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Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: Membrane topology and identification of residues involved in substrate specificity

(drug efflux pumps/protein topology/major facilitator superfamily)

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ABSTRACT **The closely related multidrug efflux pumps QacA and QacB, from the bacterial pathogen *Staphylococcus aureus*, both confer resistance to various toxic organic cations but differ in that QacB mediates lower levels of resistance to divalent cations. Cloning and nucleotide sequencing of the *qacB* gene revealed that *qacB* differs from *qacA* by only seven nucleotide substitutions. Random hydroxylamine mutagenesis of *qacB* was undertaken, selecting for variants that conferred increased resistance to divalent cations. Both QacA and the QacB mutants capable of conferring resistance to divalent cations contain an acidic residue at either amino acid 322 or 323, whereas QacB contains uncharged residues in these positions. Site-directed mutagenesis of *qacA* confirmed the importance of an acidic residue within this region of QacA in conferring resistance to divalent cations. Membrane topological analysis using alkaline phosphatase and β-galactosidase fusions indicated that the QacA protein contains 14 transmembrane segments. Thus, QacA represents the first membrane transport protein shown to contain 14 transmembrane segments, and confirms that the major facilitator superfamily contains a family of proteins with 14 transmembrane segments.**

The major facilitator superfamily (MFS) consists of various membrane transport proteins from both bacteria and eukaryotes involved in the symport, antiport, or uniport of a range of substrates (1, 2). On the basis of comparative amino acid-sequence analysis, Marger and Saier (2) have suggested that the MFS consists of five distinct clusters or families of membrane transport proteins that share a common structural motif of 12 transmembrane spanning segments (TMS). Within the MFS, cluster 1 was defined as consisting of a range of related drug resistance proteins (2). Other studies have proposed that the proteins within cluster 1 can be separated into two distinct families of resistance-conferring proteins (1, 3). Hydropathy and charge bias analyses have suggested that members of these two families differ in their predicted membrane topologies (3), with one family characteristically containing 12 TMS, in an analogous manner with other MFS transporters, and the other family potentially containing 14 TMS. The 12-TMS family is typified by the tetracycline exporter TetB from *Escherichia coli* (4), whereas perhaps the best-characterized member of the 14-TMS family is the multidrug exporter QacA from *Staphylococcus aureus* (3, 5).

Within these two proposed families of export proteins in cluster 1 of the MFS, a number of multidrug resistance efflux pumps have now been identified—namely, the *Bacillus subtilis* Bmr (6), *E. coli* EmrB (7), and *S. aureus* QacA (5) proteins (see Fig. 3, below). These proteins have been shown to be involved in conferring resistance to structurally diverse compounds by active export, in an analogous manner to the mammalian multidrug resistance protein, P-glycoprotein, which exports a broad range of cytotoxic drugs (8). However, unlike the ATP-driven P-glycoprotein pump, the multidrug efflux pumps belonging to the MFS are driven by the proton motive force (pmf) of the transmembrane proton gradient. Although an increased number of multidrug resistance efflux pumps are being identified in prokaryotic and eukaryotic organisms (3, 9, 10), the molecular basis of multidrug recognition by these pumps remains unclear.

The staphylococcal multidrug resistance gene *qacA* mediates resistance to various toxic organic cations, including intercalating dyes—e.g., ethidium bromide and a number of commonly used antiseptics and disinfectants such as cetrimide, benzalkonium chloride, and chlorhexidine (11, 12). These disparate compounds differ structurally but share the common feature of being lipophilic organic cations. Consistent with its product being a member of the MFS, transport studies have indicated that *qacA* confers resistance to ethidium bromide (11) and other organic cations (I.T.P. and R.A.S., unpublished data) via pmf-dependent efflux. The *qacA* gene from pSK1 has been cloned and expressed in *E. coli* (13, 14); its nucleotide sequence was then determined (5). A putative regulatory gene, *orf188*, was identified upstream of *qacA*, whose product shares sequence similarity with various regulatory proteins (5). Preliminary analyses of the regulation of *qacA* have supported the notion that the product of *orf188* may act as a *trans*-acting repressor of *qacA* expression (M.H.B., I.T.P., and R.A.S., unpublished data).

A closely related determinant, *qacB*, from *S. aureus* has been identified on the heavy metal resistance plasmid pSK23 and suggested to share homology with *qacA* on the basis of restriction map similarity and Southern hybridization analysis (15). However, whereas *qacA* encodes resistance to both monovalent and divalent organic cations, *qacB* characteristically differs from *qacA* by conferring lower or no resistance to divalent organic cations (11, 15).

This study was undertaken to investigate the molecular basis of the phenotypic differences observed between *qacA* and *qacB*. The complete nucleotide sequence of *qacB* was determined and compared with the previously determined sequence of *qacA*. Random chemical and site-directed mutagenesis was used to clarify the importance of specific differences between *qacA* and *qacB*. Additionally, the proposed 14-TMS topology

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Abbreviations: Dd, diamidinodiphenylamine dihydrochloride; MFS, major facilitator superfamily; Pi, propamidine isethionate; Pe, pentamidine isethionate; TMS, transmembrane segment(s).

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U22531).

of the QacA protein was investigated using a genetic fusion approach with alkaline phosphatase and β-galactosidase acting as reporters of subcellular localization, in order to corroborate the division of the export proteins in the MFS into two separate families with different membrane topologies.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* K-12 strains BHB2600 (F'803 *supE supF hsdR met*) (16) and CJ236 (*dut1 ung1 thi1 relA1* pJC105, Cm^R) (17) were used in this study. The *S. aureus* plasmids pSK1 and pSK23, and the *E. coli* plasmids pSK449, pUC18, and pBluescript SK+ have been described (11, 13, 18–20).

General Methods. Antiseptic resistance profiles were analyzed by minimum inhibitory concentration determinations, for the range of monovalent and divalent organic cations employed by Littlejohn *et al.* (11), as described (11, 18). Minimum inhibitory concentration determinations were done in at least triplicate; only data for the representative compounds—ethidium bromide, benzalkonium chloride, diamidinodiphenylamine dihydrochloride (Dd), pentamidine isethionate (Pe), and propamidine isethionate (Pi)—is displayed. Ethidium efflux was assayed indirectly with a fluorimetric assay, as described (14, 21).

Recombinant DNA Techniques. Standard molecular cloning techniques were done as described (22). PCR were done with *Pfu* DNA polymerase (Stratagene) or Pyrostase (Molecular Genetics Resources) as per the manufacturer's recommendations. Nucleotide sequences were determined on both strands by the procedure of Sanger *et al.* (23) using a T7 DNA polymerase sequencing kit (Pharmacia) and specific oligonucleotides as sequencing primers. Oligonucleotides were synthesised using a Beckman Oligo 1000 DNA synthesiser. Sequences were stored and assembled using the program SEQUENCHER (Gene Codes Corp.) and analyzed using the STADEN (24) and Genetics Computer Group (25) packages maintained by the Australian National Genomic Information Service (ANGIS). Phylogenetic analyses were done as described (3). Tn*phoA* transposon mutagenesis was done as described by Manoil and Beckwith (26).

Hydroxylamine Mutagenesis. Hydroxylamine mutagenesis of plasmid pSK537 (*qacB*) DNA was done as described (27). Mutagenized plasmid DNA was used to transform *E. coli* strain BHB2600, and transformants were selected for resistance to Dd at 150 μg/ml.

Site-Directed Mutagenesis. Site-specific oligonucleotide-directed mutagenesis of pSK4219 (*qacA* cloned as a PCR fragment, amplified from a pSK449 template, into the vector pBluescript SK+) was undertaken as per Kunkel *et al.* (17) using a single-stranded DNA template. Oligonucleotide primers employed in mutagenesis (codon changes introduced are indicated in boldface type) were as follows: D323A (CCTATGGAATAGGA**GCC**ATGGTGTTTGCACC), A291T (TTTATGACAATGTTCGCGATG**ACA**TCTGTTTTGTTATTAGC), M380T (GTTGGAGCTGGT**ACC**GCTTCACTAGCAGTTGC), and D323E (CCTATTACCTATGGCGATCGG**AGAA**ATGGTGTTTGCACC).

Analysis of Fusion Protein Activity. Alkaline phosphatase activity was determined as described (28) using *p*-nitrophenyl phosphate. β-Galactosidase activity was estimated by scoring the ability of colonies to cleave the chromogenic substrate 5-bromo-4-chloro-3-indoyl D-galactopyranoside as described (29). Alkaline phosphatase fusion proteins were detected immunologically by using rabbit anti-PhoA IgG (5 Prime → 3 Prime, Inc.) as described (26).

Table 1. Sequence differences between *orf188* and *qacA/B* on plasmids pSK1 and pSK23

Location, codon no.	pSK1*	pSK23†
orf188		
104	GTA	GCA
qacA/B		
1	CTG (Met)	ATG (Met)
26	GTT (Val)	ATT (Ile)
152	GCT (Ala)	GTT (Val)
167	TTA (Leu)	ATA (Ile)
291	GCA (Ala)	ACA (Thr)
323	GAT (Asp)	GCT (Ala)
380	ATG (Met)	ACG (Thr)

*pSK1 *qac* region sequence accession no. X56628.
†pSK23 *qac* region sequence accession no. U22531.

RESULTS

Cloning and Nucleotide Sequencing of *qacB*. The *qacB* region on the *S. aureus* plasmid pSK23 was cloned into pUC18, generating the plasmid pSK537. Nucleotide sequence analysis of this region revealed two open reading frames in opposite orientations, which were virtually identical to *qacA* and *orf188* from pSK1 (5) and hence were designated *qacB* and *orf188*, respectively (GenBank accession no. U22531). *orf188* on pSK23 differs from *orf188* on pSK1 by a single nucleotide change, and *qacB* differs from *qacA* by 7 bp (Table 1). Notable alterations between *qacA* and *qacB* include the use of different proposed start codons (CTG in *qacA* and ATG in *qacB*) and

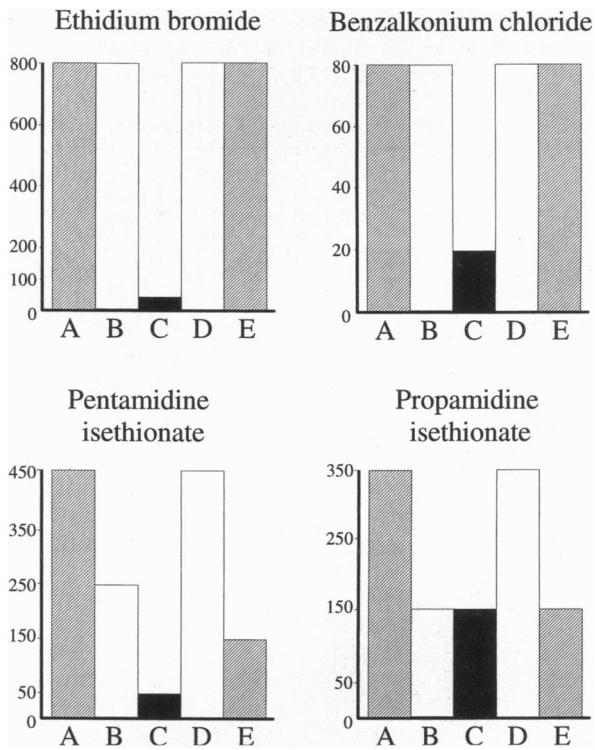

FIG. 1. Comparative resistance phenotypes conferred by *qacA*, *qacB*, and the mutants generated in this study. The vertical axes of the graphs display the minimum inhibitory concentration values (μg/ml) for the monovalent compounds, ethidium bromide and benzalkonium chloride, and the divalent compounds, Pe and Pi. Strains carrying plasmids with the following cloned genes are represented on the horizontal axes as bars: A, *qacA*; B, *qacB*; C, negative control; D, *qacB* G322E or *qacB* A323D; E, *qacA* D323A. Additionally, strains carrying the following mutant genes—*qacA* (A291T), *qacA* (M380T), and *qacA* (D322E)—displayed an identical resistance profile to that of a strain with wild-type *qacA*.

three differences resulting in nonconservative amino acid substitutions at amino acids 291, 323, and 380 in their respective products. From hydropathy analyses (30, 31), QacB, like QacA (5), is predicted to possess 14 TMS.

Isolation of *qacB* Mutants that Confer Resistance to Divalent Cations. To explore the basis of the observed differences in resistance to divalent cations conferred by *qacA* and *qacB*, hydroxylamine mutagenesis of plasmid pSK537 was used to randomly generate mutants of *qacB* that conferred high-level resistance to divalent organic cations.

From two independent experiments a total of seven mutant plasmids that conferred resistance to Dd at >150 μg/ml were obtained. After sequencing and phenotypic analysis, three distinct *qacB* mutant types were identified in which all of the changes occurred within the sequence for the putative TMS 10 of the various mutant proteins; no mutations were detected in *orf188* or elsewhere on these plasmids.

One *qacB* mutant type G322E contained a GGA (glycine, G) to GAA (glutamate, E) mutation, corresponding to amino acid 322 in the deduced QacB polypeptide. A second also contained this alteration (G322E) and additionally possessed an A323T mutation. The third mutant type contained an A323D substitution. Significantly, these three mutations each introduced an acidic residue (either aspartate or glutamate) at amino acid 322 or 323 in QacB in place of an uncharged residue at these positions; notably, QacA contains an aspartate at amino acid 323.

Antimicrobial susceptibility and transport studies indicated that the *qacB* mutants G322E and A323D conferred a similar spectrum of resistance to that of *qacA*, rather than *qacB*, and in particular conveyed resistance to the divalent compounds Dd (data not shown) Pe and Pi (Fig. 1D) bars. Interestingly, the *qacB* double mutant G322E/A323D conferred slightly lower levels of resistance to divalent compounds compared with the corresponding single G322E mutant (data not shown).

Site-Directed Mutagenesis of *qacA*. To confirm the importance of the presence of an acidic residue within putative TMS 10 of QacA and to investigate the potential significance of other substitutions between QacA and QacB, site-directed mutagenesis of *qacA* was undertaken. Single mutations were introduced into *qacA* at each of the three nonconservative amino acid positions altering the codons to the corresponding ones in *qacB*, generating the *qacA* mutants D323A, A291T, and M380T. Additionally, a *qacA* D323E mutant was constructed to examine whether glutamate could substitute effectively for aspartate at amino acid 323 in QacA.

The phenotypes conferred by these mutants were investigated by susceptibility tests (Fig. 1); due to difficulties with continuing commercial availability of Dd, these mutants could not be tested against this compound. Mutations at amino acids 291 (A291T) and 380 (M380T) in QacA had no effect on its capacity to mediate resistance to monovalent or divalent organic cations. The *qacA* D323E mutant conferred a resistance phenotype indistinguishable from that conferred by *qacA*, indicating the acidic residue in this position can be exchanged for another acidic residue without noticeably affecting the competence of the resistance pump. In contrast, the *qacA* D323A mutant conferred an analogous phenotype to *qacB*—i.e., reduced resistance to divalent cations (Fig. 1*E*), confirming the findings from the hydroxylamine mutagenesis that high-level resistance to divalent compounds depends on the presence of an acidic residue near amino acid 323 in QacA/B.

Determination of the Membrane Topology of the QacA Protein. The membrane topology of the QacA protein was elucidated using alkaline phosphatase and β-galactosidase as reporters of subcellular localization. These two bacterial enzymes characteristically show high activity when localized to

Table 2. Enzymatic activities of *qacA–phoA* and *qacA–lacZα* fusions

Plasmid*	Fusion junction†	Method of construction	Alkaline phosphatase activity‡	*lacZ* complementation§
qacA–phoA fusions				
None			0.1	
pSK699	53 (P1)	Tn*phoA*	95.8	N/A
pSK701	314 (P5)	Tn*phoA*	41.8	N/A
pSK702	107 (P2)	Tn*phoA*	73.8	N/A
pSK703	320 (TMS 10)	Tn*phoA*	42.6	N/A
pSK711	83 (C1)	Tn*phoA*	1.3	N/A
pSK712	336 (C5)	Tn*phoA*	0.4	N/A
pSK713	406 (C6)	Tn*phoA*	2.8	N/A
pSK4159	165 (P3)	PCR	100	N/A
pSK4162	451 (P7)	PCR	96.2	N/A
pSK4164	372 (P6)	PCR	118	N/A
pSK4175	228 (P4)	PCR	90.0	N/A
qacA–lacZα fusions				
pSK4222	145 (C2)	PCR	N/A	+
pSK4223	514 (COOH)	PCR	N/A	+
pSK4224	204 (C3)	PCR	N/A	−
pSK4225	274 (C4)	PCR	N/A	+
pSK4226	85 (C1)	PCR	N/A	+
pSK4227	165 (P3)	PCR	N/A	−
pSK4229	344 (C5)	PCR	N/A	+
pSK4230	228 (P4)	PCR	N/A	−
pSK4231	210 (C3)	PCR	N/A	+

*Plasmids encoding *qacA–phoA* hybrids were examined in the *E. coli* host strain CC118, whereas plasmids encoding *qacA–lacZα* fusions were resident in *E. coli* strain DH5α.

†Fusion junction lists the number of amino acids from the QacA polypeptide in the hybrid proteins, with the putative location of the fusion junction in the QacA protein (see Fig. 3) indicated in parentheses.

‡Results are the average of at least three independent assays of enzymatic activity; N/A, not applicable.

§Strains containing recombinant plasmids were scored for the ability to cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl D-galactopyranoside on LB agar plates.

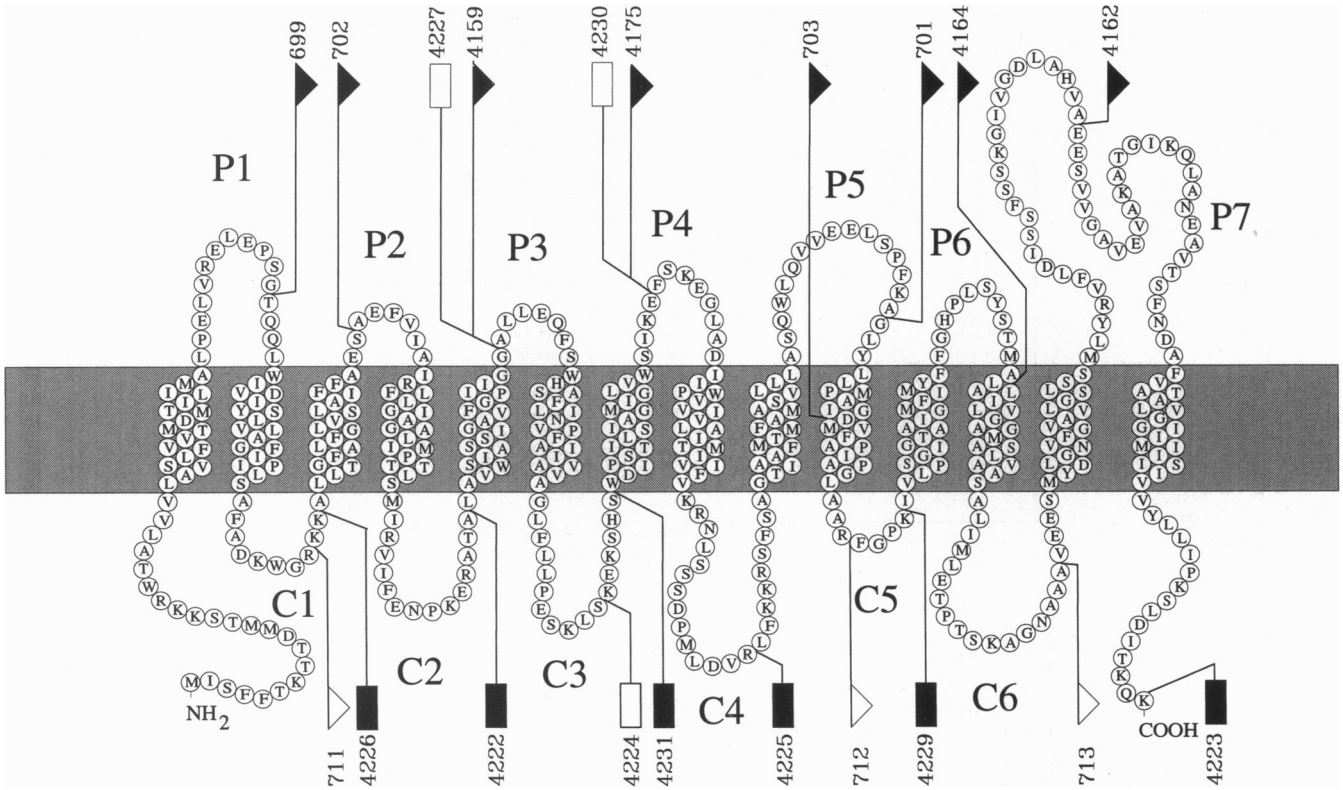

FIG. 2. Schematic representation of the QacA polypeptide showing its proposed transmembrane organization and orientation (5). The hydrophilic loops located between each TMS have been designated C1–6 and P1–7, depending on their proposed cytoplasmic or periplasmic locations. The amino (NH_2) and carboxyl (COOH) termini of the polypeptide are indicated. Positions of the fusion sites of the QacA–PhoA and QacA–LacZα hybrids are represented by the arrowheads and rectangular boxes, respectively. The shaded arrowheads/boxes correspond to fusions that mediate a high enzymatic activity, and the open arrowheads/boxes represent fusions that confer a low level of enzymatic activity (see Table 2). Each fusion is labeled with the nomenclature of the pSK plasmid encoding the particular hybrid protein.

the periplasm and cytoplasm of *E. coli* cells, respectively (32). A series of gene fusions between *qacA* and the alkaline phosphatase (*phoA*) and β-galactosidase (*lacZα*) genes were generated using a combination of *in vivo* and *in vitro* techniques.

Hybrids between *qacA* and *phoA* were constructed using Tn*phoA*, a transposon derivative of Tn*5* containing *phoA* but lacking its signal peptide sequence (26). Nucleotide sequencing identified only seven unique in-frame *qacA–phoA* hybrids (Table 2), primarily due to a very high frequency of insertion of the transposon at a single site within *qacA*, corresponding to an in-frame fusion between the first 53 codons of *qacA* and the *phoA* gene (see pSK699 in Fig. 2). The ends of the target duplication at this site contain G/C pairs, a characteristic feature of Tn*5*-like transposon insertional hotspots (33, 34), suggesting that Tn*phoA* displays a site-specific preference for insertion at this location.

To further clarify the topology of QacA, additional fusions between *qacA* and the reporter genes *phoA* and *lacZα* were constructed by cloning specific PCR fragments of *qacA* into the *phoA* vector pSK4158 (35) or the *lacZα* vector pUC118 (36). Through this approach, combined with the fusions generated by Tn*phoA* mutagenesis, *qacA–phoA* or *qacA–lacZα* hybrids were constructed to sites corresponding to all of the proposed periplasmic and cytoplasmic loops, and to the C terminus of the QacA polypeptide (Fig. 2). In all cases the fusion joints on the plasmids encoding these hybrid proteins were confirmed by nucleotide sequencing.

The alkaline phosphatase and β-galactosidase activities of cells expressing the *qacA–phoA* and *qacA–lacZα* constructs were assayed (Table 2). The activities of all of the fusions were consistent with the proposed 14-TMS model of QacA, except for the *qacA–lacZα* fusion encoded by pSK4224, corresponding to a fusion with the proposed C3 cytoplasmic loop of the QacA polypeptide. This hybrid truncates the C3 loop (Fig. 2) and does not include three positively charged residues, which may be important topogenic determinants for the proper localization of this loop. A second β-galactosidase fusion (encoded on pSK4231) located within this loop and including these charged residues did confer high enzymatic activity supporting the notion that this loop in QacA is localized to the cytoplasm.

Western analysis using anti-PhoA IgG was used to identify the hybrid proteins encoded by the *qacA–phoA* fusions. Hybrid proteins of the predicted sizes were readily identified, although in each case additional smaller products, probably corresponding to degradative products of the hybrid proteins, were also visible (data not shown). Drug susceptibility studies and transport assays (data not shown) revealed that, of the *qacA* fusions generated in this study, only the full-length *qacA–lacZα* fusion conferred resistance to typical substrates of *qacA*, indicating that all 14 TMS of QacA appear to be required for the transport function of the protein.

DISCUSSION

The staphylococcal multidrug resistance genes *qacA* and *qacB* differ in their capacity to confer resistance to divalent organic cations. Determination of the nucleotide sequence of *qacB*, generation of *qacB* mutants that conveyed extended resistance phenotypes, and site-directed mutagenesis of *qacA* has provided unequivocal evidence that the phenotypic differences between *qacA* and *qacB* are due to a single base difference within these two genes. Specifically, the presence of an acidic residue at amino acid 323 within putative TMS 10 of the QacA protein plays a critical role in conveying resistance to divalent organic cations. Additionally, it was demonstrated that QacB

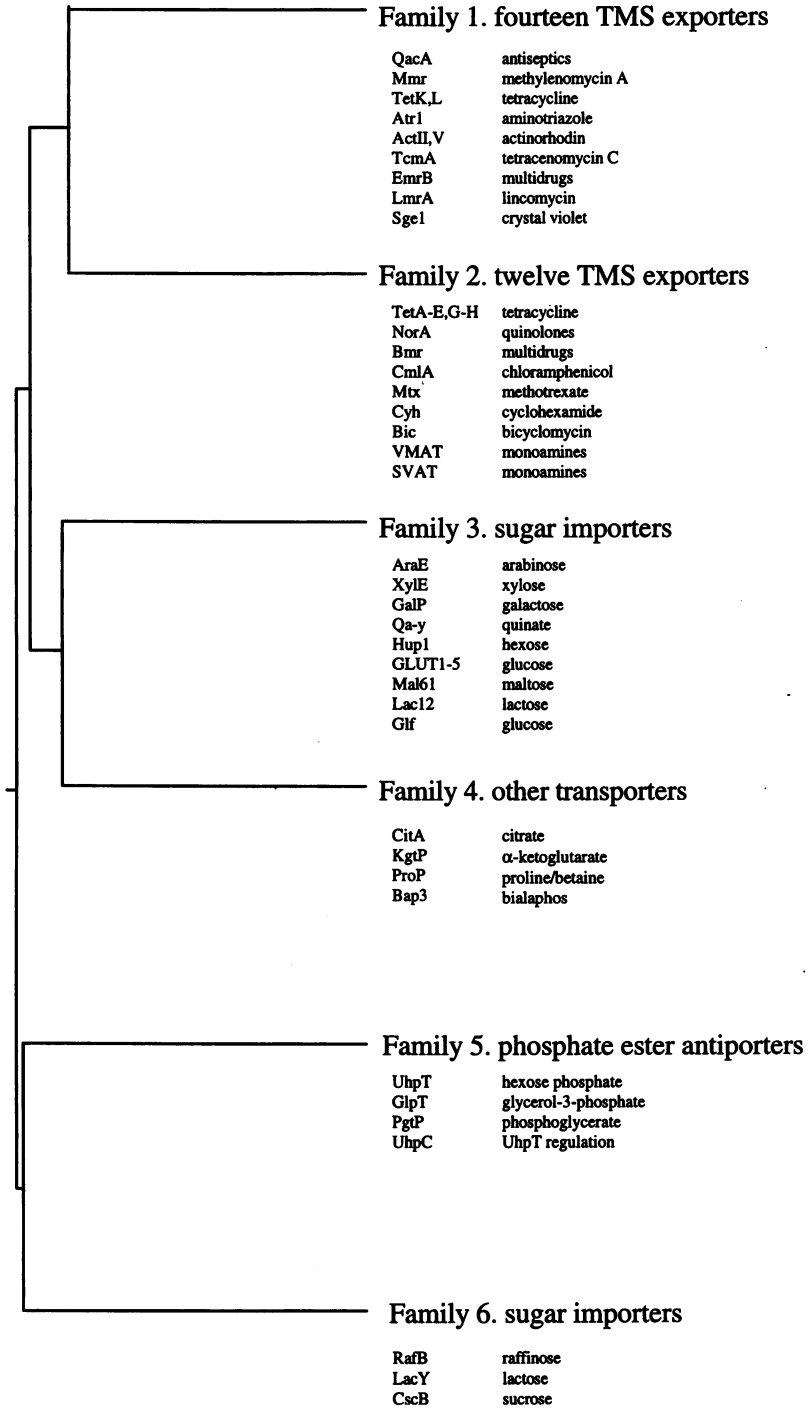

FIG. 3. A phylogenetic tree showing the proposed evolutionary relationships among the six families in the MFS. Selected transporters belonging to each family and their substrate, putative substrate, or proposed function are shown beneath each family. The tree was adapted and extended from those presented in Griffith *et al.* (1), Marger and Saier (2), and Paulsen and Skurray (3); see these for references to specific MFS family proteins. The existence of an additional family, consisting of sodium/solute antiporters, belonging to the MFS has been hypothesized (44).

mutants containing an acidic residue at amino acid 322 were also able to mediate resistance to divalent cations.

One attractive hypothesis is that the region of the QacA/B protein, containing the essential acidic residue, plays a role in substrate recognition or binding, and that the negative charge of this residue may interact directly with one of the positively charged moieties of the divalent cation. Other possibilities are that the acidic residue is involved in energizing transport of divalent cations or that the mutations indirectly affect a binding site located elsewhere in the protein via conformational alterations.

The product of the *qacB* (G322E/A323T) mutant contains an additional amino acid substitution adjacent to Glu-322, and this mutant conferred lower levels of resistance to divalent compounds than the other mutants containing only a single alteration. This observation is consistent with the above proposition that this region plays a role in substrate binding, as this additional change may decrease the affinity of the binding site for its substrates, potentially due to steric hindrance, as threonine has a larger side-chain volume than the original alanine.

Interestingly, the putative α-helix of TMS 10 of QacA contains three proline residues (Fig. 2), an unusual feature, as

proline residues typically disrupt the structure of an α-helix. Brandl and Deber (37) have previously hypothesized that intramembraneous proline residues in transport proteins may be involved in the conformational changes involved in the opening and closing of their transport channels. This raises the possibility that these three proline residues, which are located on the opposite face of the helix compared with the acidic residues at amino acid 322 or 323 in QacA or the mutants in this study, play a role in the conformational changes in the protein associated with drug export, as a consequence of substrate interaction with the acidic residue.

Analysis of a series of alkaline phosphatase and β-galactosidase fusions has provided firm support in favor of the QacA protein containing 14 TMS (see Fig. 2). Secondary transport proteins have typically been thought to contain 12 TMS, consisting of two arrays of six TMS each (38). In particular, a representative member of each of the five clusters of the MFS have been demonstrated to contain 12 TMS (39–43). To our knowledge, QacA represents the first membrane transport protein demonstrated to contain 14 TMS.

Comparative hydropathy and amino acid sequence analyses of the export proteins that form cluster 1 of the MFS have suggested that this cluster consists of two separate families of proteins with 12 and 14 TMS, respectively (3). The substantiation of the 14-TMS model of QacA provides experimental evidence endorsing the proposal that this cluster consists of two separate families distinguishable by their membrane topology, and that a family of proteins belonging to the MFS contain 14 TMS. Hence, a revised phylogeny of the MFS is presented in Fig. 3, with family 1 consisting of the 14 TMS exporters, typified by QacA, and families 2–6 representing the 12-TMS proteins from clusters 1–5, respectively.

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1. Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A. & Henderson, P. J. F. (1992) *Curr. Opin. Cell Biol.* **4,** 684–695.
2. Marger, M. D. & Saier, M. H., Jr. (1993) *Trends Biochem. Sci.* **18,** 13–20.
3. Paulsen, I. T. & Skurray, R. A. (1993) *Gene* **124,** 1–11.
4. Levy, S. B. (1992) *Antimicrob. Agents Chemother.* **36,** 695–703.
5. Rouch, D. A., Cram, D. S., DiBerardino, D., Littlejohn, T. G. & Skurray, R. A. (1990) *Mol. Microbiol.* **4,** 2051–2062.
6. Neyfakh, A. A., Bidnenko, V. E. & Chen, L. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4781–4785.
7. Lomovskaya, O. & Lewis, K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8938–8942.
8. Gottesman, M. M. & Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427.
9. Lewis, K. (1994) *Trends Biochem. Sci.* **19,** 119–123.
10. Nikaido, H. (1994) *Science* **264,** 382–388.
11. Littlejohn, T. G., Paulsen, I. T., Gillespie, M. T., Tennent, J. M., Midgley, M., Jones, I. G., Purewal, A. S. & Skurray, R. A. (1992) *FEMS Microbiol. Lett.* **95,** 259–266.
12. Leelaporn, A., Paulsen, I. T., Tennent, J. M., Littlejohn, T. G. & Skurray, R. A. (1994) *J. Med. Microbiol.* **40,** 214–220.
13. Tennent, J. M., Lyon, B. R., Gillespie, M. T., May, J. W. & Skurray, R. A. (1985) *Antimicrob. Agents Chemother.* **27,** 79–83.
14. Tennent, J. M., Lyon, B. R., Midgley, M., Jones, I. G., Purewal, A. S. & Skurray, R. A. (1989) *J. Gen. Microbiol.* **135,** 1–10.
15. Lyon, B. R. & Skurray, R. (1987) *Microbiol. Rev.* **51,** 88–134.
16. Hohn, B. (1979) *Methods Enzymol.* **68,** 299–309.
17. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
18. Lyon, B. R., May, J. W. & Skurray, R. A. (1983) *Antimicrob. Agents Chemother.* **23,** 817–826.
19. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–119.
20. Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988) *Nucleic Acids Res.* **16,** 7583–7600.
21. Midgley, M. (1986) *J. Gen. Microbiol.* **132,** 3187–3193.
22. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
23. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
24. Gleeson, T. J. & Staden, R. (1991) *Comp. Appl. Biosci.* **7,** 398.
25. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
26. Manoil, C. & Beckwith, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8129–8133.
27. Humphreys, G. O., Willshaw, G. A., Smith, H. R. & Anderson, E. S. (1976) *Mol. Gen. Genet.* **145,** 101–108.
28. Brickman, E. & Beckwith, J. (1975) *J. Mol. Biol.* **96,** 307–316.
29. Smith, R. L., Banks, J. L., Snavely, M. D. & Maguire, M. E. (1993) *J. Biol. Chem.* **268,** 14071–14080.
30. Hofmann, K. & Stoffel, W. (1992) *Comp. Appl. Biosci.* **8,** 331–337.
31. von Heijne, G. (1992) *J. Mol. Biol.* **225,** 487–494.
32. Traxler, B., Boyd, D. & Beckwith, J. (1993) *J. Membr. Biol.* **132,** 1–11.
33. Lodge, J. K., Weston-Hafer, K. & Berg, D. E. (1988) *Genetics* **120,** 645–650.
34. Lodge, J. K. & Berg, D. E. (1990) *J. Bacteriol.* **172,** 5956–5960.
35. Paulsen, I. T., Brown, M. H., Dunstan, S. J. & Skurray, R. A. (1995) *J. Bacteriol.* **177,** 2827–2833.
36. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153,** 3–11.
37. Brandl, C. J. & Deber, C. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 917–921.
38. Maloney, P. C. (1994) *Curr. Opin. Cell Biol.* **6,** 571–582.
39. Calamia, J. & Manoil, C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4937–4941.
40. Lloyd, A. D. & Kadner, R. J. (1990) *J. Bacteriol.* **172,** 1688–1693.
41. Allard, J. D. & Bertrand, K. P. (1993) *J. Bacteriol.* **175,** 4554–4560.
42. Seol, W. & Shatkin, A. J. (1993) *J. Bacteriol.* **175,** 565–567.
43. Hresko, R. C., Kruse, M., Strube, M. & Mueckler, M. (1994) *J. Biol. Chem.* **269,** 20482–20488.
44. Reizer, J., Reizer, A. & Saier, M., Jr. (1994) *Biochim. Biophys. Acta* **1197,** 133–166.