Bioenergetics of the Staphylococcal Multidrug Export Protein QacA

IDENTIFICATION OF DISTINCT BINDING SITES FOR MONOVALENT AND DIVALENT CATIONS*

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The multidrug efflux pump QacA from Staphylococcus aureus confers resistance to an extensive range of structurally dissimilar compounds. Fluorimetric analyses demonstrated that QacA confers resistance to the divalent cation 4',6-diamidino-2-phenylindole, utilizing a proton motive force-dependent efflux mechanism previously demonstrated for QacA-mediated resistance to the monovalent cation ethidium. Both the ionophores nigericin and valinomycin inhibited QacA-mediated export of ethidium, indicating an electrogenic drug/H+ (n = 2) antiport mechanism. The kinetic parameters, K_m and V_max, were determined for QacA-mediated export of four fluorescent substrates, 4',6-diamidino-2-phenylindole, 3',3'-dipropylxocarboxycyanine, ethidium, and pyronin Y. Competition studies showed that QacA-mediated ethidium export is competitively inhibited by monovalent cations, e.g. benzalkonium, and non-competitively inhibited by divalent cations, e.g. propamidine, which suggests that monovalent and divalent cations bind at distinct sites on the QacA protein. The quaternary ammonium salt, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, was used as a membrane-specific fluorescence probe and demonstrated that the amount of substrate entering the inner leaflet was significantly reduced in QacA-containing strains, supporting the notion that the substrate is extruded directly from the membrane.

The phenomenon of multidrug resistance is widespread in prokaryotic and eukaryotic organisms and is characterized by the ability of a single transmembrane protein to mediate the extrusion of a broad spectrum of structurally disparate toxic substances from the cell. Multidrug resistance pumps operate via an active efflux mechanism and are typically ATP- or PMF-dependent. Currently, all known ATP-dependent multidrug efflux systems belong to the ABC superfamily of transporters, and the best characterized of these is human P-glycoprotein, which confers resistance to an extensive array of cytotoxic, chemotherapeutic agents used in human cancer cells (1, 2). Another example is LmrA, a P-glycoprotein homolog that has been identified in Lactococcus lactis and represents the first ATP-dependent multidrug efflux system found in bacteria (3). PMF-dependent multidrug efflux systems have been classified into three distinct families of membrane transport proteins based on comparative sequence analysis: the major facilitator superfamily (4–6), e.g. Bmr from Bacillus subtilis (7) and QacA from Staphylococcus aureus (8, 9); the small multidrug resistance family (10), e.g. EmR from Escherichia coli (11) and Smr from S. aureus (12, 13); and the resistance/nodulation/cell division family (14), e.g. MexB from Pseudomonas aeruginosa (15) and AcrB from E. coli (16).

The multidrug resistance gene qacA is encoded on multi-resistance plasmids from clinical isolates of S. aureus and confers resistance to a wide range of antimicrobial organic cations, including various dyes, Qacs, diamidines, biguanidines, and guanylhydrazones (17). Sequence analysis and mutagenesis studies have revealed that the difference in substrate specificity between QacA and QacB is due to a single amino acid substitution at position 323 in TMS 10, where the presence of an acidic residue in QacA is essential for high levels of resistance to monovalent cations, including a number of intercalating dyes and Qacs. However, QacB characteristically differs from QacA in that it confers significantly reduced levels of resistance to divalent cationic drugs, such as diamidines and biguanidines (17, 18). Sequence analysis of the qacA gene reveals the presence of an acidic residue in QacA that is essential for high levels of resistance to diamidines and biguanidines (18). The wider substrate specificity of QacA compared with QacB may have evolved in response to the clinical use of divalent cations as chemotherapeutic agents (19).

In this study, fluorescence transport assays have been utilized to measure various aspects of the bioenergetics of qacA-mediated multidrug transport. Kinetic analyses of transport of monovalent and divalent fluorescent substrates show for the first time that QacA interacts with each of its substrates with high affinity. Interactions between various substrates and QacA using competition analysis suggest that there are distinct binding sites for monovalent and divalent cations. Transport assays utilizing a membrane-specific fluorescence probe provide evidence that QacA extrudes its substrates from the inner leaflet of the cytoplasmic membrane.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The S. aureus strain SK982 was used as the host strain for the qacA-encoding plasmid pSK1 and the qacB-encoding plasmid pSK23, as described previously (8). The E. coli...
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K-12 strain BHB2600 (F503 supE sup Hzd met) (21) was used as the host strain for the plasmid constructs pSK4219 and pSK4270, which contain the qacA and qacB genes cloned into pBluescript SK+ (22) (18). All strains were cultured at 37 °C in LB containing, where appropriate, 25 μg/ml gentamicin or 100 μg/ml ampicillin.

Cell membranes—DiOC<sub>3</sub>, TMA-DPH and verapamil were provided by Rhône-Poulenc Rorer (Dagenham, United Kingdom). Benzoinkon, DAPI, chlorhexidine, DiOC<sub>6</sub>, ethidium, nigericin, pyronin Y, reserpine, tetraphenylarsonium, TMA-DPH, valinomycin, and verapamil were all purchased from Sigma Aldrich. Nigericin and valinomycin were dissolved in 100% chloroform. Reserpine was prepared as a 1.0 mg/ml stock solution in 9:1 chloroform:ethanol. Verapamil and TMA-DPH were solubilized in 50% and 100% methanol, respectively. CCCP was prepared as a solution in 1 mg/ml ethanol and then diluted to 0.1 mg/ml in 10 mM NaOH as described previously (22). All other chemicals were prepared as stock solutions of 1 or 10 mg/ml in water, depending on solubility, immediately prior to use.

**Determination of Sensitivity to Potential Substrates and Inhibitors**—MICs of DAPI, DiOC<sub>6</sub>, reserpine, TMA-DPH, and verapamil were performed in 96-well microtiter plates. 100 μl of LB was added to each well together with increasing concentrations of the antimicrobial agent within the range of susceptibility and 1 × 10<sup>-4</sup> dilution of an overnight culture of the strain to be tested. Plates were incubated for 48 h at 37 °C, and the MICs were determined by scoring the lowest concentration at which no growth was observed. For each compound, the MIC analysis was carried out in triplicate.

**Fluorimetric Analysis of QacA- and QacB-mediated Efflux of DAPI, DiOC<sub>6</sub>, Ethidium, and Pyronin Y**—Fluorimetric assays of QacA- and QacB-mediated efflux of DAPI, DiOC<sub>6</sub>, ethidium, and pyronin Y were carried out essentially as described previously for ethidium (8). These substrates bind to specific cellular components and in so doing undergo a change in fluorescence that can be used to indicate how much of the substrate is retained by the cell, e.g., ethidium and DAPI bind to double-stranded DNA resulting in a substantial increase in fluorescence (22, 23), DiOC<sub>6</sub>, when bound to the cytoplasmic side of hyperpolarized membranes, is highly fluorescent (24), and pyronin Y binds to RNA resulting in fluorescence quenching (25). The S. aureus strain SK982, carrying the qacA-encoding plasmid pSK1 or the qacB-encoding plasmid pSK23, was grown for 16 h in 10 ml of LB (with selection) from which 1 ml was subcultured into 10 ml of fresh LB and grown to OD<sub>590</sub> = 0.85 (± 2.5 h). The E. coli strain BHB2600, carrying pSK4219 (qacA), pSK4270 (qacB) or the vector (pBluescript SK+), was grown for 16 h in 10 ml of LB (with selection) from which 1 ml was subcultured into 10 ml of fresh LB and grown to OD<sub>590</sub> = 0.6 (± 3.5 h). Cells were harvested by centrifugation, washed twice and resuspended in 10 ml of 20 mM HEPES (pH 7.0). Aliquots (1 ml) of cells were then loaded with each individual substrate (10 μM DAPI, 2.5 μM DiOC<sub>6</sub>, 15 μM ethidium, or 15 μM pyronin Y) by incubating for 1.5 h at 37 °C in the presence of 10 μM CCCP. Loaded cells were collected by centrifugation, washed three times and resuspended in 20 mM HEPES (pH 7.0). Energy-dependent efflux was assayed following the addition of sodium formate to a final concentration of 125 mM, 10 μM CCCP was used as an inhibitor in some experiments. Fluorimetric measurements were performed at 37 °C using a Hitachi 4500 fluorometer (slit widths of 5 nm). The excitation and emission wavelengths used for each of the fluorescent compounds are as follows: DAPI, 364 nm and 454 nm, respectively; DiOC<sub>6</sub>, 485 nm and 520 nm, respectively; ethidium, 530 nm and 610 nm, respectively; and pyronin Y, 500 nm and 570 nm, respectively.

**Determination of the Kinetic Parameters Km and Vmax**—Increasing substrate concentrations were used to establish the kinetics of QacA- and QacB-mediated drug efflux in E. coli and in S. aureus. Aliquots (1 ml) of cells were prepared as described above and loaded with the appropriate range of concentrations for each substrate: DAPI, 1–25 μM; DiOC<sub>6</sub>, 0.1–10 μM; ethidium, 0.1–25 μM; and pyronin Y, 1–50 μM. From these concentrations, a series of efflux curves were generated and the initial velocity was calculated by averaging the linear part of each curve. The range of substrate concentrations was selected to be equally spread above and below the K<sub>m</sub>, and a minimum of 10 substrate concentrations were utilized for each experiment. The non-linear least squares method ($v = V_{\text{max}} [S]/(K_m + [S])$); where [S] represents substrate concentration, was the method of choice to obtain estimates of K<sub>m</sub> and V<sub>max</sub> for all assays. The non-linear least squares method was kindly provided by Dr Ray Ritchie (School of Biological Sciences, University of Sydney) and uses a general least squares fitting technique to determine accurate estimates of K<sub>m</sub> and V<sub>max</sub> followed by matrix algebra to refine and calculate the errors of these estimations (26). K<sub>m</sub> and V<sub>max</sub> calculations were confirmed by double-reciprocal plots where 1/velocity (v) is plotted as a function of 1/[S].

**Inhibition of QacA-mediated Drug Export**—Aliquots (1 ml) of S. aureus cells were prepared as described above, loaded with 15 μM ethidium, washed three times, and resuspended in the appropriate buffer. To measure inhibition by the ionophores nigericin and valinomycin, cells were resuspended in a potassium phosphate buffer (50 mM KH<sub>2</sub>PO<sub>4</sub> and 1.0 mM MgSO<sub>4</sub>, pH 7.0). Nigericin or valinomycin, at a final concentration of 4 μM, was added to loaded cells and incubated for 1 min following the addition of sodium formate, to a final concentration of 125 mM, to energize transport. To measure inhibition by reserpine and verapamil, cells were resuspended in 20 mM HEPES (pH 7.0) and 50 μM reserpine or 150 μM verapamil. Loaded cells were washed three times and resuspended in 20 mM HEPES (pH 7.0), and fluorimetric measurements were obtained after the addition of sodium formate to energize transport.

**Competition Analysis**—Competition analyses were performed by determining the kinetics of QacA- and QacB-mediated export of ethidium (0.5–25 μM) in the presence of various fixed concentrations of non-fluorescent substrates, including the monovalent cations benzalkonium (2–10 μM) and tetraphenylarsonium (5–100 μM), and the divalent cations chlorhexidine (2–10 μM), progamidine (5–100 μM), and diaminodiphenylamine (5–150 μM). Aliquots of S. aureus cells were loaded with both substrates in the presence of 10 μM CCCP for 1.5 h and harvested by centrifugation. Export of ethidium was measured fluorimetrically as described above. The results were illustrated graphically by double-reciprocal plots using the computer program Cricket Graph (Computer Associates International) to obtain a line of best fit.

**Uptake of TMA-DPH into Whole Cells**—TMA-DPH specifically fluo-

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**Table I**

| Monovalent cations | Divalent cations |
|-------------------|-----------------|
| Aminoacridines    | Diamidines      |
| Acidine yellow    | Amicarbalide    |
| Acriflavine       | DAPI            |
| Proflavine        | Dibromopropamidine |
| Cytosin          | Diminazene      |
| Cyanines          | Hexamidine      |
| DiOC<sub>6</sub>  | Pentamidine     |
| Phenanthridines   | Phenamidine     |
| Ethidium          | Progamidine     |
| Pyronins          | Stilbamidine    |
| Quinolines        | Biguanidines    |
| Quinaldine red    | Chlorhexidine   |
| Quaternary ammonium compounds | Guanyhydrazones |
| Rhodamines        | 1i-39/JC-1–134 |
| Rhodamine 6G      | 1a-62/JC-1–127 |
| Crystal violet    | Dequainium      |

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**Figure 1**

A summary of the experimental methods used to study the bioenergetics of qacA-mediated multidrug efflux in S. aureus. The figure illustrates the relationship between the concentration of the efflux substrate and the rate of efflux, showing the inhibition of efflux by the ionophores nigericin and valinomycin. The kinetic parameters K<sub>m</sub> and V<sub>max</sub> were determined using the non-linear least squares method, and the results were confirmed by double-reciprocal plots. The figures are based on data obtained from experiments performed on the host strain K-12 strain BHB2600 (F503 supE sup Hzd met) (21).
resces in lipid environments (27) with an excitation and emission spectra of 350 nm and 425 nm, respectively (24).

S. aureus strains, harboring plasmids pSK1, pSK23, or no plasmid, were prepared as described above for fluorimetric analysis. 2.5 mM TMA-DPH was added to cell suspensions, and accumulation of this substrate was measured fluorimetrically. Cells were also preloaded with 2.5 mM TMA-DPH in the presence of 10 mM CCCP as for other fluorescent substrates and efflux generated by the addition of sodium formate to a final concentration of 125 mM. 10 mM CCCP was used as an inhibitor in some experiments.

RESULTS

QacA Mediates Export of Monovalent and Divalent Cations—The multidrug export protein QacA confers resistance to more than 30 compounds that belong to 12 distinct chemical families (Table I; Refs. 8, 9, and 17). To establish if QacA and the closely related staphylococcal protein QacB mediate the efflux of structurally dissimilar compounds via a common mechanism, fluorimetric transport analyses of the monovalent substrates ethidium and pyronin Y, and two newly identified substrates, the monovalent cation DiOC3 (S. aureus MICs: SK982, 0.25 μg/ml; pSK1 or pSK23 in SK982, 8 μg/ml) and the divalent cation DAPI (S. aureus MICs: SK982 or pSK23 in SK982, 20 μg/ml; pSK1 in SK982, >80 μg/ml), all from different chemical families (Table I), were performed (Fig. 1). Formate-driven efflux of DAPI, DiOC3, and ethidium are shown as a rapid decrease in fluorescence, whereas efflux of pyronin Y was observed as a rapid increase in fluorescence, as described under “Experimental Procedures.” Structures of each substrate are shown at the top of the appropriate graph. All efflux experiments were performed in at least triplicate.

QacA- and QacB-mediated Drug Export Conforms to Michaelis-Menten Kinetics—There are various hypotheses that attempt to explain the broad substrate range of multidrug transporters, e.g. the transporter possesses one or more high affinity binding sites, which enables it to interact directly and specifically with its substrates, (28–30); the transporter has an indiscriminate binding mechanism, which would enable it to recognize many different structures; or the transporter is involved in altering some biophysical parameter of the cell, such as the magnitude of the Δψ or the ΔpH, the result of which drives the movement of various charged compounds across the cell membrane (31). To determine if QacA and QacB interact specifically with structurally dissimilar substrates, kinetic analyses of transport were performed using various fluorescent substrates. These kinetic analyses, undertaken in whole cells, were based on the assumptions that after loading the cells in the presence of CCCP, the intracellular substrate concentration is effectively equivalent to the extracellular concentration, and that...
the use of fluorescence can accurately represent the amount of substrate inside the cell as has previously been demonstrated for ethidium, namely the amounts of ethidium retained by E. coli, calculated utilizing either flurometry or \(^{14}C\)ethidium produced identical results (22). Consistent with these assumptions was the observation that the amount of the fluorescent substrate in loaded cells was proportional to the concentration used. Similar kinetic studies of multidrug efflux systems using whole cells have been performed in P. aeruginosa (32) and Mycobacterium smegmatis (33).

Examination of the relationship between increasing substrate concentration and the transport velocity indicated that QacA- and QacB-mediated export of monovalent and divalent cations conformed to classical Michaelis-Menten kinetics (Fig. 2), with the exception that export of DAPI by QacB was not observed at any concentration (Fig. 2B and Table I). The kinetic parameters, \(K_m\) and \(V_{\text{max}}\), for transport of the fluorescent substrates DAPI, DiOC\(_3\), ethidium, and pyronin Y were determined (Table I). Similar \(K_m\) values were obtained from fluorimetric analyses performed in the natural host S. aureus, and in the heterologous host E. coli, suggesting that these proteins function similarly in both organisms. Both QacA and


### Table I

| Transporter | Compound | \(K_m\) (\(\mu\text{M}\)) | \(V_{\text{max}}\) (FU s\(^{-1}\)) |
|-------------|----------|--------------------------|--------------------------|
| E. coli     | QacA     | 13.50 (± 5.73)           | 5.03 (± 0.91)            |
|             | QacB     | NA*                     | NA*                     |
|             | QacA     | 1.48 (± 0.49)           | 37.93 (± 6.9)            |
|             | QacB     | 1.13 (± 0.77)           | 29.89 (± 5.08)           |
|             | Ethidium | 5.79 (± 1.96)           | 10.83 (± 2.67)           |
|             | Ethidium | 5.75 (± 2.35)           | 3.82 (± 0.60)            |
|             | Pyronin Y| 17.89 (± 8.09)          | 56.66 (± 8.74)           |
|             | QacB     | 13.06 (± 6.44)          | 19.26 (± 2.87)           |

Fig. 2. Michaelis-Menten kinetics of ethidium and DAPI export in S. aureus. Michaelis-Menten kinetics of export of the monovalent cation ethidium (A) and the divalent cation DAPI (B) from SK982 cells harboring plasmid pSK1 (qac, circles) or plasmid pSK23 (qacB, squares). Efflux experiments were performed utilizing a range of drug concentrations between 0.1 and 25 \(\mu\text{M}\), as described under "Experimental Procedures." Initial velocity, represented by fluorescence units per second (FU s\(^{-1}\)), was plotted as a function of substrate concentration. All efflux experiments were performed in at least triplicate.

QacB demonstrated a high affinity for their substrates with \(K_m\) values in the low micromolar range (<20 \(\mu\text{M}\); Table I). QacA-mediated export of monovalent cations, e.g. DiOC\(_3\), ethidium, and pyronin Y, displayed an increased \(V_{\text{max}}\) but not \(K_m\), compared with QacB-mediated efflux of these compounds (Table I), suggesting that although QacA and QacB bind monovalent substrates with equally high affinity, QacA may transport them at a faster rate.

**Competitive and Non-competitive Inhibition of QacA- and QacB-mediated Export of Ethidium**—Fractional inhibitory concentration analysis has indicated that QacA confers resistance to two compounds at a rate proportional to the overall amount of substrate present, thereby implying that the presence of one compound inhibits the transport of another (17). Fluorimetric competition studies were performed in S. aureus to examine QacA-mediated transport of ethidium in the presence of various non-fluorescent substrates (Fig. 3). The monovalent cations benzalkonium and tetraphenylarsonium competitively inhibited QacA-mediated export of ethidium (Fig. 3, A and B), whereas the divalent cations chlorhexidine and propamidine non-competitively inhibited QacA-mediated export of ethidium (Fig. 3, C and D). The ability of the monovalent cations benzalkonium and tetraphenylarsonium to competitively inhibit ethidium transport suggests that these substrates either share a common binding site or have unique but overlapping binding sites. In contrast, the divalent cations propamidine and chlorhexidine appear to bind at a distinct site(s) in QacA compared with ethidium.

QacB-mediated export of ethidium was competitively inhibited by the monovalent cations benzalkonium and tetraphenylarsonium within a similar concentration range as shown for QacA (data not shown). These data, together with the fact that QacA and QacB differ by only 7 amino acids (18) and display similar binding constants for monovalent cations (Table I), imply that these two transporters possess identical binding site(s) for monovalent substrates. The divalent cation propamidine non-competitively inhibited QacB-mediated ethidium efflux only at a substantially higher concentration (25 \(\mu\text{M}\)) compared with QacA (10 \(\mu\text{M}\); see Fig. 3D), and the divalent...
cation diamidinodiphenylamine did not inhibit QacB-mediated ethidium efflux even at a concentration of 150 μM. This is consistent with the resistance profile of QacB, which provides a low degree of resistance to propamidine and no resistance to diamidinodiphenylamine (8). Thus, despite the high level of similarity between QacA and QacB, they display completely different binding characteristics for divalent cations.

**Inhibition of QacA-mediated Transport by Ionophores**—To investigate which component, i.e. the ΔpH and/or the Δψ of the Δψ, is involved in energizing QacA-mediated drug efflux, transport assays were performed in the presence of the ionophores, valinomycin and nigericin. In potassium-buffered solutions, the potassium ionophore valinomycin specifically collapses the Δψ, whereas nigericin, which allows H⁺:K⁺ exchange, specifically collapses the ΔpH (34). Both nigericin and valinomycin inhibited QacA-mediated efflux of ethidium (Fig. 4A) and DAPI (data not shown), indicating that both the ΔpH and the Δψ are required to energize QacA-mediated efflux of monovalent and divalent cations. This is consistent with an electrogenic proton/antiport mechanism, e.g. an exchange of two or more protons for one molecule of substrate in the case of ethidium.

**Inhibition of QacA-mediated Ethidium Transport by Reserpine and Verapamil**—Sensitivity to reserpine and verapamil has been observed in an extensive range of multidrug efflux systems, including: members of the ABC superfamily, e.g. P-glycoprotein (35); the major facilitator superfamily, e.g. the mammalian VMAT protein (30), Bmr from *B. subtilis* (36), and LmrP from *L. lactis* (37); and the small multidrug resistance superfamily, e.g. EmrE from *E. coli* (38). MIC analysis demonstrated that QacA does not confer resistance to either reserpine or verapamil. However, both substances inhibited QacA-mediated ethidium export at a concentration of 50 μM (reserpine) or 150 μM (verapamil) (Fig. 4B).

**Analysis of Accumulation and Efflux of TMA-DPH**—The hydrophobic compound TMA-DPH fluoresces when partitioned into the membrane (27), and has been utilized to identify the sub-cellular origin of efflux for various multidrug export systems (32, 37, 39). TMA-DPH was shown to be a QacA substrate by using drug susceptibility (S. aureus MICs: SK982, 15 μg/ml)
pSK1 in SK982, 30 μg/ml), and formate-driven efflux (Fig. 5A, B) studies. TMA-DPH accumulation and efflux in cells expressing qacA were indistinguishable from cells lacking qacA, following treatment with the uncoupler CCCP (Fig. 5, A and B). It should be noted that CCCP partially quenches TMA-DPH fluorescence, resulting in the observed lower fluorescence of the CCCP-treated cells. TMA-DPH has previously been shown to display a biphasic interaction with bacterial membranes, where the first phase is represented by the intercalation of this compound into the outer leaflet of the membrane resulting in a very rapid increase in fluorescence, and the second phase is represented by the movement of this compound from the outer leaflet to the inner leaflet of the membrane resulting in a more gradual increase in fluorescence (24, 39). Fluorimetric analysis of TMA-DPH accumulation by the staphylococcal strain SK982, with and without the qacA-containing plasmid pSK1, indicated that TMA-DPH intercalates into the outer leaflet of the cytoplasmic membrane in both strains, as represented by a rapid increase in fluorescence (Fig. 5B). The first phase was observed as an almost instantaneous increase in fluorescence, occurring at approximately the 30-s point in Fig. 5B. However, the second phase, represented by a gradual increase in fluorescence, where the substrate moves from the outer leaflet to the inner leaflet of the cytoplasmic membrane, was significantly reduced in the QacA-containing strain relative to the background strain (Fig. 5B). This suggests that QacA interacts with, and expels...
TMA-DPH from the cell membrane, preventing it from entering the inner leaflet and hence the cytoplasm.

**DISCUSSION**

The staphylococcal multidrug export protein QacA confers resistance to an extensive range of monovalent and divalent, antimicrobial, lipophilic compounds (Table I). QacA-mediated PMF-dependent efflux of one divalent and three monovalent cations was demonstrated by using fluorescent transport assays (Fig. 1). This suggests that QacA utilizes a common PMF-dependent efflux mechanism for transport of all of its substrates. QacA-mediated export of the monovalent cation ethidium and the divalent cation DAPI was inhibited by the ionophores nigericin and valinomycin (Fig. 4A), which specifically dissipate the ΔΨ and the ΔpH, respectively. This indicates that the PMF-dependent mechanism utilized by QacA requires both components of the ΔΨ/* to energize transport and implies for monovalent cations, a stoichiometry of at least two protons being exchanged for one molecule of substrate. Similar energy requirements have been shown for some other multidrug efflux systems, e.g., the mammalian VMA7 proteins, which mediate monoamine antiport in exchange for two protons (40), and LmrP-mediated drug efflux from *L. lactis*, which is reliant on both the ΔΨ and the ΔpH of the ΔΨ/*, suggesting an electrogenic drug/H*+- (n = 2) antiport mechanism (37).

Fluorometric analysis of the kinetic parameters of QacA-mediated export of DAPI, DiOC<sub>3</sub>, ethidium, and pyronin Y produced *Km* values in the low micromolar range, i.e., <20 μM (Table II), indicating that QacA interacts with each structurally dissimilar substrate with high affinity. Similarly, other multidrug efflux systems have been demonstrated to possess a high affinity for their substrates, e.g., Smr-mediated efflux of triphenylmethylphosphonium (*Km* of 5 μM) (12), and MexAB-OprM-mediated efflux of dimethylaminoaryl-1-ethylpyridinium in *P. aeruginosa* (*Km* of 10 μM) (32). QacA and the closely related QacB share similar binding affinities for monovalent cations (Table II), and ethidium efflux mediated by these two proteins was competitively inhibited by other monovalent cations (Fig. 3, A and B). Taken collectively, these data are indicative that the QacA and QacB multidrug efflux proteins potentially utilize a common mechanism for the recognition of monovalent ligands. However, QacA and QacB differ in their recognition of divalent cations, reflected by the inability of QacB to transport DAPI (Fig. 2; Table II). QacA-mediated ethidium efflux was non-competitively inhibited by divalent cations (Fig. 3C and D), whereas QacB-mediated ethidium efflux was unaffected by diamidinodiphenylamine and non-competitively inhibited by propamidine, although only at high concentrations. These data are consistent with the relative resistance profiles conferred by QacA and QacB (8, 17). Thus, QacA appears to utilize a high affinity binding site(s) for the recognition of divalent cations, distinct from the site(s) responsible for recognition of monovalent cations; QacB lacks this high affinity binding mechanism, but is capable of binding some divalent cations, albeit with a lower affinity.

Previous studies have shown that a single amino acid, namely Asp-323 located in TMS 10 of QacA, is essential for conferring high levels of resistance to diamidines and the bi guanidine, chlorhexidine, implying that this region may form part of the high affinity binding site(s) for divalent cations in QacA (18). Although QacB can interact with some divalent cations with a low affinity, mutation of Asp-323 in QacA to Ala, the residue at this position in QacB, abolished all resistance to these compounds (18). There are six additional amino acid differences between QacA and QacB, some of which are likely to be involved in forming the binding site responsible for low level resistance to the diamidines mediated by QacB. Previous studies have supported the notion that QacA evolved from QacB (20). The data presented here imply that this evolutionary process entailed the acquisition of a high affinity binding mechanism for divalent cations and the concomitant loss of a low affinity binding mechanism for selected divalent cations.

TMA-DPH is a fluorescence probe that specifically interacts with the cytoplasmic membrane. TMA-DPH was shown to be a substrate of QacA (Fig. 5A), and it was demonstrated that QacA reduces the accumulation of TMA-DPH in the inner leaflet of the cytoplasmic membrane (Fig. 5B), suggesting that QacA is most likely to interact with this substrate within the membrane. Whether the findings with TMA-DPH can be extrapolated to include all QacA substrates is unknown, but this is an appealing hypothesis, since the known substrates of QacA are all hydrophobic, lipophilic cations (Table I). The multidrug transport systems LmrP (37), MexA-MexB-OprM (32), and P-glycoprotein (41, 42) have been shown to extrude substrates directly from the cytoplasmic membrane, implying this may be a general feature of multidrug efflux systems.

The results presented here provide evidence that the multidrug transport protein QacA interacts directly and specifically with its substrates and that this interaction may occur, in the first instance, in the cell membrane as the substrate is entering the cell. Such a mechanism would prevent toxic antimicrobial agents from reaching intracellular targets. Intramembranous transporter-substrate interactions imply that critical sites in the protein are accessible directly from within the lipid phase. Residues contained within TMS, such as Asp-323 in TMS 10 of QacA, are ideal candidates for such interactions.

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**REFERENCES**

1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
2. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171
3. van Veen, H. W., Venema, K., Bolhuis, H., Ossenkloek, I., Kok, J., Poolman, B., Driessen, A. J. M., and Koning, W. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10666–10672
4. Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. W., and Henderson, P. J. F. (1992) Curr. Opin. Cell Biol. 4, 286–295
5. Marler, M. D., and Saier, M. H., Jr. (1993) Trends Biochem. Sci. 18, 13–20
6. Paulsen, I. T., and Skurray, R. A. (1995) Gene (Amst.) 124, 1–11
7. Noulhal, A. A., Bidnemark, V. E., and Chen, L. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4781–4785
8. Littlejohn, T. G., Paulsen, I. T., Gillespie, M. T., Tennent, J. M., Middley, M. G., Jones, I. G., Purewal, A. S., and Skurray, R. A. (1992) FEBS Microbiol. Lett. 98, 259–266
9. Tennent, J. M., Lyon, B. R., Middley, M., Jones, I. G., Purewal, A. S., and Skurray, R. A. (1989) J. Gen. Microbiol. 135, 1–19
10. Paulsen, I. T., Skurray, R. A., Tam, R., Saier, M. H., Jr., Turner, R. J., Wiener, J. H., Goldberg, E. B., and Grinius, L. L. (1996) Mol. Microbiol. 19, 1167–1175
11. Schuldiner, S., Leendekerker, M., and Yerushalmi, H. (1997) J. Exp. Biol. 200, 335–341
12. Grinius, L. L., and Goldberg, E. B. (1994) J. Biol. Chem. 269, 29998–30004
13. Paulsen, I. T., Brown, M. H., Dunstan, S. J., and Skurray, R. A. (1995) J. Bacteriol. 177, 2927–2933
14. Dinh, T., Paulsen, I. T., and Saier, M. H., Jr. (1994) J. Bacteriol. 176, 3825–3831
15. Poole, K., Heinrichs, D. E., and Nesbit, S. (1993) Mol. Microbiol. 10, 529–544
16. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., and Hearst, J. E. (1993) J. Bacteriol. 175, 6299–6313
17. Mitchell, B. A., Brown, M. H., and Skurray, R. A. (1998) Antimicrob. Agents Chemother. 42, 475–477
18. Paulsen, I. T., Brown, M. H., Littlejohn, T. G., Mitchell, B. A., and Skurray, R. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3630–3635
19. Gkorgouri, S., Brown, M. H., Roberts, N. J., Paulsen, I. T., and Skurray, R. A. (1998) J. Biol. Chem. 273, 16665–16673
20. Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1998) J. Bacteriol. 180, 3477–3479
21. Hohn, B. (1979) Methods Enzymol. 68, 299–309
22. Lambert, B., and LePecq, J. B. (1984) Biochemistry 23, 166–176
23. Kapuscinski, J., and See, W. (1979) Nucleic Acids Res. 6, 3519–3534
24. Haughland, R. P. (ed) (1996) Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc., Eugene, OR
25. Crissman, H. A., Darynkiewicz, Z., Tobey, R. A., and Steinkamp, J. A. (1985) Science 228, 1321–1324
Bioenergetics of qacA-mediated Multidrug Efflux in S. aureus

26. Ritchie, R. J., and Prvan, T. (1996) Biochem. Educ. 24, 196–206
27. Prendergast, F. G., Haughland, R. P., and Callahan, P. J. (1981) Biochemistry 20, 7333–7338
28. Pawagi, A. B., Wang, J., Silverman, M., Reithmeier, R., and Deber, C. M. (1994) J. Mol. Biol. 235, 554–564
29. Garrigos, M., Mir, L. M., and Orlowski, S. (1997) Eur. J. Biochem. 244, 664–673
30. Yelin, R., and Schuldiner, S. (1995) FEBS Lett. 377, 201–207
31. Roepe, P. D. (1994) Trends Pharmacol. Sci. 15, 445–446
32. Ocaktan, A., Yoneyama, H., and Nakae, T. (1997) J. Biol. Chem. 272, 21961–21969
33. Liu, J., Takiff, H. E., and Nikaido, H. (1996) J. Bacteriol. 178, 3791–3795
34. Kaback, H. R. (1996) Annu. Rev. Biophys. Biomol. Chem. 15, 279–319
35. Wigler, P. W., and Patterson, F. K. (1993) Biochim. Biophys. Acta 1154, 173–181
36. Ahmed, M., Borsch, C. M., Neyfakh, A. A., and Schuldiner, S. (1993) J. Biol. Chem. 268, 11086–11089
37. Bolluis, H., van Veen, H. W., Brands, J. R., Putman, M., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1996) J. Biol. Chem. 271, 24123–24128
38. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1995) J. Biol. Chem. 270, 6856–6863
39. Bolluis, H., van Veen, H. W., Molenaar, D., Poolman, B., Driessen, A., and Konings, W. N. (1996) EMBO J. 15, 4239–4245
40. Njus, D., Kelley, P. M., and Harnadek, G. J. (1986) Biochim. Biophys. Acta 853, 237–265
41. Shapiro, A. B., and Ling, V. (1997) Eur. J. Biochem. 250, 122–129
42. Shapiro, A. B., and Ling, V. (1998) Eur. J. Biochem. 254, 181–188