A novel qacA allele results in an elevated chlorhexidine gluconate minimum inhibitory concentration in cutaneous Staphylococcus epidermidis isolates.

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Running Head (54 characters max): Novel qacA allele increases CHG MIC of S. epidermidis

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Abstract

Chlorhexidine gluconate (CHG) is a topical antiseptic widely used in healthcare 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 63 (6%) of these isolates had elevated CHG MICs (≥ 4 µg/mL). Screening of all 1,050 isolates for qacA/B by restriction fragment length polymorphism (RFLP) yielded 56 isolates with a novel qacA/B RFLP pattern, qacAB273. The CHG MIC was significantly higher for qacAB273-positive isolates (MIC50: 4 µg/mL, [range: 0.5 – 4 µg/mL]) compared to other qac groups: qacA-positive (n=559, 1 µg/mL, [0.5 – 4 µg/mL]), qacB-positive (n=17, 1 µg/mL, [0.25 – 2 µg/mL]), and qacA/B-negative (n=418, 1 µg/mL, [0.125 – 2 µg/mL], p=0.001). The qacAB273-positive isolates also displayed a high proportion of methicillin resistance (96.4%) compared to other qac groups (24.9 – 61.7%, p=0.001). Whole genome sequencing revealed that qacAB273-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 S. epidermidis clone ST2 and were collected from multiple centers across the United States and Canada. Curing an isolate of qacA4 resulted in a four-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.

Importance

Staphylococcus epidermidis is an important cause of infections in patients with implanted devices. Bathing with chlorhexidine gluconate (CHG), a topical antiseptic, has been shown to
reduce rates of device-associated infections, especially those caused by *S. epidermidis*. In *S. epidermidis*, reduced susceptibility to CHG is associated with carriage of the *qacA* gene. As part of a multicenter CHG bathing trial, we obtained cutaneous *Staphylococcus* isolates from pediatric oncology patients across the United States and Canada. We identified a group of isolates capable of surviving in higher concentrations of CHG and determined a novel allele of *qacA*, termed *qacA4* and carried on the novel plasmid pAQZ1, was responsible for the isolates’ survival in higher CHG concentrations. The *qacA4*-carrying *S. epidermidis* isolates belonged to the highly resistant and virulent ST2 clonal type. Our results highlight the need to understand the global distribution of novel *qacA* alleles, including *qacA4*, and their mechanistic effect on efflux.
Introduction

*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 of CHG have been demonstrated to significantly reduce 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 the closely related *qacB* differs from *qacA* by only 7-9 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 differing 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 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 the aminoglycoside resistance gene *aacA-aphD* (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 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 $\geq 4 \mu g/mL$.

To investigate the genetic basis of the elevated CHG MIC, we screened the isolates for the *qacA/B* genes via PCR and restriction fragment length polymorphism (RFLP). From this screening, we identified a previously undescribed RFLP pattern, termed *qacAB$_{273}$*, in a subset of isolates. We then determined whether the *qacAB$_{273}$* RFLP pattern was associated with a significantly higher CHG MIC when compared to the *qacA*-positive, *qacB*-positive, and *qacA/B*-negative isolates. We also described the sequence of the novel *qacA* allele, referred to as *qacA*4, producing the novel *qacAB$_{273}$* RFLP pattern and characterized the isolates carrying *qacA*4.

Furthermore, through curing experiments, we investigated the role of *qacA*4 in causing elevated CHG MICs in *S. epidermidis*. 
Results

Overview of study population

In total, 1050 cutaneous Staphylococcus isolates were obtained from 173 patients. The study isolates primarily consisted of coagulase negative Staphylococcus 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, four coagulase negative *Staphylococcus* isolates could not be speciated by MALDI-TOF.

A subset of *Staphylococcus* isolates have an elevated CHG MIC

Measuring the CHG MICs across all 1050 isolates yielded 63 isolates with elevated CHG MICs, defined as an MIC $\geq 4$ μg/mL (Figure 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* gene 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 (Figure 2), and is referred to as *qacAB*$_{273}$-positive isolates hereafter.

Of the 1050 isolates, 632 contained a *qacA/B* gene 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
qacAB273-positive (Table 2). The qacA/B genes were detected in 8 different coagulase-negative Staphylococcus species. When screened for carriage of smr, 279 of the 1050 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 qacAB273 RFLP pattern is associated with an elevated CHG MIC

Next, the relationship between elevated CHG MICs and detection of the qacA/B and smr genes was examined. A qacA/B gene was detected in each of the 63 isolates with an elevated CHG MIC: 54 were classified as qacAB273-positive and 9 as qacA-positive (Figure 1b). None of the isolates with an elevated CHG were classified as qacB-positive. Furthermore, 51 of the 63 isolates with elevated CHG were categorized as smr-positive and the remaining 12 as smr-negative (Figure 1c).

To further investigate if the qacAB273 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 qacAB273-positive isolates as compared to 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 compared to 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, CHG MIC distributions associated with *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 *qacAB* 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 *qacAB* positive isolates exhibited higher rates of resistance to methicillin (96.4%) and other commonly prescribed antimicrobials, including erythromycin (ERY, 92.9%), ciprofloxacin (CIP, 96.4%), gentamicin (GEN, 89.3%), and sulfamethoxazole/trimethoprim (SXT, 98.2%), as compared to the *qacA*-positive, *qacB*-positive and *qacA/B*-negative isolates (p<0.001 for all comparisons); the results did not change when restricting analyses to one randomly chosen isolate per patient per *qacA/B* group (Table 4). All *qacA/B* groups exhibited rates of resistance less than 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* gene in the *qacAB*-positive isolates, the genomes of 9 *qacAB*-positive *S. epidermidis* isolates were compared to the genomes of 10 *qacA*-positive and 4 *qacB*-positive *S. epidermidis* isolates (Table S1). All 9 of the *qacAB*-positive isolates had elevated CHG MICs, while none of the 10 *qacA*-positive and 4 *qacB*-positive isolates had elevated CHG MICs.
The sequence of qacA/B gene was highly conserved in the 9 qacAB273-positive isolates with elevated CHG MICs. As the qacA/B gene of the qacAB273-positive isolates contained the distinguishing qacA nucleotide 968A, the gene was classified as a novel allele of qacA. As shown in Figure 3a, this allele contained three SNVs (470C>G, 819G>A, and 1133C>T) compared to a reference qacA sequence (AB566410), and is referred to as qacA4 (MK040360) henceforth. The SNV at position 1133 in qacA4 resulted in the loss of an AluI digestion site, explaining the novel RFLP pattern observed in Figure 2. Two of the SNVs resulted in amino acid substitutions, Ala157Gly and Ala378Val, in transmembrane segments 5 and 12, respectively (Figure 3b).

When compared to the three previously characterized alleles of qacA (qacA1 (GU565967), qacA2 (21), and qacA3 (MK040360)) and the qacA alleles of the 10 qacA-positive S. epidermidis isolates, qacA4 differed from these sequences by at least three SNVs – including all three that distinguished qacA4 from the reference qacA sequence (Figure S1). Notably, from the 10 qacA-positive isolates we sequenced, we identified 5 additional novel qacA alleles: qacA7 (MK040363), qacA8 (MK040364), qacA9 (MK040365), qacA10 (MK040366), and qacA11 (MK040367) (Table S2; Figure S1). Similarly, comparing the sequence of qacA4 to the other sequences of qacA deposited in NCBI GenBank further confirmed the SNVs at the three positions described above were unique to qacA4.

We also sequenced the genomes of the two qacAB273-positive isolates that did not have elevated CHG MICs (MIC < 4 μg/mL) to investigate their discordant genotypic-phenotypic relationship (Table S1). Each of the qacA/B genes in these two qacAB273-positive isolates lacked the three identifying qacA4 mutations and differed from the reference qacA sequence by six SNVs (Figure
These SNVs resulted in six and seven amino acid substitutions compared to the reference qacA and qacA4, respectively (Figure 4). The qacA/B genes in these isolates were classified as two additional alleles of qacA, referred to as qacA5 (MK040361) and qacA6 (MK040362). Due to a SNV at position 1132, which resulted in the loss of an AluI digestion site, qacA5 and qacA6 displayed identical digestion patterns to qacA4.

We next sequenced 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; Figure 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 qacA4 by 2 to 8 amino acid substitutions (Figure S2). The allele qacA14, identified in isolate 96.5, contained one of the distinguishing coding changes of qacA4, 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. 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.

**Identification of the novel resistance plasmid pAQZ1 containing qacA4 allele**

The genomic context of the qacA4 allele in de novo assemblies of two separate qacAB273-positive isolates with high coverage, isolates 91.2 and 107.2, was examined to understand whether qacA4 was encoded chromosomally or on a plasmid. Both isolates carried qacA4 on a 29,431 bp circular contig with coverage that was 2.8X higher than average chromosomal coverage.
consistent with it being a plasmid (Figure 5a). The circular nature of the contig was verified by conducting PCR across the predicted junction site (data not shown). The plasmid, designated pAQZ1 (MK046687) henceforth, carrying qacA4 contained the RepA replication initiation protein with a RepA_N domain (pfam06970). Similar to other RepA_N family plasmids (26, 27), 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, and czcD, the knt kanamycin resistance gene, the ble bleomycin resistance gene, and an incomplete β-lactamase operon.

When 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 Staphylococci plasmids (CP017465 and CP023967). The complete sequence of pAQZ1, however, did not fully align with any single, previously characterized S. aureus or coagulase-negative Staphylococci plasmid. When queried against NCBI WGS, 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 (JZUM01000030.1 and QSTD01000014.1).

Curing analysis in vitro confirms qacA4 is responsible for the elevated CHG MICs

Transformations of S. epidermidis TÜ1457 with pAQZ1 was attempted, but proved unsuccessful (data not shown). Thus, to confirm the observed association between qacA4 and the elevated CHG MICs, we attempted to cure the 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<sub>cured</sub>, was identified. The CHG MIC of 107.2<sub>cured</sub> was four-fold lower
than that of 107.2 (Table 5). The 8-agent antimicrobial susceptibility profile of 107.2<sub>cured</sub> was
identical to that of the parental strain (Table 5). Sequencing of the 107.2<sub>cured</sub> (Table S1) revealed
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 (MK046688; Figure 5b), retained the RepA protein of pAQZ1. The
11.9 kb segment lost in 107.2<sub>cured</sub> not only contained $qacA4$, but also the $knt$ kanamycin
resistance gene, the $ble$ bleomycin resistance gene, the partial $\beta$-lactamase operon, and several
recombinases (Figure 5c). PCR testing further confirmed isolate 107.2<sub>cured</sub> lost the 11.9 kb
segment distinguishing pAQZ1 from pAQZ2 (data not shown). Isolate 107.2<sub>cured</sub> contained one
coding change in its chromosome when compared to 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<sub>cured</sub>
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 contained on the
eliminated segment of pAQZ1.

*Isolates carrying qacA4 belong to the highly resistant and virulent S. epidermidis sequence type*

ST2
The isolates carrying qacA4 harbored genes and mutations which confer resistance to several classes of commonly prescribed antimicrobials (Figure 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 the S. epidermidis sequence type ST2.

Five additional qacAB273-positive isolates with elevated CHG MICs, but displaying discordant susceptibility patterns (susceptible to methicillin, gentamicin, or erythromycin) were whole genome 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 (Figure 6).

qacA4-containing S. epidermidis isolates are distributed across North America

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 qacAB273-positive RFLP pattern and elevated CHG MIC. These 22 patients were enrolled at 14 study centers in 9 US states and 2 Canadian provinces (Figure 7). There was no obvious geographical clustering of the qacA4-carrying isolates.

As shown in Figure 7, isolates containing qacA4 existed outside of our study. A cutaneous S. epidermidis isolate containing qacA4 (Table S1) was obtained from a CHG bathing pilot study (13) conducted at Seattle Children’s Hospital. Four clinical S. epidermidis isolates containing qacA4 (SRA Accession Numbers: SRX761965, SRX762497, SRX762541, and SRX762777), identified through a query of the NCBI Sequence Read Archive, were obtained from a previous
study (28) conducted at the University of Washington Medical Center. Susceptibility testing of these isolates revealed that all had an elevated CHG MIC (4 μg/mL).

**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 *qacA4* was contained on the novel pAQZ1 plasmid. We demonstrated *qacA4* was the determinant for the elevated CHG MIC by curing an isolate of the gene. Additionally, our analyses revealed 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 the isolates harbored several antimicrobial resistance determinants and resistance-associated mutations. Our analyses further demonstrated these isolates contained the chromosomally-encoded biofilm formation operon, *icaADBC* (2), and belonged to the highly resistant and pathogenetic ST2 clone (29). 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 a prior study at a center without patients participating in our CHG bathing trial (28).

Previous studies have characterized three alleles of *qacA*: *qacA1*, *qacA2*, and *qacA3* (18, 21).

Additional studies, however, have suggested clinical and environmental *Staphylococcus* isolates may carry novel alleles of *qacA* (30, 31). Our identification of multiple novel *qacA* alleles supports this suggestion that considerably more *qacA* allelic variation exists within human staphylococcal populations than 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 (32–35). Despite this recognition, no 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 different alleles of qacA encode pumps with varying CHG efflux potentials. This is further supported by our identification of 4 novel qacA alleles from the nine S. epidermidis isolates with prototypical qacA restriction patterns and elevated CHG MICs. These novel alleles indicate 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 suspect 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 (32). The Ala157Gly substitution was identified in an S. epidermidis isolate with the prototypical qacA RFLP pattern and an elevated CHG MIC. This may indicate 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 is merited.
Several studies have provided contradicting results as to whether the carriage of \textit{qacA} influences the CHG MIC of \textit{Staphylococcus} isolates (16, 19, 24, 36–40). These studies, however, did not distinguish between the \textit{qacA} alleles carried by the isolates. Our findings highlight the importance of specifying the \textit{qacA} allele carried by isolates when examining associations with CHG MICs: carriage of \textit{qacA4}, as demonstrated by our curing analysis, results in a four-fold increase in the CHG MIC of an isolate, while carriage of other alleles may not increase the CHG MIC.

Screening for \textit{qacA/B} has been used as a proxy for determining whether an isolate exhibits reduced susceptibility or tolerance to CHG (39, 41–43), typically defined as a CHG MIC $\geq 4$ $\mu$g/mL (16, 39). All isolates carrying \textit{qacA4} had a CHG MIC of 4 $\mu$g/mL, compared to just 10.0% of all \textit{qacA/B}-positive isolates. Thus, screening for \textit{qacA4}, rather than indiscriminately screening for \textit{qacA/B}, may serve as a better indicator for reduced susceptibility to CHG in \textit{Staphylococcus} spp.

Similar to previously described plasmids carrying \textit{qacA/B} (22), pAQZ1 carries several genes involved in heavy metal efflux and a partial $\beta$-lactamase operon. As pAQZ1 only contains the $\beta$-lactamase transcriptional regulators, \textit{blaI} and \textit{blaR} (44), it is unclear if carriage of the plasmid influences $\beta$-lactam resistance. The kanamycin nucleotidyltransferase encoded by \textit{knt} on pAQZ1 showed high sequence similarity to the kanamycin nucleotidyltransferase of \textit{S. aureus} (X03408) and may contribute to aminoglycoside resistance (45). Since the cured isolate also contained other aminoglycoside resistant determinants, including \textit{aac(6$\prime$)-Ie/aph(2$''$)-Ia} and \textit{ant(4$'$)-Ib}, we were unable to assess the contribution of \textit{knt} to aminoglycoside resistance.
All of the \textit{qacA4}-carrying isolates we sequenced belonged to ST2, a \textit{S. epidermidis} clone frequently implicated in device-associated infections (5, 29, 46–49). Consistent with previous studies (29, 46–48), 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, 29), including the biofilm formation operon \textit{icaADBC}. These results suggest \textit{qacA4} may allow the highly resistant \textit{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 tested \textit{in vitro} (2,000 \textmu g/mL versus 4 \textmu g/mL), further study is required to fully understand the clinical implications of carriage of \textit{qacA4} by the ST2 clone.

Our results suggest \textit{qacA4} is distributed in pediatric oncology populations at centers across the United States and Canada. Four isolates carrying \textit{qacA4} were also identified from a prior study conducted at an institution without patients participating in our CHG bathing trial (28). These four isolates were collected from patients in intensive care units where CHG bathing was standard of care (Estella Whimbey, personal communication). With both the \textit{S. epidermidis} ST2 clone and \textit{qacA} widely disseminated throughout healthcare settings globally (29, 39), broader screening may reveal \textit{qacA4} follows this wide global distribution.

Our study was limited by the nature of the RFLP screening analysis. While our method of screening for \textit{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 \textit{qacA} alleles that may have been present in our study population. From just the 35 \textit{qacA}-positive and \textit{qacAB273}-positive isolates we sequenced, we identified 11 novel \textit{qacA} alleles, and, as we demonstrated, at least one of these
alleles exhibits functional difference with respect to CHG efflux. This emphasizes the necessity of using sequence 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. (50), 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 four-fold decrease in the CHG MIC exhibited by this cured isolate. Many of these genes, however, have well-described functions unrelated to CHG efflux (44, 45, 51–53). 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 differing substrate specificities of the resulting efflux pumps (18), the three SNVs of *qacA4* are associated with a four-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 highly resistant *S. epidermidis* ST2 clone (29) carries *qacA4*. Future study is required to understand if frequent usage of CHG selects for *qacA4* and this pathogenic clone of *S. epidermidis*. 
Material and Methods

Collection and identification of cutaneous Staphylococcus isolates

Skins swabs were obtained from patients between 2 months and 21 years of age undergoing allogeneic hematopoietic cell transplantation or treatment for cancer 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). The study was approved by the National Cancer Institute’s Pediatric Central Institution Review Board as well as the local review boards at participating institutions, if required.

Samples were obtained by swabbing a 3x3 cm area on the side or back of the neck and axilla regions with a sterile nylon swab (Copan Diagnostics) for 20 seconds 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 with 5% Sheep’s Blood (Remel), Chocolate (Remel), Sabouraud Dextrose (Remel), MacConkey (Remel), and Mannitol Salt (Remel). The plates were incubated at 35°C for 48 hours. Staphylococcus isolates were identified via matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).

Isolates were prepared for MALDI-TOF MS according to the manufacturer’s Direct Transfer Sample Preparation procedure (54). 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.
Identification results were interpreted according to the manufacturer’s guidelines. The isolates, and the corresponding phenotypic information, included in the study are presented in the Dataset S1.

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 (28).

**Antimicrobial susceptibility testing**

Following CLSI guidelines (55), susceptibility testing was performed by disk diffusion (Benton, 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 – 256 µg/mL) susceptibility testing was performed using the Etest (BioMérieux) MIC method. FOX was used as a surrogate for methicillin susceptibility per CLSI guidelines (55). Isolates were classified as resistant, intermediate, or susceptible to a given agent using the breakpoints specified by CLSI (56).

CHG MICs (0.0625 – 64 µg/mL) were determined via the broth microdilution method (56, 57). For the CHG MICs, the following strains were included as controls: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* (Laboratory control strain) (13), *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 (58) 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 with agarose gel electrophoresis (21). Control *qacA*-positive and *qacB*-positive strains were obtained from Dr. Nobumichi Kobayashi.

Isolates were additionally screened for the presence of *smr* via PCR with the following primers: F – 5’AAAACAATGCAACACCTACCAC3’ and R – 5’ATGCGATGTTCCGAAAATGT3’. The following reactions conditions were used: an initial denaturation for 3 minutes at 95°C, followed by 30 cycles of denaturation for 1 minute at 95°C, primer annealing for 30 seconds at 55°C, and elongation for 1 minute at 72°C, and completed with a final elongation for 10 minutes at 72°C. A control *smr*-positive strain was obtained from Dr. 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).

**Whole genome Sequencing**
In total, the genomes of 40 *Staphylococcus* spp. isolates were sequenced. An initial group of 10 qacA-positive, 4 qacB-positive, and 10 qacAB273-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 qacAB273-positive with an elevated CHG MIC 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 qacAB273-positive isolate obtained from 10 patients randomly chosen from the 24 patients with at least one qacAB273-positive isolate, and the first qacB-positive isolate from all 4 patients with at least one qacB-positive isolate. One of the initially selected qacAB273-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 qacAB273-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 qacAB273-positive isolates were chosen as they had discordant antimicrobial susceptibility patterns (susceptible to FOX, GEN, or ERY).

Isolates were grown in Brain Heart Infusion broth (Remel) for 24 hours at 37°C at a constant shaking of 150 rpm. DNA was extracted from these isolates using the QIAamp DNA Mini Kit (Qiagen) while following the manufacturer’s protocol for isolating DNA from Gram-positive bacteria (59). An initial cell lysis step was completed using a 200 µg/mL lysostaphin solution (Sigma-Aldrich).
Libraries were prepared with the KAPA Hyper Prep Kit. Isolates were sequenced to at least 27X coverage using 2x300 bp Illumina MiSeq runs. De novo assemblies were constructed with SPAdes, annotated via prokka, and visualized using Geneious v.10.2.3.

Multilocus sequence typing was completed by uploading the assemblies to PubMLST’s Staphylococcus epidermidis MLST website (https://pubmlst.org/sepidermidis/) (60). 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) (61). The sequence of the DNA gyrase A protein in our isolates was compared with the DNA gyrase A protein of S. epidermidis ATCC12228 (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 (62). The assemblies were additionally screened for virulence genes including icaADBC (U43366), aap (KJ920749), and bhp (AY028618).

Curing of qacA4

An isolate carrying qacA4, 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) in four separate curing conditions: No Selection – incubated for 24 hours at 37°C at a constant shaking of 150 rpm; Increased Temperature – incubated for 24 hours at 42°C; Increasing Sub-Inhibitory Concentrations of Sodium Dodecyl Sulfate (Sigma-Aldrich) – incubated for 24 hours at 37°C at a constant shaking of 150 rpm with 0.001% to 0.01% sodium dodecyl sulfate; and Increasing Sub-Inhibitory Concentrations of Novobiocin (Sigma-Aldrich) –
incubated for 24 hours at 37°C at a constant shaking of 150 rpm with 0.01 µg/mL to 0.1 µg/mL novobiocin.

After each passage, broths 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 hours. Screening for cured strains was completed with ultraviolet light as previously described (37). PCR was used to confirm the cured strain eliminated qacA4. Whole genome sequencing was used to confirm the cured strain contained minimal chromosomal mutations as compared to the parental strain.

Three PCR reactions 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: F – 5'GGCTACTGTGTTTTTACCTACACCACC3' and R – 5'GCATACATAACCTTTGCGTCAGTTGTC3'. To confirm curing resulted in the formation of a novel plasmid, PCR was conducted with the following primers: F – 5'CCATTGTGGCGTCATTTCACGGC3' and R – 5'CGGCGAAATCCTTGAGCCATATCTG3' and F – 5'GAAGAATCTGTAGTGGGCGCTG3' and R – 5'GATGAAAGTTGCTACTAGTGCTCC3'. The following reactions conditions were used: an initial denaturation for 3 minutes at 95°C, followed by 30 cycles of denaturation for 1 minute at 95°C, primer annealing for 30 seconds at 53°C, 57°C, or 52°C, respectively, and elongation for 1 minute at 72°C, and completed with a final elongation for 10 minutes at 72°C.

Transformation of pAQZ1 into S. epidermidis TÜ1457
In preparation for the extraction of the pAQZ1 plasmid, the *qacA*-carrying *S. epidermidis* isolate 107.2 was grown Tryptic Soy Broth (Remel) for 24 hours at 37°C at a constant shaking of 150 rpm. The plasmid was extracted using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions (63).

The pan-susceptible *S. epidermidis* TÜ1457 strain (64) was used for the transformations. Three previously described methods were used for preparing electrocompetent cells (50, 65, 66). For electroporation, 100 μL of the prepared cells were 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 1000 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 for 150 rpm for 1 hour. 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 Numbers**

The sequence of *qacA* 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 the study accession PRJNA415995. The accession numbers, as well as the phenotypic data, for the individual isolates sequenced are displayed in Table S1.
Acknowledgments:

The authors would like to thank the patients who agreed to participate in this study as well as the research teams at all the involved sites and the ACCL1034 committee. The authors would also like to thank Dr. Stephen Salipante for sharing previously collected bacterial isolates, Dr. Paul Fey for providing the *S. epidermidis* TÜ1457 strain, Dr. Nobumichi Kobayashi for supplying the qacA/B-positive control strains, and Dr. Arnold Bayer for sharing the smr-positive control strain.

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Table 1. Overview of the cutaneous *Staphylococcus* isolates (n = 1050) included in this study.

<table>
<thead>
<tr>
<th>MALDI Identification</th>
<th>Total</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>30</td>
<td>2.9%</td>
</tr>
<tr>
<td>Coagulase Negative <em>Staphylococcus</em></td>
<td>1020</td>
<td>97.1%</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>558</td>
<td>53.1%</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>267</td>
<td>25.4%</td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>62</td>
<td>5.9%</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>47</td>
<td>4.5%</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>22</td>
<td>2.1%</td>
</tr>
<tr>
<td><em>S. pasteuri</em></td>
<td>16</td>
<td>1.5%</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
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</tr>
<tr>
<td><em>S. ludgunesis</em></td>
<td>8</td>
<td>0.8%</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
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<td>0.4%</td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td>4</td>
<td>0.4%</td>
</tr>
<tr>
<td><em>S. pettenkoferi</em></td>
<td>4</td>
<td>0.4%</td>
</tr>
<tr>
<td><em>S. condiment</em></td>
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</tr>
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</tr>
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<td><em>S. simulans</em></td>
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<tr>
<td><em>Staphylococcus sp.</em></td>
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</table>

Table 2. Comparison of the CHG MIC distributions, measured in μg/mL, of the *qacA*-positive, *qacB*-positive, *qacAB*$_{273}$-positive, and *qacA/B*-negative isolates and the CHG MIC distributions of the *smr*-positive and *smr*-negative isolates.

<table>
<thead>
<tr>
<th>(μg/mL)</th>
<th>Total Isolates</th>
<th><em>qacA</em></th>
<th><em>qacB</em></th>
<th><em>qacAB</em>$_{273}$</th>
<th><em>qacA/B</em></th>
<th><em>smr</em>+</th>
<th><em>smr</em>–</th>
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<tbody>
<tr>
<td>50</td>
<td>559</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>279</td>
<td>771</td>
</tr>
<tr>
<td>90</td>
<td>418</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Range</td>
<td>0.5 – 4</td>
<td>0.25 – 2</td>
<td>0.5 – 4</td>
<td>0.125 – 2</td>
<td>0.25 – 4</td>
<td>0.125 – 4</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Comparison of the CHG MIC distributions, measured in μg/mL, associated with the eight CHG resistance gene combinations detected in our isolates.

<table>
<thead>
<tr>
<th>qacA</th>
<th>qacA</th>
<th>qacB</th>
<th>qacB</th>
<th>qacAB273</th>
<th>qacAB273</th>
<th>qacA/B</th>
<th>qacA/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>smr+</td>
<td>smr–</td>
<td>smr+</td>
<td>smr–</td>
<td>smr+</td>
<td>smr–</td>
<td>smr+</td>
<td>smr–</td>
</tr>
<tr>
<td>Total Isolates</td>
<td>101</td>
<td>458</td>
<td>5</td>
<td>12</td>
<td>50</td>
<td>6</td>
<td>123</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
<td>1</td>
<td>0.75</td>
<td>4</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>2</td>
<td>2</td>
<td>1.6</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Range</td>
<td>0.5 – 4</td>
<td>0.5 – 4</td>
<td>0.5 – 2</td>
<td>0.25 – 1</td>
<td>4 – 4</td>
<td>0.5 – 4</td>
<td>0.25 – 1</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the proportion of qacA-positive, qacB-positive, qacAB<sub>273</sub>-positive, and qacA/B-negative isolates resistant to commonly prescribed antimicrobials: cefoxitin (FOX), erythromycin (ERY), ciprofloxacin (CIP), gentamicin (GEN), sulfamethoxazole/trimethoprim (SXT), linezolid (LZD), rifampin (RIF), and vancomycin (VAN).

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>qacA</th>
<th>qacB</th>
<th>qacAB&lt;sub&gt;273&lt;/sub&gt;</th>
<th>qacA/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOX*</td>
<td>61.7</td>
<td>52.9</td>
<td>96.4</td>
<td>22.7</td>
</tr>
<tr>
<td>ERY*</td>
<td>76.0</td>
<td>88.2</td>
<td>92.9</td>
<td>30.1</td>
</tr>
<tr>
<td>CIP*</td>
<td>25.0</td>
<td>5.9</td>
<td>96.4</td>
<td>8.4</td>
</tr>
<tr>
<td>GEN*</td>
<td>15.0</td>
<td>0</td>
<td>89.3</td>
<td>2.6</td>
</tr>
<tr>
<td>SXT*</td>
<td>60.1</td>
<td>35.3</td>
<td>98.2</td>
<td>22.7</td>
</tr>
<tr>
<td>LZD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RIF</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VAN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

Table 5. Comparison of the CHG resistance gene combinations and resistance patterns of isolates 107.2 and 107.2<sub>cured</sub>. The CHG MIC is measured in μg/mL. “R” denotes the isolate is resistant to the specified antimicrobial and “S” indicates the isolate is susceptible to the specified antimicrobial.
| Isolate   | MADLI ID | qacA? | smr? | CHG MIC | FOX | ERY | CIP | GEN | SXT | LZD | RIF | VAN |
|----------|----------|-------|------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 107.2    | S. epidermidis | Yes   | No   | 4       | R   | R   | R   | R   | R   | S   | S   | S   |
| 107.2cured | S. epidermidis | No    | No   | 1       | R   | R   | R   | R   | R   | S   | S   | S   |

**Figure 1.** CHG MIC distribution of the 1050 cutaneous *Staphylococcus* isolates included in our study grouped by a) species, b) qacA/B PCR and RFLP patterns, and c) smr PCR results. The dashed line indicates the concentration we defined as an elevated CHG MIC (≥ 4 μg/mL).

**Figure 2.** RFLP patterns observed from the AluI restriction digest of the qacA/B PCR amplicon. Isolates were classified as qacA-positive, qacAB273-positive, or qacB-positive based on the presence of a 198 bp, 273 bp, or 165 bp fragment, respectively (Alam et al., 2003). Ladder: 100 bp (Promega).

**Figure 3.** Comparison of a) the sequence of qacA4 (MK040360), a reference qacA sequence (AB566410), and a reference qacB sequence (AF053772). 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. Structure of b) the predicted efflux pump encoded by qacA4, adapted from previous representations of QacA (Paulsen et al., 1996) (Brown et al., 1998). The residues which distinguish QacA4 from the reference QacA are highlighted in red. Those which distinguish QacA from QacB are displayed in blue.
Figure 4. Comparison of the sequences of \textit{qacA4} (MK040360), \textit{qacA5} (MK040361), and \textit{qacA6} (MK040362) and the reference \textit{qacA} sequence (AB566410). The associated \textit{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 \textit{qacA4} from the reference \textit{qacA}, \textit{qacA5}, and \textit{qacA6} are highlighted in red. Those which distinguish \textit{qacA5} and/or \textit{qacA6} from \textit{qacA4} and the reference \textit{qacA} are displayed in green.

Figure 5. Schematic representations of a) the plasmid pAQZ1 containing \textit{qacA4} obtained from the \textit{de novo} assemblies of isolates 91.2 and 107.2, b) the plasmid pAQZ2 retained in isolate 107.2_{cured}, and c) the 11,934 bp segment of pAQZ1 eliminated through the curing experiments. 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.

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

Figure 7. Geographic distribution of the patients with at least one \textit{S. epidermidis} isolate carrying \textit{qacA4}. The size of the circles represents the total number of patients enrolled in each state or province, and shade of the circle represents the proportion of the total patients with at least one \textit{S}.
*epidermidis* isolate. The green marker (Roach et al., 2015; Soma et al., 2012) represents isolates carrying *qacA4* collected outside of our study.
Figure 1. CHG MIC distribution of the 1050 cutaneous Staphylococcus isolates included in our study grouped by a) species, b) qacA/B PCR and RFLP patterns, and c) smr PCR results. The dashed line indicates the concentration we defined as an elevated CHG MIC (≥ 4 µg/mL).
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Figure 5. Schematic representations of a) the plasmid pAQZ1 (MK046687) containing qacA4 obtained from the de novo assemblies of isolates 91.2 and 107.2, b) the plasmid pAQZ2 (MK046687) retained in isolate 107.2 cured, and c) the 11,934 bp segment of pAQZ1 eliminated through the curing experiments. 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.
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### Table 1. Overview of the cutaneous *Staphylococcus* isolates (n = 1050) included in this study.

<table>
<thead>
<tr>
<th>MALDI Identification</th>
<th>Total</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>30</td>
<td>2.9%</td>
</tr>
<tr>
<td>Coagulase Negative <em>Staphylococcus</em></td>
<td>1020</td>
<td>97.1%</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>558</td>
<td>53.1%</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>267</td>
<td>25.4%</td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>62</td>
<td>5.9%</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>47</td>
<td>4.5%</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>22</td>
<td>2.1%</td>
</tr>
<tr>
<td><em>S. pasteuri</em></td>
<td>16</td>
<td>1.5%</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>9</td>
<td>0.9%</td>
</tr>
<tr>
<td><em>S. ludgunesis</em></td>
<td>8</td>
<td>0.8%</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>4</td>
<td>0.4%</td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td>4</td>
<td>0.4%</td>
</tr>
<tr>
<td><em>S. pettenkoferi</em></td>
<td>4</td>
<td>0.4%</td>
</tr>
<tr>
<td><em>S. condiment</em></td>
<td>3</td>
<td>0.3%</td>
</tr>
<tr>
<td><em>S. schleiferi</em></td>
<td>3</td>
<td>0.3%</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>3</td>
<td>0.3%</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>2</td>
<td>0.2%</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>2</td>
<td>0.2%</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>1</td>
<td>0.1%</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>1</td>
<td>0.1%</td>
</tr>
<tr>
<td><em>Staphylococcus</em> sp.</td>
<td>4</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of the CHG MIC distributions, measured in μg/mL, of the *qacA*-positive, *qacB*-positive, *qacAB*<sub>273</sub>-positive, and *qacA/B*-negative isolates and the CHG MIC distributions of the *smr*-positive and *smr*-negative isolates.

<table>
<thead>
<tr>
<th></th>
<th><em>qacA</em></th>
<th><em>qacB</em></th>
<th><em>qacAB</em>&lt;sub&gt;273&lt;/sub&gt;</th>
<th><em>qacA/B</em>&lt;sup&gt;−&lt;/sup&gt;</th>
<th><em>smr</em>&lt;sup&gt;+&lt;/sup&gt;</th>
<th><em>smr</em>&lt;sup&gt;−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Isolates</td>
<td>559</td>
<td>17</td>
<td>56</td>
<td>418</td>
<td>279</td>
<td>771</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Range</td>
<td>0.5 – 4</td>
<td>0.25 – 2</td>
<td>0.5 – 4</td>
<td>0.125 – 2</td>
<td>0.25 – 4</td>
<td>0.125 – 4</td>
</tr>
</tbody>
</table>
### Table 3. Comparison of the CHG MIC distributions, measured in μg/mL, associated with the eight CHG resistance gene combinations detected in our isolates.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Isolates</td>
<td>101</td>
<td>458</td>
<td>5</td>
<td>12</td>
<td>50</td>
<td>6</td>
<td>123</td>
<td>295</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.75</td>
<td>4</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>2</td>
<td>2</td>
<td>1.6</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Range</td>
<td>0.5 – 4</td>
<td>0.5 – 4</td>
<td>0.5 – 2</td>
<td>0.25 – 1</td>
<td>4 – 4</td>
<td>0.5 – 4</td>
<td>0.25 – 1</td>
<td>0.125 – 2</td>
</tr>
</tbody>
</table>

### Table 4. Comparison of the proportion of qacA-positive, qacB-positive, qacAB<sub>273</sub>-positive, and qacA/B-negative isolates resistant to commonly prescribed antimicrobials: ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT), cefoxitin (FOX), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), rifampin (RIF), and vancomycin (VAN).

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>qacA</th>
<th>qacB</th>
<th>qacAB&lt;sub&gt;273&lt;/sub&gt;</th>
<th>qacA/B –</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOX*</td>
<td>61.7</td>
<td>52.9</td>
<td>96.4</td>
<td>22.7</td>
</tr>
<tr>
<td>ERY*</td>
<td>76.0</td>
<td>88.2</td>
<td>92.9</td>
<td>30.1</td>
</tr>
<tr>
<td>CIP*</td>
<td>25.0</td>
<td>5.9</td>
<td>96.4</td>
<td>8.4</td>
</tr>
<tr>
<td>GEN*</td>
<td>15.0</td>
<td>0</td>
<td>89.3</td>
<td>2.6</td>
</tr>
<tr>
<td>SXT*</td>
<td>60.1</td>
<td>35.3</td>
<td>98.2</td>
<td>22.7</td>
</tr>
<tr>
<td>LZD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RIF</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VAN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* P-value <0.001 for all comparisons. Results were generated using all isolates; results did not change when restricting analyses to one randomly chosen isolate per patient per qacA/B group.
Table 5. Comparison of the CHG resistance gene combinations and resistance patterns of isolates 107.2 and 107.2\textsubscript{cured}. The CHG MIC is measured in μg/mL. “R” denotes the isolate is resistant to the specified antimicrobial and “S” indicates the isolate is susceptible to the specified antimicrobial.

| Isolate  | MADLI ID   | qacA4? | smr? | CHG MIC | FOX | ERY | CIP | GEN | SXT | LZD | RIF | VAN |
|----------|------------|--------|------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 107.2    | S. epidermidis | Yes    | No   | 4       | R   | R   | R   | R   | R   | S   | S   | S   |
| 107.2\textsubscript{cured} | S. epidermidis | No     | No   | 1       | R   | R   | R   | R   | R   | S   | S   | S   |