Multidrug resistance to antiseptics and disinfectants in coagulase-negative staphylococci

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Summary. The occurrence of resistance to antiseptics and disinfectants in clinical isolates of coagulase-negative staphylococci (CNS) was examined. Of 164 clinical strains of CNS isolated in the early 1980s, 65 were resistant to cationic antimicrobial compounds such as cetyltrimethylammonium bromide. Further characterisation of 40 resistant isolates by DNA–DNA hybridisation analysis and phenotypic resistance studies revealed that this resistance was mediated by the multidrug export genes qacA and qacC, characterised previously in Staphylococcus aureus. Of the resistant CNS isolates, 50% contained only qacA, 10% contained only qacC, and the remaining 40% contained both qacA and qacC. Both qacA and qacC genes resided on plasmids in all cases, with qacA located on plasmids of >10 kb, whereas qacC was located primarily on plasmids of 2–3 kb. Representative qacA and qacC plasmids were characterised by restriction endonuclease mapping, and were found to be similar in some cases, but different in others, to those plasmids on which these genes are found in S. aureus.

Introduction

Multidrug resistance to organic cationic antiseptic and disinfectant compounds, such as cetyltrimethylammonium bromide (Ct) and benzalkonium chloride (Bc), has been reported in clinical isolates of Staphylococcus aureus from Australia, Europe, Asia and North America.1,2 Resistance to these compounds has also been characterised in various other organisms, including Bacillus subtilis,3 Escherichia coli4,5 and Klebsiella aerogenes.6 This multidrug resistance is mediated by active export of the toxic compounds from the cell by means of the proton motive force.5,6,7 The wide distribution and broad substrate specificity of these resistance determinants suggest that they may be important elements of an organism’s potential to survive in a hospital environment.6

Antiseptic and disinfectant resistance in S. aureus is encoded by one of at least three separate multidrug resistance determinants, designated qacA, qacB and qacC.1,9 The qacA gene confers the broadest resistance phenotype, including resistance to four classes of compounds: the intercalating dyes such as ethidium bromide (Eb) and proflavine (Pf); the quaternary ammonium compounds such as Ct and Bc; the diamidines such as propamidine isethionate (Pi) and diaminodiphenylamine dihydrochloride (Dd); and the biguanidines such as chlorhexidine (Ch).7 The qacA gene is located predominantly on members of the pSK1 family of multiresistance plasmids,10,11 but has been found also on β-lactamase and heavy-metal resistance plasmids, such as pSK5,12 and may be located chromosomally in some S. aureus clinical isolates.13 The qacB gene is almost identical at a nucleotide sequence level to qacA (personal unpublished results), but specifies resistance only to the intercalating dyes and quaternary ammonium compounds.7 The qacC gene has been found on β-lactamase and heavy-metal resistance plasmids such as pSK23.1 The qacC gene, which is identical to ebr14 and smr15 encodes resistance to Eb and some quaternary ammonium compounds,7 and is located typically either on small (<3-kb) plasmids, such as pSK89, or on large (c. 50-kb) conjugative plasmids, such as pSK41.9,16

In recent years, coagulase-negative staphylococci (CNS) have been recognised increasingly as important causative organisms of infections associated with implanted foreign bodies—such as prosthetic heart valves, prosthetic joints, intravenous catheters, cerebrospinal fluid shunts and peritoneal dialysis catheters—and in patients with compromised host defences.17,18 The mortality rate of CNS infection in some cases, e.g., bacteraemia, is similar to the mortality rate reported with S. aureus.19 While multidrug
resistance to cationic antiseptic has been well-characterised in \textit{S. aureus}, no comprehensive investigations have been completed on antiseptic resistance in CNS. The present study addressed this issue by characterising the occurrence, distribution and phenotype of the \textit{qac} genes in clinical isolates of CNS from Australian hospitals during the period 1979–1984.

Materials and methods

Bacterial strains and plasmids

In total, 164 CNS strains were selected from a collection of multiresistant clinical isolates obtained from Australian hospitals during 1979–1984.\textsuperscript{21} The plasmid-free \textit{S. aureus} strain SK982\textsuperscript{22} was used as a recipient in the plasmid transfer experiments and as a control in the MIC determinations and DNA hybridisation experiments. \textit{S. aureus} strains SK2355,\textsuperscript{23} carrying pSK1 (\textit{qac}A), SK2725,\textsuperscript{2} carrying pSK23 (\textit{qac}B), and SK2721,\textsuperscript{2} carrying pSK89 (\textit{qac}C), were also used as controls in the MIC determinations and in the DNA hybridisation experiments.

General procedures

The media and method used for the determination of susceptibility to antimicrobial compounds have been described previously.\textsuperscript{24} MICs of Be, Ch, Ct, Dd, Eb, Pf, Pi, which is mediated characteristically by \textit{qac}A, were observed in 36 of the 40 strains. These 36 strains also displayed levels of resistance to the intercalating dyes Eb and Pf, to the quaternary ammonium compounds Be and Ct, and to the biguanidine Ch, that were comparable to the resistance phenotype specified by \textit{qac}A in \textit{S. aureus} (table I). The resistance profiles of the other four CNS strains resembled the \textit{qac}C phenotype expressed in \textit{S. aureus}—i.e., resistance to the quaternary ammonium compounds and low-level resistance to Eb—with the exception that two of these strains had Ch MICs of 6 mg/L. The activity of a \textit{qac}C probe was performed as described by Lyon \textit{et al.}\textsuperscript{25} Plasmid DNA transfer to the recipient strain SK982 was performed either by mixed culture transfer\textsuperscript{26} or by electroporation with a BioRad Gene Pulser apparatus. Cells were prepared for electroporation as follows. Cultures in 200 ml of brain heart infusion (BHI) broth were incubated at 37°C until an OD\textsubscript{600} of 0.7 was reached. Cells were harvested, washed once in 80 ml of cold (4°C) distilled water and resuspended in 2.4 ml cold glycerol 10% v/v. Plasmid DNA was added to 250-\mu l cell samples in 0.2-cm cuvettes (BioRad). The Gene Pulser parameters used were: capacitance 25 \mu FD; voltage 2.5 kV; and pulse controller 1000 ohms. After a single pulse, 1 ml of BHI broth was added to the cells. Samples (100 \mu l) were then either plated directly on selective BHI agar, or diluted in 10 ml of BHI broth and incubated overnight at 37°C before plating on selective BHI agar.

DNA manipulation procedures

Isolation of plasmid DNA, digestion with restriction endonucleases, agarose gel electrophoresis, and DNA–DNA hybridisation analysis were all performed as described previously.\textsuperscript{24,26} A probe specific for the closely related genes \textit{qac}A and \textit{qac}B was prepared by labelling the 0.33-kb \textit{PvuII} fragment, internal to \textit{qac}A, from pSK449\textsuperscript{11} by nick translation\textsuperscript{27} with [\textit{z-32}P]dATP. A \textit{qac}C probe was prepared by end-labelling\textsuperscript{27} a 26-mer oligonucleotide (5'-CTTCCAATACAATCAATTGAGC-3') specific to \textit{qac}C\textsuperscript{9} with [\textit{y-33}P]dATP.

Results

Resistance to antiseptics and disinfectants in CNS

In total, 164 clinical isolates of CNS were examined for susceptibility to Eb, Be and Ct. Resistance to one or more of these compounds, as defined in Materials and methods, was observed in 65 (40%) of the 164 strains tested. The MICs of a range of cationic compounds were determined for 40 of the resistant CNS strains (table I). Most of the strains were resistant to high concentrations of nearly all of the agents tested. High-level resistance to the diamidines Dd and Pi, which is mediated characteristically by \textit{qac}A, was observed in 36 of the 40 strains. These 36 strains also displayed levels of resistance to the intercalating dyes Eb and Pf, to the quaternary ammonium compounds Be and Ct, and to the biguanidine Ch, that were comparable to the resistance phenotype specified by \textit{qac}A in \textit{S. aureus} (table I). The resistance profiles of the other four CNS strains resembled the \textit{qac}C phenotype expressed in \textit{S. aureus}—i.e., resistance to the quaternary ammonium compounds and low-level resistance to Eb—with the exception that two of these strains had Ch MICs of 6 mg/L. The activity of a 1:10 mixture of Ch and Ct against the 40 CNS strains did not differ significantly from that of Ct alone.

Detection of the \textit{qac}A and \textit{qac}C multidrug resistance determinants in CNS

DNA–DNA hybridisation analysis was used to investigate whether the \textit{S. aureus} multidrug resistance genes \textit{qac}A–C were responsible for the resistance to antiseptics observed in CNS in this study. Probes specific to \textit{qac}A/B or \textit{qac}C were prepared as described in Materials and methods, and were hybridised under high stringency conditions with total genomic DNA from the 40 resistant clinical isolates of CNS. Hybridisation of these probes with DNA from the control strains—SK982 (negative control), SK2355 (\textit{qac}A), SK2721 (\textit{qac}C) and SK2725 (\textit{qac}B)—confirmed the specificity of the probes (data not shown). DNA from 20 strains hybridised with only the \textit{qac}A/B probe, from four strains with only the \textit{qac}C probe, and from the remaining 16 CNS strains with both of the probes (table I). Table II lists examples of plasmids and \textit{qac} determinants carried by representative CNS strains. The hybridisation profiles of 11 of these strains are
Table I. Characterisation of multidrug resistance in CNS isolates

<table>
<thead>
<tr>
<th>qac-specific probe</th>
<th>Number of isolates*</th>
<th>Plasmid sizes (kb)t</th>
<th>MICs (mg/L)‡</th>
<th>Dyes</th>
<th>QACs</th>
<th>DD</th>
<th>BIG</th>
</tr>
</thead>
<tbody>
<tr>
<td>qacA/B</td>
<td>20</td>
<td>10.8–30.5</td>
<td>100–300</td>
<td>80–320</td>
<td>1–6</td>
<td>2–6</td>
<td>150–400</td>
</tr>
<tr>
<td>qacA + qacC</td>
<td>16</td>
<td>11.5–28.0</td>
<td>100–250</td>
<td>80–320</td>
<td>4–6</td>
<td>4–6</td>
<td>200–400</td>
</tr>
<tr>
<td>qacC</td>
<td>4</td>
<td>2.3–2.7, 11</td>
<td>&lt;20–50</td>
<td>4–6</td>
<td>4–6</td>
<td>25–50</td>
<td>50</td>
</tr>
</tbody>
</table>

* Number of CNS isolates examined in this study that hybridised with the various qac-specific probes.
† Range of sizes of plasmids that hybridised with each of the qac-specific probes in this study.
‡ Range of MICs observed in strains within each hybridisation group. Compounds tested: dyes (Eb, ethidium bromide; Pf, proflavine); QACs, quaternary ammonium compounds (Bc, benzalkonium chloride; Ct, cetyltrimethylammonium bromide); DD, diamidines (Dd, diamidinodiphenylamine dihydrochloride; Pi, propamidine isethionate); BIG, biguanidine (Ch, chlorhexidine diacetate).

Fig. 1. DNA–DNA hybridisation analysis of the distribution of the qac genes in representative CNS strains. (A), agarose gel electrophoresis of total genomic DNA from CNS clinical isolates. Lanes: a, SK30; b, SK50; c, SK53; d, SK62; e, SK76; f, SK91; g, SK95; h, SK252; i, SK275; j, SK281; k, SK352. Plasmid size markers are indicated on the left, as is the position of the chromosomal band (chr). B and C, DNA from gel A transferred to nitrocellulose and hybridised with (B) a qacA/B-specific probe and (C) a qacC-specific probe. Hybridisation of these probes to the open circular (upper) and covalently closed circular (lower) forms of plasmids is particularly evident with the qacA-containing plasmids (B).

In all cases, the probes specific for qacA/B and qacC hybridised with plasmid DNA rather than chromosomal DNA. The qacA/B-specific probe hybridised only with plasmids larger than 10 kb, e.g., those from strains SK30, SK53, SK62, SK76, SK91, SK95, SK252, SK275, SK281, SK352 (fig. 1; lanes a, c, d, e, f, g, h, i, j, k) and SK398 (data not shown). In contrast, qacC was located primarily on small plasmids of 2.3–2.9 kb, e.g., those from strains SK50, SK95, SK252, SK281, SK352 (fig. 1; lanes b, g, h, j and k) and SK2001 (data not shown). However, qacC was also detected on plasmids of c. 11 kb in...
strains SK275 (fig. 1; lane i) and c. 21 kb in strain SK977 (data not shown).

Comparison of the hybridisation results with the MIC data for the 40 CNS strains indicated that the observed resistance levels correlated well with the expected phenotypes of the qac determinants (table I). The 36 CNS strains that displayed resistance to the diamidines Dd and Pi, as well as other characteristics typical of the resistance conferred by qacA (see above), all hybridised with the qacA/B-specific probe. This suggests that these strains contain qacA rather than qacB, since qacB does not confer resistance to the diamidines or to the biguanidines in S. aureus. Strains that contained both qacA and qacC were resistant to higher concentrations of the quaternary ammonium compounds compared with strains containing only qacA. The four strains that contained only qacC displayed resistance to the quaternary ammonium compounds and low-level resistance to the intercalating dyes, but were sensitive to Dd and Pi. Interestingly, two of these strains (SK50 and SK2001) were resistant to Ch, a feature not usually associated with expression of qacC.

Characterisation of representative CNS plasmids encoding qacA and qacC

Plasmid elimination and transfer experiments were performed to confirm that the observed resistance phenotypes were specified by plasmid-encoded qac genes. Plasmids pSK105, pSK107, pSK638, pSK4032 and pSK4769 (table II), which all hybridised with the qacA-specific probe, could be eliminated at frequencies of 50–85% from their respective host clinical strains. DNA–DNA hybridisation and MIC determination analysis of these cured derivatives confirmed that there was a concomitant loss of qacA with each plasmid.

Similarly, transfer of these plasmids to the S. aureus recipient strain SK982 by electroporation or mixed culture transfer, or both, resulted in transfer of the qacA determinant. Transfer of plasmid pSK108, which hybridised with the qacC-specific probe, to strain SK982, followed by analysis of the transciipients by hybridisation and phenotypic characterisation, confirmed that this plasmid carries qacC. The probes specific for qacA and qacC both hybridised to a plasmid or plasmids of c. 11–11.5 kb in CNS strain SK275. Elimination studies revealed that this strain contained two plasmids of 11.5 kb (pSK696) and 11 kb (pSK697), encoding qacA and qacC, respectively, since curing of the latter plasmid resulted in a concomitant loss of qacC, as judged by hybridisation analysis (data not shown).

Selected CNS plasmids were characterised further by restriction endonuclease mapping and DNA–DNA hybridisation analysis of plasmid digests to investigate their possible relationship with qac plasmids from S. aureus. Restriction maps and the location of the qacA or qacC gene, as determined by hybridisation, on each plasmid are presented in fig. 2. The qacA gene is found typically on members of the pSK1 family of plasmids in S. aureus.10 The 23.5-kb CNS plasmid pSK105, which contains qacA, shares extensive restriction map identity with pSK1 (fig. 2), but contains the gentamicin-tobramycin-kanamycin resistance transposon Tn400112 inserted at a different site compared with other plasmids belonging to the pSK1 family. In contrast, the 13.5-kb qacA CNS plasmids pSK107, pSK4032 and pSK4769, which were virtually indistinguishable, did not share detectable restriction map similarity with members of either the pSK1 or the β-lactamase/heavy-metal resistance family of plasmids. Likewise, the 10.5-kb qacA plasmid pSK638 did not show obvious restriction map similarity with other qacA-containing plasmids.

The qacC gene in S. aureus is located commonly on small plasmids, such as pSK89 (fig. 2), or on large conjugative plasmids, such as pSK41.9 Although the CNS qacC plasmid pSK108 was approximately the same size as pSK89, it appeared to be structurally distinct with no apparent restriction map similarity. The larger CNS qacC plasmid pSK697 did not show obvious restriction map similarity with any plasmids characterised previously from S. aureus.

Discussion

The multidrug export gene qacA is located on the pSK1 family of multiresistance plasmids10 which have been isolated frequently from strains associated with hospital outbreaks of S. aureus infection in Australia and the UK during the 1980s.1,10 The qacC resistance gene has been found on large, conjugative plasmids in S. aureus clinical strains isolated since the mid-1970s in Europe, USA and Japan,9,14,15 and on small plasmids in S. aureus strains from Australia.9,15 The data

<p>| Table II. Examples of plasmids and qac determinants carried by representative CNS strains |
|---------------------------------|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>CNS clinical isolate</th>
<th>Plasmid</th>
<th>Plasmid size (kb)</th>
<th>qac determinant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK30</td>
<td>pSK638</td>
<td>10.5</td>
<td>qacA</td>
</tr>
<tr>
<td>SK30</td>
<td>pSK826</td>
<td>2.4</td>
<td>qacC</td>
</tr>
<tr>
<td>SK31</td>
<td>pSK107</td>
<td>13.5</td>
<td>qacA</td>
</tr>
<tr>
<td>SK62</td>
<td>pSK827</td>
<td>15.0</td>
<td>qacA</td>
</tr>
<tr>
<td>SK76</td>
<td>pSK4769</td>
<td>13.5</td>
<td>qacA</td>
</tr>
<tr>
<td>SK91</td>
<td>pSK828</td>
<td>18.0</td>
<td>qacA</td>
</tr>
<tr>
<td>SK95</td>
<td>pSK829</td>
<td>13.5</td>
<td>qacA</td>
</tr>
<tr>
<td>SK98</td>
<td>pSK830</td>
<td>28</td>
<td>qacC</td>
</tr>
<tr>
<td>SK252</td>
<td>pSK831</td>
<td>20.0</td>
<td>qacA</td>
</tr>
<tr>
<td>SK252</td>
<td>pSK832</td>
<td>26</td>
<td>qacC</td>
</tr>
<tr>
<td>SK275</td>
<td>pSK696</td>
<td>11.5</td>
<td>qacA</td>
</tr>
<tr>
<td>SK398</td>
<td>pSK697</td>
<td>11</td>
<td>qacC</td>
</tr>
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<td>SK281</td>
<td>pSK833</td>
<td>22.0</td>
<td>qacA</td>
</tr>
<tr>
<td>SK352</td>
<td>pSK834</td>
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<td>qacC</td>
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<tr>
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<td>qacA</td>
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<tr>
<td>SK2001</td>
<td>pSK836</td>
<td>2.7</td>
<td>qacC</td>
</tr>
<tr>
<td>* The qac gene carried on each plasmid as determined in this study by hybridisation and phenotypic analysis.</td>
<td></td>
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</tr>
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</table>
**qacA-containing plasmids**

Fig. 2. Comparative restriction maps of qac plasmids from selected CNS and S. aureus strains. The following restriction endonuclease sites were mapped for all of the above plasmids and are indicated by: □, BglII; ○, EcoRI; ▼, EcoRV; ▲, HindII; ●, HindIII; ●, HpaII; △, PvuII; ◊, SalI. Additional restriction endonuclease sites were mapped for pSK89 and pSK108 and are shown on the enlarged maps of these plasmids: △, AccI; ▲, MboI; △, PstI; ●, RsaI; ◊, TaqI. The following genes are represented in this figure: dfrA, trimethoprim-resistant dihydrofolate reductase qacA and qacC, multidrug export genes, investigated in this report. The aminoglycoside resistance transposon Tn4001 is also indicated; the IS256 and IS257 repeats associated with these elements are represented by hatched and solid boxes, respectively. The dashed lines on the map of pSK105 correspond to regions which are not present on pSK105 compared with the closely related plasmid pSK1. The maps of pSK105 and pSK89 were as published previously.

Presented in this study indicate that CNS strains isolated from Australian hospitals in the early 1980s contained either or both qacA and qacC. The prevalence of these multidrug export genes in clinical isolates of S. aureus and CNS is probably a result of selective pressure imposed by the use of agents such as acriflavine, Bc, Ch, cetylpyridinium chloride, Cc (cetrime) and Dd as part of commonly employed antiseptic and disinfectant formulations. The observation that qac genes are often located on plasmids...
encoding multiple antibiotic resistance, and that these plasmids are found typically in hospital isolates rather than community isolates, supports the suggestion that resistance to these cationic antimicrobial compounds is maintained because of selective pressures in the hospital environment.

In this study, 40% of the CNS isolates contained both \textit{qac}A and \textit{qac}C. The combination of two \textit{qac} genes in the same isolate has not been reported previously in \textit{S. aureus}, and suggests that there may be a selective advantage in having both \textit{qac}A and \textit{qac}C compared to only \textit{qac}A. A range of MICs of each compound was observed in different isolates containing the same \textit{qac} genes (table I). This resulted, presumably, either from differential expression of the respective \textit{qac} genes in the different clinical strains, or from variations in the levels of background resistance in these strains. The basis of the resistance to Ch observed in the two CNS strains that harboured small \textit{qac}C-carrying plasmids remains to be clarified. This resistance may result from an alteration in the substrate binding site of the \textit{qac}C protein that enables it to recognise Ch. Alternatively, Ch resistance may be a consequence of an as yet uncharacterised resistance determinant, possibly located on the chromosome in these strains.

The \textit{qac}A and \textit{qac}C genes were plasmid-encoded in all CNS strains examined in this study. The \textit{qac}A gene was detected on pSK105, a plasmid belonging to the pSK1 family, and also on a number of previously uncharacterised plasmids (fig. 2). The \textit{qac}C gene was located in CNS strains on a range of small plasmids, and the relationship between these plasmids and the \textit{S. aureus} \textit{qac}C plasmid pSK89 remains to be determined. The identification of \textit{qac}A and \textit{qac}C genes on plasmids which were similar in some cases, but different in others, to those plasmids on which these genes are found in \textit{S. aureus}, also highlights the mobility of genetic information among the staphylococci, and further supports growing evidence \cite{21-23} that \textit{S. aureus} and CNS strains share a common pool of resistance determinants.

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