Glycine-Rich Transmembrane Helix 10 in the Staphylococcal Tetracycline Transporter TetA(K) Lines a Solvent-Accessible Channel†

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ABSTRACT: The staphylococcal TetA(K) tetracycline exporter is classified within the major facilitator superfamily of transport proteins and contains 14 α-helical transmembrane segments (TMS). Using cysteine-scanning mutagenesis, 27 amino acid residues across and flanking putative TMS 10 of the TetA(K) transporter were individually replaced with cysteine. The level of solvent accessibility to each of the targeted amino acid positions was determined as a measure of fluorescein maleimide reactivity and demonstrated that TMS 10 of TetA(K) has a cytoplasmic boundary at G313 and is likely to extend from at least V298 on the periplasmic side. TMS 10 was found to be amphiphilic containing at least partially solvent accessible amino acid residues along the length of one helical face, suggesting that this helix may line a solvent-exposed channel. Functional analyses of these cysteine mutants demonstrated a significant role for a number of amino acid residues, including a predominance of glycine residues which were further analyzed by alanine substitution. These residues are postulated to allow interhelical interactions between TMS 10 and distal parts of TetA(K) that are likely to be required for the tetracycline transport mechanism in TetA(K) and may be a general feature required by bacterial tetracycline transporters for activity.

The major facilitator superfamily (MFS)[1] of transport proteins is the largest characterized superfamily of secondary transporters, currently comprising more than 50 classified transporter families (*1*) (http://www.tcdb.org). MFS family transport proteins are arranged in 12 or 14 α-helical transmembrane segments (TMS), and their activities are powered by electrochemical membrane concentration gradients, typically the proton motive force (*1*). Three families within the MFS, the drug:H^+ antiporter (DHA) 1−3 families, function exclusively in drug efflux (*2*, *3*) (http://www.tcdb.org). Transporters within these three families display overlapping substrate specificities; each contains both substrate specific and multidrug transporters that recognize a number of unrelated substrates but can be separated on the basis of primary and secondary structure. As seen in the majority of MFS transporters, DHA1 family proteins, including the well-characterized *Escherichia coli* tetracycline (Tc) transporter TetA(B) (*4*) and the multidrug transporter EmrD (*5*), as well as DHA3 family proteins, contain 12 TMS (*2*, *3*). In contrast, DHA2 family members are comprised of 14 TMS and include the TetA(K) (*6*) and TetA(L) (*7*) Tc transporters identified in *Staphylococcus aureus* and *Bacillus subtilis*, respectively, in addition to the *S. aureus* multidrug transporter QacA (*8*). Recently, the first high-resolution structure of a DHA family transporter was reported, that of the multidrug transporter EmrD (*5*). In comparison to the crystal structures available for the MFS *E. coli* lactose permease (LacY) (*9*) and glycerol-3-phosphate transporter (GlpT) (*10*), the EmrD structure shows this transporter in a conformation which is less open to the cytoplasm. Despite this, the overall tertiary organization of the 12 TMS in EmrD is comparable to that of LacY and GlpT. In each of these three transporters, the six N-terminal and six C-terminal TMS form two domains, which interface primarily between TMS 2 and 11 and TMS 5 and 8. These helices, along with TMS 1, 4, 7, and 10, line an internal cavity which is the likely avenue for substrate transport, whereas TMS 3, 6, 9, and 12 are present at the periphery. Transport by these proteins, and possibly all 12-TMS MFS proteins, is proposed to occur via an alternating access mechanism, where substrate(s) bound by amino acid residues lining the internal cavity is (are) able to access the opposite side of the membrane after pivoting between the N- and C-terminal domains (*11*, *12*).

Despite the accumulating structural data for 12-TMS MFS transporters, there is currently little information available for the structure or mechanism of 14-TMS MFS transporters. Sequence similarities within the six N-terminal and six C-terminal TMS of and between 12- and 14-TMS MFS transporters suggest that 14-TMS MFS proteins evolved from 12-TMS precursors after two additional TMS (6 + 2 + 6) were formed by mutation and integration of a central loop region into the lipid bilayer (*13*). Given this likely evolution-

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[1] Abbreviations: Cys, cysteine; CLTetA(K), cysteine-less TetA(K); DHA, drug:H^+ antiporter; MFS, major facilitator superfamily; MIC, minimum inhibitory concentration; Tc, tetracycline; TMS, transmembrane segment.

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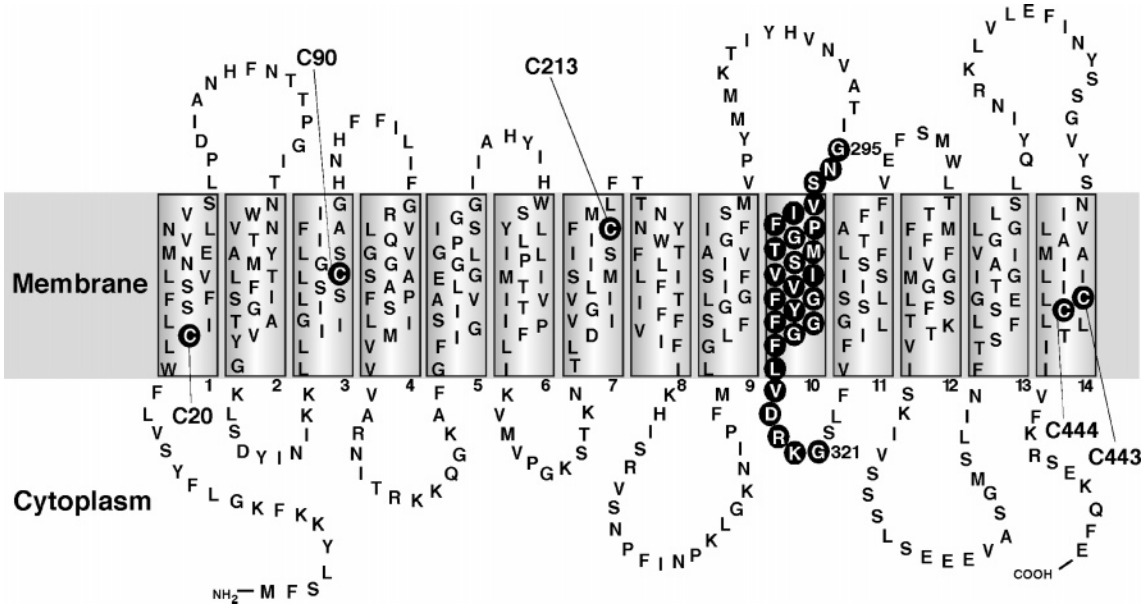

FIGURE 1: Predicted transmembrane topology of the TetA(K) Tc efflux protein. The 14 TMS are indicated by boxes and are numbered 1−14. The positions of the five Cys residues and the TMS 10-spanning residues targeted in this study are indicated by black circles.

ary relationship and the common Tc-divalent metal ion:H^+ antiport reactions catalyzed by 12- and 14-TMS MFS Tc transporters (TetA proteins), it has been hypothesised that both 12- and 14-TMS TetA proteins possess common tertiary structures within their catalytically active regions (*14*, *15*). This hypothesis has been explored using a 12-TMS version of the TetA(L) transporter; however, further studies are required before firm conclusions can be drawn (*14*).

The TetA(K) transporter has been shown via hydropathy and limited gene fusion studies to consist of 14 TMS; however, the exact limits and level of solvent accessibility to each of these TMS have not been defined (*16*, *17*). To this end, we have undertaken cysteine- (Cys-) scanning mutagenesis studies of amino acid positions in and flanking putative TMS 10 of TetA(K) (Figure 1). Helix 10 is of particular interest in analyzing the structure and function of TetA(K), since the corresponding TMS in the QacA multidrug transporter has been demonstrated to contain amino acid residues which directly interact with substrates during transport (*18*, *19*). Additionally, if structural relatedness between corresponding helices of 12- and 14-TMS MFS transporters exists, TMS 10 in TetA(K) may display a similar structure and function to TMS 8 of related 12-TMS proteins. High-resolution structures of EmrD, LacY, and GlpT show TMS 8 to line the substrate translocation region and interact with TMS 5 in the N-terminal domain (*5*, *9*, *10*). Second-site suppressor studies, as well as solvent accessibility and intermolecular cross-linking studies, support this position for TMS 8 in 12-TMS DHA1 family Tc exporters (*20*, *21*). Thus, interactions between TMS 8 and TMS 5 may accommodate large molecular motions between the N- and C-terminal domains as implied by the proposed alternating access transport mechanism. In this context, glycine residues, which possess unique structural properties, including the potential for mediating close interhelical contacts (*22*, *23*) within the targeted region of TetA(K), were of particular interest and were also subjected to alanine mutagenesis. The results of the current study provide important insight into the operation of TMS 10 in TetA(K) and allow further evaluation of hypotheses that this helix is structurally and functionally equivalent to TMS 8 of related 12-TMS MFS transport proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Reagents. The *E. coli* strains used were BL21(DE3)/pLysS [F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *dcm gal* λ(DE3)pLysS] (*24*), DH5α [*supE*44 Δ*lac*U169(ϕ 80*lac*ZΔM15) *hsdR*17*recA*1*endA*1 *gyrA*96 *thi*-1*relA*1] (*25*), and TOP10 [F^- *mcr*A (*mrr-hsd*RMS-*mcr*BC) 80*lac*ZM15 *lac*X74 *rec*A1 *ara*139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G] (Invitrogen). The plasmids used were pBluescript II SK (Stratagene), pBAD (Invitrogen), pTTQ18 (*26*), and the pBluescript-based *tetA*(K)-encoding plasmid pSK4646 (*27*). *E. coli* strains were grown using Luria−Bertani media unless otherwise stated. Ampicillin was used at a concentration of 100 μg/mL for plasmid selection where required. Ampicillin, Tc, IPTG, L-arabinose, *n*-dodecyl β-D-maltoside, and *N*-ethylmaleimide were obtained from Sigma, [^{3}H]-Tc was purchased from Perkin-Elmer, and fluorescein maleimide was from Pierce. Other chemicals were of reagent grade and purchased from commercial sources.

DNA Manipulations. Plasmid DNA was isolated via an alkali lysis method (*28*) or using the Quantum Prep plasmid miniprep kit (Bio-Rad). Restriction endonucleases were obtained from New England Biolabs and were used in accordance with the manufacturer's instructions. Plasmid subcloning and bacterial plasmid transformations were conducted using standard molecular biological techniques (*28*). Oligonucleotides were obtained from GeneWorks (Australia). Nucleotide sequencing was performed at the Australian Genomic Research Facility (Brisbane, Australia) using BDT V2 chemistry. Sequences were assembled and stored using SEQUENCHER V3.1.1 (Gene Codes Corp.).

Construction of tetA(K) Mutants. Site-directed mutants were made via the QuikChange method (Stratagene) using *Pfu* DNA polymerase (Stratagene). Pairs of complementary

oligonucleotide primers (GeneWorks) were designed to incorporate the desired amino acid change and a silent mutation, which either introduced or removed an endonuclease restriction site to aid in identification of mutant clones.

The *tetA*(K) gene of pSK4646 was isolated from the staphylococcal Tc resistance plasmid pT181 (*29*) and cloned via PCR with a C-terminal 6His epitope tag (*27*). This *tetA*(K) gene encodes five Cys residues at amino acid positions 20, 90, 213, 443, and 444 that were replaced individually with serine via site-directed mutagenesis. The pBluescript-based plasmid pSK6005 encodes an entirely Cys-less TetA(K) derivative [CLTetA(K)], where all five Cys residues have been replaced with serine, and was constructed via a combination of site-directed mutagenesis and subcloning portions from single Cys-substituted mutant templates. Subsequently, pSK6005 was used as the mutagenesis template for incorporation of Cys changes at each amino acid position from 295 to 321 and single alanine substitutions for each of the six glycine residues in this region of TetA(K), viz., G295, G302, G310, G313, G314, and G321. TetA(K) protein expression from these pBluescript-based vectors was too low to be purified at a level sufficient for solvent accessibility studies. Therefore, the Cys-less *tetA*(K) [*cltetA*(K)] gene was amplified from pSK6005 via PCR and inserted into a pBAD-based (Invitrogen) expression vector (*30*) encoding C-terminal 1D4 (TETSQVAPA) and 6His epitope tags to generate the plasmid pSK7554. Fragments of *cltetA*(K) mutant genes encompassing the Cys substitutions for each amino acid from G295 to G321 were subcloned into pSK7554, replacing the wild-type sequence, to generate a series of clones for protein purification and use in fluorescein maleimide labeling studies. The DNA sequence of each mutant plasmid was verified to ensure that no secondary mutations had been introduced.

Radioactive Protein Labeling of TetA(K) Proteins. A method modified from Maneewanakul et al. (*31*) was used for the detection of mutant proteins expressed from pBluescript-based plasmids in *E. coli* BL21(DE3)/pLysS as previously described (*27*).

Minimum Inhibitory Concentration Analysis of TetA(K) Mutants. *E. coli* DH5α cells containing the plasmids of interest at OD_{600} of 0.1 were diluted 1:10 and spotted using a multipoint replicator onto Mueller Hinton agar media containing Tc at concentrations ranging from 2 to 48 μg/mL. Cells were incubated at 35 °C and the MIC determined as the lowest concentration of Tc necessary to fully inhibit bacterial growth.

Assays of TetA(K)-Mediated Tetracycline Transport. Everted membrane vesicles were made from *E. coli* DH5α cells freshly transformed with the plasmids of interest and assayed for [^{3}H]-Tc uptake essentially as described elsewhere (*27*, *32*). The amount of [^{3}H]-Tc uptake by the vesicles (nanomoles per milligram of protein) was determined by measuring the radioactivity of each filter using a Tri-Carb liquid scintillation analyzer (Packard) and subtracting the radioactivity retained by the filter alone (*27*).

Generation of an Anti-TetA(K) Antibody for Use in Western Blot Analysis. A peptide composed of the immunogenic 95 C-terminal amino acids of TetA(K) [CTTetA(K)] was used as antigen to raise rabbit anti-TetA(K) antibodies. The portion of *tetA*(K) encoding this region was amplified from pSK4646 by PCR and cloned in-frame to the C-terminal RGSH6 tag of a modified pTTQ18 expression vector (*26*). The CTTetA(K) peptide was overexpressed in *E. coli* DH5α cells by induction with 0.1 mM IPTG and found via Western analysis to be localized within the membrane fraction. The CTTetA(K) peptide was extracted from the membrane fraction by solubilization with 1% *n*-dodecyl β-D-maltoside and purified by nickel-chelation chromatography and had a molecular weight of approximately 12 kDa. The CTTetA(K) peptide antigen was administered to a rabbit in five 500 μg doses at the Institute of Medical and Veterinary Science, Australia. The antiserum collected was found to be immunoreactive with both the CTTetA(K) peptide and the entire TetA(K) polypeptide.

TetA(K) Protein Expression and Fluorescein Maleimide Labeling of TetA(K) Cys Derivatives. Cultures of *E. coli* TOP10 cells harboring pSK7554 or plasmids encoding Cys-replacement TetA(K) derivatives were grown overnight, subcultured 1:20 in 500 mL of fresh media, and grown to an OD_{600} of 0.8. TetA(K) protein expression was induced with 0.0005% L-arabinose, and growth was allowed to proceed for a further 21 h. Three TetA(K) mutants, G295C, P301C, and G302C, displayed low protein yields, and therefore three aliquots were pooled for subsequent steps. Cells were resuspended in 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 10% glycerol and disrupted by four passages through a French pressure cell (SLM Aminco, Spectronic Instruments) at 25000 psi. Remaining intact cells were removed by centrifugation, and the membrane fraction was collected by ultracentrifugation and resuspended in 300 μL of 20 mM Tris-HCl (pH 7.5) and 300 mM NaCl.

Membrane vesicles (150 μL) were labeled with 0.37 mM fluorescein maleimide for 10 min at 37 °C. Labeling was also conducted in the presence of Tc for selected mutants, where 1 mM Tc was incubated with membrane vesicles for 10 min at 37 °C prior to the addition of fluorescein maleimide. Reactions were terminated by addition of 1 mL of 20 mM Tris-HCl (pH 8.0) and 20 mM *N*-ethylmaleimide. Vesicles were solubilized with 1% *n*-dodecyl β-D-maltoside, and TetA(K) protein was purified by nickel-chelation chromatography. Protein samples were separated using SDS−PAGE, and fluorescence was detected with a Molecular Imager FX (Bio-Rad). TetA(K) protein was detected via Western blot analysis using an anti-TetA(K) antibody (described above), and blots were scanned with a GS-710 calibrated imaging densitometer (Bio-Rad). Images were visualized and analyzed using Quantity One software (Bio-Rad).

RESULTS

Construction and Expression of a Cys-less TetA(K) Derivative. The pT181-encoded *S. aureus* TetA(K) protein contains five Cys residues (C20, C90, C213, C443, and C444) that are all predicted to lie within the TMS (*17*) (Figure 1). In generating a Cys-less TetA(K) derivative, requisite for Cys-scanning mutagenesis, the importance of these residues in TetA(K)-conferred Tc resistance and transport was assessed. Initially, five TetA(K) derivatives, each with a single different Cys to serine substitution, were constructed via site-directed mutagenesis. No single substitution was found to seriously affect the level of TetA(K) protein expression (data not shown) nor the level of TetA-

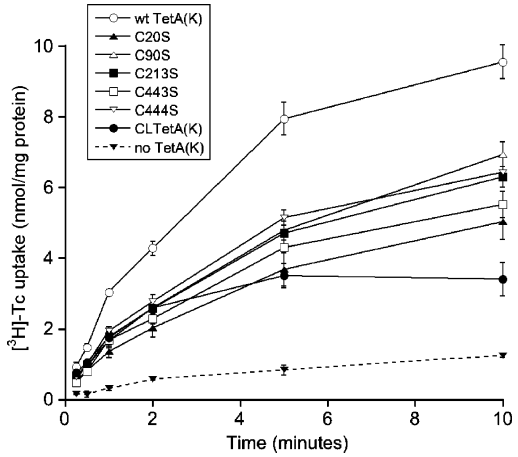

FIGURE 2: Tc transport mediated by TetA(K) mutants containing serine substitutions for Cys residues. Curves represent the average uptake of [^{3}H]-Tc from at least three repeat experiments using everted membrane vesicles of *E. coli* DH5α cells expressing no TetA(K), wild-type TetA(K), C20S, C90S, C213S, C443S, C444S, or CLTetA(K).

(K)-mediated Tc resistance which was maintained at 32 μg/mL. However, the Tc transport capacities of the five single Cys to serine mutants were less than that of wild-type TetA-(K), indicating that these residues have a small influence on TetA(K)-mediated Tc transport in *E. coli* membranes (Figure 2). The relative dispensability of Cys residues in Tc transporters has been previously demonstrated in both 12-TMS (*33*) and 14-TMS (*34*) TetA proteins. However, in the distally related 12-TMS *Clostridium perfringens* TetA(P) protein Cys residues were seen to possess a significant but yet undefined functional role (*35*).

The expression level of the CLTetA(K) protein was approximately 95% of wild-type TetA(K), further suggesting that the five Cys residues of TetA(K) do not heavily influence protein stability. As with the single Cys-replaced TetA(K) derivatives, the CLTetA(K) mutant maintained wild-type Tc resistance levels. The CLTetA(K) protein also exhibited a significant level of Tc transport activity at approximately 50% of wild-type TetA(K) and slightly lower than any single Cys-substituted mutant (Figure 2).

Mutagenesis and Expression of TetA(K) Mutants. In order to establish the functional significance and solvent accessibility of amino acid residues across the putative TMS 10 of TetA(K) and the immediate adjacent loop regions, 27 residues, from G295 to G321, were individually mutated to Cys via site-directed mutagenesis of the CLTetA(K) coding sequence (Figure 1). Due to their potential structural significance in TetA(K), the six glycine residues in this region were also targeted for alanine substitution. All 33 TetA(K) mutants were expressed in *E. coli* BL21 cells at significant levels. With the exception of Y311C and F312C, the expression level of the mutant proteins was no less than one-third that of CLTetA(K), with an average expression of 72% of CLTetA(K) (Table 1).

Tetracycline Resistance and Transport Capacities of TetA-(K) Mutants. MIC analysis demonstrated that CLTetA(K) was able to confer resistance to Tc at concentrations up to 32 μg/mL on solid media (Table 1). With the exception of V298C, all Cys-scanning mutations across TMS 10 of TetA-(K) resulted in a reduction of Tc resistance below this level, possibly indicating a general importance for the TMS 10

Table 1: MIC and Expression Levels of CLTetA(K) Mutants

mutation from CLTetA(K)	Tc MIC (μg/mL)[a]	TetA(K) protein expression[b]
pBluescript SK II	2	
TetA(K)/CLTetA(K)	32	N/A
G295C	2	+++
N296C	12	+++
S297C	24	+++
V298C	32	+++
I299C	8	++
F300C	16	++
P301C	2	++
G302C	2	++
T303C	16	+++
M304C	16	+++
S305C	16	++
V306C	16	++
I307C	16	++
V308C	16	+++
F309C	8	+++
G310C	8	++
Y311C	12	+
F312C	20	+
G313C	2	++
G314C	2	++
F315C	24	++
L316C	4	++
V317C	20	+++
D318C	2	+++
R319C	8	++
K320C	24	+++
G321C	4	+++
G295A	24	+++
G302A	12	+++
G310A	8	+++
G313A	2	+++
G314A	2	+++
G321A	2	++

[a] MIC levels are determined as the lowest concentration of Tc necessary to fully inhibit bacterial growth. [b] Expression relative to CLTetA(K) encoded from plasmid pSK6005 in *E. coli* BL21(DE3)/pLysS and normalized against a host-encoded protein (see ref *27*). Key: (+++) above 2/3 CLTetA(K) expression; (++) 1/3−2/3 CLTetA(K) expression; (+) below 1/3 CLTetA(K) expression.

region in TetA(K)-mediated Tc resistance (Table 1). Of particular note were the effects of substituting glycine residues across the targeted region. The importance of the six glycine residues, G295, G302, G310, G313, G314, and G321 within TetA(K), was examined via Cys and alanine substitution. Due to their lack of a side chain, glycine residues allow the peptide backbone greater conformational freedom than other amino acid residues, and their presence can be highly significant within the TMS of transport proteins, such as shown with the TetA(B) transporter where 8 of the 13 essential amino acid residues within TMS were found to be glycine (*36*). MIC analysis revealed that the G295C, G302C, G313C, and G314C TetA(K) mutants were incapable of facilitating Tc resistance above background levels (Table 1). The Tc transport capacities of these mutants were also reduced to, or close to, background levels (Figure 3A,B,D,E). However, unlike the Cys derivative, the alanine mutant of G295 was able to maintain both Tc resistance and transport capacities at levels close to that observed for CLTetA(K) (Table 1 and Figure 3A). The G302A derivative also possessed a greater capacity for Tc resistance and transport than its Cys-substituted counterpart (Table 1 and Figure 3B). The G313A and G314A mutants displayed a lack of TetA-(K)-mediated Tc resistance and low Tc transport function,

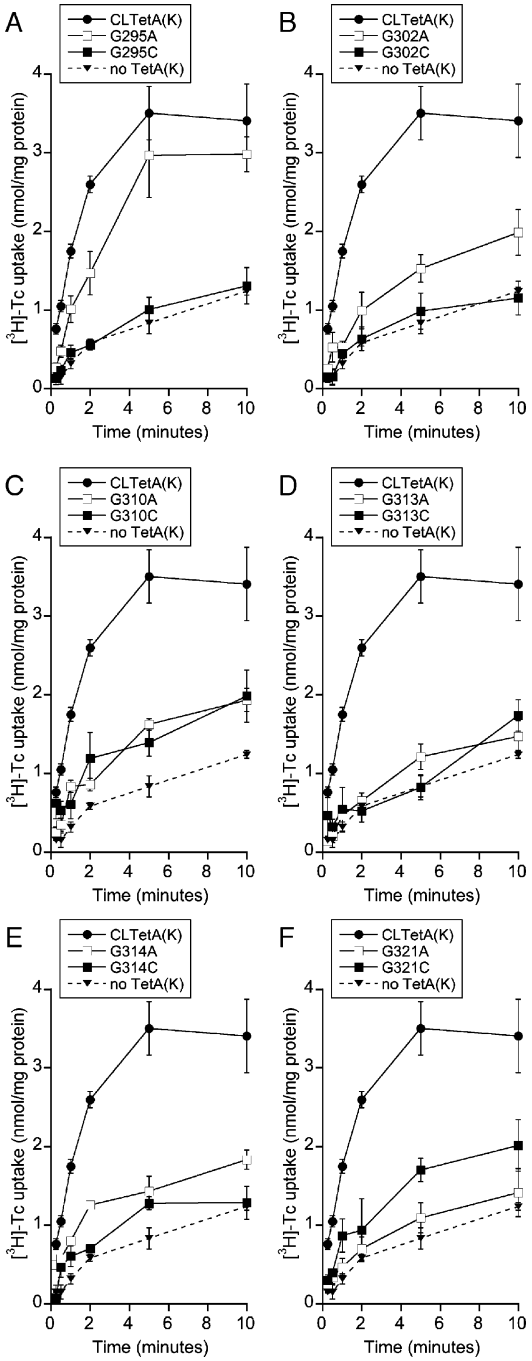

FIGURE 3: Tc transport mediated by CLTetA(K) mutants containing Cys or alanine substitutions for G295 (A), G302 (B), G310 (C), G313 (D), G314 (E), and G321 (F). Curves represent the average uptake of [^{3}H]-Tc from at least three repeat experiments using everted membrane vesicles of DH5α cells expressing no TetA(K), CLTetA(K), alanine-substituted mutants, and Cys-substituted mutants.

suggesting an absolute need for glycines at these positions in TetA(K) (Table 1 and Figure 3D,E). Moderate levels of Tc resistance and transport were maintained after both Cys and alanine substitutions of G310 (Table 1, Figure 3C). The G321C TetA(K) mutant retained a weak capacity for Tc resistance and transport; however, the G321A mutant did not confer any Tc resistance or mediate Tc transport, suggesting an important functional role for this glycine residue (Table 1 and Figure 3F). These results suggest a significant functional role for the majority of targeted glycine residues.

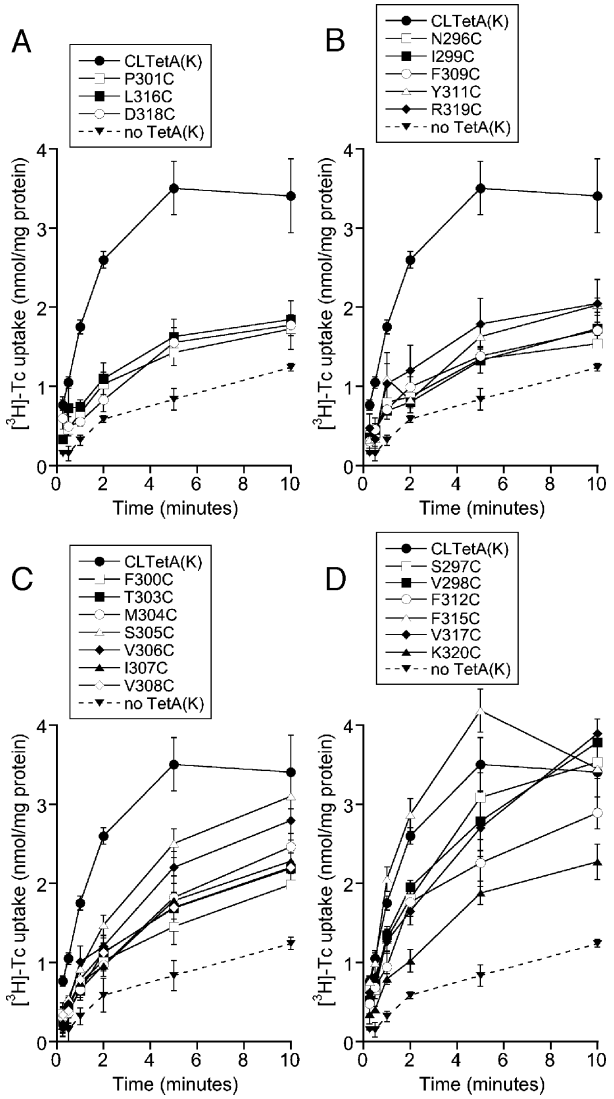

FIGURE 4: Tc transport mediated by CLTetA(K) mutants containing Cys substitutions for residues across the putative TMS 10 and immediately adjacent loops of TetA(K). Curves represent the average uptake of [^{3}H]-Tc from at least three repeat experiments using everted membrane vesicles of DH5α cells. TetA(K) mutants are grouped according to Tc resistance levels: those displaying an MIC of 2−4 μg/mL Tc (A), those displaying an MIC of 8−12 μg/mL Tc (B), those displaying an MIC of 16 μg/mL Tc (C), and those displaying an MIC of 20−32 μg/mL Tc (D).

With the exception of the glycine-substituted mutants described above, only three TetA(K) mutants were incapable of mediating Tc resistance above background (P301C and D318C) or basal (L316C) levels (Table 1). The Tc transport capacities of these mutants were also significantly reduced from that of CLTetA(K) (Figure 4A). Following the trend seen with the glycine-substituted mutants, the Tc transport capacities of the remaining Cys-scanning mutants were mostly reflective of their Tc resistance levels. Relatively high transport capacities of greater than 50% of CLTetA(K) were always observed for mutants conferring resistance to greater than 20 μg/mL Tc, whereas lower transport levels were recorded for the majority of other TetA(K) mutants (Figure 4).

Solvent Accessibility of Amino Acid Positions across TMS 10 of TetA(K). The level of reactivity of fluorescein maleimide with the single Cys-substituted TetA(K) TMS 10 mutants was tested to determine the solvent accessibility of

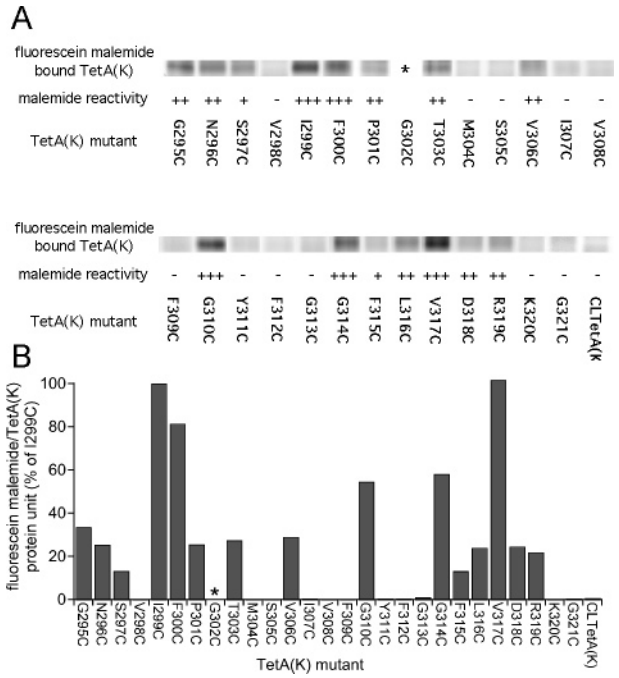

FIGURE 5: Fluorescein maleimide reactivity of CLTetA(K) mutants. (A) Membrane vesicles containing single Cys-substituted CLTetA-(K) mutant proteins were treated with fluorescein maleimide, and the fluorescence of purified TetA(K) proteins was visualized after separation on SDS−PAGE gels. The level of fluorescein maleimide reactivity per TetA(K) protein unit is shown: (−) below 5% of I299C, (+) 5−20% of I299C, (++) 20−50% of I299C, and (+++) above 50% of I299C. (B) The level of fluorescence label bound per protein unit was determined using densitometry and expressed as a percentage of the highly reactive I299C CLTetA(K) mutant. These experiments were conducted in at least duplicate, and the results of a representative sample are shown. (*) A reliable measure of fluorescein maleimide reactivity could not be obtained for the G302C TetA(K) mutant.

these amino acid positions in TetA(K). A pBAD-based system was employed to obtain TetA(K) protein expression at levels facilitating this analysis. However, expression of the G295C, P301C, and G302C mutant proteins in this system was reduced compared to CLTetA(K), a trend not seen in the pBluescript-based clones. For these mutants, fluorescein maleimide labeling experiments were conducted using increased amounts of starting material; however, a measure of reactivity with the G302C mutant could still not be reliably obtained.

With the exception of positions 306 and 310, amino acid positions from 304 to 313 within TetA(K) did not react with fluorescein maleimide and are likely to be buried within the membrane or adjacent helices, forming the core region of TMS 10 in TetA(K) (Figure 5). Cys residues from position 314 to position 319 were reactive with fluorescein maleimide to different degrees, whereas the K320C and G321C mutants were nonreactive (Figure 5). Of the mutants containing Cys substitutions at positions N-terminal to M304, only V298C was not reactive with fluorescein maleimide. Cys residues at the remaining amino acid positions, 295, 296, 297, 299, 300, 301, and 303, were reactive with fluorescein maleimide to differing degrees and therefore unlikely to be fully membrane embedded (Figure 5). To test for Tc protection of solvent-accessible residues within the membrane-spanning region, the fluorescein maleimide reactivity of the I299C, F300C, T303C, V306C, G310C, and G314C mutants was also determined in the presence of Tc. An approximately 2-fold increase in reactivity to fluorescein maleimide was observed for position 299, a 2-fold reduction for position 303, and a 4-fold reduction for position 306 in the presence of Tc (data not shown).

DISCUSSION

Hydropathy analyses and gene fusion studies have confirmed the presence of 14 TMS within TetA(K) and have defined potential boundaries of these TMS (*17*) (Figure 1). One goal of the current study was to experimentally determine the exact boundaries of TMS 10 in TetA(K). Solvent accessibility analysis using thiol-specific maleimide derivatives has been used to gather this type of information for TMS 10 in the QacA multidrug exporter (*19*) and for the entire polypeptide chain of the TetA(B) Tc transporter (*36*). The fluorescein maleimide reactivity of mutant TetA-(K) proteins containing single Cys residues at amino acid positions from 295 to 321 demonstrated that TMS 10 of TetA(K) contains a core region which extends from M304 to G313 and includes two partially solvent accessible residues, V306 and G310. TMS 10 of TetA(K) is likely to extend beyond these 10 amino acid residues, since they would be insufficient to completely span the lipid bilayer as the average length of an α-helix within a polytopic membrane protein is greater than 17 amino acids (*37*). Within the membrane-spanning region of a polytopic membrane protein at least one face of each TMS would be expected to face either the membrane or an adjacent TMS. Therefore, within a TMS the number of fluorescein maleimide-reactive amino acid positions in a single stretch should not exceed three or four, which are required for a full helical turn. A stretch of six amino acid residues was seen to react with fluorescein maleimide in the region C-terminal to G313. Therefore, these six reactive amino acid positions, from 314 to 319 inclusive, are unlikely to be membrane embedded, leading to the assignment of G313 as the C-terminal boundary of TMS 10 in TetA(K) and possibly including G314 if this residue faces a solvent-filled channel (Figure 6). The non-fluorescein maleimide-reactive positions of K320 and G321 may constitute part of a re-entrant loop or could form the N-terminal region of TMS 11. It is difficult to assign a definitive N-terminal boundary for TMS 10 of TetA(K) due to the number of fluorescein maleimide-reactive positions N-terminal to M304. The only nonreactive position in this region was V298, which could form the N-terminal boundary, or TMS 10 of TetA(K) may extend beyond the region tested in this study to possibly include I294, as previously suggested by alternative hydropathy analyses of TetA(K) (*16*). Similar difficulties in defining TMS limits were observed for corresponding TMS in other DHA family transporters: TMS 10 of the QacA multidrug transporter (*19*) and TMS 8 of TetA(B) (*36*).

Several amino acid positions within TMS 10 of TetA(K) were reactive with fluorescein maleimide, demonstrating that this helix is amphiphilic. Interestingly, these solvent-accessible positions extended along the entire length of TMS 10. Exhaustive solvent accessibility analyses of the TetA(B) transporter demonstrated that only 4 of the 12 TMS similarly contained maleimide-reactive amino acid positions along their full length (*36*). The reactive amino acid positions in these helices, TMS 2, 5, 8, and 11, were typically clustered

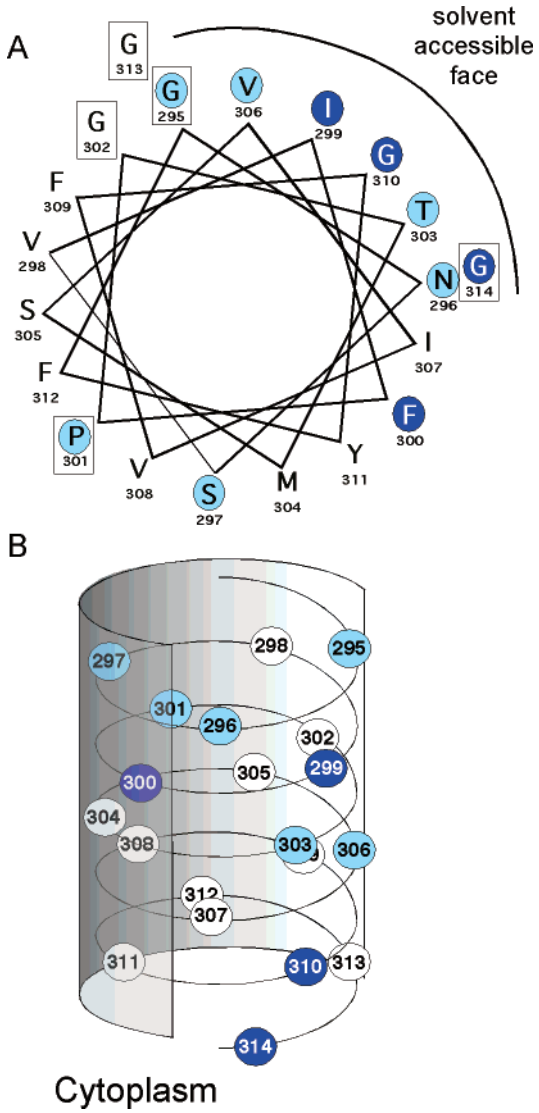

FIGURE 6: Pattern of fluorescein maleimide reactivity across TMS 10 of TetA(K). (A) Helical wheel projection of TetA(K) TMS 10. Each amino acid within the helical wheel is offset by 100° from the one preceding it. Amino acid positions found to be highly reactive (above 50% of I299C levels) or partially reactive (5−50% of I299C levels) with fluorescein maleimide are indicated by light or dark blue ovals, respectively. A semicircle indicates the face of TMS 10 that is at least partially solvent accessible from either the periplasmic or cytoplasmic ends. Amino acids found to be functionally important for Tc transport and resistance are boxed. (B) Schematic representation of TMS 10 in TetA(K) showing the relative orientation of solvent-exposed and buried amino acid positions. White shading indicates membrane-buried amino acid residues (below 5% of I299C fluorescein maleimide reactivity), light blue shading indicates partially solvent accessible residues (5−50% of I299C fluorescein maleimide reactivity), and dark blue shading indicates solvent-accessible residues (above 50% of I299C fluorescein maleimide reactivity).

on one helical face, leading to the suggestion that they line a water-filled channel, acting as the Tc translocation pathway (*36*, *38*−*40*). The reactive amino acid positions in TMS 10 of TetA(K) are also localized primarily to one helical face, with the highly reactive 299 and 310 positions juxtaposed on a helical wheel projection but separated by three helical turns (Figure 6). Therefore, TMS 10 of TetA(K) may also line part of a water-filled Tc translocation region, which could pass through the center of the TetA(K) protein. The addition of Tc caused approximately 2- and 4-fold reductions to the level of fluorescein maleimide reactivity seen at amino acid positions 303 and 306, respectively. While these positions were not highly reactive to fluorescein maleimide in the absence of Tc, these changes could be indicative of substrate protection. Since these mutants catalyzed significant levels of Tc transport, they are unlikely to be directly involved in Tc binding. However, the Tc binding site may be located on adjacent helices, also lining the solvent-accessible channel. The approximate 2-fold increase in fluorescein maleimide reactivity observed for amino acid position 299 in the presence of Tc may be due to gross conformational changes of the TetA(K) transporter which occur upon substrate binding.

A substrate translocation pathway lined by TMS 10 could be a general feature of all 14-TMS MFS transporters. The observation of amino acid residues involved in substrate recognition on TMS 10 of the QacA multidrug transporter supports this proposition, but the pattern of solvent accessibility across TMS 10 in this transporter is not consistent with it lining a solvent-accessible channel (*19*). Given the hydrophobic nature of QacA substrates and the fact that these substrates can be recruited from the inner leaflet of the lipid bilayer, it is possible that unlike Tc transporters the substrate translocation region within QacA is primarily lipid-filled (*18*, *41*). Future comparative functional and structural studies of the QacA and TetA(K) drug exporters will help to resolve this issue.

Mutagenic analysis across TMS 10 of TetA(K) examined the functional contribution of 27 amino acid residues for TetA(K)-mediated Tc transport. Both MIC and Tc transport studies revealed that the majority of targeted residues played at least a small role in TetA(K)-mediated function, suggesting an overall importance of the TMS 10 region. However, several amino acid residues were identified as being essential for Tc transport, including a predominance of amino acids known for their unique structural properties in α-helices, specifically several glycines and a proline residue (Table 1, Figures 3 and 4). One charged residue, D318, was also found to be essential for TetA(K) function, confirming the results of previous mutagenesis studies (*42*).

Glycine and proline residues can exert a strong influence on protein structure, particularly within α-helices. Due to their lack of a bulky side group, glycine residues allow helical flexibility and close helix−helix interactions (*22*, *23*). Proline residues lack an amide proton for regular α-helical H-bonding and may also add to helical flexibility or assist in the formation of interhelical contacts (*43*−*45*). The G295C, P301C, and G302C TetA(K) mutant proteins were poorly expressed in the pBAD-based expression system relative to CLTetA(K) and also failed to confer TetA(K)-mediated resistance or transport. The glycine residues at G295 and G302 of TetA(K) are well conserved within related DHA family transporters and are equivalent to G247 and G254 in the TetA(B) transporter (*46*). Mutational and second-site suppressor studies have shown these two glycines to be essential for TetA(B) function and have implicated these residues in helical interactions with TMS 5 (*20*, *36*, *47*). In light of their conservation, G295 and G302 of TetA(K) might possess a similar functional role to G247 and G254 of TetA(B) and also participate in close interhelical interactions with distal parts of the TetA(K) transporter. This possibility is supported by the observation that G295 and G302 of TetA-

(K) form a GxxxxxxG motif, which is highly represented in the TMS of transporters where it is likely to mediate close interhelical contacts (*48*). Additionally, alanine substitutions, which are less likely to impose steric disturbances than Cys substitutions, are better accommodated at the G295 and G302 positions for the maintenance of Tc transport and resistance. The PG dipeptide formed by P301 and G302 of TetA(K) may also be integral to the formation of structures required for interhelical interactions. This PG dipeptide has been suggested to be involved in different conformational states of the TetA(L) transporter, which shares a high level of sequence identity with TetA(K) (*49*). Therefore, the P301/G302 dipeptide, along with G295 in TetA(K), may facilitate changes in conformation required for Tc transport by mediating interactions between distant parts of TetA(K). In addition to being nonfunctional, TetA(K) variants containing nonconservative mutations of these amino acid residues appear to be less stable and inappropriate for high-level overexpression.

The G313A/C, G314A/C, and G321A TetA(K) substitutions were not tolerated for the maintenance of TetA(K)-mediated function, and only basal levels of Tc resistance and transport were retained by the G321C derivative. G314 and G321 are conserved within all TetA transporters, and G313 and G310 are conserved within the 14-TMS TetA(K) and TetA(L) proteins (*46*). The G310, G313, and G314 glycines are likely to be within or on the boundary of TMS 10 in TetA(K). Interestingly, these residues are found on the same face of TMS 10 as G295 and G302 and are therefore positioned appropriately for interactions with regions of TetA(K) in proximity to G295 and G302 (Figure 6). The helical localization of these glycine residues could also produce a region of heightened flexibility along one side of TMS 10, important for the docking or movement of Tc. Alternatively, the G313/G314 dipeptide may be required for the termination of TMS 10 in TetA(K). G321 of TetA(K) is not likely to reside within TMS 10 but within either a reentrant loop region or the adjacent helix. Speculation as to the functional significance of this residue awaits future investigation.

CONCLUSION

This study examined the solvent accessibility and the functional contributions of a series of amino acid residues across TMS 10 of the *S. aureus* Tc transporter TetA(K). Fluorescein maleimide reactivity experiments demonstrated that this TMS is likely to have a cytoplasmic boundary of G313, extending from V298 or N-terminal to G295 on the periplasmic side. The amino acids identified as being of high functional significance on TMS 10 of TetA(K) included a predominance of glycine residues. Several of these glycine residues are conserved within related 12-TMS transporters where they are functionally essential, possibly in mediating N- and C-terminal domain interactions. The conservation of these residues allows speculation that they mediate similar long-distance conformational interactions within TetA(K) in accordance with the hypothesis that a similar tertiary structure and mechanism of transport operate within 12-TMS and 14-TMS MFS transporters.

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