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, $qacAB_{273}$. The CHG MIC was significantly higher for $qacAB_{273}$-positive isolates (MIC_{50}: 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 $qacAB_{273}$-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 $qacAB_{273}$-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 *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 ≥ 4 μg/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 *qacA4*, producing the novel $qacAB_{273}$ 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, 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 ≥ 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

123 *qacAB*$_{273}$-positive (Table 2). The *qacA/B* genes were detected in 8 different coagulase-negative
124 *Staphylococcus* species. When screened for carriage of *smr*, 279 of the 1050 isolates were
125 classified as *smr*-positive (Table 2). In total, 12 unique coagulase-negative *Staphylococcus*
126 species carried *smr*. Notably, the *qacA/B* genes and *smr* were not detected in any of the *S. aureus*
127 isolates.
128
129 *The qacAB*$_{273}$ *RFLP pattern is associated with an elevated CHG MIC*
130 Next, the relationship between elevated CHG MICs and detection of the *qacA/B* and *smr* genes
131 was examined. A *qacA/B* gene was detected in each of the 63 isolates with an elevated CHG
132 MIC: 54 were classified as *qacAB*$_{273}$-positive and 9 as *qacA*-positive (Figure 1b). None of the
133 isolates with an elevated CHG were classified as *qacB*-positive. Furthermore, 51 of the 63
134 isolates with elevated CHG were categorized as *smr*-positive and the remaining 12 as *smr*-
135 negative (Figure 1c).
136
137 To further investigate if the *qacAB*$_{273}$ RFLP pattern was associated with an elevated CHG MIC,
138 differences in the CHG MIC distributions of the *qacA/B* containing isolates were assessed. The
139 CHG MIC was significantly higher for the *qacAB*$_{273}$-positive isolates as compared to the *qacA*-
140 positive, *qacB*-positive, and *qacA/B*-negative isolates ($p = 0.001$); the results did not change
141 when restricting the analyses to one randomly chosen isolate per patient per *qacA/B* group (Table
142 2). In addition, the CHG MIC distributions of the *smr*-positive and *smr*-negative isolates were
143 compared. The CHG MIC was significantly higher for the *smr*-positive isolates compared to the
144 *smr*-negative isolates ($p=0.02$); however, this comparison was no longer significant when the
145 analyses were restricted to one randomly chosen isolate per patient per *smr* group as one
146 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 $qacAB_{273}$ 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 $qacAB_{273}$-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 $qacAB_{273}$-positive isolates, the genomes of 9 $qacAB_{273}$-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 $qacAB_{273}$-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 $qacAB_{273}$-positive isolates with elevated CHG MICs. As the *qacA/B* gene of the $qacAB_{273}$-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), *qacA*10 (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 $qacAB_{273}$-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 $qacAB_{273}$-positive isolates lacked the three identifying *qacA4* mutations and differed from the reference *qacA* sequence by six SNVs (Figure

194 4). These SNVs resulted in six and seven amino acid substitutions compared to the reference
195 *qacA* and *qacA4*, respectively (Figure 4). The *qacA/B* genes in these isolates were classified as
196 two additional alleles of *qacA*, referred to as *qacA5* (MK040361) and *qacA6* (MK040362). Due
197 to a SNV at position 1132, which resulted in the loss of an AluI digestion site, *qacA5* and *qacA6*
198 displayed identical digestion patterns to *qacA4*.
199
200 We next sequenced genomes from the nine *qacA*-positive isolates that had elevated CHG MICs.
201 From these isolates, we identified 4 *qacA* alleles: *qacA10*, *qacA12*, *qacA13*, and *qacA14* (Table
202 S2; Figure S2). The sequences of the *qacA* genes in these isolates differed from the reference
203 *qacA* sequence by 1 to 5 amino acid substitutions, and from *qacA4* by 2 to 8 amino acid
204 substitutions (Figure S2). The allele *qacA14*, identified in isolate 96.5, contained one of the
205 distinguishing coding changes of *qacA4*, Ala157Gly, but, not the other coding change. This
206 allele encoded a unique amino acid substitution Pro328Leu, which distinguished the allele from
207 the reference *qacA* and *qacA4*. Another isolate, 86.4, with an elevated CHG MIC carried the
208 same *qacA10* allele as isolate 110.3, which did not have an elevated CHG MIC. The amino acid
209 substitutions in these novel *qacA* alleles occurred in transmembrane segments 5, 6, 9, 10, 12, and
210 13 and in the extracellular loop between transmembrane segments 5 and 6.
211
212 *Identification of the novel resistance plasmid pAQZ1 containing qacA4 allele*
213 The genomic context of the *qacA4* allele in *de novo* assemblies of two separate $qacAB_{273}$-positive
214 isolates with high coverage, isolates 91.2 and 107.2, was examined to understand whether *qacA4*
215 was encoded chromosomally or on a plasmid. Both isolates carried *qacA4* on a 29,431 bp
216 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_{cured}, was identified. The CHG MIC of 107.2_{cured} was four-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 the 107.2_{cured} (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_{cured} not only contained *qacA4*, but also the *knt* kanamycin resistance gene, the *ble* bleomycin resistance gene, the partial β-lactamase operon, and several recombinases (Figure 5c). PCR testing further confirmed 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 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_{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 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 *qacAB*$_{273}$-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 *qacAB*$_{273}$-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

288 study (28) conducted at the University of Washington Medical Center. Susceptibility testing of
289 these isolates revealed that all had an elevated CHG MIC (4 µg/mL).
290
291 **Discussion**
292 In this study, we identified a novel *qacA* allele, termed *qacA4*, associated with an elevated CHG
293 MIC in cutaneous *S. epidermidis* isolates and determined *qacA4* was contained on the novel
294 pAQZ1 plasmid. We demonstrated *qacA4* was the determinant for the elevated CHG MIC by
295 curing an isolate of the gene. Additionally, our analyses revealed isolates carrying *qacA4*
296 displayed high rates of resistance to methicillin and other commonly prescribed antimicrobials,
297 including erythromycin, ciprofloxacin, gentamicin, and sulfamethoxazole/trimethoprim. Whole
298 genome sequencing revealed the isolates harbored several antimicrobial resistance determinants
299 and resistance-associated mutations. Our analyses further demonstrated these isolates contained
300 the chromosomally-encoded biofilm formation operon, *icaADBC* (2), and belonged to the highly
301 resistant and pathogenetic ST2 clone (29). We identified isolates proven or presumed to carry
302 *qacA4* from 22 patients enrolled in a multicenter, randomized controlled CHG bathing trial at 14
303 participating study centers across the United States and Canada. Furthermore, we identified
304 *qacA4* in clinical *S. epidermidis* isolates collected in a prior study at a center without patients
305 participating in our CHG bathing trial (28).
306
307 Previous studies have characterized three alleles of *qacA*: *qacA1*, *qacA2*, and *qacA3* (18, 21).
308 Additional studies, however, have suggested clinical and environmental *Staphylococcus* isolates
309 may carry novel alleles of *qacA* (30, 31). Our identification of multiple novel *qacA* alleles
310 supports this suggestion that considerably more *qacA* allelic variation exists within human
311 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 *qacA* influences the CHG MIC of *Staphylococcus* isolates (16, 19, 24, 36–40). 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 four-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 (39, 41–43), typically defined as a CHG MIC ≥ 4 µg/mL (16, 39). All isolates carrying *qacA4* had a CHG MIC of 4 µg/mL, compared to just 10.0% of all *qacA/B*-positive isolates. 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 only contains the β-lactamase transcriptional regulators, *blaI* and *blaR* (44), 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* (X03408) and may contribute to aminoglycoside resistance (45). Since the cured isolate also contained other aminoglycoside resistant determinants, including *aac(6')-Ie/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 we sequenced belonged to ST2, a *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 *icaADBC*. These results suggest *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 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 *qacA4* is distributed in pediatric oncology populations at centers across the United States and Canada. Four isolates carrying *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 *S. epidermidis* ST2 clone and *qacA* widely disseminated throughout healthcare settings globally (29, 39), broader screening may reveal *qacA4* follows this wide global distribution.

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 $qacAB_{273}$-positive isolates we sequenced, we identified 11 novel *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

(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 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 $qacAB_{273}$-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 $qacAB_{273}$-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 $qacAB_{273}$-positive isolate obtained from 10 patients randomly chosen from the 24 patients with at least one $qacAB_{273}$-positive isolate, and the first *qacB*-positive isolate from all 4 patients with at least one *qacB*-positive isolate. One of the initially selected $qacAB_{273}$-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 $qacAB_{273}$-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 $qacAB_{273}$-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) –

524 incubated for 24 hours at 37°C at a constant shaking of 150 rpm with 0.01 µg/mL to 0.1 µg/mL
525 novobiocin.
526
527 After each passage, broths were plated onto Tryptic Soy agar plates (Remel) containing 0.375
528 µg/mL of filter-sterilized ethidium bromide (VWR). The plates were incubated at 35°C for 48
529 hours. Screening for cured strains was completed with ultraviolet light as previously described
530 (37). PCR was used to confirm the cured strain eliminated *qacA4*. Whole genome sequencing
531 was used to confirm the cured strain contained minimal chromosomal mutations as compared to
532 the parental strain.
533
534 Three PCR reactions were conducted on the plasmids predicted from whole genome sequencing
535 of the isolates. To confirm the circular nature of the contig presumed to be a plasmid, PCR was
536 conducted with the following primers: F – 5'GGCTACTGTTGTTTTACCTACACCACC3' and
537 R – 5'GCATACATAACCTTTGCGTCAGTTGTC3'. To confirm curing resulted in the
538 formation of a novel plasmid, PCR was conducted with the following primers: F –
539 5'CCATTGTGGCGTCATTTCACGGC3' and R – 5'CGGCGAAATCCTTGAGCCATATCTG3'
540 and F – 5'GAAGAATCTGTAGTGGGCGCTG3' and R –
541 5'GATGAAAGTTGCTACTAGTGCTCC3'. The following reactions conditions were used: an
542 initial denaturation for 3 minutes at 95°C, followed by 30 cycles of denaturation for 1 minute at
543 95°C, primer annealing for 30 seconds at 53°C, 57°C, or 52°C, respectively, and elongation for 1
544 minute at 72°C, and completed with a final elongation for 10 minutes at 72°C.
545
546 **Transformation of pAQZ1 into *S. epidermidis* TÜ1457**

In preparation for the extraction of the pAQZ1 plasmid, the *qacA4*-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 *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 the study accession PRJNA415995. The accession numbers, as well as the phenotypic data, for the individual isolates sequenced are displayed in Table S1.

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767

768 **Legends/Tables**

769

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

MALDI Identification	Total	Percent
S. aureus	30	2.9%
Coagulase Negative *Staphylococcus*	1020	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. ludgunesis	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 sp.	4	0.4%

771

772 **Table 2.** Comparison of the CHG MIC distribrutions, measured in µg/mL, of the *qacA*-positive,

773 *qacB*-positive, *qacAB*$_{273}$-positive, and *qacA/B*-negative isolates and the CHG MIC distributions

774 of the *smr*-positive and *smr*-negative isolates.

	qacA	*qacB*	*qacAB*$_{273}$	*qacA/B* –	*smr* +	*smr* –
Total Isolates	559	17	56	418	279	771
MIC_{50}	1	1	4	1	1	1
MIC_{90}	2	1	4	1	2	1
Range	0.5 – 4	0.25 – 2	0.5 – 4	0.125 – 2	0.25 – 4	0.125 – 4

775

776 **Table 3.** Comparison of the CHG MIC distributions, measured in μg/mL, associated with the
777 eight CHG resistance gene combinations detected in our isolates.

	qacA *smr* +	*qacA* *smr* –	*qacB* *smr* +	*qacB* *smr* –	*qacAB*$_{273}$ *smr* +	*qacAB*$_{273}$ *smr* –	*qacA/B* – *smr* +	*qacA/B* – *smr* –
Total Isolates	101	458	5	12	50	6	123	295
MIC_{50}	1	1	1	0.75	4	4	0.5	1
MIC_{90}	2	2	1.6	1	4	4	1	1
Range	0.5 – 4	0.5 – 4	0.5 – 2	0.25 – 1	4 – 4	0.5 – 4	0.25 – 1	0.125 – 2

778

779 **Table 4.** Comparison of the proportion of *qacA*-positive, *qacB*-positive, *qacAB*$_{273}$-positive, and
780 *qacA/B*-negative isolates resistant to commonly prescribed antimicrobials: cefoxitin (FOX),
781 erythromycin (ERY), ciprofloxacin (CIP), gentamicin (GEN), sulfamethoxazole/trimethoprim
782 (SXT), linezolid (LZD), rifampin (RIF), and vancomycin (VAN).

Antimicrobial	*qacA*	*qacB*	*qacAB*$_{273}$	*qacA/B* –
FOX*	61.7	52.9	96.4	22.7
ERY*	76.0	88.2	92.9	30.1
CIP*	25.0	5.9	96.4	8.4
GEN*	15.0	0	89.3	2.6
SXT*	60.1	35.3	98.2	22.7
LZD	0	0	0	0
RIF	0.9	0	0	0
VAN	0	0	0	0

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

785

786

787 **Table 5.** Comparison of the CHG resistance gene combinations and resistance patterns of
788 isolates 107.2 and 107.2$_{cured}$. The CHG MIC is measured in μg/mL. "R" denotes the isolate is
789 resistant to the specified antimicrobial and "S" indicates the isolate is susceptible to the specified
790 antimicrobial.

791

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_{cured}	*S. epidermidis*	No	No	1	R	R	R	R	R	S	S	S

792

793

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

797

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

802

803 **Figure 3.** Comparison of a) the sequence of *qacA4* (MK040360), a reference *qacA* sequence
804 (AB566410), and a reference *qacB* sequence (AF053772). The associated AluI restriction sites
805 are shown below the nucleotide sequences and the corresponding amino acid sequences are
806 displayed in the boxes above the nucleotide sequences. Structure of b) the predicted efflux pump
807 encoded by *qacA4*, adapted from previous representations of QacA (Paulsen et al., 1996) (Brown
808 et al., 1998). The residues which distinguish QacA4 from the reference QacA are highlighted in
809 red. Those which distinguish QacA from QacB are displayed in blue.

810

Figure 4. Comparison of the sequences of *qacA4* (MK040360), *qacA5* (MK040361), and *qacA6* (MK040362) and the reference *qacA* sequence (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 *qacA4* from the reference *qacA*, *qacA5*, and *qacA6* are highlighted in red. Those which distinguish *qacA5* and/or *qacA6* from *qacA4* and the reference *qacA* are displayed in green.

Figure 5. Schematic representations of a) the plasmid pAQZ1 containing *qacA4* obtained from the *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 elimated 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 *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 *S. epidermidis* isolate carrying *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 *S.*

834 *epidermidis* isolate. The green marker (Roach et al., 2015; Soma et al., 2012) represents isolates

835 carrying *qacA4* collected outside of our study.

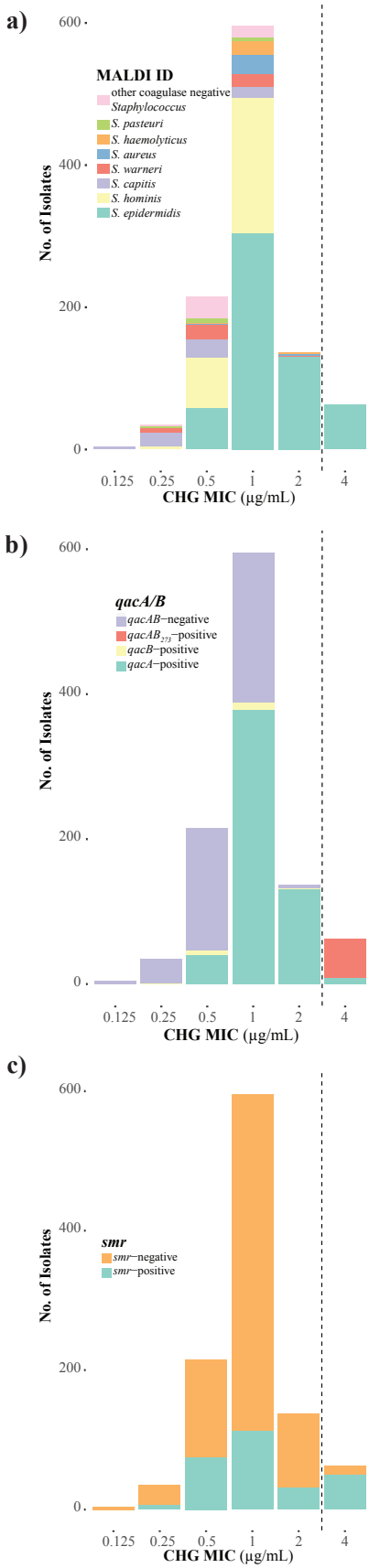


Figure 1. CHG MIC distribution of the 1050 cutaneous Staphylococcus isolates included in our study grouped by a) species, b) *qacA/B* PCR and RFLP patterns, and c) *smr* PCR results. The dashed line indicates the concentration we defined as an elevated CHG MIC (≥ 4 µg/mL).

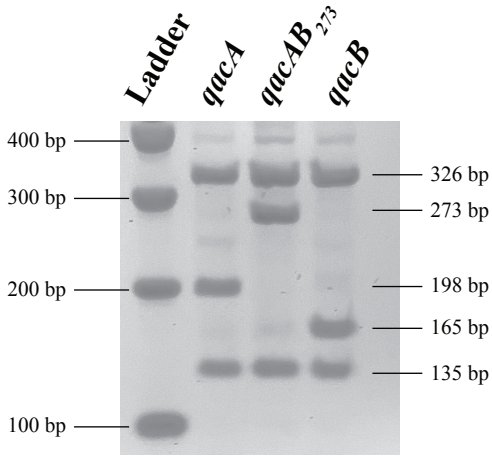
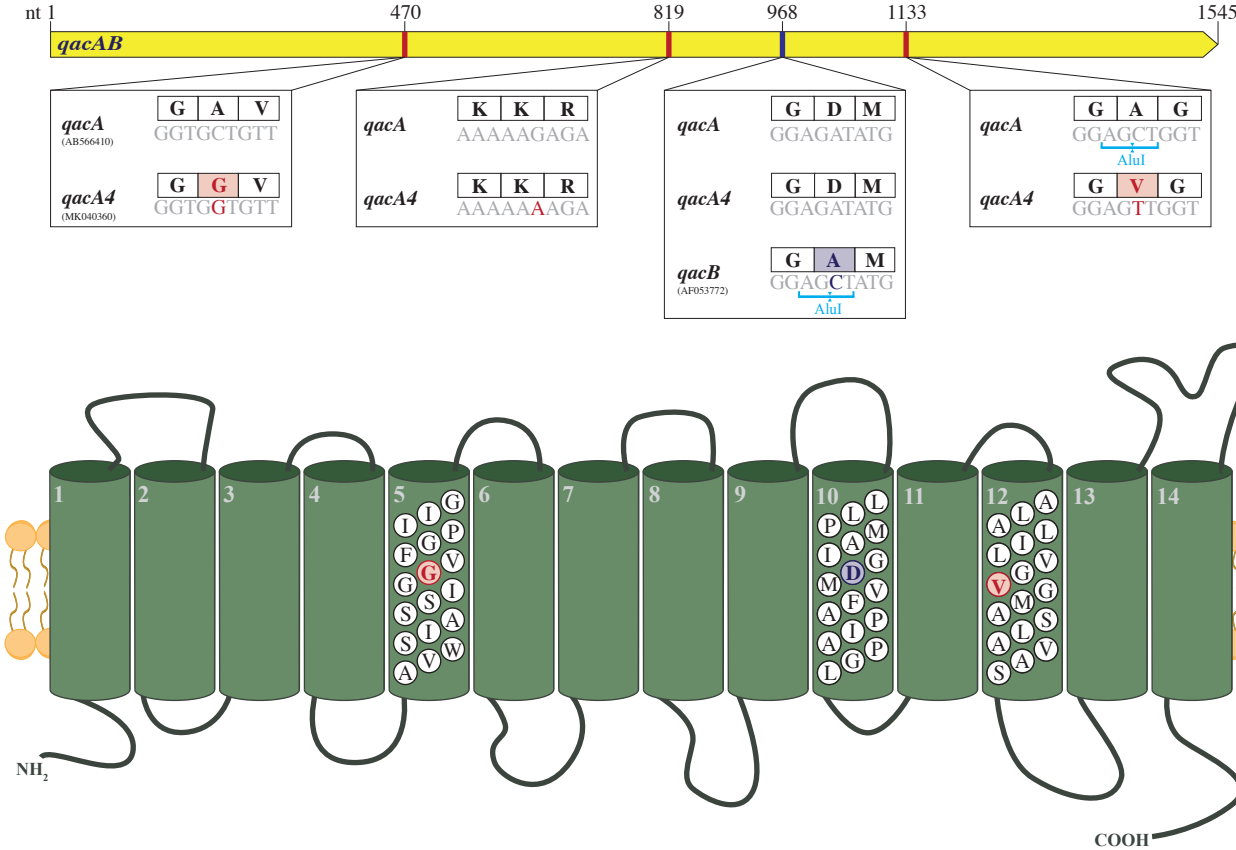


Figure 2. RFLP patterns observed from the AluI restriction digest of the *qacA/B* PCR amplicon. Isolates were classified as *qacA*-postive, $qacAB_{273}$-positve, or *qacB*-positive based on the presence of a 198 bp, 273 bp, or 165 bp fragment, respectively (Alam et al., 2003). Ladder: 100 bp (Promega).



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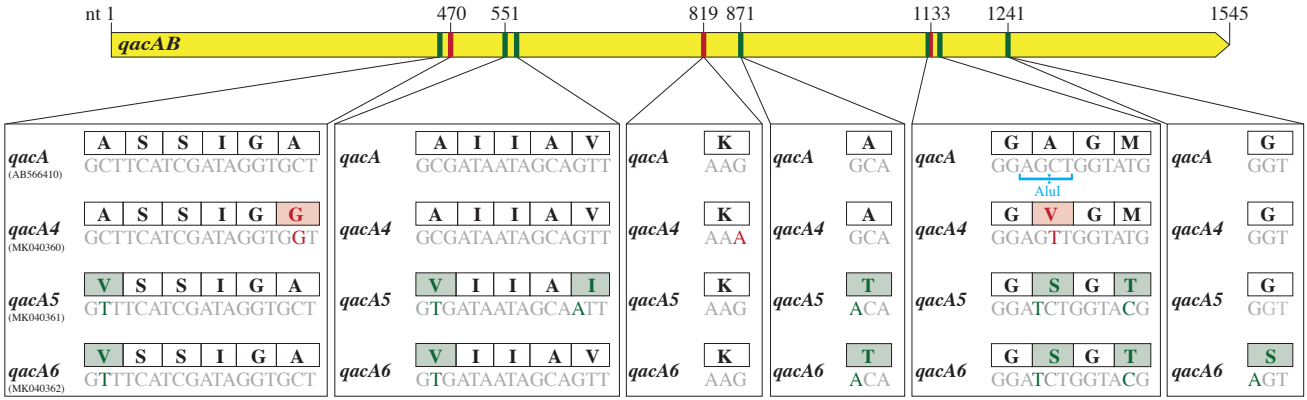


Figure 4. Comparison of the sequences of *qacA4* (MK040360), *qacA5* (MK040361), and *qacA6* (MK040362) and the reference *qacA* sequence (AB566410). The associated AluI restriction sites are shown below the nucleotide sequences and the corresponding amino acid sequences are displayed in the boxes above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

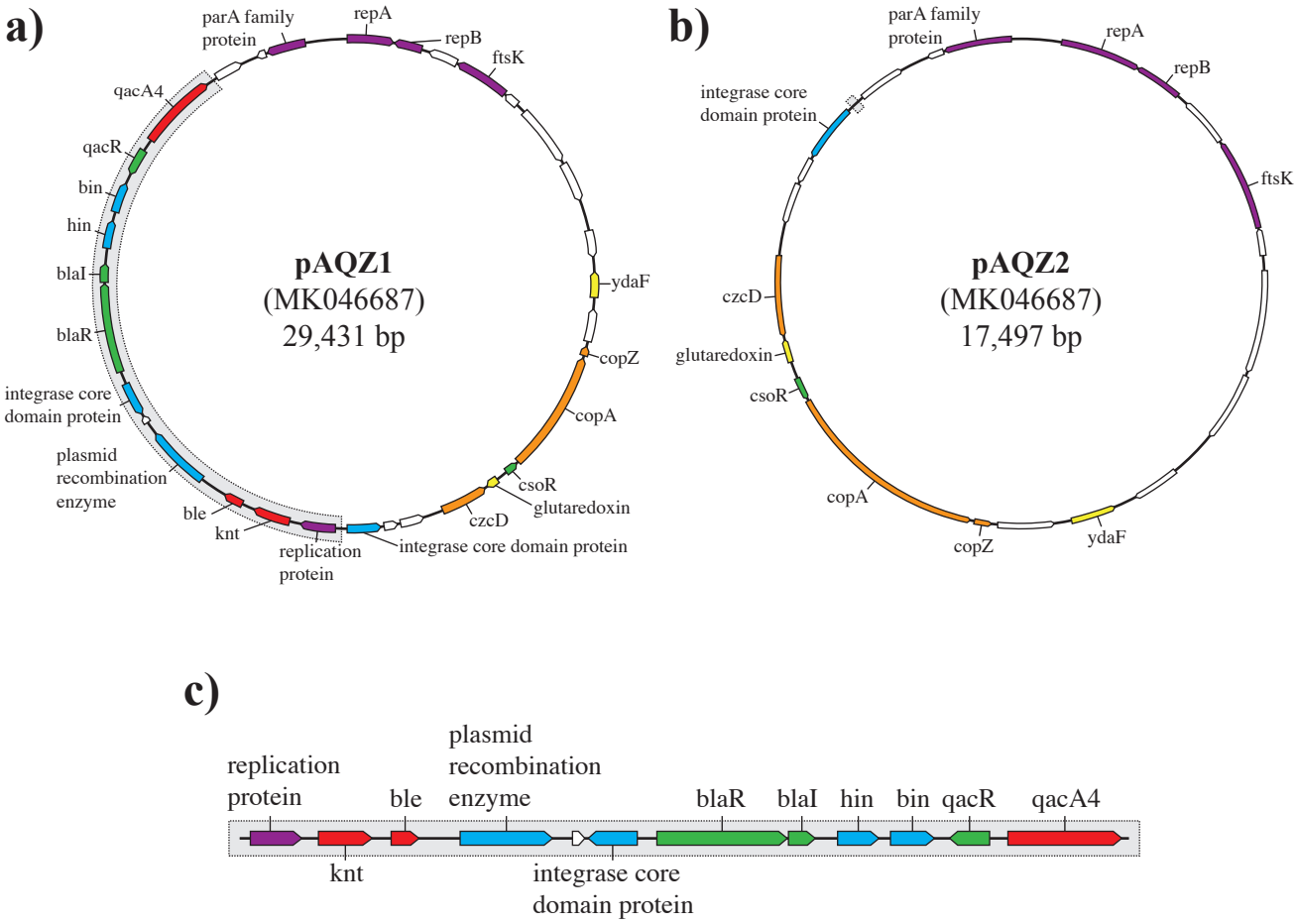
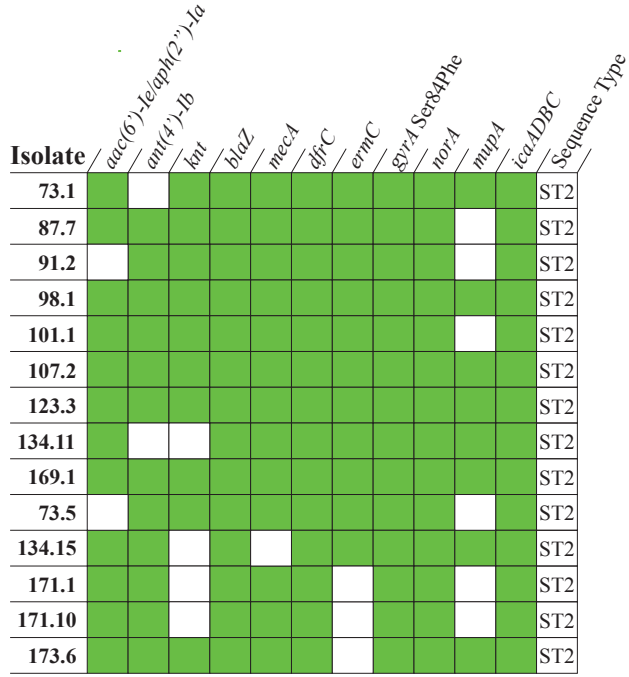


Figure 5. Schematic representations of a) the plasmid pAQZ1 (MK046687) containing *qacA4* obtained from the *de novo* assemblies of isolates 91.2 and 107.2, b) the plasmid pAQZ2 (MK046687) retained in isolate 107.2$_{cured}$, and c) the 11,934 bp segment of pAQZ1 eliminated through the curing experiments. 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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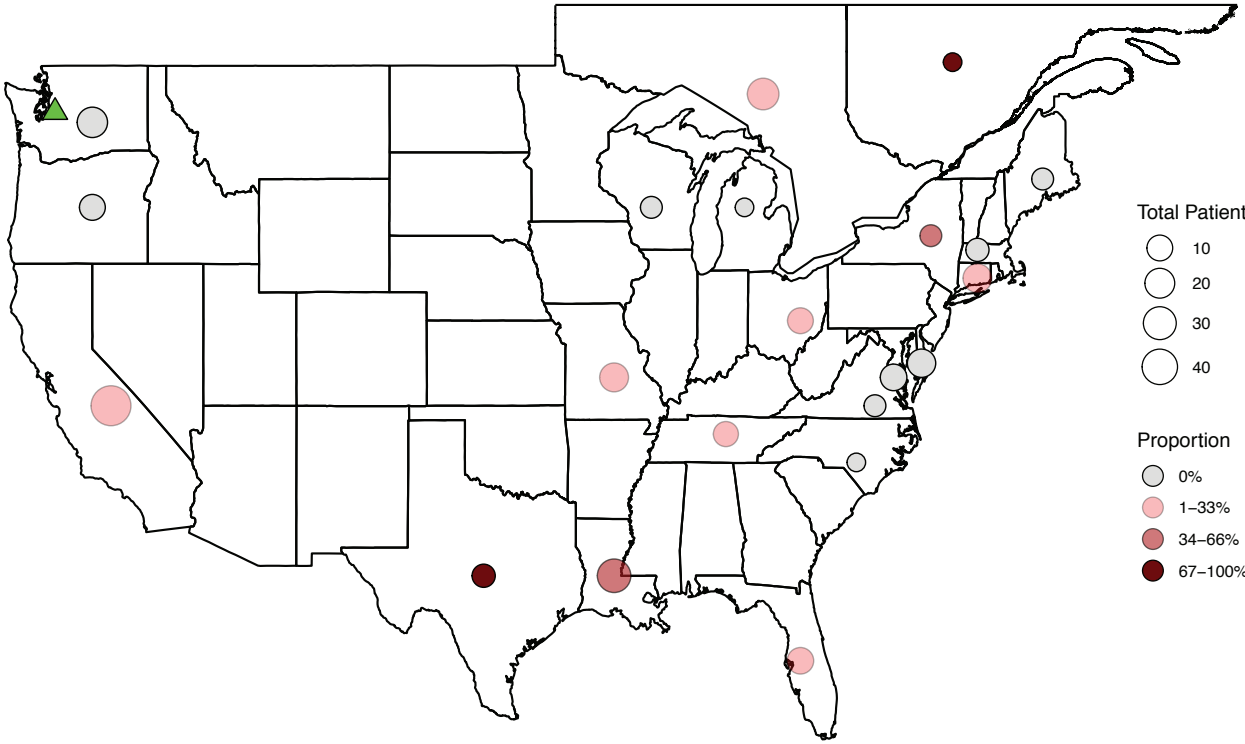

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Table 1. Overview of the cutaneous *Staphylococcus* isolates (n = 1050) included in this study.

MALDI Identification	Total	Percent
S. aureus	30	2.9%
Coagulase Negative *Staphylococcus*	1020	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. ludgunesis	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 sp.	4	0.4%

Table 2. Comparison of the CHG MIC distribrutions, 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.

	qacA	*qacB*	*qacAB*$_{273}$	*qacA/B* –	*smr* +	*smr* –
Total Isolates	559	17	56	418	279	771
MIC_{50}	1	1	4	1	1	1
MIC_{90}	2	1	4	1	2	1
Range	0.5 – 4	0.25 – 2	0.5 – 4	0.125 – 2	0.25 – 4	0.125 – 4

Table 3. Comparison of the CHG MIC distributions, measured in μg/mL, associated with the eight CHG resistance gene combinations detected in our isolates.

	qacA smr +	*qacA smr –*	*qacB smr +*	*qacB smr –*	*qacAB*$_{273}$ *smr +*	*qacAB*$_{273}$ *smr –*	*qacA/B – smr +*	*qacA/B – smr –*
Total Isolates	101	458	5	12	50	6	123	295
MIC_{50}	1	1	1	0.75	4	4	0.5	1
MIC_{90}	2	2	1.6	1	4	4	1	1
Range	0.5 – 4	0.5 – 4	0.5 – 2	0.25 – 1	4 – 4	0.5 – 4	0.25 – 1	0.125 – 2

Table 4. Comparison of the proportion of *qacA*-positive, *qacB*-positive, *qacAB*$_{273}$-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).

Antimicrobial	*qacA*	*qacB*	*qacAB*$_{273}$	*qacA/B –*
FOX*	61.7	52.9	96.4	22.7
ERY*	76.0	88.2	92.9	30.1
CIP*	25.0	5.9	96.4	8.4
GEN*	15.0	0	89.3	2.6
SXT*	60.1	35.3	98.2	22.7
LZD	0	0	0	0
RIF	0.9	0	0	0
VAN	0	0	0	0

* 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$_{cured}$. 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$_{cured}$	*S. epidermidis*	No	No	1	R	R	R	R	R	S	S	S