

The TetA(K) Tetracycline/H⁺ Antiporter from *Staphylococcus aureus*: Mutagenesis and Functional Analysis of Motif C

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Conserved motif C, identified within members of the major facilitator superfamily (MFS) of transport proteins that mediate drug export, was examined in the tetracycline resistance efflux protein TetA(K) from *Staphylococcus aureus*; motif C is contained within transmembrane segment 5. Using site-directed mutagenesis, the importance of the conserved glycine (G151, G155, G159, and G160) and proline (P156) residues within this motif was investigated. Over 40 individual amino acid replacements were introduced; however, only alanine and serine substitutions for glycine at G151, G155, and G160 were found to retain significant levels of tetracycline resistance and transport activity in cells expressing mutant proteins. Notably, P156 and G159 appear to be crucial, as amino acid replacements at these positions either significantly reduced or abolished tetracycline/H⁺ activity. The highly conserved nature of motif C and its distribution throughout drug exporters imply that the residues of motif C play a similar role in all MFS proteins that function as antiporters.

The major facilitator superfamily (MFS), also known as the uniporter-symporter-antiporter family, comprises over 500 membrane-bound transport proteins which are present in all classes of living organisms (21, 26). These proteins perform a wide variety of cellular processes, including the uptake of essential ions and nutrients and the removal of toxic compounds, and typically utilize the proton motive force to drive the transport process (10). Seventeen families can be identified within the MFS based on sequence homology, where a correlation exists between each phylogenetic family and the class of compound transported (26). Despite conveying substrates vastly different in structure, MFS proteins are alike in their predicted membrane topologies. Fourteen out of the 17 families comprise proteins which have been shown or are predicted to possess 12 transmembrane-spanning segments (TMS), and the remaining three MFS families contain proteins with 14 TMS (26). The two families consisting of drug efflux systems, DHA12 and DHA14, possess 12 and 14 TMS, respectively. These families include the well-characterized *Staphylococcus aureus* multidrug efflux protein QacA (24, 25, 28) and the tetracycline export proteins TetA(B) (22) and TetA(K) (9, 12), from *Escherichia coli* and *S. aureus*, respectively, which in comparison to QacA display more limited substrate specificities.

Transport proteins of the MFS contain a number of conserved amino acid sequence motifs which are either ubiquitous within the MFS or family specific (21, 28). Conservation of such motifs among proteins responsible for the transport of a wide variety of structurally disparate compounds implies that they play some vital structural or functional role rather than directly interacting with their substrate(s) (21). Of these, motif C, also known as the antiporter motif (33), is common to all members of the DHA12 and DHA14 families (29). This motif is positioned in TMS 5 of drug export proteins and is typified by the amino acid sequence gxxxGPxiGGxl (28, 30), where x represents any amino acid, residues in uppercase are conserved

in more than 90% of export proteins, and residues in lowercase occur at a frequency of at least 40% (see Fig. 1 below).

The *S. aureus* tetracycline resistance efflux protein TetA(K) is comprised of 14 TMS (9) and has been shown to function as a metal-tetracycline/H⁺ antiporter in both everted membrane vesicles (38) and proteoliposomes (J. Cheng, A. A. Guffanti, and T. A. Krulwich, Abstr. 1997 Meet. Microb. Pathog. Host Response, p. 144, 1997). Additionally, TetA(K) can mediate net potassium (K⁺) ion uptake (14, 15) in a manner analogous to that of TetA(C), encoded by the *E. coli* plasmid pBR322 (7). However, the highly related TetA(B) protein, encoded by the transposon Tn10, is unable to transport K⁺, thereby demonstrating that the function is not common to tetracycline resistance determinants (7).

The present study examined the role of the proline residue and four glycine residues of motif C that are conserved among members of the DHA12 and DHA14 families of the MFS (see Fig. 1). Using site-directed mutagenesis, these residues were altered to a number of different amino acids, including conservative and nonconservative amino acid replacements, and the ability of mutant proteins to transport tetracycline was determined in everted membrane vesicles. Hybrid TetA(K) proteins in which TMS 5 was replaced by the corresponding helix from the tetracycline efflux protein TetA(B) or the multidrug exporter QacA, members of the DHA12 and DHA14 MFS families, respectively, were constructed and analyzed. Taken together, these studies indicate that all conserved residues of motif C are essential for TetA(K)-mediated tetracycline transport.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and growth media. The *E. coli* strains used were BL21(DE3)/pLysS [F⁻ ompT hsdS_B(T₁₈⁻ m_B⁻) dcm gal λ(DE3) pLysS (Cm^r)] (32), CJ236 [F⁺ cat (=pCJ105; M13⁺) dut-1 ung-1 thi-1 relA1 pCJ105 (Cm^r)] (18), DH5α [*supE44* Δ*lacU169* (φ80*dlacZ*ΔM15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] (16), TG1 [F⁺ (*traD36* *proAB*⁺ *lacI*^q *lacZ*ΔM15) *supE* *hsdΔ5* *thi* Δ(*lac-proAB*)] (31), and TK2205 (F⁻ *thi* *lacZ* *rha* *nagA* *kdpABC5* *trkA405* *trkD1*) (7). Plasmids used were pBluescript II SK (Stratagene), pBR322 (22), pNK81 (36), pSK4236 (27), and pSK4646 (this study). The bacteriophage M13K07 (34) was employed in the creation of site-directed mutants. *E. coli* strains were grown in Luria-Bertani medium unless otherwise stated. Antibiotics for plasmid selection were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml.

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Measurement of bacterial growth in the presence of tetracyclines. Inhibitory concentrations (IC) of antibiotics were determined by an agar plate method. Duplicate Luria-Bertani agar plates containing the desired concentrations of tetracycline or the tetracycline analogs chlortetracycline, doxycycline, or oxytetracycline were inoculated with approximately 10^4 CFU of a stationary-phase *E. coli* strain TG1 culture, harboring the plasmid to be tested, using a multipoint replicator. Plates were incubated at 37°C for 24 h. Each test was performed in triplicate to ensure reproducibility of the data. The IC was defined as the lowest concentration of antibiotic (in micrograms per milliliter) which inhibited bacterial growth.

Potassium uptake complementation by TetA(K) and derivatives. To test the ability of TetA(K) and various TetA(K) mutants to phenotypically complement the K^+ uptake defect of *E. coli* strain TK2205, plasmids carrying a wild-type or mutant *tetA(K)* gene were transformed into this strain. Minimal medium plates (37) supplemented with K^+ at various concentrations (0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 10 mM) were inoculated with the strains as described above for the measurement of bacterial resistance to the tetracyclines, incubated, and scored for growth. Bacterial growth on plates containing at least 1.5 mM K^+ was taken as an ability of the *tetA(K)* gene to overcome the K^+ transport defect of *E. coli* strain TK2205.

DNA manipulations. Single-stranded DNA was prepared as described by Sambrook et al. (31). Plasmid DNA was isolated from *E. coli* by using either an alkaline lysis method (31) or the Quantum Prep plasmid miniprep kit (Bio-Rad). Restriction endonucleases were used in accordance with the manufacturers' instructions. Primers for mutagenesis and nucleotide sequencing were made using a Beckman Oligo 1000 synthesizer. Nucleotide sequencing was performed by using the SequiTherm cycle sequencing kit (Epicentre Technologies) as recommended by the manufacturer or by the Sydney University and Prince Alfred Macromolecular Analysis Centre, using the ABI ready reaction kit. Sequences were assembled and stored using the program SEQUENCHER (Gene Codes Corp.). The multiple-amino-acid-sequence alignment of motif C was prepared by using PILEUP (5) and SEQVU (Garvan Institute of Medical Research, Sydney, Australia).

Mutagenesis of *tetA(K)*. Site-directed mutants were constructed by using oligonucleotide-directed mutagenesis according to the method of Kunkel et al. (18). The following oligonucleotide primers were employed in the mutagenesis procedure: G151X, 5'-GCCTTTGGTTTTATAGGATCGATTGTAGCTTTA (ACG)(ACGT)(CG)GAAGGGTTAGTCCCTCAATAGG-3'; G155X, 5'-GT AGCTTTAGGTGAAGGGTTA(ACG)(CG)(CT)CCTTCGATCGGGGAAT AATAGCACATTATATTC-3'; P156X, 5'-GCTTTAGGTGAAGGGTTAGG T(ACG)(ACGT)(CG)TCGATCGGGGAATAATAGCACATTATATTC-3'; G159X, 5'-GCTTTAGGTGAAGGGTTAGGTCATCGATA(ACG)(ACGT) (CG)GGAATAATAGCACATTATATTC-3'; and G160X, 5'-GCTTTAGGTG AAGGGTTAGGTCCATCGATAGGG(ACG)(ACGT)(CG)ATAATAGCAC ATTATATTCATTGG-3'. Underlining indicates mismatching of base pairs with the wild-type *tetA(K)* sequence so as to introduce a restriction endonuclease site without altering the coding sequence, and parentheses indicate degeneracy introduced into each oligonucleotide, resulting in the alteration of the amino acid sequence at that position. As a first step in the creation of TetA(K) hybrid proteins, *StuI* and *NsiI* sites were introduced into *tetA(K)*, flanking the region encoding TMS 5 at amino acid positions 138 and 166, by using the QuickChange site-directed mutagenesis kit (Stratagene) and the oligonucleotides (i) 5'-CAA GAAAAACAAGGCAAGGCCTTTGGTTTTATAGGATC-3' and its complement and (ii) 5'-GGGGGAATAATAGCACATTATATGCATTGGTCTTA CCTACTTATAC-3' and its complement, respectively. Oligonucleotide primers used for the amplification of TMS 5 of QacA (nucleotides 1254 to 1358, GenBank accession no. X56628; 5'-CGCAAGGCCTTAGCTGTATGGTCAATCG C-3' and 5'-CGCATGCAATTGCTCAAGTAAAGCTCCTC-3') and TMS 5 of TetA(B) (nucleotides 1998 to 2072, GenBank accession no. J01830; 5'-CGCAA GGCCTTAGCTGTATGGTCAATCGC-3' and 5'-CGCATGCAATTGCTCAA GTAAAGCTCCTC-3') incorporated *StuI* and *NsiI* sites at the 5' and 3' ends, respectively. Chemical modification of *tetA(K)* was achieved utilizing hydroxylamine as described by McNicholas et al. (23). Second-site suppressor mutations of *E. coli* DH5 α cells harboring the *tetA(K)* mutant, pSK4541, were obtained by using the method of Kimura et al. (17). The DNA sequence of the *tetA(K)* gene for each mutant constructed was confirmed and analyzed to ensure that no spurious mutations had been introduced.

Radioactive labeling of mutant proteins. A modified method of Maneewanakul et al. (20) was used to detect mutant proteins. *E. coli* BL21(DE3)/pLysS cells harboring the desired plasmid were grown to an optical density at 600 nm of 0.7, harvested, and resuspended in 1 volume of assay medium (5% 18-amino-acid mixture, 0.2% glucose, 2 mM MgSO₄, 1 × M9 salts). Cells were incubated at 37°C for 1 h before the addition of 0.25 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and incubation was continued for a further 1 h at 37°C. Rifampin was added to a final concentration of 200 μ g/ml, and the incubation temperature was switched to 42°C for 10 min and then returned to 37°C for 40 min. One milliliter of cells was transferred to an Eppendorf tube, and proteins were radioactively labeled for 10 min at 37°C by the addition of 10 μ Ci of Tran³⁵S-label (ICN) per ml of cells. The cells were then collected by centrifugation and resuspended in sodium dodecyl sulfate gel-loading buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). Following electrophoresis, the gel was incubated in 1 M sodium salicylate, and

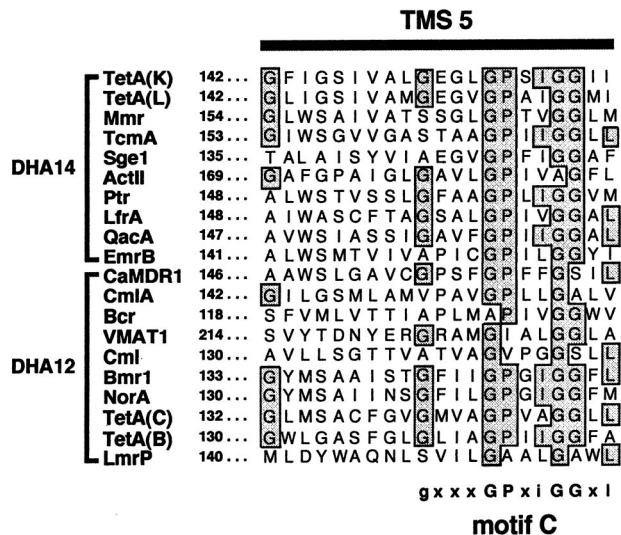


FIG. 1. Multiple-amino-acid-sequence alignment of TMS 5 from representative members of the MFS DHA14 and DHA12 drug efflux families (sequence names are shown on the left). GenBank (GB), Swiss-Prot (SP), or EMBL (EM) accession numbers for each protein are as follows: TetA(K), EM M16217; TetA(L), SP P07561; Mmr, GB M18263; TcmA, GB M80674; Sge1, SP P33335; ActII, GB M64683; Ptr, GB X84072; LfrA, GB U40487; QacA, EM X56628; EmrB, SP P27304; CaMDR1, SP P28873; CmlA, SP 32482; Bcr, SP P28246; VMAT1, GB M97380; Cml, SP P31141; Bmr1, SP P33449; NorA, SP P21191; TetA(C), EM J01749; TetA(B), EM J01830; LmrP, GB X89779. Numbers on the left refer to the position of the leftmost amino acid residue for each protein. Shaded and boxed residues represent amino acids that are conserved in at least 40% of proteins. The highly conserved motif C consensus sequence is displayed below the alignment, where x represents any amino acid, residues in uppercase are conserved in more than 90% of export proteins, and residues in lowercase occur at a frequency of at least 40%.

proteins were identified by fluorography (2); fluorographs were scanned with a Nikon ScanTouch Controller AX-1200. Quantitation of TetA(K) proteins was performed by using the software package Quantity One (Bio-Rad). A host-encoded protein, present in all samples, was used as an internal standard and assigned a value of 100; this was subsequently used to normalize the level of TetA(K) expression. To evaluate the reproducibility of the technique and to ensure that any deviations observed in protein level expression were not due to variations caused either by the labeling procedure or by plasmid loss, experiments were performed twice, and plasmids from all samples were isolated and analyzed.

Preparation of membrane vesicles. Everted membrane vesicles were prepared from *E. coli* TG1 cells harboring wild-type or mutant plasmids essentially as described by Yamaguchi et al. (38). Everted vesicles were prepared by disruption of cells by a single passage at 4,500 lb/in² through an Aminco French pressure cell, and unlysed cells were removed by centrifugation. Vesicles were sedimented by using ultracentrifugation and gently resuspended by using a glass rod in 50 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 7.0) containing 100 mM KCl. The vesicles were then frozen in a dry ice-ethanol bath and stored at -70°C. The protein concentration in membrane vesicles was determined by using the DC protein assay (Bio-Rad) as described by the manufacturer.

Tetracycline transport assays. A suspension of membrane vesicles in 50 mM MOPS-KOH buffer (pH 7.0) containing 100 mM KCl and 500 μ M CoCl₂ was preincubated at 31°C for 10 min with shaking in the presence of 5 mM NADH as an energy source. The uptake of tetracycline was initiated by the addition of [³H]tetracycline (DuPont New England Nuclear) to a final concentration of 10 μ M. Aliquots were removed at the required time intervals and immediately mixed with 5 ml of 50 mM MOPS-KOH (pH 7.0) buffer containing 150 mM LiCl and filtered through a 0.45- μ m-pore-size Gelman Metricel filter, followed by a 2-ml wash with the LiCl-containing buffer. The radioactivity on the filter was determined by using a Tri-Carb liquid scintillation analyzer (Packard), and tetracycline accumulation (nanomoles per milligram of protein) was calculated by subtracting the radioactivity retained by the filter alone.

RESULTS

Mutagenesis of motif C. To identify functionally important amino acids within motif C of TetA(K) (151-GEGLGPSIG

GII-162) (Fig. 1), random and site-directed mutageneses were performed on the cloned *tetA(K)* gene carried by pSK4646. This construct, a derivative of pSK4607 (9), incorporated a C-terminal tag which encodes six consecutive histidine residues prior to the termination codons. The residues targeted for mutagenesis were the glycines G151, G155, G159, and G160 and the proline P156 of the TetA(K) protein. The majority of these represent the highly conserved residues of motif C. However, more variation among DHA12 and DHA14 MFS members exists at G151; this residue is conserved in at least 40% of drug exporters (28) (lowercase in Fig. 1). By utilizing the mutagenic oligonucleotides listed in Materials and Methods, a number of substitutions were obtained at each of these positions (Table 1). Additionally, a random mutant created by hydroxylamine treatment, which expressed a G155D substitution (pSK4400), was included in this analysis.

Based on IC data, it appears that at positions 151, 155, and 160 the only substitutions that preserved wild-type tetracycline resistance levels were alanine and serine (Table 1). In contrast, no substitutions for G159 maintained any significant resistance to tetracycline. This indicated that there is an absolute requirement for the glycine residue at this position in TetA(K); G159A and G159S mutations conferred only marginal tetracycline resistance (8 μ g/ml) (Table 1). Although this seems to be true for TetA(K), it is not the case for all MFS drug export proteins, because other amino acids naturally occur at this position; e.g., the actinohordin efflux protein from *Streptomyces coelicolor*, ActII, has an alanine residue at the equivalent position (Fig. 1 and see reference 28). The requirement for P156 also appears to be vital for TetA(K)-mediated tetracycline resistance; despite the numerous replacements introduced at P156, only the P156G mutation retained tetracycline resistance, albeit at a low level (12 μ g/ml) (Table 1).

Expression of TetA(K) mutants. To determine whether any of the introduced mutations affected the expression level of TetA(K), proteins were radioactively labeled and visualized as described in Materials and Methods. With the wild type and all TetA(K) mutants, a band corresponding to a size of approximately 43 kDa was detected (Fig. 2 and data not shown). This is considerably smaller than the predicted size of 50.7 kDa for TetA(K) (12). However, this phenomenon has also been observed for the highly related *Bacillus subtilis* TetA(L) protein, which, although having a predicted size of 49.9 kDa, was determined experimentally to be 37.5 kDa (3); the difference between the observed and predicted molecular masses might be due to the high hydrophobicity of these membrane proteins. Radioactive labeling of mutant TetA(K) proteins indicated that, in general, the level of protein detected did not deviate greatly from that expressed by cells carrying the wild-type TetA(K) (Fig. 2 and data not shown). However, one exception was the introduction of a proline residue at G159 (pSK4574), which did not confer tetracycline resistance (Table 1) and resulted in a less intense TetA(K) band (24.7%) (Fig. 2, lane 8), reflecting either reduced protein expression or reduced stability.

It has been demonstrated that the TetA(K) polypeptide can complement *E. coli* cells defective in K^+ import (14, 15). This collateral phenotype has been exploited in the present study as an indication of whether mutant TetA(K) proteins were inserted into the membrane in a functional conformation. It was found that the presence of either wild-type TetA(K) or any of its mutant derivatives enabled the growth of the K^+ transport-defective *E. coli* strain TK2205 on medium supplemented with as little as 1.5 mM K^+ . As a positive control, *E. coli* TK2205 cells harboring the plasmid pBR322, which encodes the TetA(C) protein, were able to grow on medium containing low

TABLE 1. Tetracycline resistance levels conferred by TetA(K) motif C mutants^a

Plasmid	Mutation	IC (μ g/ml)
pBluescript II SK		5
pBR322		96
pSK4646		48
G151 mutations		
pSK4591	G151R	5
pSK4592	G151V	10
pSK4594	G151E	5
pSK4595	G151D	5
pSK4596	G151L	5
pSK4597	G151K	5
pSK4598	G151A	48
pSK4599	G151S	48
G155 mutations		
pSK4400	G155D	5
pSK4538	G155A	48
pSK4539	G155R	5
pSK4541	G155P	5
pSK4543	G155S	48
pSK4544	G155T	5
pSK4549	P155S-P156S	32
P156 mutations		
pSK4551	P156A	5
pSK4552	P156R	5
pSK4555	P156L	5
pSK4556	P156T	5
pSK4559	P156V	5
pSK4560	P156N	5
pSK4561	P156Q	5
pSK4562	P156G	12
pSK4563	P156H	5
pSK4564	P156I	5
pSK4565	P156K	5
pSK4566	P156S	5
G159 mutations		
pSK4568	G159A	8
pSK4570	G159R	5
pSK4571	G159D	5
pSK4572	G159N	5
pSK4573	G159E	5
pSK4574	G159P	5
pSK4575	G159S	8
pSK4576	G159T	5
pSK4577	G159V	8
G160 mutations		
pSK4579	G160V	10
pSK4580	G160T	5
pSK4581	G160L	5
pSK4582	G160I	5
pSK4583	G160E	5
pSK4584	G160Q	5
pSK4585	G160R	5
pSK4586	G160A	48
pSK4588	G160S	32

^a All mutants were constructed by site-directed mutagenesis procedures, with the exception of the hydroxylamine-generated G155 mutation of pSK4400 and the second-site suppressor mutation of pSK4541, pSK4549 (see Materials and Methods for details).

K^+ concentrations, as previously reported (11); *E. coli* TK2205 cells either with or without the vector pBluescript II SK were unable to grow at a K^+ concentration of 3 mM. The finding that all mutant TetA(K) proteins, including cells expressing the

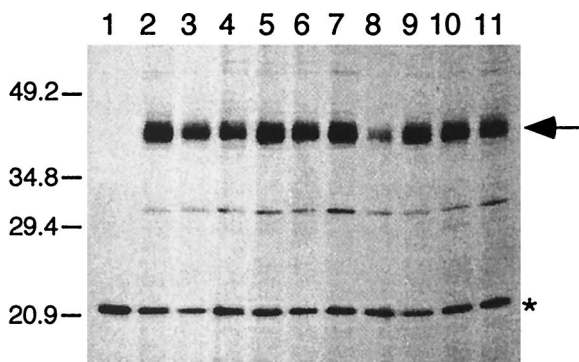


FIG. 2. Fluorograph of radioactively labeled TetA(K) proteins prepared from *E. coli* BL21(DE3)/pLysS cell extracts containing plasmids encoding mutations at G159 in TetA(K). Lanes [extracts from cells harboring the following plasmids, with the normalized value for detected TetA(K) protein denoted as a percentage of the wild-type value]: 1, pBluescript II SK; 2, pSK4646 (wild type; 100%); 3, pSK4568 (G159A; 101.0%); 4, pSK4570, (G159R; 63.6%); 5, pSK4571 (G159D; 102.6%); 6, pSK4572 (G159N; 96.1%); 7, pSK4573 (G159E; 97.9%); 8, pSK4574 (G159P; 24.7%); 9, pSK4575 (G159S; 87.7%); 10, pSK4576 (G159T; 78.5%); and 11, pSK4577 (G159V; 64.8%). The positions of migration and sizes (in kilodaltons) of coelectrophoresed standard proteins are shown on the left. Products corresponding to the TetA(K) protein (43 kDa) are indicated on the right by the arrow, and a host-encoded protein, used for normalization of TetA(K) expression levels, is indicated by the asterisk. Only the relevant portion of the fluorograph is shown. Similar studies were performed for all TetA(K) mutant proteins; only one representative data set is presented here.

G159P mutation, which resulted in the production of a less-intense TetA(K) band (Fig. 2, lane 8), appeared to be able to complement the K⁺ uptake defect at a level reminiscent of the wild type suggested that the introduced changes did not sig-

nificantly affect the folding of mutant proteins into the cytoplasmic membrane.

Tetracycline transport assays in everted membrane vesicles. IC analyses revealed that most *E. coli* cells expressing mutant TetA(K) proteins were unable to sustain growth in the presence of as little as 5 μg of tetracycline per ml (Table 1), suggesting that these mutant derivatives are incapable of extruding tetracycline from the cell. This was confirmed for selected mutants, carrying representative amino acid substitutions, by direct measurements of tetracycline accumulation in *E. coli* TG1 everted membrane vesicles (Fig. 3). Relative tetracycline transport activities of the mutant TetA(K) proteins, as compared to that of the wild type and corrected for protein expression levels as determined by radioactive labeling, are shown in Table 2. Usually, a high tetracycline IC was reflected by significant TetA(K)-mediated transport activity (Fig. 3 and Table 1). For example, replacements at position 151, where glycine was replaced by either alanine or serine, maintained essentially wild-type tetracycline transport levels (Fig. 3A and Table 2), with some activity also being observed when valine was introduced at this position, consistent with the IC data (Table 1). In cases where no tetracycline resistance was observed, it was demonstrated that the active uptake of tetracycline was negligible (Fig. 3 and Table 1). These findings, together with the potassium uptake complementation data and the radioactive labeling studies, which indicate normal expression, support the notion that the inability to transport tetracycline as seen with a number of mutations (Fig. 3) is likely to be due solely to the introduced amino acid substitutions within motif C.

All amino acid substitutions of G159, including those with similar chemical properties, resulted in the complete loss of tetracycline transport activity (Fig. 3D), and, with the excep-

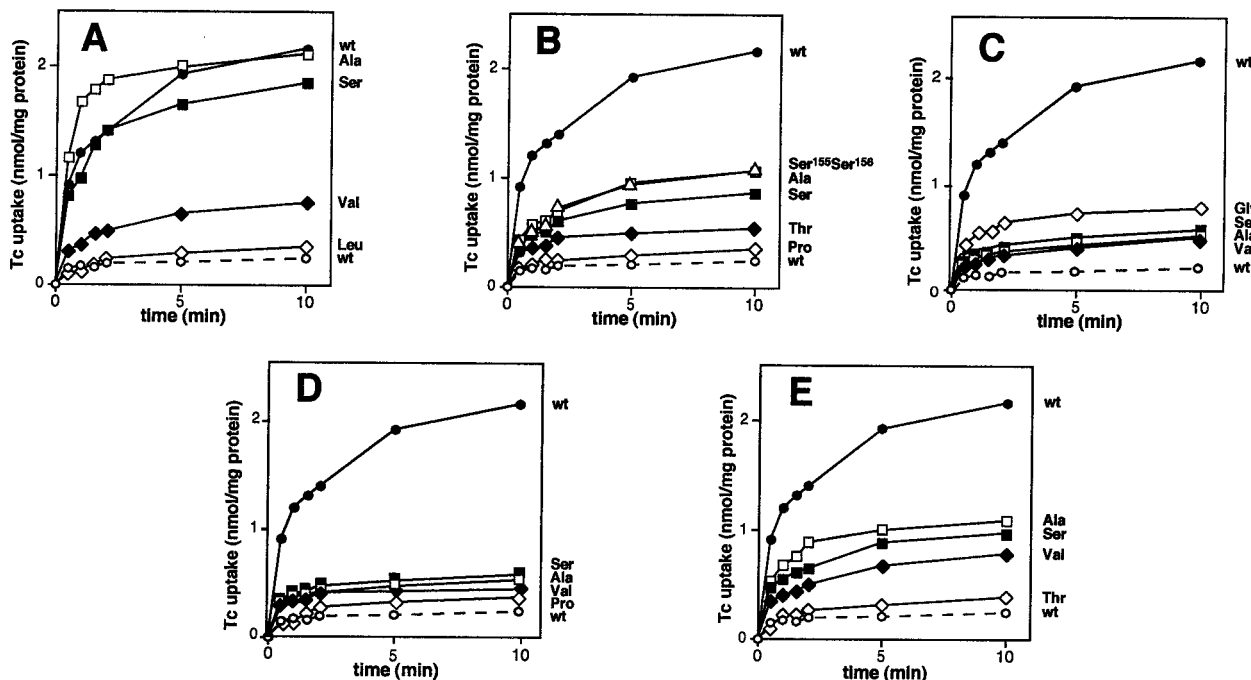


FIG. 3. Tetracycline (Tc) accumulation by everted membrane vesicles prepared from *E. coli* TG1 cells harboring plasmids encoding mutations at the following amino acid positions in TetA(K): A, G151; B, G155; C, P156; D, G159; and E, G160. Data for cells carrying plasmids encoding the wild-type (wt) or mutant TetA(K) proteins are labeled with the three-letter code corresponding to the incorporated amino acid. Solid and dashed lines indicate the uptake of tetracycline by vesicles in the presence and absence, respectively, of NADH as an energy source.

TABLE 2. Relative protein expression levels and transport activities of representative TetA(K) motif C mutants

Mutation	TetA(K) protein expression (%) ^a	Relative tetracycline transport activity (%) ^b
G151 mutations		
G151V	92.0	28.0
G151L	117.4	4.3
G151A	110.1	88.3
G151S	80.6	103.3
G155 mutations		
G155A	141.4	30.0
G155P	82.8	6.3
G155S	143.4	22.2
G155T	112.1	13.1
P155S-P156S	134.6	31.6
P156 mutations		
P156A	91.1	16.1
P156V	104.0	13.8
P156G	82.7	27.2
P156S	93.0	12.5
G159 mutations		
G159A	101.1	14.4
G159P	24.7	24.3
G159S	87.7	20.1
G159V	64.9	15.9
G160 mutations		
G160V	142.4	19.5
G160L	110.7	5.0
G160A	103.6	42.9
G160S	120.1	31.1

^a TetA(K) expression levels were normalized using a host-encoded protein (see Materials and Methods for details) and are expressed as a percentage of the level detected with the wild-type TetA(K) encoded by pSK4646 (Table 1).

^b Tetracycline transport activity, corrected for the TetA(K) protein expression levels shown, is represented as a percentage of wild-type TetA(K) transport activity.

tion of glycine, no changes at P156 were tolerated (Fig. 3C). However, replacements at positions 155 and 160 with alanine or serine, or also valine in the case of G160, while conferring near-wild-type levels of tetracycline resistance (Table 1), displayed only 19.5 to 42.9% of wild-type TetA(K) transport function, suggesting that even minor structural changes affect the tetracycline/H⁺ antiport activity (Fig. 3B and E and Table 2). Therefore, although a good correlation exists between the levels of tetracycline resistance and transport activity of cells expressing mutant TetA(K) proteins in general, the data obtained for replacements at G155 and G160 demonstrated that the transport assay system was more sensitive than IC analysis for determining the effect of amino acid replacements in TetA(K).

Isolation of a suppressor mutation to TetA(K) G155P. Replacement of G155 with proline (pSK4541) caused a complete loss of both tetracycline resistance (Table 1) and transport function compared to wild-type TetA(K) (Fig. 3B and Table 2). A spontaneous revertant (pSK4549) exhibiting near-wild-type levels of tetracycline resistance (32 µg/ml) (Table 1) was isolated from *E. coli* DH5α cells carrying the *tetA(K)* gene with this mutation. Sequence analysis of pSK4549 revealed that two amino acid replacements had occurred within motif C of TetA(K). The first changed the original G155P mutation to serine, and the second substitution occurred at the adjacent residue, P156, also replacing this amino acid with serine. Analysis of this double serine (S155-S156) mutant in everted mem-

brane vesicles revealed that it possessed 31.6% of wild-type tetracycline transport function (Fig. 3B and Table 2). Not only is this level greater than that obtained for cells carrying the single G155S mutation (Table 2), it is significantly greater than that observed for cells with the P156S mutation, for which no tetracycline resistance or significant tetracycline transport activity was observed (Fig. 3C and Tables 1 and 2).

Construction of hybrid TetA(K) proteins. In general, individual replacement of the conserved residues that comprise motif C within TetA(K) resulted in the loss of tetracycline resistance and tetracycline/H⁺ antiport activity. Topological studies performed on TetA(K) demonstrated that all 14 membrane segments of this protein were required to confer tetracycline resistance (9). Therefore, studies were undertaken to determine whether TMS 5 of the TetA(K) polypeptide could be replaced with the corresponding membrane segments from other drug export proteins also containing motif C, a strategy we term TMS replacement mutagenesis. To achieve this, unique restriction endonuclease recognition sites were introduced into the cloned *tetA(K)* gene carried by pSK4646, as described in Materials and Methods, such that these sites flanked the region encoding TMS 5. Although the introduction of the *SuuI* recognition site did not alter the coding sequence of the TetA(K) protein, the incorporation of the *NsiI* recognition sequence resulted in an I166M mutation within TetA(K). Cells that carried this construct, pSK4410, had wild-type tetracycline resistance (48 µg/ml) and, in addition to complementation of *E. coli* cells defective in K⁺ import, radioactive labeling demonstrated that the level of TetA(K) protein detected was not affected (98.0% of the wild-type level).

By using PCR, the regions encoding TMS 5 from the tetracycline exporter TetA(B) and the multidrug resistance efflux protein QacA were amplified from pNK81 (36) and pSK4236 (27), respectively, and cloned into pSK4410 after removal of its cognate TMS 5. The resulting plasmids, pSK4411 and pSK4412, encode TetA(K)-QacA and TetA(K)-TetA(B) chimeras, respectively. IC analysis revealed that *E. coli* cells expressing these hybrid proteins conferred only background levels of resistance to tetracycline and the tetracycline analogs chlortetracycline, doxycycline, and oxytetracycline, which are also substrates of TetA(K) and TetA(B) (13). Additionally, cells expressing the TetA(K)-QacA hybrid were unable to grow on plates containing ethidium bromide, a substrate of the QacA polypeptide (30). Radioactive labeling of these chimeras, as described for Fig. 2, revealed that, in comparison to cells expressing wild-type TetA(K), the TetA(K)-QacA and TetA(K)-TetA(B) hybrid proteins were present at reduced levels (14.5 and 9.6%, respectively).

DISCUSSION

Motif C, a motif in TMS 5 of the TetA(K) polypeptide, is conserved throughout members of the DHA12 and DHA14 drug export families of the MFS. Conservation of this motif within these two families and its absence from proteins that function as importers suggest that it plays an important role in linking proton translocation to the antiport, but not symport, of the substrate or in influencing the polarity of substrate transport (29, 33). Previously it has been postulated that the G155-P156 dipeptide of motif C influences the conformation of the TMS 5 helix and, in conjunction with the other glycine residues, forms a pocket on one face of TMS 5 (33). Indeed, in proteins where glycine residues do not naturally occur in motif C, alanine and serine residues are prevalent (Fig. 1); like glycine, alanine and serine possess small side chains. By using site-directed mutagenesis combined with IC analysis and the

more sensitive tetracycline transport assay system, we have demonstrated experimentally that G155, along with the glycine residues G151 and G160, is important for tetracycline/H⁺ antiport activity, although alanine and serine substitutions in lieu of glycine are well tolerated at these positions (Fig. 3A, B, and E and Tables 1 and 2). In contrast to these glycine residues, glycine residue G159 and proline residue P156 appear to be crucial, since all single-amino-acid replacements at these positions either significantly reduced or abolished TetA(K)-mediated tetracycline transport activity (Fig. 3C and D and Tables 1 and 2). The ability of the double serine mutant with mutations at positions 155 and 156 to mediate significant levels of tetracycline resistance (Table 1) suggests that the tetracycline transport defect conferred by the P156S single mutant can be partially compensated for by also replacing the adjacent residue with serine.

Proline residues are frequently embedded in the membrane-spanning portions of ion channels and transporters but are absent from the transmembrane segments of proteins with no transport function. This has led to the proposition that membrane-bound proline residues play an important functional role in transport proteins (1). However, proline residues are unfavorable in α -helical structures due to the steric hindrance caused by their ring structure; their backbone nitrogen is unavailable for normal hydrogen bonding (4). Consequently, proline residues introduce kinks into α -helices, with the convex side of the kink packed away from the lipid, potentially forming a binding site or channel vestibule (35). The ability of the proline-to-glycine substitution at position 156 to retain partial tetracycline resistance (12 μ g/ml) (Table 1) may be explained by the hypothesis that both proline and glycine act as helix-breaking residues (4). Thus, the introduced glycine residue may preserve the natural secondary structure of TMS 5. Indeed, this role has been suggested for the proline residue within motif C, where this residue may insert a bend in the α -helix (33). It is worth noting that in drug export proteins lacking a proline residue at this position, such as the chloramphenicol export protein Cml from *Streptomyces lividans* (6), one is often located at the adjacent position (Fig. 1).

Based on the results of TMS replacement mutagenesis, TMS 5 of the TetA(K) polypeptide was unable to be replaced by the corresponding helix from the drug export protein TetA(B) or QacA. Radioactive-labeling experiments demonstrated that these hybrid proteins were present at reduced levels in comparison to the wild-type TetA(K) polypeptide. Fujihira and coworkers (8) have shown that the glutamic acid residue E152 in TMS 5 of TetA(K) is important for tetracycline transport function; only a charge-conserved replacement to aspartic acid retained transport activity. This raises the possibility that the inability of TetA(K) chimeras to confer tetracycline resistance may be due to the fact that TetA(B) and QacA possess the neutral residues leucine and alanine, respectively, at the corresponding position (Fig. 1). Thus, if E152 plays an important structural role within the TetA(K) polypeptide (for example, if it was involved in an ionic interaction), a reduction in the amount of TetA(K) hybrid proteins detected would not be unexpected.

Examination of a helical-wheel projection of TMS 5 from the TetA(K) polypeptide revealed that the conserved residues of motif C are all located on the same face of the helix (Fig. 4), furthering the proposition that these residues are close to each other and form a pocket in drug export proteins (33). Interestingly, the glutamic acid residue E152, shown previously to be crucial for TetA(K)-mediated tetracycline resistance (8), appears to be embedded within the center of the recess formed by these glycine residues, between P156 and G159 (Fig. 4),

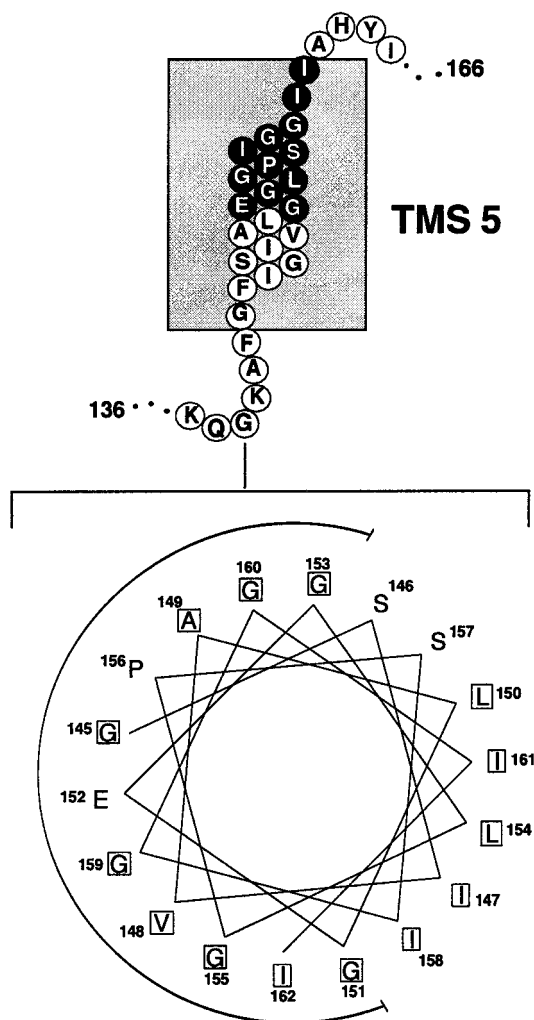


FIG. 4. Schematic diagram of the proposed topology of TMS 5 from the TetA(K) polypeptide (9); residues comprising motif C are depicted in reverse type. Shown at the bottom is a helical-wheel projection of 18 amino acid residues from TetA(K) TMS 5 created using the Helicalwheel program of the Genetics Computer Group package (5). Each residue within the helical wheel is offset from the preceding one by 100°; hydrophobic amino acids are boxed. The glycine-rich face of TMS 5 is indicated by the semicircle.

residues which are both vital for high-level transport activity. In addition to the conserved glycines that comprise motif C, TetA(K) contains another three glycine residues on the same helical face, located at positions 142, 145, and 153 (Fig. 4). Thus, in TetA(K) the glycine residues comprise one-third of the amino acids of TMS 5, seven glycine residues in total (Fig. 1); other TMS of TetA(K) contain between zero and four glycine residues. Significantly, the high glycine content of TMS 5 is not unique to TetA(K); the drug efflux proteins TemA, ActII, BmrI, and NorA all contain six glycine residues within this TMS (Fig. 1). Conformational transitions, which occur within or adjacent to membrane domains of membrane transport proteins during substrate flux, are likely to require regions of the protein structurally responsive to the immediate environment. The abundance of glycine residues in TMS 5 may confer such conformational plasticity to the MFS drug export proteins.

ACKNOWLEDGMENTS

We thank A. M. George for many useful discussions regarding transport assays, N. Firth for critical reading of the manuscript, and J. K. Griffith for providing the *E. coli* strain TK2205.

This work was supported in part by a grant from the Australian Research Council. S.L.G. is the recipient of an Australian Postgraduate Award.

REFERENCES

- Brandl, C. J., and C. M. Deber. 1986. Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc. Natl. Acad. Sci. USA* **83**:917–921.
- Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132–135.
- Cheng, J. B., D. B. Hicks, and T. A. Krulwich. 1996. The purified *Bacillus subtilis* tetracycline efflux protein TetA(L) reconstitutes both tetracycline-cobalt/H⁺ and Na⁺ (K⁺)/H⁺ exchange. *Proc. Natl. Acad. Sci. USA* **93**:14446–14451.
- Chou, P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**:45–148.
- Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
- Dittrich, W., M. Betzler, and H. Schrempf. 1991. An amplifiable and deletable chloramphenicol-resistance determinant of *Streptomyces lividans* 1326 encodes a putative transmembrane protein. *Mol. Microbiol.* **5**:2789–2797.
- Dosch, D. C., F. F. Salvacion, and W. Epstein. 1984. Tetracycline resistance element of pBR322 mediates potassium transport. *J. Bacteriol.* **160**:1188–1190.
- Fujihira, E., T. Kimura, Y. Shiina, and A. Yamaguchi. 1996. Transmembrane glutamic acid residues play essential roles in the metal-tetracycline/H⁺ antiporter of *Staphylococcus aureus*. *FEBS Lett.* **391**:243–246.
- Ginn, S. L., M. H. Brown, and R. A. Skurray. 1997. Membrane topology of the metal-tetracycline/H⁺ antiporter TetA(K) from *Staphylococcus aureus*. *J. Bacteriol.* **179**:3786–3789.
- Griffith, J. K., M. E. Baker, D. A. Rouch, M. G. P. Page, R. A. Skurray, I. T. Paulsen, K. F. Chater, S. A. Baldwin, and P. J. F. Henderson. 1992. Membrane transport proteins: implications of sequence comparisons. *Curr. Opin. Cell. Biol.* **4**:684–695.
- Griffith, J. K., T. Kogoma, D. L. Corvo, W. L. Anderson, and A. L. Kazim. 1988. An N-terminal domain of the tetracycline resistance protein increases susceptibility to aminoglycosides and complements potassium uptake defects in *Escherichia coli*. *J. Bacteriol.* **170**:598–604.
- Guay, G. G., S. A. Khan, and D. M. Rothstein. 1993. The *tet(K)* gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices. *Plasmid* **30**:163–166.
- Guay, G. G., and D. M. Rothstein. 1993. Expression of the *tetK* gene from *Staphylococcus aureus* in *Escherichia coli*: comparison of substrate specificities of TetA(B), TetA(C), and TetK efflux proteins. *Antimicrob. Agents Chemother.* **37**:191–198.
- Guay, G. G., M. Tuckman, P. McNicholas, and D. M. Rothstein. 1993. The *tet(K)* gene from *Staphylococcus aureus* mediates the transport of potassium in *Escherichia coli*. *J. Bacteriol.* **175**:4927–4929.
- Guffanti, A. A., J. Cheng, and T. A. Krulwich. 1998. Electrogenic antiport activities of the Gram-positive Tet proteins include a Na⁺(K⁺)/K⁺ mode that mediates net K⁺ uptake. *J. Biol. Chem.* **273**:26447–26454.
- Hanahan, D. 1983. Studies on transformation in *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
- Kimura, T., T. Sawai, and A. Yamaguchi. 1997. Remote conformational effects of the gly-62→leu mutation of the Tn10-encoded metal-tetracycline/H⁺ antiporter of *Escherichia coli* and its second-site suppressor mutation. *Biochemistry* **36**:6941–6946.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Maneewannakul, K., S. Maneewannakul, and K. Ippen-Ihler. 1992. Sequence alterations affecting F plasmid transfer gene expression: a conjugation system dependent on transcription by the RNA polymerase of phage T7. *Mol. Microbiol.* **6**:2961–2973.
- Marger, M. D., and M. H. Saier, Jr. 1993. A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. *Trends Biochem. Sci.* **18**:13–20.
- McMurry, L. M., R. E. Petrucci, Jr., and S. B. Levy. 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **77**:3974–3977.
- McNicholas, P., I. Chopra, and D. M. Rothstein. 1992. Genetic analysis of the *tetA(C)* gene on plasmid pBR322. *J. Bacteriol.* **174**:7926–7933.
- Mitchell, B. A., M. H. Brown, and R. A. Skurray. 1998. QacA multidrug efflux pump from *Staphylococcus aureus*: comparative analysis of resistance to diamidines, biguanidines, and guanlylhydrazones. *Antimicrob. Agents Chemother.* **42**:475–477.
- Mitchell, B. A., I. T. Paulsen, M. H. Brown, and R. A. Skurray. 1999. Bioenergetics of the staphylococcal multidrug export protein QacA: identification of distinct binding sites for monovalent and divalent cations. *J. Biol. Chem.* **274**:3541–3548.
- Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr. 1998. The major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62**:1–32.
- Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray. 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. *Proc. Natl. Acad. Sci. USA* **93**:3630–3635.
- Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1996. Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**:575–608.
- Paulsen, I. T., and R. A. Skurray. 1993. Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes—an analysis. *Gene* **124**:1–11.
- Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray. 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. *Mol. Microbiol.* **4**:2051–2062.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
- Varela, M. F., C. E. Sansom, and J. K. Griffith. 1995. Mutational analysis and molecular modelling of an amino acid sequence motif conserved in antiporters but not symporters in a transporter superfamily. *Mol. Membr. Biol.* **12**:313–319.
- Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153**:3–11.
- von Heijne, G. 1991. Proline kinks in transmembrane α -helices. *J. Mol. Biol.* **218**:499–503.
- Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner. 1984. New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* **32**:369–379.
- Willets, N. S., and D. J. Finnegan. 1970. Characteristics of *E. coli* K12 strains carrying both an F prime and an R factor. *Genet. Res.* **16**:113–122.
- Yamaguchi, A., Y. Shiina, E. Fujihira, T. Sawai, N. Noguchi, and M. Sasatsu. 1995. The tetracycline efflux protein encoded by the *tet(K)* gene from *Staphylococcus aureus* is a metal-tetracycline/H⁺ antiporter. *FEBS Lett.* **365**:193–197.