The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance

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Abstract

The prevalence of disinfectant-resistant food-related microorganisms is of concern to the food industry. The *Staphylococcus saprophyticus* strain ST2H6 isolated from a poultry processing plant contained a 2.4-kb plasmid (p2H6) harbouring *qacH*, which encodes resistance to disinfectants based on quaternary ammonium compounds. The complete p2H6 nucleotide sequence revealed an open reading frame encoding a putative protein of 107 amino acid residues with strong similarity to members of the small multidrug resistance protein family. QacH also conferred high-level ethidium bromide resistance and low-level proflavine resistance and thus differed phenotypically from the similar proteins Smr and QacG. Fluorimetry indicated that the high-level ethidium bromide resistance was due to improved efflux energised by the proton motive force. Site-directed mutagenesis substituting the Asp-24 residue with Glu-24 had no effect on resistance characteristics. An additional open reading frame on p2H6 encoded a putative protein with similarity to rolling circle replication proteins.

Keywords: Quaternary ammonium compound resistance; Efflux; SMR family; Staphylococcus

1. Introduction

The focus on safer foods and longer shelf-life has led to more frequent use of disinfectants in the food industry. Disinfectants based on quaternary ammonium compounds (QAC) are widely used in the food industry as well as in clinical environments due to their surface-active, non-corrosive and low-toxic properties. However, QAC-resistant clinical strains of *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) have emerged in many countries ([1,2] and references therein, [3]). More recently, QAC-resistant staphylococci isolated from different kinds of food products and food processing industries have been reported [4,5]. The encoded proteins of three different genes *qacA*, *qacB* and *smr* (also known as *qacC*, *qacD*, and *ebr*) confer efflux-mediated resistance to lipophilic cationic compounds including QAC and dyes (e.g. ethidium bromide) in staphylococci (for a review see [6]). The *qacA* and *qacB* gene are nearly identical at the nucleotide level and are commonly found on large multiresistance plasmids. Both encode transmembrane efflux proteins belonging to the major facilitator superfamily with putatively 12–14 transmembrane segments. The *smr* gene has been detected on small, non-conjugative plasmids and on large conjugative plasmids [1,2].
The encoded Smr protein belongs to the small multidrug resistance protein family (SMR) consisting of only four predicted transmembrane segments [7]. Also included in the SMR protein family are the putative staphylococcal qacG gene products different from the smr gene product in that they confer a higher resistance to ethidium bromide (Eb) in addition to resistance to other dyes like proflavine (Pro), crystal violet and rhodamine 6G. The QacA protein also confers resistance to chlorhexidine and diamidines [2]. The mechanism of resistance to Eb mediated by qacA, qacB and smr has been determined to be due to active efflux driven by the proton motive force (PMF) [2,8]. Here we report the cloning and characterisation of a new multidrug resistance determinant, qacH, residing on the 2.4-kb plasmid p2H6. The protein encoded by qacH shows extensive sequence similarity to members of the SMR protein family.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The QAC-resistant strain ST2H6 was isolated from a poultry processing plant in Norway and identified as *S. saprophyticus* after biochemical analysis and comparative 16S rDNA sequencing (E. Heir, G. Sundheim and A.L. Holck, unpublished). *S. aureus* RN4220 [9] was used as a host in transformation experiments. Staphylococci were grown in brain heart infusion (BHI) broth at 37°C with shaking unless otherwise stated. The *S. aureus* RN4220 transformants with p2H6 were selected on BHI agar containing benzalkonium chloride (BC) and Eb (5 and 0.5 μg ml⁻¹, respectively). *S. aureus* RN4220 transformants containing pSK265 were selected by adding chloramphenicol (6 μg ml⁻¹) to the growth medium. *E. coli* DH5α transformants were grown at 37°C on BHI agar with ampicillin (100 μg ml⁻¹), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactopyranoside, 40 μg ml⁻¹ each).

2.2. Curing of resistance plasmid p2H6

Strain ST2H6 was grown in Mueller-Hinton (MH) broth containing 15 μg ml⁻¹ novobiocin and subcultured every day in increasing sublethal concentrations of novobiocin. Cells were spread onto MH agar containing 0.5 μg ml⁻¹ Eb. Fluorescent colonies accumulating Eb and non-fluorescent colonies not accumulating Eb were analysed for plasmid content and resistance to BC.

2.3. Plasmid DNA isolation and molecular techniques

Standard DNA cloning techniques were performed as described [10]. Plasmids from staphylococci were isolated by a modified alkaline lysis procedure [5,11] following purification by the Wizard Clean up kit (Promega, Madison, WI, USA). Direct shotgun transformation of *S. aureus* RN4220 with p2H6 plasmid DNA was done by electroporation [12]. Overlapping p2H6 DNA fragments were obtained by digestion with *Hin*III, *Taq*I and *Sau*3AI in separate reactions and ligation into pGEM7zf(+) (Promega, Madison, WI, USA). Direct shotgun transformation of *E. coli* DH5α. Both DNA strands were sequenced using a primer walking strategy. The *S. aureus* RN4220 pSK265(qacH) clones containing the *qacH* gene with its native promoter region in plasmid vector pSK265 [13] were constructed by standard molecular techniques using PCR with the flanking primers QH-F (5’-ATCGGATCCCTTTATCTTTACATCTGAGG-3’, nt 1569-1587) and QH-R (5’-TCAGTCGACGAGCTCCTGATTCTTAGTGATGATCCG-3’, nt 2185-2170) containing 5' -connected linkers with *Bam*HI and *Eco*RI restriction sites (underlined), respectively. (Localisation of the primers on the p2H6 plasmid is according to numbering in Fig. 1). Site-specific mutagenesis of the *qacH* gene was accomplished using a two-step PCR strategy [14]. Overlapping mutagenic primers (5’-GGATATTTTTTGAAACCTTCGAGATT-3’, 5’-AATCTTCAGAAGGTCTCAAAATTATACT-3’), each containing a single bp substitution (bold letters) to cause a Asp to Glu amino acid replacement in position 24, were combined with the primers QH-F and QH-R in two PCR reactions, respectively. In a second PCR reaction, the overlapping PCR products of the above reactions were used as template DNA to produce the muta-
genic qacH gene by the flanking primers QH-F and QH-R. The mutagenic qacH gene was ligated in BamHI/EcoRI digested pSK265 vector and transformed into S. aureus RN4220 by electroporation. The mutation was verified by DNA sequencing of both strands.

2.4. Minimal inhibitory concentrations

Minimal inhibitory concentrations (MIC) to the QAC BC, to the dyes Eb, Pro and rhodamine 6G, to the commercial disinfectants TP99 (Henkel-Ecolab, Düsseldorf, Germany), Betane (Agma, Northumberland, UK) and Tego (Th. Goldschmidt, Essen, Germany) and to the antibiotics ampicillin, penicillin G, tetracycline, chloramphenicol, gentamicin, norfloxacin, kanamycin, erythromycin, trimethoprim and vancomycin were determined in a microtitre plate assay [4]. An inoculum of $10^4 - 10^5$ cells ml$^{-1}$ was used. The lowest concentration of antimicrobial agent totally preventing growth after 24 h was taken to be the MIC.

2.5. Ethidium bromide efflux experiments

The fluorescence technique used by Jones and Midgley [15] and Midgley [16] was adapted to measure the efflux of Eb. Cultures grown overnight (16 h) in 50 ml MHB were used as inoculum to 200 ml MHB and grown for 90–120 min to an OD$_{600}$ = 0.3. Aliquots of 10 ml were harvested by centrifugation (3000×g) and were washed twice in 20 mM HEPES buffer (pH 7.0). Loading of the cells was done by resuspending and incubating the cells in 10 ml 20 mM HEPES (pH 7.0) containing Eb (5 $\mu$M) in the presence of (20 $\mu$M) carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 37°C with agitation for 30 min. The cell suspension was chilled on ice and the cells were washed twice in 20 mM HEPES (pH 7.0) containing Eb (5 $\mu$M) and were resuspended in 1 ml ice-cold 20 mM HEPES (pH 7.0) containing Eb (5 $\mu$M) and stored on ice until efflux measurement. Efflux of Eb was initiated by addition of glucose (10 mM). All fluorescence measurements were performed at 37°C with a Perkin-Elmer Luminescence spectrophotometer LS 50B. Fluorescence was integrated every 1 s with excitation and emission wavelengths of 520 and 590 nm, respectively.

3. Results

3.1. Identification of the QAC resistance plasmid p2H6

Curing of the QAC-resistant S. saprophyticus ST2H6 resulted in Eb-accumulating colonies lacking the 2.4-kb p2H6 plasmid. The Eb-accumulating cells were also more sensitive to BC than non-accumulating cells harbouring p2H6. S. saprophyticus ST2H6 plasmid DNA was transformed by electroporation into the BC-sensitive S. aureus RN4220 and QAC-resistant transformants were selected by plating on BHI agar containing BC and Eb (5 and 0.5 $\mu$g ml$^{-1}$, respectively). Isolated transformants contained a plasmid of 2.4 kb (p2H6) and showed a typical BC- and Eb-resistant phenotype.

3.2. Nucleotide sequence of the QAC resistance plasmid p2H6

The complete nucleotide sequence of the 2.4-kb p2H6 plasmid contained two open reading frames (ORFs) transcribed in opposite directions (Fig. 1). The smaller ORF, designated qacH, encoded a putative protein of 107 amino acid residues with similarity to the QAC resistance determinants smr and qacG (76 and 70% identity to qacH, respectively, at the nucleotide level). The potential promoter region of qacH contained putative -35 and -10 regions with the optimal 17-bp spacing [17]. Just downstream of qacH a region with partial homology to single strand origins (SSOs) was evident (Fig. 1). In addition to being the initiator site of lagging strand replication, this region with dyad symmetry (inverted repeats) may also have the potential to serve as a transcriptional terminator for the qacH transcriptional unit. A 28-bp region comprising the 5′-terminal region of the SSO was found directly repeated at bp 122–149. The larger ORF (rep) encoded a putative replication initiator protein of 330 amino acid residues. The initiation codon of the rep2H6 gene is presumed to be the less commonly used GTG codon. A candidate ribosomal binding site together with potential -35 and -10 regions with the optimal 17-bp spacing was also associated with the rep2H6 gene. Regions of dyad symmetry, possibly functioning as rho-independent transcription terminators, are located in a
region approximately 100–250 bp downstream of rep2H6. Upstream of rep is a region with extensive homology to regions found to contain the nick site of the leading strand origin (+ori) of plasmids belonging to the pC194 family [18]. Comparison of the rep2H6 nucleotide sequence with sequences in the
GenEMBL databank revealed similarity (>60%) with putative replication protein encoding sequences of plasmids replicating by a rolling circle mechanism. Highest similarity (80%) to the rep2H6 gene was shown for the putative rep gene of the smr-harbouring plasmid pST827 [5]. Further nucleotide sequence analysis revealed similarity in several hundred bp of rep2H6 flanking regions to corresponding regions of the smr plasmid pST827 although regions unique to both plasmids were also present (data not shown).

### 3.3. Resistance to antimicrobial agents

Strains expressing the QacH protein showed significantly higher MIC values to BC, Eb and Pro than corresponding susceptible strains (Table 1). The QacH, Smr and QacG expressing cells showed small differences in MIC values to BC (8–10 μg ml⁻¹).

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<tr>
<th>Strain</th>
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<td>QAC</td>
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<td></td>
<td>BC</td>
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<tr>
<td>S. saprophyticus ST2H6⁵</td>
<td>10</td>
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<tr>
<td>S. saprophyticus ST2H6⁶</td>
<td>–</td>
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<tr>
<td>S. aureus RN4220</td>
<td>p2H6</td>
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<tr>
<td>S. aureus RN4220</td>
<td>pSK265(qacH)⁷</td>
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<td>S. aureus RN4220</td>
<td>pSK265(smr)⁷</td>
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<td>S. aureus RN4220</td>
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<td>S. aureus RN4220</td>
<td>pSK265⁸</td>
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⁺Minimal inhibitory concentration (MIC) for the QAC benzalkonium chloride (BC) and the dyes ethidium bromide (Eb) and proflavine (Pro). All MIC values are averages of at least three repeated experiments.

⁵Wild-type S. saprophyticus ST2H6.

⁶Negative control, wild-type S. saprophyticus ST2H6 cured for qacH-harbouring plasmid p2H6.

⁷qacH, smr and qacG with their native promoters cloned in pSK265 plasmid vector.

⁸Negative control.
However, the QacH-expressing cells (S. aureus RN4220) showed significantly higher resistance to Eb (440 μg ml⁻¹) than corresponding cells expressing Smr or QacG (80 μg ml⁻¹). In addition, QacH conferred low-level resistance to the dye Pro, a characteristic not expressed by Smr and QacG (Table 1). Resistance to Eb and Pro varied depending on host strain (wild-type ST2H6 vs S. aureus RN4220) and plasmid (p2H6 vs high copy number pSK265). No difference in susceptibility was observed for cured and p2H6-harbouring S. saprophyticus ST2H6 wild-type strain to the other disinfectants, dyes and antibiotics tested (see Section 2.4 for compounds applied).

3.4. Site-directed mutagenesis

The ability of the qacH gene product to confer high-level Eb resistance compared to the observed low-level Eb resistance conferred by Smr and QacG warranted further investigations of this phenomenon. Mutational analysis performed by Grinius and Goldberg [8] on smr showed that expression of the Asp-24 mutant protein made E. coli cells 60% more resistant to Eb than the wild-type Glu-24 protein. Sequence analysis of QacH verified an Asp residue at position 24. To determine if the observed high-level Eb resistance expressed by QacH was due to this residue, Asp was substituted for Glu by site-directed mutagenesis at this position. Interestingly, this substitution had no effect on resistance (MIC values) to Eb, BC or Pro (data not shown).

3.5. Ethidium bromide efflux in staphylococci expressing different Qac proteins

The S. aureus RN4220 host cells expressing either QacH, mutated QacH (see above), Smr or QacG were compared for their ability to efflux Eb. The uncoupler CCCP had a similar inhibiting effect on the efflux of Eb in cells expressing either QacH, QacG or Smr (Fig. 2A,D,E). In the absence of CCCP and the presence of a carbon source (glucose), efflux was initiated (Fig. 2I,G,H). This indicated that QacH, like QacG and Smr, energised efflux by the PMF electrochemical gradient. QacH-expressing cells had a quicker response to the efflux of Eb than cells expressing QacG or Smr (Fig. 2I,G,H).

The efflux pattern of cells expressing the mutated QacH protein was identical to cells expressing the wild-type QacH protein (Fig. 2I,J).

4. Discussion

A new staphylococcal gene, qacH, encoding resistance to QAC comparable to that of smr and qacG has been detected. Unlike Smr and QacG, QacH also conferred low-level Pro resistance (Table 1). Although cloned in the same plasmid vector, the qac genes were expressed from their native promoters. Thus, the phenotypic differences observed could be due to different levels of expression of the genes. However, the resistance characteristics observed suggest that QacH confers a broader resistance phenotype compared with QacG and Smr. The 107-amino acid QacH protein showed 78% identity to the staphylococcal Smr and 70% identity to the QacG protein (Fig. 3). Significant similarity was also evident to other members of the SMR protein family [7,8] including QacE (41%) and EmrE (40%), proteins expressed by Gram-negative bacteria. The efflux patterns observed indicated that QacH-expressing cells had a quicker response to efflux after the addition of glucose and a higher Eb efflux activity. The uncoupler CCCP had a similar inhibitory effect on Eb efflux for cells expressing either QacH, Smr or QacG. Overall, the amino acid sequence identity and Eb efflux patterns confirm that QacH, like other members of the SMR protein family, confers an efflux mechanism energised by the proton motive force.

The Smr protein has been extensively studied regarding the potential roles of specific amino acid residues in protein conformation, substrate recognition and/or drug efflux [8,19]. A number of specific amino acid residues have been shown to possess key functions in this conjunction. The alignment (Fig. 3) showed that QacH in various sites contained amino acid residues not present in many corresponding positions of other members of the SMR protein family. These positions represent potential sites responsible for the altered phenotypic characteristics observed for QacH compared with Smr and QacG. The most conspicuous difference observed was the QacH Asp residue in position 24, in place of Glu
for the other related efflux proteins. Site-directed mutagenesis on smr, performed by Grinius and Goldberg [8], showed that the replacement of Glu-24 with Asp made cells more resistant to Eb without affecting the expression level of the protein. Thus, the Asp-24 residue of QacH was suspected to contribute to the high-level Eb resistance phenotype observed for cells expressing this protein. However, the resistance phenotype to any of the compounds tested of mutants expressing the Glu-24 QacH protein was indistinguishable from that of cells expressing wild-type QacH. Neither could any difference in efflux properties be observed when these cells were tested in a fluorimetric assay for Eb export. This suggests that other residues and/or domains of QacH may be responsible for the phenotype observed. The observation that QacH-expressing cells have a higher resistance towards Eb and Pro while no difference in resistance was observed for the structurally different substrate BC suggests that increased Eb resistance is due to specific interaction of Eb with QacH. This is also consistent with studies of other resistance proteins like Bmr [20], QacA and QacB [21] suggesting that specific single amino acid residues are critical for the recognition of specific drugs. Amino acid residues in other positions may indirectly affect the substrate recognition site and/or the binding site by conformational alteration of the QacH protein. Further mutational analyses of these proteins are required to obtain a better understanding of multidrug recognition.

The qacH-harbouring plasmid p2H6 showed similarity to other plasmids originating from a variety of species and genera. The larger ORF (rep2H6) showed extensive homology to replication protein encoding regions of the pC194 family of plasmids. The p2H6 plasmid also contained a conserved region of the leading strand origin (+ori). These sequence features strongly suggest that the p2H6 plasmid replicates by a rolling circle mechanism [18,22].

All previously characterised plasmids harbouring the smr or the qacG genes show remarkable identity in their putative SSOs (also known as palA) only differing in a few nucleotide positions and always located just downstream of the qac resistance termination codon. The SSO sequences of plasmids belonging to the same family are generally not homologous [22]. This supports the cassette-like structure often observed for plasmids of the pC194 family [18] and indicates that the qac gene and the SSO together represent a cassette element. A candidate SSO was also located just downstream of qacH. Although partial homology was observed, this region differed from previous SSO sequences detected. The extensive secondary structure typical for SSOs [22] suggests that this region is a functional SSO. Which effect these SSO differences have on the replication, and consequently on the copy number of p2H6, is not clear. Overall, the cassette-like plasmid structure indicates that the various smr/qacG/qacH plasmids have evolved through deletions and insertions of DNA elements.

Concerning QAC resistance, an interesting finding is the prevalence of smr in enterococci as well as in different species of staphylococci [23]. Recently, a multidrug-resistant S. aureus isolate from Korea harboured a smr-containing plasmid [24] with 99.7% sequence identity to a plasmid first reported to be present in an Australian S. epidermidis strain [25]. Together with other reports, this indicates that gene transfer both between different genera and between species within a genus may occur with the possible spread of resistance genes to pathogenic strains. The spread and maintenance of QAC resistance genes in staphylococci isolated from clinical environments and the food industry may be due to selective pressure caused by the use of cationic biocides [26]. The detection of three genes (smr, qacG, qacH) encoding staphylococcal proteins with extensive similarity (≥70%) is comparable to other efflux protein families that also have members of highly similar proteins (QacA/QacB (see above), AcrB/AcrF (77% identity), CnrA/NccA (89% identity) [6] and references therein). These highly similar proteins often differ in substrate specificity.

References


