Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*

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1. SUMMARY

Clinical isolates of *Staphylococcus aureus* carry various antiseptic and disinfectant resistance determinants (qac genes) on a variety of plasmids. The biochemistry and specificity of these resistance genes in *S. aureus* is the subject of this report. The qac genes were separated into two families on the basis of resistance profiles and DNA homology. Isotopic and fluorimetric assays demonstrated that the qac genes encode efflux systems that rely on proton motive force.

2. INTRODUCTION

Multiresistant strains of *Staphylococcus aureus* (MRSA) frequently encode resistance to antiseptics and disinfectants such as quaternary ammonium compounds (Qac) and chlorhexidine, and to intercalating dyes such as ethidium bromide (Eb) [1–3]. These compounds are structurally unrelated, although all are cationic, lipophilic agents. Resistance to a broad range of antiseptics may be of particular significance to an organism's potential to survive in the hospital environment.

Four determinants encoding antiseptic resistance have been detected in MRSA strains. The *qacA* gene has been widely detected on pSK1-like plasmids [4,5] as well as on the β-lactamase/heavy metal resistance plasmid pSK57 [6]. The *qacB* gene has also been located on β-lactamase/heavy metal resistance plasmids (pSK21 and pSK23) [7] as well as on pSK84 and pSK156. *qacC* has only been located on small molecular mass plasmids such as pSK89 [8], while *qacD* has been exclusively located on large conjugative plasmids such as pJE1 [9,10] and pSK41 [11].
These determinants are widely distributed geographically as they have been identified in MRSA strains from Australia, Europe, Japan, and North America [6,8,10,12–15].

A number of resistance mechanisms in bacteria rely on the energised efflux of their substrate. The tetA-D tetracycline resistance gene products of Gram-negative bacteria are known to use proton motive force (pmf) to energise the transport of tetracycline from the cell [16]. In contrast, some resistance determinants, such as that for arsenate, require ATP to energise transport [17]. While the qacA and qacD determinants are thought to specify efflux mechanisms [4,9], it has not yet been established whether pmf or ATP is required to energise this transport.

We report here a survey of the levels of resistance conferred by the various qac genes to a number of antiseptic and disinfectant compounds. The qac genes were then separated into two families on the basis of their resistance profiles and DNA homology. Also, the mechanism of resistance to ethidium encoded by each of the qac genes was determined to be due to active efflux and the energetics of this transport was further examined.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

A description of the S. aureus strains and plasmids used in this study is presented in Table 1. The E. coli strains employed were AN418 (F- ,uncA401,argH,pyrE) [24] and BHB2600 (F-,supE,supF,hsdR,met) [25]. The Escherichia coli plasmids used were pSK449 (qacA cloned into pBR322) [3], pSK503 (qacC cloned into pUC18) [20], pSK534 (qacD cloned into pUC18) [20], and pSK552 (qacB from pSKI56 cloned into pUC18) (I.T.P. and R.A.S., unpublished data).

3.2. General methods

The media used and the method for the determination of minimum inhibitory concentrations (MICs) have been previously described [3,18]. MIC tests were performed in at least triplicate. The transfer of plasmids by mixed culture was performed as previously described [19].

### Table 1

S. aureus strains and plasmids

<table>
<thead>
<tr>
<th>Clinical strain</th>
<th>Year isolated</th>
<th>Plasmid carried</th>
<th>Plasmid size (kb)</th>
<th>qac gene</th>
<th>Plasmid phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK18</td>
<td>1980</td>
<td>pSK1</td>
<td>28.4</td>
<td>qacA</td>
<td>Gm; Tm; Km; Tp</td>
<td>18, 19</td>
</tr>
<tr>
<td>SK449</td>
<td>1981</td>
<td>pSK57</td>
<td>28.8</td>
<td>qacA</td>
<td>Hg; Cd; Pc</td>
<td>6</td>
</tr>
<tr>
<td>SK413</td>
<td>1981</td>
<td>pSK21</td>
<td>35.3</td>
<td>qacB</td>
<td>Hg; Cd; Pc</td>
<td>This study</td>
</tr>
<tr>
<td>SK654</td>
<td>1982</td>
<td>pSK23</td>
<td>38.0</td>
<td>qacB</td>
<td>Gm; Tm; Km; Hg; Cd</td>
<td>This study</td>
</tr>
<tr>
<td>SK1265</td>
<td>1951</td>
<td>pSK84</td>
<td>32.8</td>
<td>qacB</td>
<td>Cd</td>
<td>This study</td>
</tr>
<tr>
<td>SK1275</td>
<td>1951</td>
<td>pSK156</td>
<td>45.4</td>
<td>qacB</td>
<td>Cd; Pc</td>
<td>This study</td>
</tr>
<tr>
<td>SK1448</td>
<td>1960</td>
<td>pSK89</td>
<td>2.4</td>
<td>qacC</td>
<td></td>
<td>20, This study</td>
</tr>
<tr>
<td>Spradlin</td>
<td>1977</td>
<td>pSK41</td>
<td>47.8</td>
<td>qacD</td>
<td>Gm; Tm; Km; Nm; Pm; Tra</td>
<td>21, 22</td>
</tr>
<tr>
<td>L3626</td>
<td>1979</td>
<td>pUW3626</td>
<td>54.4</td>
<td>qacD</td>
<td>Gm; Tm; Km; Nm; Pm; Pc; Tra</td>
<td>19, 22</td>
</tr>
<tr>
<td>A118</td>
<td>1979</td>
<td>pJE1</td>
<td>50</td>
<td>qacD</td>
<td>Gm; Tm; Km; Nm; Pm; Tp; Tra</td>
<td>10, 23</td>
</tr>
<tr>
<td>SK982</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
</tr>
</tbody>
</table>

*Clinical strain refers to strain designation of the S. aureus clinical isolate.

Year isolated refers to the year the clinical strain was isolated for all strains with the SK prefix, or the year first reported for the remaining strains.

*Other than the phenotype specified by the qac gene (see Table 3.), the plasmids may additionally encode resistance to gentamicin (Gm); tobramycin (Tm); karamycin (Km); neomycin (Nm); paromomycin (Pm); mercury (Hg); cadmium (Cd); penicillin (Pc); trimethoprim (Tp) as well as transfer functions (Tra).

NA: not applicable
3.3. Measurement of ethidium efflux

Fluorimetric and isotopic assays were employed to measure ethidium efflux as previously described [4,26], except that fluorescence was measured at 20°C using a Perkin Elmer 204A Fluorescence Spectrophotometer. Cells were loaded with ethidium bromide by treatment with 20 μM carbonyl cyanide m-chlorophenylhydrazone [4]. Substrate-driven efflux was assayed as previously described [26]. In radiochemical experiments, [6-14C] ethidium bromide was used at a specific activity of 3.3 μCi μmol⁻¹ (122 kBq μmol⁻¹).

3.4. Measurement of respiration

Rates of respiration were measured using an oxygen electrode as described previously [26].

4. RESULTS

Previous work has indicated that the *S. aureus* qac determinants specify resistance to a range of antiseptics and disinfectants [3,4,12–14,27,28]. In order to characterise the resistance profiles encoded by each of the various qac determinants, a range of plasmids (Table 1) carrying representatives of qacA-D were transferred to the *S. aureus* recipient strain SK982 in mixed culture experiments. The MICs of a range of compounds were determined for each of these strains and the results are presented in Table 2. Resistance to four different classes of compounds, the intercalating dyes, quaternary ammonium compounds, diamidines, and the biguanidines, was detected. The characteristic phenotypes of the qac genes can be defined and summarized as follows: qacA encodes resistance to all four classes of compounds and confers the highest levels of resistance to all compounds tested except for cetyltrimethylammonium bromide (Ct) and tetraphenylarsonium chloride (Ta). qacB confers similar levels of resistance to the quaternary ammonium compounds and to the dyes compared to qacA. However, qacB characteristically differed from qacA in that it conferred very low levels of resistance to the diamidines, and no resistance to chlorhexidine (Ch). qacC and qacD, on the other hand, demonstrated identical resistance profiles; viz, high resistance to some of the quaternary

<table>
<thead>
<tr>
<th>qac gene</th>
<th>Plasmid</th>
<th>Compounds</th>
<th>MIC values for strains of <em>S. aureus</em> carrying various qac genes</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Dyes</td>
<td>BIG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eb</td>
<td>Pf</td>
</tr>
<tr>
<td>A</td>
<td>pSK1</td>
<td>300</td>
<td>&gt;640</td>
</tr>
<tr>
<td>A</td>
<td>pSK57</td>
<td>300</td>
<td>&gt;640</td>
</tr>
<tr>
<td>B</td>
<td>pSK21</td>
<td>250</td>
<td>320</td>
</tr>
<tr>
<td>B</td>
<td>pSK23</td>
<td>250</td>
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<td>pSK84</td>
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<tr>
<td>C</td>
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<td>pUW3626</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>pJE1</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>SK982</td>
<td>&lt;25</td>
<td>40</td>
<td>0.25</td>
</tr>
</tbody>
</table>

- qac resistance genes designated A to D.
- All plasmids were transferred to the host strain SK982 for MIC testing.
- Compounds tested: Dyes (Eb, ethidium bromide; Pf, proflavine; Cv, crystal violet; R6, rhodamine 6G); BIG, biguanidine (Ch, chlorhexidine diacetate); Diamidines (Dd, diamidinodiphenylamine dihydrochloride; Pt, pentamidine isethionate; Pi, propamidine isethionate); OAC, quaternary ammonium compounds (Ct, cetyltrimethylammonium bromide; Be, benzalkonium chloride; Ce, cetylpyridinium chloride; De, dequalinium chloride; Ta, tetraphenylarsonium chloride). Values are shown as μg/ml.
ammonium compounds (benzalkonium chloride (Be), cetlypyridinium chloride (Cp) and Ct), low levels of resistance to the dye ethidium bromide (Eb), and no resistance to the other dyes, the diamidines or the biguanidine, chlorhexidine.

The qacA and qacD genes have previously been suggested to encode energy-dependent ethidium efflux systems [4,9]. The antiseptic resistance mechanisms encoded by qacB and qacC were subject to a similar analysis using an isotopic assay that monitored the efflux of radiolabelled ethidium from S. aureus cells. qacB-(pSK23) and qacC- (pSK89) containing cells were loaded with radiolabelled ethidium, washed, and then analyzed for efflux of the radiolabelled substrate. When formate (Fig. 1) or glucose (not shown) was added to the cells to energise transport, a rapid efflux of ethidium was observed which was not seen in the absence of a carbon source (Fig. 1a for qacB, Fig. 1b for qacC). Substrate promoted efflux of ethidium from the background strain SK982 was not detected (data not shown) indicating that the qac determinants were responsible for the observed efflux. Comparable rates of formate respiration were observed in the various strains, demonstrating that differences in ethidium efflux were not attributable to differences in respiration (data not shown).

To determine the energetics of the ethidium efflux systems encoded by the qac genes, we examined, using a fluorimetric assay, the effects on efflux of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), and of N,N-di-cyclohexylcarbodiimide (DCCD), an inhibitor of the F_{0}F_{1} ATPase. The formate-driven efflux en-

![Graph](image-url)
coded by qacA in S. aureus was abolished by CCCP but not affected by DCCD (Fig. 2a). This suggested that qacA-mediated efflux is dependent on pmf and not upon ATP. Similar results were obtained in an E. coli host (BHB2600) containing the cloned qacA gene on pSK449 [3] (data not shown). Experiments with the E. coli host strain BHB2600 also showed that under the same experimental conditions used, the chromosomal ethidium efflux system in E. coli [29,30] produced a very slow rate of efflux which was comparable to the rate of efflux observed under de-energized conditions (data not shown). Thus, the chromosomal efflux system is unlikely to have contributed to the results observed with qacA in this host.

To confirm that qacA-mediated efflux is dependent on pmf, pSK449 was transformed into the E. coli uncA mutant strain AN418 that cannot produce ATP by oxidative phosphorylation [24]. The observation that formate-driven ethidium efflux occurred under these conditions (Fig. 2d) supports the notion that the qacA-encoded efflux system is dependent on the pmf. Formate-driven efflux was not observed in the background strain AN418 (data not shown). Similar fluorimetric experiments were performed with S. attreus strains carrying qacB on pSKI56 (Fig. 2b), qacC on pSK89 (data not shown) and qacD on pSK41 (Fig. 2c). Identical rates of efflux were observed with strains carrying qacC or qacD. In all instances, formate driven efflux was inhibited by CCCP but remained unaffected by DCCD. The same results were observed for S. aureus isolates containing qacB on pSK23 or pSK84 (data not shown). qacB on pSK552, cloned from pSK156 (I.T.P. and R.A.S., unpublished data), qacC on pSK503 and qacD on pSK534 [20] were also transformed into the E. coli uncA strain AN418, and the data presented in Fig. 2e and 2f show that formate was sufficient to drive qacB- and qacD-mediated export of ethidium in these strains. Identical rates of efflux were again observed for qacC- and qacD-mediated efflux (data not shown). These results further support the conclusion that the qac gene products do not require ATP to energise the transport of their substrates.

5. DISCUSSION

The qac genes can be classified into two families based on substrate specificity and DNA sequence identity. qacC and qacD have been shown to have identical reading frames at the nucleotide level although they utilise different promoters [20]. The data reported in this paper show that qacC and qacD exhibit identical phenotypes and identical mechanisms and rates of pmf-promoted efflux. On the basis of these identities qacD has been redesignated qacC. The nucleotide sequence of qacC (pSK1) has also been determined [31], but it appears to be unrelated to qacC as shown by the lack of DNA sequence homology [20]. The qacA and qacB genes constitute a family of antiseptic/disinfectant resistance genes which share the property of conferring resistance to high levels of dyes. qacA differs from qacB in that it also confers resistance to higher levels of the diamidines and to chlorhexidine. qacA and qacB share restriction map identity [8] and determination of the nucleotide sequence of qacB suggests that they share a high degree of homology (I.T.P., T.G.L., and R.A.S., unpublished data). The genes of the qacA/B family share a number of resistance specificities with qacC but differ in that the former specify resistance to a broader range of compounds.

A number of antimicrobial resistance genes depend upon energized efflux of their substrate as the mechanism of resistance. The reliance of the qac genes on pmf was demonstrated in this study. Nucleotide sequence analysis of the qacA gene product indicated that it shared significant homology with other transporters known to be dependent on pmf [31], a result which supports the proposed mechanism. In contrast, the nucleotide sequence of the qacC gene showed no similarity with other known transporters [20]. This suggests that QacC is a member of a new family of pmf-dependent transporters.

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REFERENCES