JOURNAL OF BACTERIOLOGY, May 1995, p. 2827–2833 Vol. 177, No. 10
0021-9193/95/$04.00+0

Molecular Characterization of the Staphylococcal Multidrug Resistance Export Protein QacC

IAN T. PAULSEN,[1,2] MELISSA H. BROWN,[1] SARAH J. DUNSTAN,[2] AND RONALD A. SKURRAY[1,2]*

School of Biological Sciences, University of Sydney, Sydney, New South Wales 2006,[1] and Department of Microbiology, Monash University, Clayton, Victoria 3168,[2] Australia

Received 10 November 1994/Accepted 10 March 1995

The QacC polypeptide is a member of a family of small membrane proteins which confer resistance to toxic compounds. The staphylococcal *qacC* gene confers resistance to toxic organic cations via proton-dependent export. The membrane topology of the QacC polypeptide was investigated by constructing and analyzing a series of *qacC-phoA* and *qacC-lacZ* fusions. From these analyses, most of the predicted features of the QacC protein were verified, although data regarding the possible orientation of the COOH region were not conclusive. The role of the sole cysteine residue, Cys-42, in QacC was studied by using the sulfhydryl reagent *N*-ethylmaleimide and site-directed mutagenesis. *N*-Ethylmaleimide was shown to inhibit *qacC*-mediated ethidium export. Multiple amino acid substitutions were made for Cys-42, and mutations at this location had various effects on resistance specificity. This suggests that the Cys-42 residue may be located near a region of QacC that is involved in substrate recognition. Mutagenesis of conserved residues in QacC indicated that Tyr-59 and Trp-62 also play an essential structural or functional role in QacC.

Staphylococcus aureus is an opportunistic pathogen which has become a serious clinical problem because of the emergence of strains that are resistant to a large number of antimicrobial compounds and consequently may be intransigent to chemotherapy (20). Resistance to antiseptics and disinfectants in *S. aureus* isolates is typically due to the presence of any one of three determinants, designated *qacA*, *qacB*, and *qacC*, which vary in ability to confer resistance to a range of toxic organic cations (11, 15, 20). *qacA* specifies resistance to intercalating dyes (such as ethidium bromide), quaternary ammonium compounds (such as cetrimide), and divalent cations (such as chlorhexidine). *qacB*, which is closely related to *qacA* (27), characteristically differs from this determinant by conferring low-level or no resistance to divalent cations (15), whereas *qacC*, which is genetically unrelated to *qacA* and *gacB*, mediates resistance to quaternary ammonium compounds and some dyes (15).

The *qac* determinants confer resistance to structurally diverse compounds via export, driven by the proton motive force of the transmembrane electrochemical proton gradient (7, 15, 30). Most active export systems deal with a single substrate or a small group of structurally related substrates, e.g., tetracycline extrusion is mediated by TetB in *Escherichia coli* (12). In contrast, a number of multidrug export systems which can handle a range of structurally disparate substrates have been identified (13, 26, 28). Examples of bacterial multidrug export systems include those encoded by *bmr* in *Bacillus subtilis* (25), *emrB* in *E. coli* (16), and the *qac* genes in *S. aureus* (15).

The *qacA* (33, 40), *qacB* (15, 27), and *qacC* (14) genes have been cloned and expressed in *E. coli*, and their nucleotide sequences have been determined. The deduced products of the *qacA* and *qacB* genes have been predicted to be membrane proteins with 14 transmembrane segments (TMS) (27, 29, 33) and exhibit sequence similarity with import and export proteins of the major facilitator superfamily (6, 22, 29). The *qacC* gene, which has also previously been called *qacD* (14) and is identical in sequence to the staphylococcal *smr* (7) and *ebr* (37) genes, codes for a much smaller membrane protein which is a member of a family of prokaryotic multidrug resistance proteins (7, 14, 28). This family includes the proposed multidrug exporters Ebr from *E. coli* (24, 31) and QacE and its semifunctional derivative QacEΔ1 from integrons in gram-negative bacteria (28). Members of this family typically contain four highly hydrophobic regions which may form alpha-helical TMS (7, 28) (see Fig. 1).

In this study, we have investigated the membrane topology of the QacC protein by genetic fusion, with alkaline phosphatase and β-galactosidase as reporters of subcellular localization (for a review, see reference 42). Mutagenesis and biochemical analyses were utilized to investigate the potential roles of specific residues within QacC in multidrug export.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* SK982 (19) and *E. coli* K-12 strains DH5α (*endA hsdR17 supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* ϕ80*dlacZ*ΔM15) (Bethesda Research Laboratories), CC118 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rspE rpoB argE recA1*) (21), and CJ236 [*dut-1 ung-1 thi-1 relA1* pJC105 (Cm^r)] (9) were employed in this study. The *S. aureus* plasmids used were pSK1 (*qacA*) (41), pSK41 (*qacC*) (14), pSK89 (*qacC*) (14), and pJE1 (*qacC*) (2, 3). *E. coli* plasmid vectors pUC118 (45), pPHO7 (8), pSK4158 (this study), and pSU2718 (23) and *qacC*-encoding plasmids pSK503 and pSK530 (14) were utilized in this study.

General methods. The media used and the method for MIC determinations have been described previously (15, 18). MIC tests were performed at least in triplicate.

DNA methodology. Standard molecular cloning techniques were performed as previously described (34). Transposon mutagenesis with transposon Tn*phoA* was performed as previously described (21). PCRs were performed with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's recommendations. Nucleotide sequences were determined by the procedure of Sanger et al. (35), with a T7 DNA polymerase sequencing kit (Pharmacia) and specific oligonucleotides as sequencing primers.

Ethidium transport assays. Ethidium accumulation and efflux were assayed indirectly by fluorimetric assay as previously described (24, 41). All fluorimetric assays were performed in at least triplicate. *N*-Ethylmaleimide (NEM) (1 mM) and carbonyl cyanide *m*-chlorophenylhydrazone (5 μM) were added as previously described (4, 41).

Enzymatic assays. Alkaline phosphatase activities were determined as previously described (1) with *p*-nitrophenyl phosphate. β-Galactosidase activities were estimated by scoring the ability of colonies to cleave the chromogenic substrate

* Corresponding author. Mailing address: School of Biological Sciences, University of Sydney, Macleay Building A12, Sydney, New South Wales 2006, Australia. Phone: (61) (2) 3512376. Fax: (61) (2) 3514771. Electronic mail address: skurray@extro.ucc.su.oz.au.

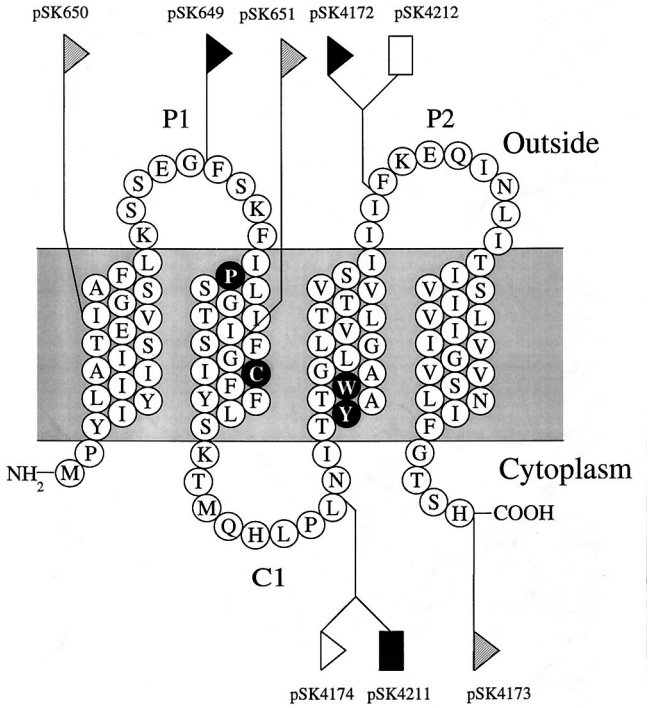

FIG. 1. Schematic representation of the QacC polypeptide, showing its proposed transmembrane organization and orientation. The proposed model includes four membrane-spanning alpha-helices that are separated by two periplasmic loops (P1 and P2) and one cytoplasmic loop (C1). The amino (NH_2) and carboxyl (COOH) termini of this polypeptide are indicated. The positions of alkaline phosphatase (triangles) and β-galactosidase (squares) fusions, as determined by nucleotide sequencing of fusion plasmids (Table 1), are indicated. Each fusion is labelled with the nomenclature of the plasmid that encodes that particular chimeric protein. Solid triangles and squares, fusions with high-level enzymatic activities; hatched triangles, intermediate activities; open triangles and squares, fusions with low-level alkaline phosphatase or β-galactosidase activities, respectively (Table 1). The locations of residues within QacC which were targeted by site-directed mutagenesis in this study are highlighted.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as described previously (38, 39).

Western (immunoblot) analysis. Proteins from whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (10). Western transfer of proteins to a nitrocellulose membrane was performed by using a Bio-Rad TransBlot semidry transfer cell according to the manufacturer's recommendations. Alkaline phosphatase fusion proteins were detected immunologically with rabbit anti-PhoA immunoglobulin G (5 Prime → 3 Prime, Inc.) as previously described (21).

Site-directed mutagenesis. Site-specific oligonucleotide-directed mutagenesis of *qacC* was undertaken as described by Kunkel et al. (9), with a single-stranded DNA template. The following oligonucleotide primers were employed in mutagenesis: C42X (ACAATAATTTCATTTGGAATT**ZXX**TTCTATTTTTTAA GTAAAACA), P32G (CAAAATTTATA**GGA**TCCTTAGGAAC), P32A (CAA AATTTATA**GCA**TCCTTAGGAAC), Y59W (CTAAATATAAC**TTG**GGCAA CTTGGGC), Y59S (CTAAATATAAC**AGT**GGCAACTTGGGC), W62F (CT TATGCAACT**TTC**GCGGGACTAGG), and W62Y (CTTATGCAACT**TAC** GCGGGACTAGG). The introduced codon changes are indicated in boldface; X represents any base, and *Z* represents any base except T. Oligonucleotides were synthesized on a Beckman Oligo 1000 DNA synthesiser.

RESULTS

Construction of *qacC-phoA* and *qacC-lacZα* fusions. The proposed four-TMS topological model of the QacC polypeptide (14, 28) (Fig. 1) was investigated by constructing a series of gene fusions between *qacC* and the alkaline phosphatase gene (*phoA*) without its signal peptide sequence by a combination of in vivo and in vitro techniques. This approach is based on the observation that alkaline phosphatase is highly active when exported across the cell membrane but exhibits low-level activity in the cytoplasm. Hence, in-frame fusions of the alkaline phosphatase gene to a target membrane protein gene can differentiate between regions that code for cytosolic domains and those that code for periplasmic domains of the membrane protein on the basis of the observed levels of alkaline phosphatase activity (42). The *qacC*-encoded multidrug export system functions in *E. coli* as well as in *S. aureus*, conferring similar resistance spectra in both organisms (15). Hence, the QacC polypeptide presumably maintains the same orientation and topology in the membrane of each of these organisms. Therefore, for ease of analysis, these *qacC-phoA* chimeras were expressed and analyzed in *E. coli*. Similarly, other membrane proteins from a wide variety of organisms have been studied by expression of *phoA* fusions in *E. coli* (for examples, see references 43, 46, and 48).

TABLE 1. Alkaline phosphatase activities encoded by *qacC-phoA* fusions[a]

Plasmid	No. of amino acids (fusion junction)[b]	Method of construction	Alkaline phosphatase activity[c]
pSK649	25 (P1)	Tn*phoA*	261
pSK650	14 (TMS I)	Tn*phoA*	161
pSK651	37 (TMS II)	Tn*phoA*	155
pSK4172	76 (P2)	PCR	288
pSK4173	107 (COOH)	PCR	170
pSK4174	55 (C1)	PCR	33
None			0.1

[a] Plasmids that encoded *qacC-phoA* fusions were examined in *E. coli* CC118.
[b] Putative location of each fusion junction in the QacC protein (Fig. 1).
[c] Data are averages of at least three independent assays of enzymatic activity.

Initially, *qacC-phoA* gene fusions were constructed by in vivo transposition of transposon Tn*phoA* (21) into *qacC*-containing plasmids pSK503 and pSK530. However, only three unique in-frame *qacC-phoA* fusions were isolated by this procedure, and nucleotide sequencing of these constructs with a *phoA*-specific oligonucleotide as a primer revealed that they encoded hybrid QacC-PhoA proteins that contained residues 1 to 14 (pSK650), 1 to 25 (pSK649), and 1 to 37 (pSK651) of QacC (Table 1 and Fig. 1). In order to clarify the topology of QacC, additional *qacC-phoA* fusions were constructed in vitro by cloning specific PCR products from *qacC* into the *phoA* vector pSK4158.

Cm^r vector pSK4158, which contains the *phoA* gene preceded by a unique multiple cloning site and carries a P15A origin of replication, was constructed as shown in Fig. 2. Plasmid pPHO7, which contains the *phoA* gene flanked by two essentially identical multiple cloning sites (8), was digested with restriction enzyme *Xho*I, blunt ended by filling in the ends with the Klenow fragment of DNA polymerase I, and digested with *Hin*dIII. The resulting 2.6-kb *Hin*dIII blunt fragment that contained the *phoA* gene was gel purified and cloned into the *Hin*dIII-*Sma*I sites of Cm^r vector pSU2718 to yield vector pSK4158.

Specific PCR fragments amplified from template DNA (pSK503) that contained *qacC* were digested with *Bam*HI and cloned into the multiple cloning site of pSK4158 to generate *qacC-phoA* hybrids that encoded residues 1 to 55 (pSK4174), 1 to 76 (pSK4172), and 1 to 107 (pSK4173) of the QacC protein (Table 1 and Fig. 1). The DNA sequence at the fusion joint of each of these constructs was confirmed by nucleotide sequencing. The locations of the PhoA fusions in these hybrid QacC-PhoA proteins are shown in Fig. 1 (triangles). As can be seen from this figure, these hybrid proteins represent fusions to putative hydrophilic loops C1, P1, and P2, putative intramembranous regions TMS I and TMS II, and the C terminus (COOH) of QacC.

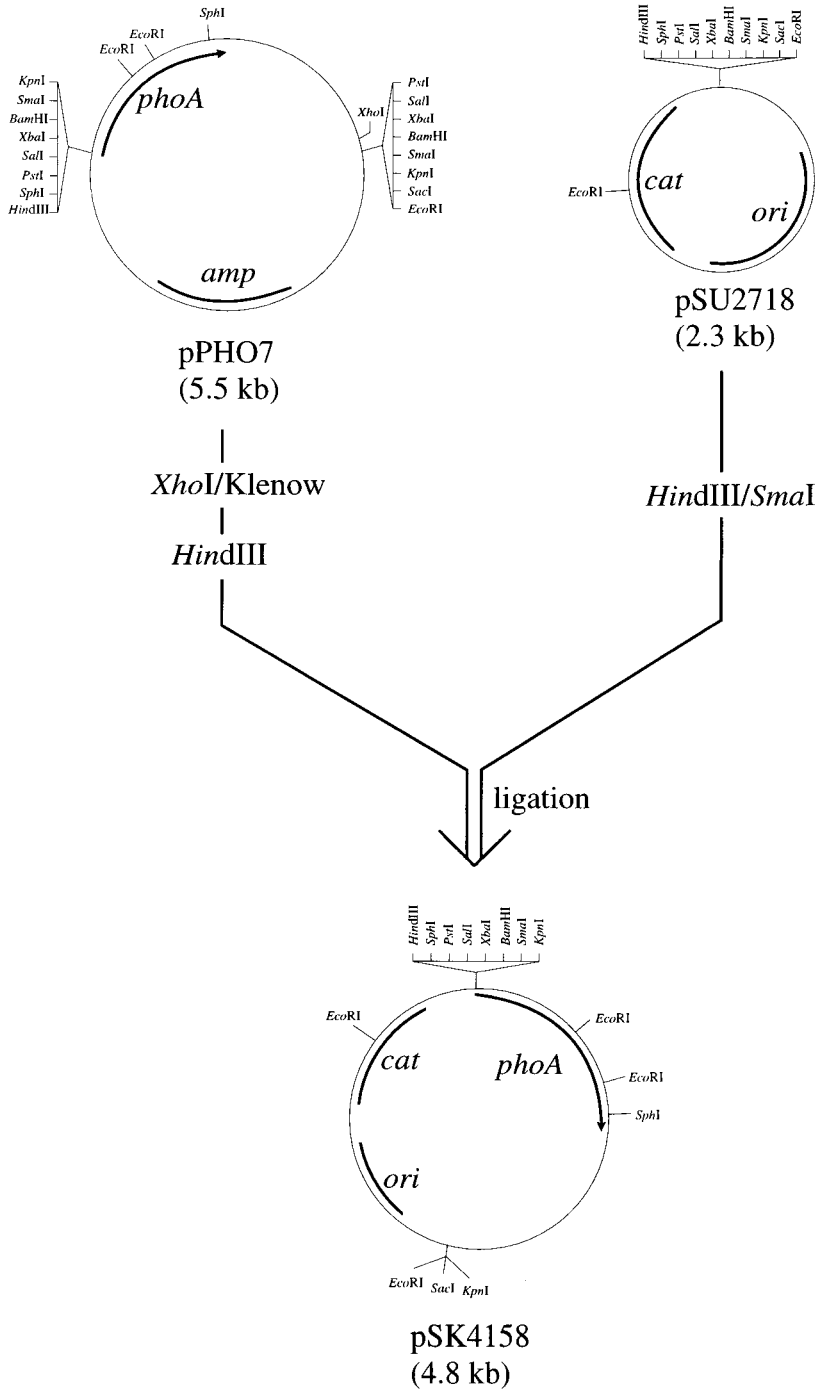

FIG. 2. Construction of *phoA* fusion vector pSK4158. The *phoA* gene from plasmid pPHO7 (8) was cloned into Cm^r vector pSU2718 (23), as described in Results, generating 4.8-kb vector pSK4158. The *amp* and *cat* genes, which confer resistance to ampicillin and chloramphenicol, respectively, and *ori*, the minimal replication region of pSU2718, are shown.

To obtain information that was complementary to that provided by alkaline phosphatase studies, fusions between *qacC* and an alternative reporter gene, *lacZ*, were also constructed. The LacZα peptide had previously been used as a reporter of subcellular localization (38, 39), providing complementary information to that from alkaline phosphatase studies, with high β-galactosidase activity indicative of a cytoplasmic location. Specific *qacC* PCR fragments were digested with *Bam*HI and cloned into the multiple cloning site of pUC118 to generate *qacC-lacZ* chimeras pSK4211 and pSK4212, which correspond to fusions between the C1 and P2 regions of QacC and the α fragment of β-galactosidase (Fig. 1 [squares]), at positions that were identical to the *qacC-phoA* fusions encoded by pSK4174 and pSK4172, respectively. Repeated attempts to generate a *qacC-lacZ* hybrid that corresponded to a fusion between the COOH terminus of QacC and the LacZα peptide by this ap-

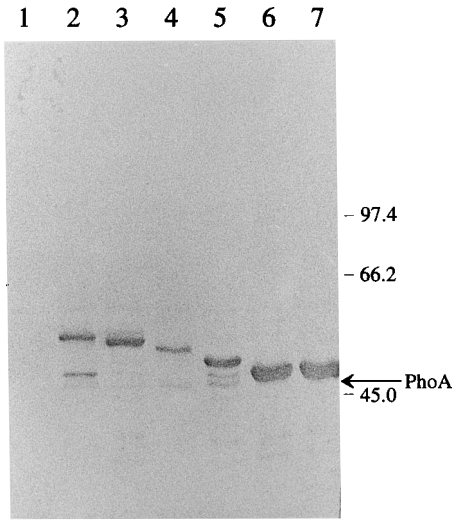

FIG. 3. Western hybridization analysis of products from cells that expressed *qacC-phoA* fusions with anti-PhoA antisera. Whole-cell extracts were separated by electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose, and immunostained (see Materials and Methods). The results with extracts from *E. coli* CC118 cells that harbored the indicated plasmid are shown in the following lanes (with the putative subcellular location of the fusion junction within the hybrid protein encoded by each of these constructs indicated in parentheses): 1, no plasmid; 2, pSK4173 (COOH); 3, pSK4172 (P2); 4, pSK4174 (C1); 5, pSK651 (TMS II); 6, pSK650 (TMS I); 7, pSK649 (P1). In lanes 2 through 7, the prominent upper band corresponds in size to that expected of hybrid QacC-PhoA proteins. Additionally, faint bands are evident in lanes 2 through 7 (most clearly visible in lanes 4 and 5); they correspond in size to the alkaline phosphatase moiety, PhoA, and are indicated by an arrow on the right. The migrations and sizes (in kilodaltons) of coelectrophoresed standard proteins are also shown on the right.

proach proved to be unsuccessful, suggesting that overexpression of such a hybrid may be deleterious for cells.

Characterization of QacC-PhoA and QacC-LacZ hybrids. The relative alkaline phosphatase (Table 1 and Fig. 1) and β-galactosidase (Fig. 1) activities of cells that harbored various *qacC-phoA* or *qacC-lacZ* constructs were assayed. Cells which contained *qacC-phoA* fusions that corresponded to the projected periplasmic regions, P1 and P2, exhibited high-level alkaline phosphatase activities (261 to 288 U), whereas the *qacC-phoA* fusion which codes for PhoA fused to the predicted cytoplasmic region C1 of QacC conferred low-level alkaline phosphatase activities (33 U). The two *qacC-lacZ* hybrids that corresponded to fusions to C1 and P2 of QacC conferred reciprocal enzymatic activities compared with those of the equivalent *qacC-phoA* hybrids (Fig. 1). Cells that harbored *qacC-phoA* fusions that corresponded to the proposed intramembrane regions, TMS I and TMS II, of the QacC polypeptide displayed intermediate levels of alkaline phosphatase activity (155 to 161 U), as did cells that contained pSK4173, which encodes a hybrid protein that consists of the C terminus of QacC fused to PhoA (170 U). With the exception of pSK4173, the enzymatic activities conferred by *qacC-phoA* and *qacC-lacZ* fusions were consistent with the proposed topological model of QacC (Fig. 1). The possible reasons for the high-level activity of pSK4173 are considered below.

To identify the hybrid proteins that are encoded by these *qacC-phoA* constructs, whole-cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with anti-PhoA immunoglobulin G. Hybrid proteins of the predicted sizes, encoded by each of these constructs, were readily detected by immunostaining (Fig. 3); in each case, a smaller faint band that corresponded in size to the PhoA moiety (~47 kDa) was also observed. In some cases, additional products were observed; e.g., in the case of the C-terminal QacC-PhoA fusion encoded by pSK4173, a product of ~49 kDa, which may be responsible for the observed alkaline phosphatase activity, was also observed (Fig. 3, lane 2). These additional products were most probably derived from proteolysis of hybrid proteins, as previously observed with other alkaline phosphatase fusion proteins (43).

The multidrug resistance activities of the various gene fusions were assessed by MIC analysis and ethidium transport assays. None of the plasmids that carried *qacC-phoA* or *qacC-lacZ* chimeras conferred resistance to ethidium bromide or quaternary ammonium compounds, with the exception of pSK4173, which codes for a hybrid QacC-PhoA fusion that contains all 107 amino acids of QacC. pSK4173 conveyed resistance to a range of *qacC* substrates at levels that were approximately 50 to 75% of those of wild-type *qacC* (data not shown). Similarly, proton motive force-dependent ethidium efflux was observed in *E. coli* cells that contained pSK4173 at rates that were approximately 50% of the rate of *qacC*-mediated export (data not shown). This suggests that the hybrid protein encoded by pSK4173 is properly localized in the membrane.

Effect of NEM on *qacC*-encoded ethidium efflux. NEM is a membrane-permeable thiol reagent which covalently bonds to the sulfhydryl groups of cysteine residues (44). The roles of cysteine residues in various transport systems, such as the *E. coli* lactose permease LacY (4) and glucose-6-phosphate transporter UhpT (47), have been evaluated by using sulfhydryl-specific reagents and subsequent site-directed mutagenesis. An analogous approach was utilized to examine the role of the sole QacC cysteine residue, Cys-42, in ethidium efflux.

The addition of 1 mM NEM was observed to inhibit ethidium export in *S. aureus* cells that harbored *qacC* plasmid pSK89 (Fig. 4a), pSK41 (data not shown), or pJE1 (data not shown). Similarly, ethidium accumulation in *qacC*-encoding strains was increased following NEM treatment (Fig. 4b). As controls, the effects of 1 mM NEM on ethidium transport in the background *S. aureus* strain and in cells that expressed *qacA*, whose product does not contain any cysteine residues, were examined. NEM was found to have no detectable effect on ethidium accumulation and efflux in either *S. aureus* SK982 or cells that expressed the *qacA* encoded on pSK1 (Fig. 4a and b). These data suggest that NEM does not exert a generalized effect on ethidium transport or on proton motive force generation in *S. aureus* under the experimental conditions used but, instead, specifically inhibits *qacC*-encoded ethidium export.

A number of NEM-sensitive transport proteins have been shown to be partially protected from NEM inhibition by their appropriate substrates, such as the lactose permease LacY (4). In other instances (e.g., human erythrocyte D-glucose transporter), the transport substrate accelerates the rate of NEM inhibition (32). The effect of excess substrate on NEM inhibition of *qacC*-encoded ethidium export was examined indirectly by assaying the relative rates of ethidium accumulation by cells that contained pSK89 in the presence of a 10-fold excess of ethidium bromide (50 μM). Under these conditions, the addition of 1 mM NEM induced only a slight increase in the rate of ethidium accumulation (Fig. 4c), indicating that excess substrate may partially protect the *qacC*-encoded ethidium export system from the inhibitory effects of NEM. As a control, the addition of an alternative inhibitor, such as the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, resulted in a rapid increase in ethidium accumulation (data not shown).

Generation of *qacC* (Cys-42) mutants. The possible role of

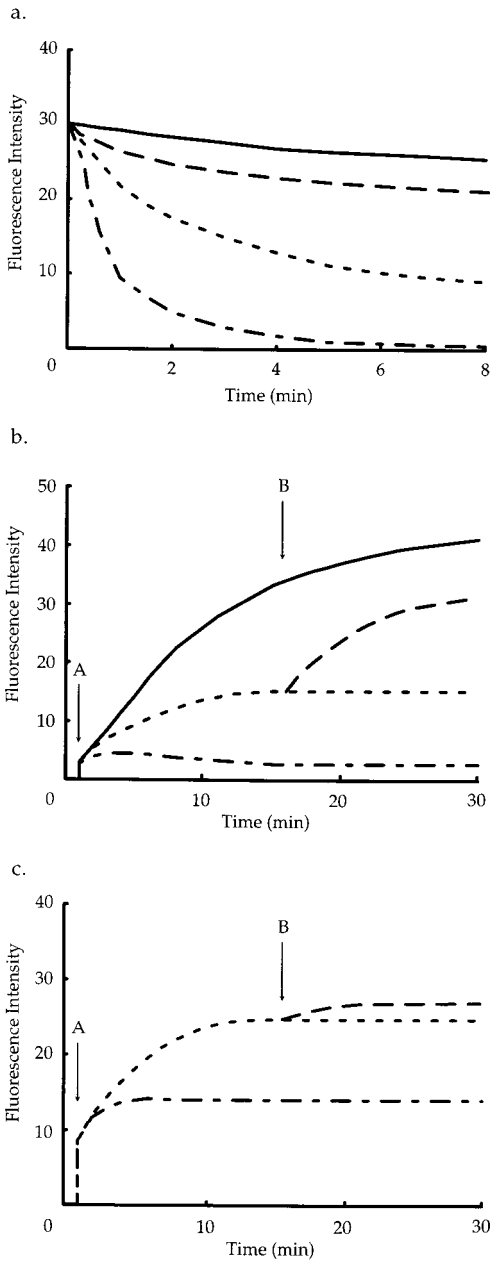

FIG. 4. Effects of the sulfhydryl reagent NEM on ethidium export and accumulation by *qacC*-encoding cells. (a) Ethidium export in *S. aureus* SK982 cells that harbored the following plasmids was examined under the conditions noted: ——, no plasmids and addition or no addition of 1 mM NEM; – – – –, pSK89 (*qacC*) with no addition; — —, pSK89 (*qacC*) with addition of 1 mM NEM; — – —, pSK1 (*qacA*) with no addition of 1 mM NEM. (b) Ethidium accumulation in *S. aureus* SK982 cells that harbored the indicated plasmid. Ethidium bromide (5 μM) was added at the time indicated by arrow A; in the instances indicated, 1 mM NEM was added at the time indicated by arrow B. ——, no plasmids and addition or no addition of 1 mM NEM; – – – –, pSK89 (*qacC*) with no addition; — —, pSK89 (*qacC*) with addition of 1 mM NEM; — – —, pSK1 (*qacA*) with no addition of 1 mM NEM. (c) Ethidium accumulation, in the presence of excess ethidium, in *S. aureus* SK982 cells that harbored the indicated plasmid was examined. Ethidium bromide (50 μM) was added at the time indicated by arrow A; in the instances indicated, 1 mM NEM was added at the time indicated by arrow B. – – – –, pSK89 (*qacC*) with no addition; — —, pSK89 (*qacC*) with addition of 1 mM NEM; — – —, pSKl (*qacA*) with addition or no addition of 1 mM NEM.

TABLE 2. Resistance specificities conferred by *qacC* mutants on *E. coli* DH5α

qacC mutation[a]	MIC (μg/ml)[b]							
	Dye				Quaternary ammonium compound			
	Eb	Pf	Cv	R6	Ct	Bc	Cc	Dc
None	50	20	5	600	40	20	20	20
Wild type	800	60	20	>800	100	80	80	40
C42T	800	60	20	>800	100	80	80	40
C42G, A	200	20	10	>800	100	80	80	20
C42S	50	20	5	>800	100	80	80	20
C42V, I, L, P, D, E, K, R	50	20	5	600	40	20	20	20
P31G, A	100	60	10	ND	100	80	80	40
Y59W	50	20	5	ND	40	20	20	20
Y59S	75	40	10	ND	40	20	40	30
W62F	50	40	10	ND	40	20	20	30
W62Y	50	20	5	ND	40	20	20	20

[a] None, negative control (DH5α alone). When different mutations at the same site in *qacC* confer identical phenotypes, these mutations are listed sequentially.
[b] Eb, ethidium bromide; Pf, proflavine; Cv, crystal violet; R6, rhodamine 6G; Ct, cetyltrimethylammonium bromide; Bc, benzalkonium chloride; Cc, cetylpyridinium chloride; Dc, dequalinium chloride; ND, not determined.

Cys-42 in the QacC polypeptide was investigated by utilizing site-directed mutagenesis to construct a series of *qacC* mutants with an altered cysteine codon at this position. The *qacC*-encoding plasmid pSK89 was linearized by digestion with *Pst*I and cloned into the *Pst*I site of vector pUC118 to generate pSK660. By using single-stranded DNA derived from phagemid pSK660 as a template, *qacC* mutants were constructed by the method of Kunkel et al. (9). Changes were introduced by using the degenerate oligonucleotide C42X, in which the cysteine codon (TGT) had been replaced by ZXX (see Materials and Methods). Potential mutants were screened phenotypically and by nucleotide sequencing, and 12 mutants which contained different single amino acid alterations in place of Cys-42 in QacC were identified.

In each case, completion of the nucleotide sequences on both strands of the mutant *qacC* genes verified that no spurious mutations had been introduced. The relative resistance specificities to organic cations conferred by each of these mutants were investigated by MIC analyses (Table 2). Strains that harbored the *qacC* cysteine-to-isoleucine at amino acid 42 (C42I), C42L, C42V, C42P, C42D, C42E, C42K, or C42R mutant were sensitive to all of the compounds tested. All of these mutations result in radical substitutions, such as the introduction of a charged residue (D, E, K, or R), an alpha-helix-breaking residue (P), or a residue with a large side chain volume (V, I, or L). In contrast, the *qacC* C42T mutant, whose product contains a conservative cysteine-to-threonine substitution, retained full activity, conferring resistance to both quaternary ammonium compounds and intercalating dyes. The remaining *qacC* mutants, C42A, C42G, and C42S, conveyed resistance to quaternary ammonium compounds but conferred only reduced or no resistance to intercalating dyes, such as ethidium bromide and crystal violet, compared with that of *qacC*.

In support of the susceptibility data, fluorimetric transport assays of ethidium export indicated that the *qacC* C42T mutant conferred a rate of ethidium efflux that was similar to that of wild-type *qacC*, the C42A and C42G mutants conveyed reduced rates of ethidium efflux, and no detectable ethidium efflux was observed with the remaining mutants (Fig. 5 and

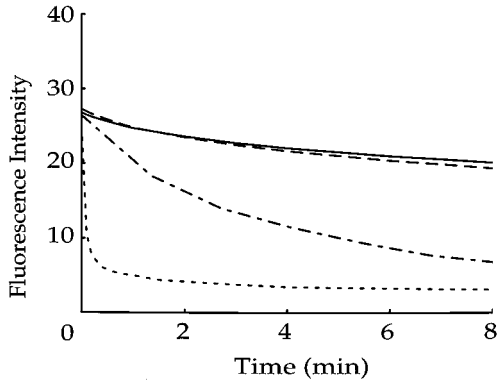

FIG. 5. Fluorimetric assays of ethidium export conferred by *qacC* and selected *qacC* (Cys-42) mutants. *E. coli* DH5α cells that harbored plasmids that encode the following *qacC* variants were loaded with ethidium bromide, and ethidium export was energized by the addition of 50 mM sodium formate at time zero. ——, negative control (DH5α alone); – – – –, wild-type *qacC*; — – —, *qacC* C42G; — —, *qacC* C42S. In this experiment, ethidium export in *E. coli* DH5α cells that expressed the *qacC* C42T mutant was indistinguishable from that observed for wild-type *qacC*.

data not shown). To confirm that the observed NEM inhibition of *qacC*-encoded ethidium export was due to interaction between NEM and the sulfhydryl group of Cys-42, the effect of NEM on ethidium export conferred by the *qacC* C42T mutant was assessed. The addition of 1 mM NEM had no detectable effect on the rate of ethidium export in cells that harbored pSK687, which encodes the *qacC* C42T mutant (data not shown).

Site-directed mutagenesis of residues located within conserved motifs. QacC shares four highly conserved regions with other related proteins, such as QacE (28). In particular, it has been proposed that the conserved motifs which are located in TMS II and III of these proteins may be involved in substrate recognition (28). To investigate the potential role of these two regions in QacC-mediated drug export, site-directed mutagenesis of *qacC* was undertaken to target the residues, Pro-31, Tyr-59, and Trp-62, which are absolutely conserved among the members of this family of proteins. By utilizing the mutagenic oligonucleotides listed in Materials and Methods, two substitutions were introduced for each of these three residues within QacC (Table 2).

MIC analyses (Table 2) and transport assays (data not shown) indicated that the *qacC* Pro-31 mutants (P31G and P31A) conferred a phenotype that was similar to that of the *qacC* Cys-42 mutants (C42G and C42A), viz., reduced resistance to ethidium bromide and crystal violet and high-level resistance to quaternary ammonium compounds (Table 2). In contrast, the Tyr-59 mutants (Y59W and Y59S) and Trp-62 mutants (W62Y and W62F) displayed negligible levels of resistance against all of the compounds tested (Table 2), suggesting that these residues play a critical role in the structure or function of the QacC polypeptide.

DISCUSSION

A series of *qacC-phoA* and *qacC-lacZ* gene fusions were constructed and analyzed to test the proposed four-TMS topology of QacC. The enzymatic activities conferred by these constructs were consistent with the proposed topology and orientation of TMS I through III of QacC (Fig. 1), with the N terminus of QacC located cytoplasmically. However, the location of the C terminus of QacC was not positively assigned. Cells that harbored pSK4173, which encodes a hybrid protein that consists of PhoA fused to the C terminus of QacC, exhibited alkaline phosphatase activities that were approximately 60% of those of cells that encode hybrid proteins with PhoA fused to proposed periplasmic regions of QacC (Table 1 and Fig. 1).

Immunostaining of extracts from cells that carried pSK4173 with anti-PhoA antisera identified two prominent products (Fig. 3, lane 2). One of these products corresponded with the expected size of the chimeric protein encoded by pSK4173, and a smaller product migrated to approximately 49 kDa. The smaller product is presumably derived from proteolysis of the QacC-PhoA fusion protein, as no potential restart codon exists at an appropriate position within *qacC*. In this case, it may represent a polypeptide that consists of the entire PhoA moiety (47 kDa) fused to the C-terminal 20 to 30 amino acids of QacC. This product may be largely or entirely responsible for the observed alkaline phosphatase activities in cells that harbor pSK4173, as the hydrophobic C-terminal 20 to 30 amino acids of QacC may act as a signal sequence to promote translocation of the PhoA moiety across the membrane. On this basis, the full-length chimeric protein encoded by pSK4173 may exhibit only low-level activity in accord with its predicted cytoplasmic location. Further studies are required to clarify this possibility and confirm the location of the C terminus of QacC.

qacC-encoded ethidium export was demonstrated to be sensitive to the effects of the sulfhydryl reagent NEM, and the presence of excess ethidium appeared to partially protect against such NEM inhibition. Site-directed mutagenesis with a degenerate oligonucleotide was employed to generate a range of *qacC* (Cys-42) mutants, whose phenotypes were subsequently analyzed. The replacement of cysteine with threonine (which is similar in side chain volume and H-bonding ability) resulted in a mutant whose phenotype was indistinguishable from that of wild-type *qacC*, suggesting that Cys-42 does not play a direct role in QacC-mediated drug export or in the formation of disulfide bonds. However, other substitutions of residues with similar side chain volumes (Ala, Gly, and Ser) at this location specifically affected the ability to confer resistance to compounds such as ethidium and crystal violet (Table 2). The ability of these mutations to modulate the activity and substrate specificity of the *qacC*-encoded multidrug export system implies that these substitutions affect regions of the protein that are involved in substrate recognition or binding. The notion that this residue may be located near a region that is involved in substrate binding is supported by indirect evidence that suggests that partial protection from NEM inhibition of *qacC*-encoded ethidium export is provided by excess ethidium bromide (Fig. 4c).

The roles of the conserved QacC residues Pro-31, Tyr-59, and Trp-62 were also investigated by site-directed mutagenesis. Replacement of Pro with either Gly or Ala, both of which have similar side chain volumes, led to a reduction in resistance to ethidium and crystal violet in a manner that was analogous to that observed with *qacC* C42A, C42G, and C42S mutants, suggesting that this residue is also located near a region that is involved in determining substrate specificity in QacC. As these mutations affected the levels of resistance and export conferred by *qacC* to a specific class of substrates, there may be differences in the requirements for binding or export of particular substrates by QacC. A similar situation has been observed for the mammalian multidrug resistance P-glycoprotein, in which a number of point mutations have been observed to affect transport of particular classes of substrates (5, 17). There may be separate but potentially overlapping binding sites in these proteins for different classes of substrates.

Substitutions at Tyr-59 and Trp-62 abolished or greatly reduced the levels of resistance to all of the compounds tested, suggesting that these residues play a crucial structural or func-

tional role in QacC. Speculatively, the aromatic nature of these Tyr and Trp residues and their predicted locations on approximately the same face of the alpha-helix of TMS III of QacC (Fig. 1) pose the possibility that these residues interact with the hydrophobic regions of substrates of QacC, such as ethidium. Further biochemical characterization of the Tyr-59 and Trp-62 mutants may clarify whether these residues play a role in substrate binding.

Previous studies have shown that the *qacC* gene encodes a product involved in mediating multidrug export but have not established that the QacC protein acts as a multidrug exporter in its own right (7, 14, 15, 28, 36). The identification in this study of a number of specific point mutations in QacC which affect the substrate specificity of multidrug export provides support for the notion that QacC does function as a multidrug exporter.

ACKNOWLEDGMENTS

This work was supported in part by a project grant from the National Health and Medical Research Council (Australia). I.T.P. was a recipient of the Ernest Fields Scholarship, Faculty of Medicine, Monash University.

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