHG resistance gene combinations and resistance patterns of isolates 107.2 and 107.2$_{cured}$

Isolate[a]	Presence of:		CHG MIC (μg/ml)	Susceptibility to[b]:							
	qacA4	*smr*		FOX	ERY	CIP	GEN	SXT	LZD	RIF	VAN
107.2	Yes	No	4	R	R	R	R	R	S	S	S
107.2$_{cured}$	No	No	1	R	R	R	R	R	S	S	S

[a]The isolates were determined to be *S. epidermidis* by MALDI-TOF MS.
[b]CIP, ciprofloxacin; SXT, sulfamethoxazole-trimethoprim; FOX, cefoxitin; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; RIF, rifampin; VAN, vancomycin; R, the isolate is resistant to the specified antimicrobial; S, the isolate is susceptible to the specified antimicrobial.

pAQZ1 was attempted but was unsuccessful (data not shown). Thus, to confirm the observed association between *qacA4* and the elevated CHG MICs, we attempted to cure *qacA4*-carrying, *smr*-negative *S. epidermidis* isolate 107.2 of the pAQZ1 plasmid. We took advantage of the ability of QacA to efflux ethidium bromide (18) to screen for colonies which lost *qacA4*. Cells without *qacA4* accumulate ethidium bromide in their cytoplasm, and the resulting colonies fluoresce under UV radiation. Those retaining *qacA4* do not accumulate ethidium bromide, and thus, the resulting colonies do not fluoresce.

After 11 successive passages in Trypticase soy broth without selection, an isolate cured of *qacA4*, referred to as isolate 107.2$_{cured}$, was identified. The CHG MIC of 107.2$_{cured}$ was 4-fold lower than that of 107.2 (Table 5). The 8-agent antimicrobial susceptibility profile of 107.2$_{cured}$ was identical to that of the parental strain (Table 5). Sequencing of 107.2$_{cured}$ (Table S1) revealed that recombination, presumably catalyzed by the recombinases on the plasmid, led to pAQZ1 eliminating an 11,934-bp segment and resulted in the formation of a new 17,497-bp plasmid. This new plasmid, pAQZ2 (GenBank accession number MK046688; Fig. 5b), retained the RepA protein of pAQZ1. The 11.9-kb segment lost in 107.2$_{cured}$ contained not only *qacA4* but also the *knt* kanamycin resistance gene, the *ble* bleomycin resistance gene, the partial β-lactamase operon, and several recombinases (Fig. 5c). PCR testing further confirmed that isolate 107.2$_{cured}$ lost the 11.9-kb segment, distinguishing pAQZ1 from pAQZ2 (data not shown). Isolate 107.2$_{cured}$ contained one coding change in its chromosome compared to the sequence of the 107.2 parental strain. This coding change occurred in a GCN5-related *N*-acetyltransferase family protein (Gly225Glu).

With the exception of *qacA4*, each of the genes present on the segment lost in isolate 107.2$_{cured}$ was identified in at least one of the *qacA*-positive control isolates without elevated CHG MICs. One of these *qacA*-positive isolates, 110.3, contained all of these other 11 genes present on the eliminated segment of pAQZ1.

Isolates carrying *qacA4* belong to highly resistant and virulent *S. epidermidis* ST2. The isolates carrying *qacA4* harbored genes and mutations which confer resistance to several classes of commonly prescribed antimicrobials (Fig. 6). Additionally, all of the sequenced *qacA4*-carrying isolates contained the biofilm formation operon, *icaADBC*. When classified by multilocus sequence typing (MLST), all these isolates belonged to *S. epidermidis* ST2.

Next, we performed a core genome analysis with 9 *qacA4*-carrying and 9 non-*qacA4*-carrying *S. epidermidis* ST2 isolates using the 2,793,003-bp chromosome of an *S. epidermidis* ST2 isolate from Australia (Genbank accession number GCA_900086615.1) as the reference genome (26). Our isolates differed from the reference strain by 1,904 to 2,186 SNVs (median, 2,148 SNVs). There was no apparent clustering based on the isolates' collection location or date (Fig. S3). The *qacA4*-carrying isolates were more closely related to one another rather than the other non-*qacA4*-carrying ST2 isolates, with the exception of isolate 96.5. We included multiple isolates (isolates 36.3 and 36.4 and isolates 125.1, 125.3, and 125.8) collected at different time points from two patients in our core genome analysis, as these isolates had unusual *qacA/B* genotypes and CHG MICs, as described in Materials and Methods. These isolates differed from their corresponding coisolates by 0 to 3 SNVs (median, 2 SNVs) (Table S3).

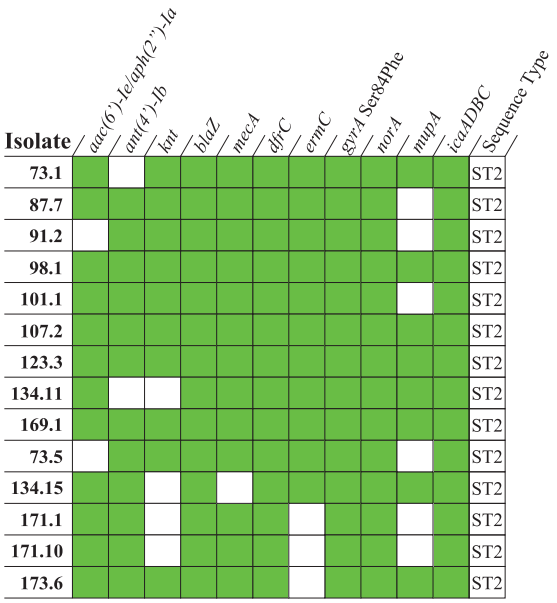

FIG 6 Presence-absence matrix displaying the antimicrobial resistance genes, resistance-associated mutations, and virulence genes identified in the isolates carrying *qacA4*. A green shaded box indicates that the resistant or virulence determinant was identified in a given isolate. The sequence type of each isolate, as determined by MLST, is shown.

The whole genomes of five additional *qacA/B*$_{273}$-positive isolates that had elevated CHG MICs but that displayed discordant susceptibility patterns (susceptible to methicillin, gentamicin, or erythromycin) were sequenced (Table S1). Each of the isolates carried *qacA4* and belonged to ST2. The divergent susceptibility patterns were explained by the absence of one or more resistance genes (Fig. 6).

***qacA4*-containing *S. epidermidis* isolates are globally distributed.** In total, 22 patients carried at least one cutaneous *S. epidermidis* isolate containing *qacA4*, as confirmed by sequencing or as presumed through the isolate's *qacA/B*$_{273}$-positive RFLP pattern and elevated CHG MIC. These 22 patients were enrolled at 14 study centers in 9 U.S. states and 2 Canadian provinces (Fig. 7). There was no obvious geographical clustering of the *qacA4*-carrying isolates.

We also identified isolates containing *qacA4* that existed outside of our study (Fig. S4). A cutaneous *S. epidermidis* isolate containing *qacA4* (Table S4) was identified from a CHG bathing pilot study (13) conducted at Seattle Children's Hospital. In addition, we searched through the sequence reads of 922 isolates deposited in the NCBI Sequence Read Archive as part of several large *S. epidermidis* sequencing projects (BioProject accession numbers PRJEB12090, PRJNA239883, PRJNA382527, PRJNA433155, PRJNA434275, PRJNA470534, and PRJNA470752) (29–33). We further queried the NCBI WGS database and all of the bacterial whole-genome sequencing data sets in the European Nucleotide Archive through December 2016 (34). Including the isolate from the CHG bathing pilot study (13), we identified *qacA4* in 39 additional *S. epidermidis* isolates collected at 10 sites in 5 different countries, including Australia, Belgium, Brazil, the United Kingdom, and the United States (Table S4). We obtained four local clinical *S. epidermidis* isolates containing *qacA4* (isolates 6_SEPI, 642_SEPI, 872_SEPI, and 1295_SEPI) from a previous study (35) conducted at the University of Washington Medical Center that were identified during our search through publicly available sequencing data. Susceptibility testing of these isolates revealed that all four isolates had an elevated CHG MIC (4 μg/ml).

One of the 39 *qacA4*-carrying isolates (isolate MB591) contained the two coding changes but not the synonymous mutation (819G>A) of *qacA4*. Another *qacA4*-carrying isolate (isolate MB2095) appeared to contain *qacA4* as well as an additional *qacA* allele.

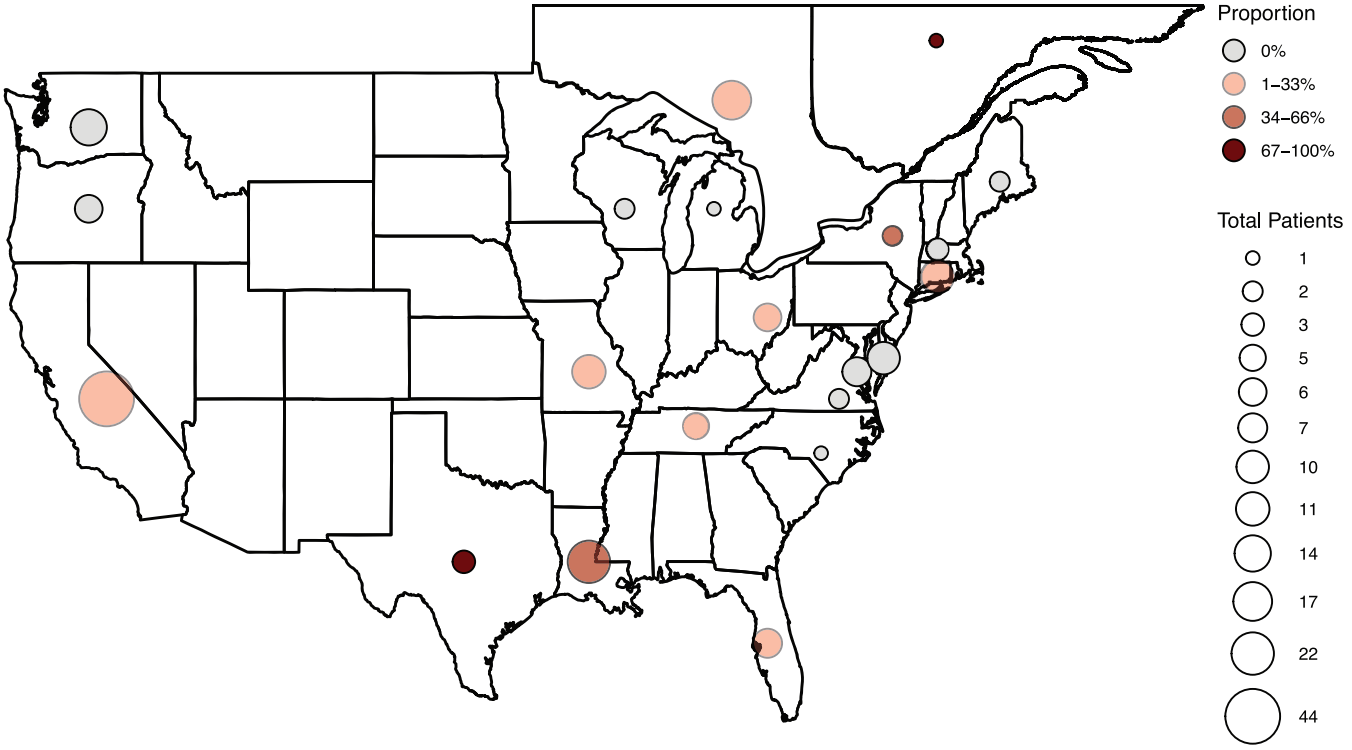

FIG 7 Geographic distribution of patients enrolled with the proportion of *qacA4*-carrying isolates within each geographic location. The size of the circles represents the total number of patients enrolled in each state or province, and the shade of the circle represents the proportion of the total patients with at least one *qacA4*-carrying *S. epidermidis* isolate.

Thirty-three of the 39 isolates contained reads that mapped to pAQZ1. Of these, 27 isolates contained reads that mapped to the entirety of pAQZ1. Five other isolates (isolates AUS16, BEL14, MB1595, 795_SEPI, and 798_SEPI) lacked reads corresponding to a 4.6-kb segment of pAQZ1 that contains the *knt* kanamycin resistance gene and the *ble* bleomycin resistance gene. One additional isolate (isolate 386_SEPI) lacked reads corresponding to a 10.4-kb segment of pAQZ1 that contains the *copA* and *czcD* heavy metal export genes, the *knt* kanamycin resistance gene, and the *ble* bleomycin resistance gene. Of note, we were unable to *de novo* assemble a single circular contig containing *qacA4* in any these 33 isolates as a result of the short read length or low coverage. Furthermore, due to the lack of availability of sequencing reads and the quality of the genome assemblies, we were unable to determine if the remaining 6 isolates carried *qacA4* on pAQZ1.

Of the 39 *qacA4*-carrying isolates from other sequencing projects, 35 belonged to ST2. The other 4 isolates (isolates 795_SEPI, 798_SEPI, 802_SEPI, and 803_SEPI) belonged to the novel ST847. ST847 differs from ST2 by a single SNV in the *arcC* loci (12A>T) and is therefore likely a descendant of ST2 and may share many characteristics of ST2.

DISCUSSION

In this study, we identified a novel *qacA* allele, termed *qacA4*, associated with an elevated CHG MIC in cutaneous *S. epidermidis* isolates and determined that *qacA4* was contained on the novel pAQZ1 plasmid. We demonstrated that *qacA4* is the determinant for the elevated CHG MIC by curing an isolate of the gene. Additionally, our analyses revealed that isolates carrying *qacA4* displayed high rates of resistance to methicillin and other commonly prescribed antimicrobials, including erythromycin, ciprofloxacin, gentamicin, and sulfamethoxazole-trimethoprim. Whole-genome sequencing revealed that the isolates harbored several antimicrobial resistance determinants and resistance-associated mutations. Our analyses further demonstrated that

these isolates contained the chromosomally encoded biofilm formation operon, *icaADBC* (2), and belonged to the highly resistant and pathogenic ST2 clone (36). We identified isolates proven or presumed to carry *qacA4* from 22 patients enrolled in a multicenter, randomized controlled CHG bathing trial at 14 participating study centers across the United States and Canada. Furthermore, we identified *qacA4* in clinical *S. epidermidis* isolates collected in prior studies at centers without patients participating in our CHG bathing trial (29, 33, 35, 37, 38).

Previous studies have characterized three alleles of *qacA*: *qacA1*, *qacA2*, and *qacA3* (18, 21). Additional studies, however, have suggested that clinical and environmental *Staphylococcus* isolates may carry novel alleles of *qacA* (39, 40). Our identification of multiple novel *qacA* alleles supports this suggestion that the *qacA* allelic variation that exists within human staphylococcal populations is considerably greater than that previously appreciated.

The efflux pumps encoded by *qacA1*, *qacA2*, and *qacA3* do not exhibit any functional differences (21). However, the efflux potential of QacA has been shown to vary with its amino acid sequence (41–44). Despite this recognition, no previous studies have examined the functional differences associated with the sequence variation of *qacA* observed in clinical and environmental *Staphylococcus* isolates. As the CHG MIC of the *qacA4*-carrying isolates was significantly higher than the CHG MICs of the isolates carrying other alleles of *qacA*, our results suggest that different alleles of *qacA* encode pumps with various CHG efflux potentials. This is further supported by our identification of four novel *qacA* alleles from the nine *S. epidermidis* isolates with prototypical *qacA* restriction patterns and elevated CHG MICs. These novel alleles indicate that other unique mutations may result in elevated CHG MICs and further underscore the importance of exploring the allelic variation of *qacA* in clinical and environmental *Staphylococcus* isolates.

It is tempting to speculate which of the two amino acid substitutions in QacA4, Ala157Gly and Ala378Val, is causal for the elevated CHG MIC observed in *qacA4*-carrying isolates. The Ala378Val substitution is particularly intriguing, as this mutation occurs in transmembrane segment 12. Transmembrane segment 12 is noteworthy when discussing the CHG efflux potential of QacA, since a previous study demonstrated that this segment lines the CHG binding pocket (41). The Ala157Gly substitution was identified in an *S. epidermidis* isolate with the prototypical *qacA* RFLP pattern and an elevated CHG MIC. This may indicate that the Ala157Gly substitution has a more causal role in the elevated CHG MICs. Future *in vitro* characterizations examining the structure-function relationship of the amino acid substitutions are merited.

Several studies have provided contradicting results as to whether the carriage of *qacA* influences the CHG MIC of *Staphylococcus* isolates (16, 19, 24, 45–49). These studies, however, did not distinguish between the *qacA* alleles carried by the isolates. Our findings highlight the importance of specifying the *qacA* allele carried by isolates when examining associations with CHG MICs: carriage of *qacA4*, as demonstrated by our curing analysis, results in a 4-fold increase in the CHG MIC of an isolate, while carriage of other alleles may not increase the CHG MIC.

Screening for *qacA/B* has been used as a proxy for determining whether an isolate exhibits reduced susceptibility or tolerance to CHG (48, 50–52), typically defined as a CHG MIC of $\geq 4\ \mu$g/ml (16, 48). All isolates carrying *qacA4* but just 10.0% of all *qacA/B*-positive isolates had a CHG MIC of 4 μg/ml. Thus, screening for *qacA4*, rather than indiscriminately screening for *qacA/B*, may serve as a better indicator for reduced susceptibility to CHG in *Staphylococcus* spp.

Similar to previously described plasmids carrying *qacA/B* (22), pAQZ1 carries several genes involved in heavy metal efflux and a partial β-lactamase operon. As pAQZ1 contains only the β-lactamase transcriptional regulators *blaI* and *blaR* (53), it is unclear if carriage of the plasmid influences β-lactam resistance. The kanamycin nucleotidyltransferase encoded by *knt* on pAQZ1 showed high sequence similarity to the kanamycin nucleotidyltransferase of *S. aureus* (GenBank accession number X03408.1) and may contribute to aminoglycoside resistance (54). Since the cured isolate also con-

tained other aminoglycoside resistance determinants, including *aac(6′)-Ie* plus *aph(2″)-Ia* and *ant(4′)-Ib*, we were unable to assess the contribution of *knt* to aminoglycoside resistance.

All of the *qacA4*-carrying isolates that we sequenced belonged to ST2, an *S. epidermidis* clone frequently implicated in device-associated infections (5, 36, 55–58). Consistent with the findings of previous studies (36, 55–57), our ST2 isolates contained genes and mutations which confer resistance to several classes of commonly prescribed antimicrobials. Additionally, our isolates contained genes associated with binding to foreign materials (5, 36), including the biofilm formation operon, *icaADBC*. These results suggest that *qacA4* may allow the highly resistant *S. epidermidis* ST2 clone to better persist following topical application of CHG and, thus, further succeed as an opportunistic pathogen. However, as the concentration of CHG used in clinical settings (8, 10) is much higher than that tested *in vitro* (2,000 μg/ml versus 4 μg/ml), further study is required to fully understand the clinical implications of carriage of *qacA4* by the ST2 clone.

Our results suggest that *qacA4* is distributed in pediatric oncology populations at centers across the United States and Canada. In addition, we identified 39 isolates carrying *qacA4* collected at institutions in 5 countries (29, 33, 35, 37, 38). This suggests that *qacA4* follows the wide distribution of both the *S. epidermidis* ST2 clone and *qacA* (36, 48) and is likely disseminated throughout health care settings globally.

Our study was limited by the nature of the RFLP screening analysis. While our method of screening for *qacA/B* allowed us to identify all the isolates with a mutation at positions 1131 to 1134, we were unable to easily detect the other novel *qacA* alleles that may have been present in our study population. From just the 35 *qacA*-positive and *qacA/B*$_{273}$-positive isolates that we sequenced, we identified 11 novel *qacA* alleles, and, as we demonstrated, at least one of these alleles exhibits a functional difference with respect to CHG efflux. This emphasizes the necessity of using sequencing to screen for allelic variation in resistance determinants, especially in those determinants in which allelic variation has been underappreciated. Furthermore, reflecting the difficulty of performing transformations in *Staphylococcus* spp. (59), we were unable to perform a gain-of-function analysis for *qacA4*, despite trying three different methods of preparing electrocompetent cells and two separate electroporation conditions for each cell preparation. Despite this limitation, we were able to perform a loss-of-function analysis to confirm the role of *qacA4* in the elevated CHG MIC. It is remarkable that the loss of function was achieved by recombination and that the cured isolate retained more than half of pAQZ1. Beyond *qacA4*, each of the 11 other genes contained on the segment lost in the cured isolate may explain the 4-fold decrease in the CHG MIC exhibited by this cured isolate. Many of these genes, however, have well-described functions unrelated to CHG efflux (53, 54, 60–62). Additionally, we identified each of the other 11 genes in an isolate without an elevated CHG MIC. Thus, the decrease in the CHG MIC observed in the cured isolate is most consistent with the loss of *qacA4*.

Our results highlight the importance of screening for allelic variation in *qacA*. Just as a single SNV between *qacA* and *qacB* accounts for the different substrate specificities of the resulting efflux pumps (18), the three SNVs of *qacA4* are associated with a 4-fold increase in the CHG MIC. Further study should focus on understanding the functional differences of the various *qacA* alleles identified in clinical and environmental *Staphylococcus* isolates. Moreover, our results indicate the *qacA4* is carried by the highly resistant *S. epidermidis* ST2 clone and related strains. Future study is required to understand if the use of CHG selects for *qacA4* and this pathogenic clone of *S. epidermidis*.

MATERIALS AND METHODS

Collection and identification of cutaneous *Staphylococcus* isolates. Skin swab specimens were obtained from patients between 2 months and 21 years of age who were undergoing allogeneic hematopoietic cell transplantation for any reason or treatment for a cancer diagnosis that required at least 3 months of additional chemotherapy from the time of enrollment and who were enrolled in a randomized double-blind placebo-controlled trial of CHG bathing versus control bathing conducted at 37 centers in the United States and Canada from January 2014 to April 2017 (Children's Oncology Group

ACCL1034; ClinicalTrials.gov identifier NCT01817075). The study was approved by the National Cancer Institute's Pediatric Central Institution Review Board as well as the local review boards at the participating institutions, if required.

After enrollment, patients were followed for 90 days both during hospitalizations and while they were outpatients. Cutaneous swab samples were obtained by swabbing a 3- by 3-cm area on the side or back of the neck and axilla regions with a sterile nylon swab (Copan Diagnostics) for 20 s and transported in 1 ml of the accompanying liquid Amies medium. The swab and Amies medium were vigorously vortexed, and the medium was plated on the following agar plates: tryptic soy agar with 5% sheep's blood (Remel), chocolate agar (Remel), Sabouraud dextrose agar (Remel), MacConkey agar (Remel), and mannitol salt agar (Remel). The plates were incubated at 35°C for 48 h. *Staphylococcus* isolates were identified via MALDI-TOF MS.

Isolates were prepared for MALDI-TOF MS according to the manufacturer's direct transfer sample preparation procedure (63). A MicroFlex LT mass spectrometer (Bruker Daltonics, Inc.) operated in the positive linear mode with FlexControl software (version 3.4; Bruker) was used to obtain spectra. The resulting spectra were processed and classified using Biotyper software (version 3.2; Bruker). Identification results were interpreted according to the manufacturer's guidelines. The isolates and the corresponding phenotypic information included in the study are presented in Data Set S1 in the supplemental material.

Five additional *qacA4*-carrying isolates were obtained for phenotypic testing: one was collected during a pilot study conducted at Seattle Children's Hospital (13), and the other four were collected in a previous study at the University of Washington Medical Center (35).

Antimicrobial susceptibility testing. Following CLSI guidelines (64), susceptibility testing was performed by disk diffusion (Becton, Dickinson and Company) for the following antimicrobials: ERY (15 μg), CIP (5 μg), GEN (10 μg), LZD (30 μg), cefoxitin (FOX; 30 μg), RIF (5 μg), and SXT (23.75/1.25 μg). Additionally, VAN (0.016 to 256 μg/ml) susceptibility testing was performed using the Etest (bioMérieux) MIC method. FOX was used as a surrogate for determination of methicillin susceptibility per CLSI guidelines (64). Isolates were classified as resistant, intermediate, or susceptible to a given agent using the breakpoints specified by CLSI (65).

CHG MICs (0.0625 to 64 μg/ml) were determined via the broth microdilution method (65, 66). For the CHG MICs, the following strains were included as controls: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* (a laboratory control strain) (13), and *Escherichia coli* ATCC 25922.

Detection of *qacA/B* and *smr*. Isolates were tested for the presence of *qacA/B* via PCR using AmpliTaq DNA polymerase (Applied Biosystems) following the manufacturer's instruction for the reaction mixture (67) with the previously described primers and reaction conditions (21). To distinguish between *qacA* and *qacB*, the PCR products were digested with AluI, and the resulting fragments were visualized by agarose gel electrophoresis (21). Control *qacA*-positive and *qacB*-positive strains were obtained from Nobumichi Kobayashi.

Isolates were additionally screened for the presence of *smr* via PCR with the following primers: forward primer 5′-AAAACAATGCAACACCTACCAC-3′ and reverse primer 5′-ATGCGATGTTCCGAAAATGT-3′. The following reaction conditions were used: an initial denaturation for 3 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, primer annealing for 30 s at 55°C, and elongation for 1 min at 72°C, with completion with a final elongation for 10 min at 72°C. A control *smr*-positive strain was obtained from Arnold Bayer.

Statistical testing. Kruskal-Wallis or Wilcoxon rank-sum tests were used to assess differences in the distribution of CHG MICs between *qac* groups. Fisher's exact test was used to assess the proportion of isolates resistant to commonly used antimicrobials by *qac* group. All analyses were first performed on all isolates and then repeated using one randomly chosen isolate per patient per *qac* group. Analyses were performed using STATA (version 14; College Station, TX) and R (version 3.3.2; R Core Team) software.

Whole-genome sequencing. In total, the genomes of 40 *Staphylococcus* species isolates were sequenced. An initial group of 10 *qacA*-positive, 4 *qacB*-positive, and 10 *qacA/B*$_{273}$-positive isolates was selected for whole-genome sequencing. The pool of *qacA*-positive and *qacB*-positive isolates was restricted to those identified as *S. epidermidis* since all of the *qacA/B*$_{273}$-positive isolates with elevated CHG MICs were identified as *S. epidermidis*. The pool of isolates was then further restricted to the first *qacA*-positive isolate from 10 patients randomly chosen from the 91 patients with at least one *qacA*-positive isolate, the first *qacA/B*$_{273}$-positive isolate obtained from 10 patients randomly chosen from the 24 patients with at least one *qacA/B*$_{273}$-positive isolate, and the first *qacB*-positive isolate from all 4 patients with at least one *qacB*-positive isolate. One of the initially selected *qacA/B*$_{273}$-positive isolates contained two alleles of *qacA*, with one being *qacA4*. As a result, this isolate was excluded from all subsequent analyses. Additional isolates were selected for whole-genome sequencing based on their antimicrobial susceptibility phenotypes: 2 *qacA/B*$_{273}$-positive isolates were chosen as they did not have elevated CHG MICs, 9 *qacA*-positive isolates were selected as they had elevated CHG MICs, and 5 *qacA/B*$_{273}$-positive isolates were chosen as they had discordant antimicrobial susceptibility patterns (susceptible to FOX, GEN, or ERY). Of note, selection of isolates with divergent *qacA* and *qacB* genotypes and antimicrobial susceptibility phenotypes was not restricted to the first *S. epidermidis* isolate per patient. As a result, 5 patients contributed multiple isolates.

Isolates were grown in brain heart infusion broth (Remel) for 24 h at 37°C at a constant shaking of 150 rpm. DNA was extracted from these isolates using a QIAamp DNA minikit (Qiagen) while following the manufacturer's protocol for isolating DNA from Gram-positive bacteria (68). An initial cell lysis step was completed using a 200-μg/ml lysostaphin solution (Sigma-Aldrich).

Libraries were prepared with a Kapa Hyper preparation kit. Isolates were sequenced to at least 27× coverage using 2 × 300-bp Illumina MiSeq runs. *De novo* assemblies were constructed with the SPAdes program, annotated by use of the prokka program, and visualized using Geneious (version 10.2.3) software.

Multilocus sequence typing was completed by uploading the assemblies to PubMLST's *Staphylococcus epidermidis* MLST website (https://pubmlst.org/sepidermidis/) (69). A core genome analysis was performed as previously described (70, 71) using BWA-MEM, SAMtools, and VCFtools (parameters, minDP 10, minQ 200, and minGQ 10), based on mapping to an *S. epidermidis* ST2 reference genome (GCA_900086615.1) (26). Resistance genes, limited to only those matching "perfect" and "strict" criteria, were detected with the Comprehensive Antibiotic Resistance Database's Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) (72). The sequence of the DNA gyrase A protein in our isolates was compared with that of the DNA gyrase A protein of *S. epidermidis* ATCC 12228 (GenBank accession number AE015929), a ciprofloxacin-sensitive strain, to determine the identity of the residue at position 84. Isolates containing the Ser84Phe mutation were determined to contain a *gyrA* gene conferring resistance to fluoroquinolones (73). The assemblies were additionally screened for virulence genes, including *icaADBC* (GenBank accession number U43366), *aap* (GenBank accession number KJ920749), and *bhp* (GenBank accession number AY028618).

Curing of *qacA4*. An isolate carrying *qacA4*, isolate 107.2, was selected for the curing analysis, as this isolate did not contain *smr*, which can efflux ethidium bromide. The isolate was successively passaged in tryptic soy broth (Remel) under four separate curing conditions: with no selection, in which it was incubated for 24 h at 37°C at a constant shaking of 150 rpm; at an increased temperature, in which it was incubated for 24 h at 42°C; in the presence of increasing subinhibitory concentrations of sodium dodecyl sulfate (Sigma-Aldrich), in which it was incubated for 24 h at 37°C at a constant shaking of 150 rpm with 0.001% to 0.01% sodium dodecyl sulfate; and in the presence of increasing subinhibitory concentrations of novobiocin (Sigma-Aldrich), in which it was incubated for 24 h at 37°C at a constant shaking of 150 rpm with 0.01 μg/ml to 0.1 μg/ml novobiocin.

After each passage, broth samples were plated onto tryptic soy agar plates (Remel) containing 0.375 μg/ml of filter-sterilized ethidium bromide (VWR). The plates were incubated at 35°C for 48 h. Screening for cured strains was completed with UV light as previously described (46). PCR was used to confirm that the cured strain eliminated *qacA4*. Whole-genome sequencing was used to confirm that the cured strain contained minimal chromosomal mutations compared to the sequence of the parental strain.

Three PCRs were conducted on the plasmids predicted from whole-genome sequencing of the isolates. To confirm the circular nature of the contig presumed to be a plasmid, PCR was conducted with the following primers: forward primer 5′-GGCTACTGTTGTTTTACCTACACCACC-3′ and reverse primer 5′-GCATACATAACCTTTGCGTCAGTTGTC-3′. To confirm that the curing resulted in the formation of a novel plasmid, PCR was conducted with the following primers: forward primer 5′-CCATTGTGGCGTCAT TTCACGGC-3′ and reverse primer 5′-CGGCGAAATCCTTGAGCCATATCTG-3′ and forward primer 5′-GAAG AATCTGTAGTGGGCGCTG-3′ and reverse primer 5′-GATGAAAGTTGCTACTAGTGCTCC-3′. The following reactions conditions were used: an initial denaturation for 3 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C; primer annealing for 30 s at 53°C, 57°C, or 52°C for the three sets of primers, respectively; and elongation for 1 min at 72°C, with completion with a final elongation for 10 min at 72°C.

Transformation of pAQZ1 into *S. epidermidis* TÜ1457. In preparation for the extraction of the pAQZ1 plasmid, *qacA4*-carrying *S. epidermidis* isolate 107.2 was grown in tryptic soy broth (Remel) for 24 h at 37°C at a constant shaking of 150 rpm. The plasmid was extracted using a QIAprep Spin miniprep kit (Qiagen) following the manufacturer's instructions (Qiagen, Hilden, Germany).

The pan-susceptible *S. epidermidis* TÜ1457 strain (74) was used for the transformations. Three previously described methods were used for preparing electrocompetent cells (59, 75, 76). For electroporation, 100 μl of the prepared cells was mixed with 100 ng of pAQZ1 DNA in a 1-mm electroporation cuvette (Bio-Rad). Two electroporation conditions were used for each preparation of electrocompetent cells: 21 kV/cm, 100 Ω, and 25 μF and 23 kV/cm, 100 Ω, and 25 μF. The pulsed cells were resuspended in 1,000 μl of broth, with the type of broth being selected based on the previously described methods, and incubated at 37°C at a constant shaking of 150 rpm for 1 h. The cells were plated onto tryptic soy agar plates (Remel) containing either 2 μg/ml CHG (Sigma-Aldrich), 15 μg/ml ethidium bromide (VWR), or 10 μg/ml of kanamycin (Sigma-Aldrich) and incubated overnight at 37°C.

Accession number(s). The sequence of *qacA4* was deposited in GenBank under accession number MK040360. The accession numbers for the additional 10 novel *qacA* alleles identified in this study are listed in Table S2. The sequences of pAQZ1 and pAQZ2 were deposited under accession numbers MK046687 and MK046688, respectively. Draft genome assemblies are available in GenBank under study BioProject accession number PRJNA415995. The accession numbers as well as the phenotypic data for the individual isolates sequenced are displayed in Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02607-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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