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Membrane Topology of the Metal-Tetracycline/H^+ Antiporter TetA(K) from *Staphylococcus aureus*

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A series of fusions to the reporter proteins alkaline phosphatase and β-galactosidase have been constructed in the predicted periplasmic and cytoplasmic loops of TetA(K), a protein responsible for efflux-mediated tetracycline resistance in *Staphylococcus aureus*. The results support a topological model of 14 transmembrane segments for TetA(K).

In *Staphylococcus aureus*, resistance to tetracycline is commonly mediated by *tetA*(K), which encodes the 50.7-kDa TetA class K membrane protein, TetA(K) (8). TetA(K) has been shown to function as a metal-tetracycline/H^+ antiporter (27) in a manner analogous to that of the TetA tetracycline efflux proteins of classes A to H from gram-negative bacteria (14). These TetA proteins belong to the major facilitator superfamily, which is comprised of a large number of prokaryotic and eukaryotic uniporters, symporters, and antiporters, all of which either have been shown or are predicted to utilize energy from the proton motive force for transport function (7, 16). Members of this superfamily can be identified by characteristic sequence motifs, which are more commonly located throughout the first six transmembrane segments (TMS) than the C-terminal halves of these proteins (7, 16, 20, 21, 22).

The major facilitator superfamily was originally divided into five groups based on sequence homology (16). However, it was later proposed that the first group of proteins, comprised of drug exporters, be subdivided further into 12- and 14-TMS families based on their predicted membrane topologies (7, 20, 21). The gram-negative tetracycline resistance proteins TetA(B) and TetA(C) have been shown experimentally to contain 12 TMS (1, 4), whereas the gram-positive proteins TetA(K) and the structurally related TetA(L) (10) are predicted to contain 14 TMS based on hydropathy analysis (8, 21). To date, the multidrug resistance efflux protein QacA from *S. aureus* (22) is the only member of the 14-TMS family for which the membrane topology has been demonstrated experimentally (19). Therefore, it was the aim of this study to clarify the topology of another export protein from the 14-TMS family, namely, TetA(K), by using fusions with alkaline phosphatase and β-galactosidase as indicators of subcellular localization (17, 24).

Isolation of *tetA*(K). The tetracycline resistance determinant *tetA*(K) was isolated from the naturally occurring *S. aureus* plasmid pT181 (11) by standard PCR techniques. In designing the PCR primers, the native start codon and ribosomal binding site were changed from TTG to ATG and GAGG to GGAGG, respectively; *tetA*(K) was subsequently cloned into the vector pBluescript II SK (Stratagene). The sequence of the cloned *tetA*(K) was compared to that reported by Guay et al. (8) to ensure that no errors were introduced during the amplification or cloning procedures.

Construction of TetA(K) fusions to alkaline phosphatase. Fusions to the reporter protein alkaline phosphatase (PhoA) were constructed in two ways. Firstly, selected regions of *tetA*(K) were amplified by PCR and cloned into the promoterless *phoA*-containing vector pSK4158 (18). Fusions created in this way encode hybrid proteins comprised of TetA(K) proteins with various N-terminal lengths fused in frame with PhoA. Secondly, via site-directed mutagenesis (12), *Pst*I recognition sequences were introduced into *tetA*(K) into which a *phoA* gene cartridge was inserted. The *phoA* cartridge was amplified from the template pPHO7 (9) and engineered to contain compatible *Pst*I termini. The method of construction and predicted cellular location of fusions are listed in Table 1; fusion junctions were confirmed by DNA sequencing and are represented on the predicted 14-TMS topological model for the TetA(K) polypeptide (Fig. 1) by lines joined to arrowheads.

Activity and stability of TetA(K)-PhoA hybrid proteins. Since PhoA is required to be exported from the cytoplasm for high enzymatic activity, the level of PhoA activity of a hybrid protein is an indicator of its subcellular location (17). The PhoA activities of cells harboring TetA(K)-PhoA hybrids were highest when PhoA was fused to the predicted periplasmic regions of the TetA(K) polypeptide, viz., P1, P2, P4, P5, P6, and P7, or to amino acids located within TMS, e.g., pSK4547 (TMS 6), pSK4532 (TMS 7), and pSK4698 (TMS 8) (Table 1; Fig. 1). Low activity was observed with PhoA fusions to the C terminus (pSK4645) and cytoplasmic loops (C1 to C6) and also to the third periplasmic loop, P3 (pSK4629 and pSK4640).

To identify hybrid proteins encoded by *tetA*(K)-*phoA* fusions, whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunostained with rabbit anti-PhoA immunoglobulin G (5 Prime → 3 Prime), as described previously (15). Products conforming to the calculated size of hybrid proteins or breakdown products, some equivalent in size to the PhoA moiety (47 kDa) (15), were identifiable in all cases with the exception of cells harboring the plasmids pSK4629 and pSK4640. Figure 2 shows an immunostained Western blot of extracts from selected fusion-carrying cells. The absence of detectable hybrid-PhoA proteins in cells carrying the plasmids pSK4629 and pSK4640 (Fig. 2, lanes 4 and 5, respectively) provides an explanation for the low PhoA activity observed with these plasmids (Table 1; Fig. 1). This may well reflect their instability rather than their cellular location. In these fusions to periplasmic loop P3, C-terminal TetA(K) sequences may additionally be required for the correct insertion of TMS 5 into the cytoplasmic membrane; a similar suggestion has been

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TABLE 1. Activities of *tetA*(K)-*phoA* and *tetA*(K)-*lacZ*α fusions

Plasmid	Method of construction[a]	Fusion junction[b]	PhoA activity[c]	LacZα complementation[d]
tetA(K)-*phoA* fusions				
None			0.3	
pSK4628	PCR	P2 (104)	159.7	NA[e]
pSK4629	PCR	P3 (164)	6.2	NA
pSK4547	PCR	TMS 6 (178)	163.4	NA
pSK4532	PCR	TMS 7 (212)	191.0	NA
pSK4533	PCR	P4 (217)	170.8	NA
pSK4698	PCR	TMS 8 (229)	107.1	NA
pSK4631	PCR	P5 (289)	198.0	NA
pSK4632	PCR	P5 (297)	172.1	NA
pSK4633	PCR	P6 (347)	136.9	NA
pSK4634	PCR	P7 (418)	69.8	NA
pSK4635	PCR	C1 (73)	13.7	NA
pSK4636	PCR	C4 (254)	0.3	NA
pSK4637	PCR	C5 (320)	14.4	NA
pSK4639	*phoA* cartridge	P1 (50)	86.3	NA
pSK4640	*phoA* cartridge	P3 (167)	0.0	NA
pSK4642	*phoA* cartridge	C2 (137)	0.7	NA
pSK4643	*phoA* cartridge	C3 (196)	0.4	NA
pSK4644	*phoA* cartridge	C6 (386)	0.6	NA
pSK4645	*phoA* cartridge	C terminus (459)	0.0	NA
tetA(K)-*lacZ*α fusions				
pSK4656	PCR	P2 (104)	NA	−
pSK4657	PCR	P3 (164)	NA	−
pSK4699	PCR	TMS 8 (229)	NA	−
pSK4659	PCR	P5 (289)	NA	−
pSK4660	PCR	P5 (297)	NA	−
pSK4661	PCR	P6 (347)	NA	−
pSK4662	PCR	P7 (418)	NA	−
pSK4663	PCR	C1 (73)	NA	+
pSK4548	PCR	C3 (196)	NA	+
pSK4664	PCR	C4 (254)	NA	+
pSK4665	PCR	C5 (320)	NA	+
pSK4666	PCR	C terminus (459)	NA	+

[a] See text for details.
[b] The predicted cellular location of fusion junctions in the TetA(K) polypeptide (Fig. 1) are indicated: P (periplasmic), C (cytoplasmic). The amino acid position of each fusion in the TetA(K) polypeptide, as determined by nucleotide sequencing, is shown in parentheses.
[c] Plasmids encoding *tetA*(K)-*phoA* fusions were examined in the *E. coli* host CC118 (*phoA*Δ20). PhoA activities, expressed as hydrolysis of micromoles of *p*-nitrophenylphosphate per minute per milligram of total cellular protein, determined as previously described (2), are the average of three independent enzymatic assays.
[d] *E. coli* DH5α (*lacZ*ΔM15) cells carrying *tetA*(K)-*lacZ*α fusions were scored for the ability (+) to cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside on Luria-Bertani agar plates as previously described (19, 23).
[e] NA, not applicable.

made for TMS 1, TMS 3, TMS 9, and TMS 10 of the TetA(C) protein (1). Indeed, the high PhoA activity observed when PhoA was fused to the predicted TMS 6 region of the TetA(K) polypeptide (cells harboring the plasmid pSK4547) offers support to this proposition.

Construction of TetA(K) fusions to β-galactosidase. Specific PCR fragments of *tetA*(K) were cloned into the *lacZ*α-containing vector pUC119 (25) with the exception of the fragment contained within the plasmid pSK4548 which was cloned into pUC8. The positions of fusion junctions are listed in Table 1 and represented on the 14-TMS topological model of TetA(K) (Fig. 1) by lines joined to rectangles. Characteristically, β-galactosidase shows high enzymatic activity when located in the cytoplasm of *Escherichia coli* cells (24). High activity was observed with cells carrying pSK4663, pSK4548, pSK4664, pSK4665, and pSK4666 in which fusions were predicted to be cytoplasmically located (Table 1; Fig. 1). Conversely, β-galactosidase activity is low when localized to the periplasm, and such activity was found with all fusions to predicted periplasmic loops. This low β-galactosidase activity was also seen with cells harboring the plasmid pSK4657. This finding, taken with the high PhoA activity of cells carrying the plasmid pSK4547, supports the conclusion that the predicted hydrophilic loop P3 of TetA(K) is located in the periplasm.

It should be noted that tetracycline resistance was only observed in cells carrying pSK4645 or pSK4666, which encode the full-length TetA(K)-PhoA or TetA(K)-LacZα hybrid protein, respectively. This suggests that all of the predicted 14 TMS of TetA(K) are required for transport function.

Topology of TetA(K). Although TetA(K) had previously been predicted to possess 14 TMS (8, 21), it has recently been proposed by Fujihira et al. (6) to contain only 12 TMS, with the α-helices of TMS 7 and TMS 8 (as shown in the 14-TMS model in Fig. 1) located in the cytoplasm. Our hydropathy analyses, performed by using the program TopPred II (3) and various hydropathy scales (5, 13, 26), all predicted a 14-TMS model for TetA(K), which was confirmed by the results obtained with fusions to the reporter proteins PhoA and LacZα (Table 1 and Fig. 1). In particular, the high PhoA activities observed with cells carrying the fusion plasmids pSK4532, pSK4533, and

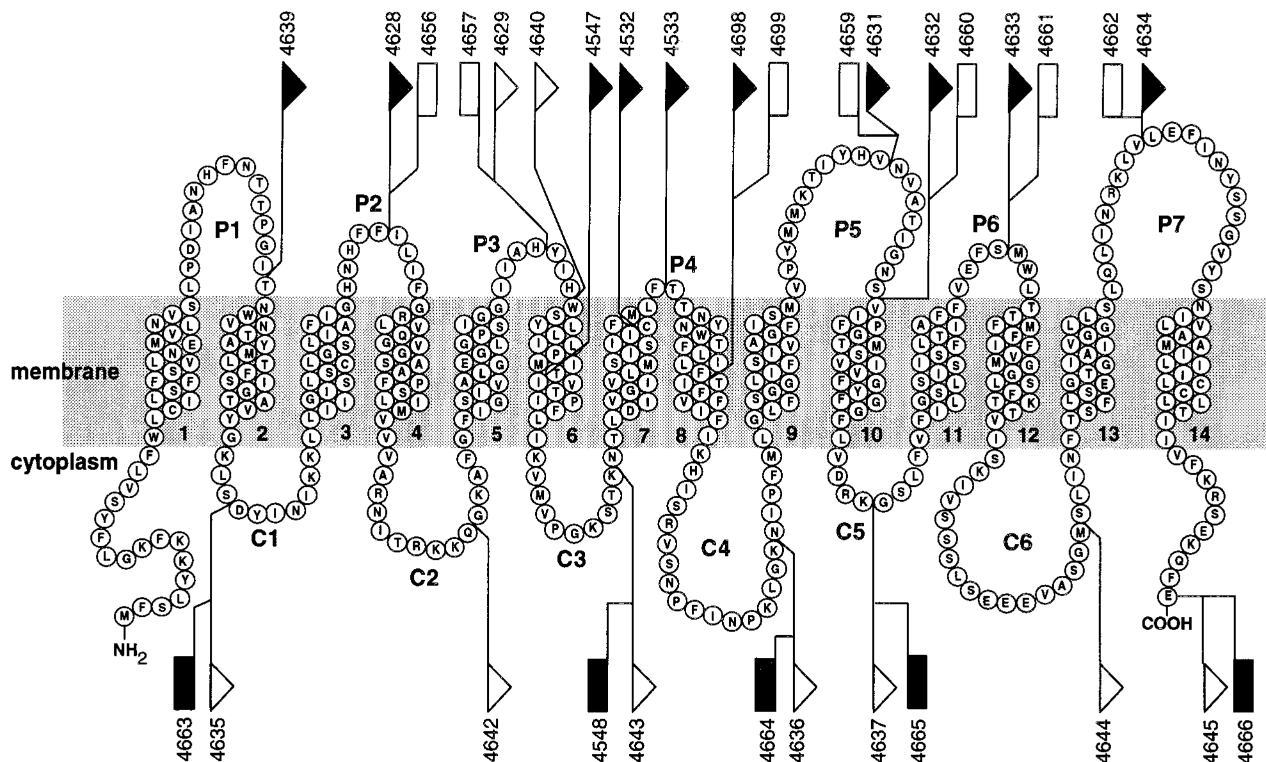

FIG. 1. Proposed 14-TMS topology of the TetA(K) polypeptide in the cytoplasmic membrane, as determined by hydropathy analysis using the program TopPred II (3). Fusion sites are represented by lines joined to arrowheads [*tetA*(K)-*phoA* fusions] or rectangular boxes [*tetA*(K)-*lacZ*α fusions], labelled with the pSK plasmid number as shown in Table 1. High and low enzymatic activities are represented, respectively, by filled or open arrowheads and rectangular boxes. The amino (NH_2) and carboxy (COOH) termini of the TetA(K) polypeptide are indicated, with the proposed TMS (TMS 1 to 14), periplasmic loops (P1 to P7) and cytoplasmic loops (C1 to C6) also shown.

pSK4698 (191.0, 170.8, and 107.1 U, respectively [Table 1; Fig. 1]) fully support the positioning of TMS 7 and TMS 8 in the membrane, albeit separated by the short periplasmic loop P4. Finally, the amino acid sequence conservation between TetA(K) and other members of the 14-TMS family of export proteins, particularly across motif E of TMS 7 and motif F of TMS 13 (7, 20, 21), both of which are specific for 14-TMS family members and absent from the 12-TMS family, adds further to the strong evidence for a 14-TMS topological model for the metal-tetracycline/H^+ antiporter TetA(K).

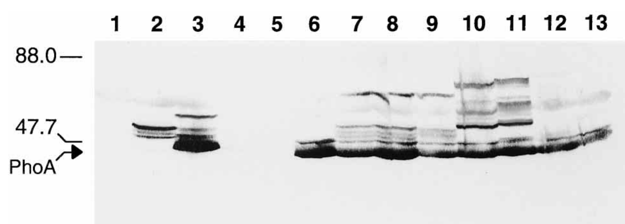

FIG. 2. Western hybridization analysis, using anti-PhoA immunoglobulin G, of the products produced by cells expressing *tetA*(K)-*phoA* fusions, examined in the *E. coli* host CC118. Extracts from cells harboring the indicated plasmids, with the cellular location of the fusion as described in Fig. 1 shown in parentheses, are contained in lanes as follows: 1, no plasmid; 2, pSK4639 (P1); 3, pSK4628 (P2); 4, pSK4629 (P3); 5, pSK4640 (P3); 6, pSK4547 (TMS 6); 7, pSK4532 (TMS 7); 8, pSK4533 (P4); 9, pSK4698 (TMS 8); 10, pSK4631 (P5); 11, pSK4632 (P5); 12, pSK4633 (P6); and 13, pSK4634 (P7). The positions of migration and sizes (in kilodaltons) of coelectrophoresed standard proteins are shown on the left. Breakdown products corresponding in size to the alkaline phosphatase moiety, PhoA (47 kDa), are indicated by the arrow on the left.

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