Transmembrane glutamic acid residues play essential roles in the metal-tetracycline/H^+ antiporter of *Staphylococcus aureus*

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Abstract Three transmembrane aspartyl residues play essential roles in the transposon Tn10-encoded metal-tetracycline/H^+ antiporter (Tet(B)) [Yamaguchi, A. et al. (1992) J. Biol. Chem. 267, 7490–7498]. The *tet*K gene-encoding tetracycline resistance protein (Tet(K)) of *Staphylococcus aureus* mediates metal-tetracycline/H^+ antiport similarly to Tet(B); however, it has no transmembrane aspartyl residue. On the other hand, Tet(K) has three glutamyl residues, Glu-30, Glu-152 and Glu-397, in the putative transmembrane regions. In the present work, *tet*(K) gene was expressed in *Escherichia coli* and the transport activity was measured in everted membrane vesicles. When these glutamyl residues were replaced with Gln, the tetracycline transport activity was almost completely lost, indicating the important roles of these residues in Tet(K). In the case of Glu-397, even the charge-conserved mutation to Asp caused complete loss of the activity. On the other hand, the mutation of Glu-30 and Glu-152 to Asp resulted in significant retention of transport activity. These results are similar to those on the mutation of the three transmembrane aspartyl residues in Tet(B), indicating that the transmembrane glutamyl residues in Tet(K) play roles similar to those of the transmembrane aspartyl residues in Tet(B).

Key words: Tetracycline; Tetracycline/H^+ antiporter; Tet K; *Staphylococcus aureus*

1. Introduction

The *tet*(K) gene is responsible for tetracycline resistance of *Staphylococcus aureus* [2,3] as well as the *tet*(M) gene which encodes the ribosomal protection system [4,5]. The Tet(K) protein is closely related to the Tet(L) protein found among Gram-positive pathogens [6–8]. They confer resistance by the active efflux of tetracycline. The substrate transported by Tet(K) is a tetracycline-divalent-cation chelation complex [9]. The drug efflux is driven by an antiport with protons. These mechanisms are very similar to those of the Tet proteins of Gram-negative enteric bacteria [10]. In Gram-negative bacteria, tetracycline resistance is mostly assigned to the drug efflux systems, which are classified into A–H [11]. They have a conserved sequence motif, G(K/R)XSD(R/K)XGR(R/K), in the first cytoplasmic loop (named loop2–3), which is common not only in bacterial drug export proteins [12,13] but also in the secondary transporters and belongs to a major facilitator family [14,15]. Tet(K) and Tet(L) also contain a derivative of this motif, GKXSDXX(X/G)XK(K/R), in the corresponding loop, indicating that they belong to a major facilitator family.

In spite of such similarity in the function and sequence motif between Tet proteins of Gram-positive and Gram-negative microorganisms, there are large apparent differences in their molecular features. Tet(B) and Tet(C), which are well-studied Tet proteins in Gram-negative bacteria [16], are integral membrane proteins of about 43 kDa which consist of 12 membrane-spanning segments with a large central loop [17,18]. On the other hand, the molecular sizes of Tet(K) and Tet(L) are greater than those of Tet(B) and Tet(C). On the basis of hydropathy analysis and the odd-number-spanning rule of membrane transporters, Guay et al. [19] predicted that Tet(K) contains 14 transmembrane helices. However, there is no distinct hydrophilic region between hydrophobic segments 7 and 8. Thus, an alternative topology is possible, with Tet(K) consisting of 12-transmembrane segments with a large central loop (Fig. 1). The long hydrophobic part of the central loop may form α-helices partially embedded in the membrane locus but not passing through the membrane (Fig. 1).

The most striking difference between the Tet proteins of Gram-positive and Gram-negative bacteria is the absence of aspartic acid in the putative transmembrane region of Tet(K) and Tet(L) (Fig. 1). In the Tet proteins of enteric bacteria, the three aspartic acid residues are conserved in their membrane-spanning regions and play essential roles for tetracycline binding [20] and translocation across the membrane [1], probably through ionic interaction with the positively charged metal-tetracycline chelation complex. These three aspartic acid residues are located in the putative transmembrane helices 1, 3, and 9 [1]. On the other hand, three glutamic acid residues are conserved in the transmembrane regions of Tet(K) and Tet(L) (Fig. 1). These three glutamic acid residues in Tet (K) are Glu-30, Glu-152 and Glu-397, which are located in transmembrane segments I, V and XI, respectively (Fig. 1). In this study, we constructed site-directed mutants in which these three glutamic acid residues were replaced in turn with a glutamine or aspartic acid residue. The results indicated that the glutamic acid residues in Tet(K) play similar roles to the essential aspartic acid residues in Tet(B).

2. Materials and methods

2.1. Materials

[7-^{3}H(N)]Tetracycline was purchased from Du Pont-New England Nuclear. All other chemicals were of reagent grade and obtained from commercial sorces.

2.2. Bacterial strains and plasmids

E. coli W3104 [21] was used as the host strain expressing the *tet*(K) gene and for the preparation of inverted membrane vesicles. pTZ1252, which was a gift from N. Noguchi, Tokyo College of Pharmacy, was constructed by insertion of a 2.3 kb *Hin*dIII fragment of pNS1, which

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carries the *tet*(K) gene of *S. aureus* [3], into pUC119, in which the initiation codon and the ribosome binding sequence (RBS) were changed from TTG to ATG and from GAGG to GGAGG, respectively, and the distance between the RBS and the initiation codon was altered from 4 to 11 bases [22]. pUC118RV [1] was constructed from pUC118 [23] by changing the *Sac*I site to an *Eco*RV site. pTKN1 and pTKC2 are the plasmids constructed by subcloning of the 5'- and 3'-halves of the *tet*(K) gene, respectively, into pUC118 and pUC118RV, respectively. pTKN1 was constructed by insertion of the *Eco*RI-*Kpn*I fragment of pTZ1252 into the corresponding cloning site of pUC118. pTKC2 was constructed by insertion of the *Hin*dIII-*Eco*RV fragment of pTZ1252 into the corresponding cloning site of pUC118RV.

2.3. Site-directed mutagenesis

Mutagenesis at codons 30 and 152, and at codon 397 in the *tet*(K) gene was performed using pTKN1 and pTKC2, respectively, as a template. The method employed for mutagenesis was mainly that of Kunkel [24], however, for mutagenesis at codon 152, we employed the procedure of Taylor et al. [25]. The mutagenic primers used were as follows: E30Q, 5'-ATTAAATCAAATGGTCCTAAATG-3'; E30D, 5'-TTAAATGACATGGTCCTAAATG-3'; E152Q, 5'-CTTTAGGCCAAGGGTT-3'; E152D, 5'-TTAGGTGACGGCCTAGGTCCTTC-3'; E397Q, 5'-TTTATCCCAAGGAACA-3'; E397D, 5'-TTATCAGACGGTACCGGTATAGC-3'. In the above oligonucleotide sequences, the underlined letters indicate the mismatched bases which generate the replacement of amino acid residues or the new restriction sites without amino acid change. The mutation was first detected by restriction analysis and then confirmed by DNA sequencing. The mutant *tet*(K) gene was reconstructed from the mutant pTKN1 or pTKC2 by the corresponding fragment exchange with pTZ1252.

2.4. Preparation of inverted membrane vesicles and transport assay

Inverted membrane vesicles were prepared from *E. coli* W3104 harboring pTZ1252 or the mutant plasmid as described in our previous paper [9]. Respiration-driven [^{3}H]tetracycline uptake by inverted membrane vesicles were measured in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ as described in [9].

3. Results and discussion

3.1. Distribution of conserved acidic residues and putative topology of the Tet(K) protein

There are 9 acidic residues conserved throughout the sequences of Tet(K) and Tet(L) proteins isolated from six different bacterial species [26]. The distribution of these acidic residues is shown in Fig. 1. Among them, Asp-200 is conserved except for Tet(L) encoded by pSTE1, in which Asp is replaced by Gly [26]. The Tet(L) protein encoded by a chromosome of *B. subtilis* has Asp-345 in place of Glu-345 [26]. The other 7 acidic residues are completely conserved in all of the Tet(K) and Tet(L) proteins [26]. The conserved aspartic acid residues are Asp-39, Asp-74, Asp-200, and Asp-318. Out of these aspartyl residues, Asp-74 and Asp-318 are located in the first and second conserved sequence motifs, respectively, common to the major facilitator family [27], thus, these two aspartic acid residues are likely to be located in the cytoplasmic surface. Asp-39 and Asp-200 are located in the middle or the edge of the large distinct hydrophilic domains; thus, it is not likely that these aspartyl residues are embedded in the membrane. Therefore, there is no candidate for the transmembrane aspartyl residues in the putative topology of Tet(K) in either the 14- or 12-transmembrane structure.

There are 5 conserved glutamic acid residues, Glu-30, Glu-152, Glu-345, Glu-381, and Glu-397. Out of these residues, only Glu-381 is located in the middle of the large hydrophilic

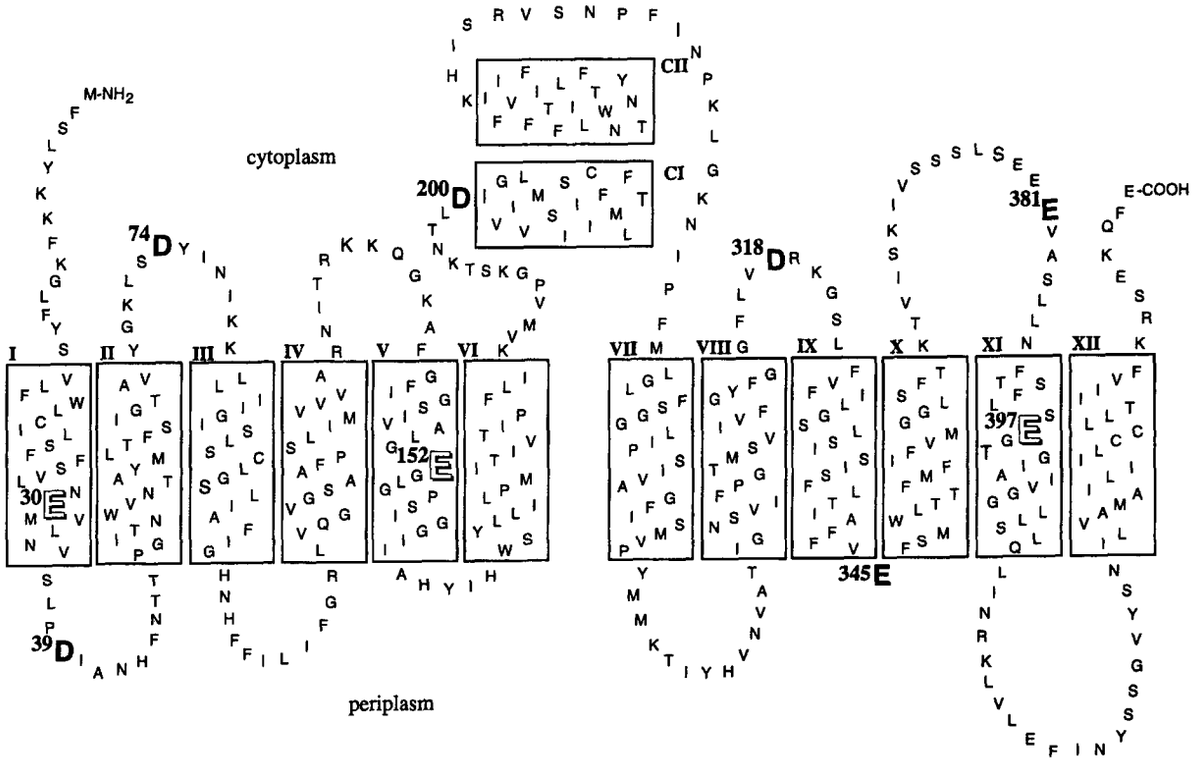

Fig. 1. Putative 12-transmembrane domain structure of the Tet(K) protein. The hydrophobic segments are enclosed in boxes. Roman numerals indicate the putative transmembrane segments. CI and CII are the predicted central hydrophobic segments not spanning the membrane but probably being folded in the protein interior or buried in the membrane. The conserved acidic residues in the transmembrane regions and the hydrophilic loop regions are indicated by shadowed and bold letters, respectively.

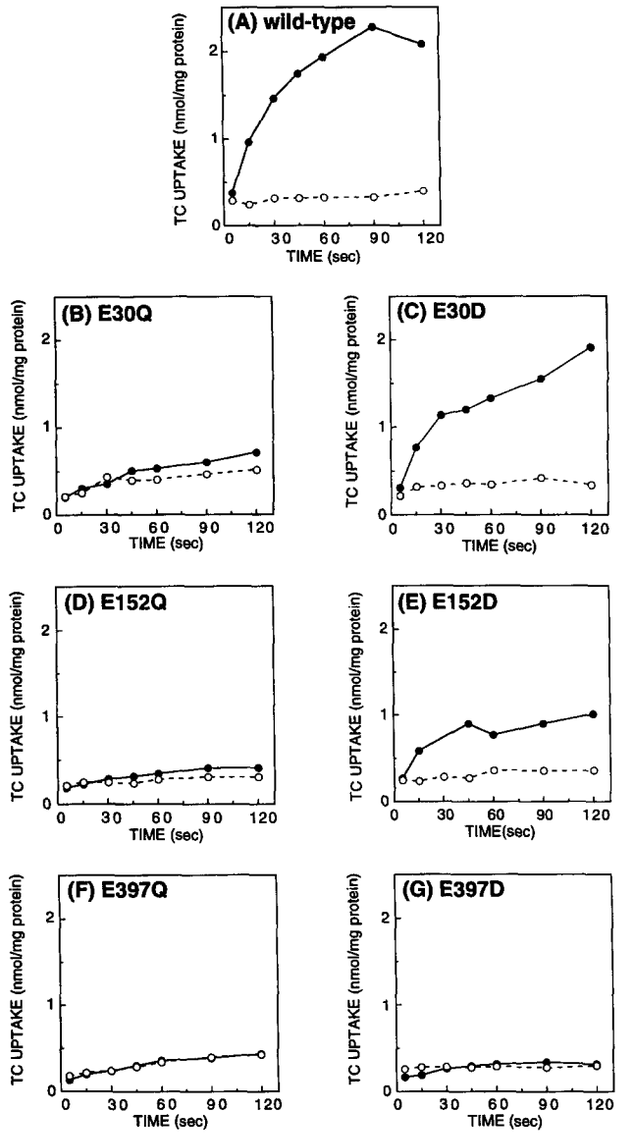

Fig. 2. Tetracycline uptake by everted membrane vesicles prepared from *E. coli* W3104 cells harboring pTZ1252 or a mutant plasmid: (A) pTZ1252, (B) pTZE30Q, (C)pTZE30D, (D) pTZE152Q, (E) pTZE152D, (F) pTZE397Q, (G) pTZE397D. Closed and open circles indicate the uptake by the vesicles in the presence or absence of NADH as an energy source, respectively.

loop, indicating localization in the water-extruding loop. The other four glutamic acid residues are located in the middle of the hydrophobic domains. Glu-30, Glu-152, and Glu-397 are located in the hydrophobic segments composed of about 20 amino acid residues sufficient to span once across the membrane, whereas Glu-345 is located at the center of a large hydrophobic segment containing about 43 amino acid residues enough to span twice across the membrane. In the cases of Tet(B) and Tet(C), evidence has been obtained that the 3-membered short loop containing a glutamic acid residue between the transmembrane segments IX and X is located on the periplasmic surface ([18]; our unpublished observation). Glu-345 in Tet(K) is exactly located in the loop between the transmembrane segments IX and X in the 12-membrane-spanning structure (Fig. 1). Thus, it is likely that Glu-345 is a member of the periplasmic short loop. Therefore, the acidic residues clearly located in the transmembrane segments of Tet(K) are Glu-30, Glu-152 and Glu-397.

3.2. *Effect of mutations in membrane-embedded glutamic acid residues*

Firstly, the three membrane-embedded glutamic acid residues were replaced one by one with a glutamine residue. As shown in Fig. 2, all the mutants showed a large decrease in tetracycline transport activity. Among them, the Glu-397'Gln (E397Q) mutant completely lost activity (Fig. 2F). The Glu-30'Gln (E30Q) and Glu-152'Gln (E152Q) mutants retained less than 5 and 1%, respectively, of the wild-type transport activity (Fig. 2B,D). Therefore, it seems that Glu-30 and Glu-152 are very important and that Glu-397 is essential for the tetracycline transport function of Tet(K).

These glutamic acid residues were then replaced by a charge-conserved aspartyl residue. When Glu-30 and Glu-152 were replaced by Asp, the resulting E30D and E152D mutants retained about two-thirds and one-third of the wild-type activity, respectively (Fig. 2C,E), indicating that the aspartyl side chain works more or less well in place of a glutamyl side chain at these positions. In contrast, when Glu-397 was replaced by Asp, the transport activity was completely lost (Fig. 2G), as with the D397Q mutant. Thus, the strict configuration of a carboxyl group at position 397 is necessary for the transport function.

In comparison with the site-directed mutants of the transmembrane aspartyl residues in Tet(B) [1], the results of the mutagenesis of Glu-30 and Glu-152 were similar to those of Asp-15 and Asp-84 in Tet(B). At these positions, a negatively charged side-chain is necessary and the aspartyl and glutamyl side chains are interchangeable with each other. The result of the mutagenesis of Glu-397 in Tet(K) corresponds to that of Asp-285 in Tet(B) [1]. That is, even charge-conserved mutations at these positions resulted in the almost complete loss of transport activity, indicating the requirement for a particular configuration of the carboxyl group. Glu-30 and Glu-152 are located in the N-terminal half of Tet(K) as well as Asp-15 and Asp-84 in Tet(B). On the other hand, Glu-397 is located in the C-terminal half of Tet(K), as is Asp-285 in Tet(B). The location of Glu-30 in Tet(K) exactly corresponds to that of Asp-15 in Tet(B). The two residues are in the middle of the putative transmembrane helix I. On the other hand, the locations of Glu-152 and Glu-397 are different from those of Asp-84 and Asp-285. The latter are in transmembrane helices III and IX, respectively, the former being in transmembrane helices V and XI, respectively, in the putative 12-transmembrane structure (Fig. 1). However, there is an important similarity in their distribution. That is, both Glu-152 and Glu-397 are located in the 5th transmembrane helices in the N- and C-terminal halves, respectively, in the 12-transmembrane structure of Tet(K) (Fig. 1), as well as Asp-84 and Asp-285 being located in the 3rd transmembrane helices in the N- and C-terminal halves of Tet(B), respectively. Such a symmetrical distribution of the functionally important residues and the correspondence of the mutational effects between Tet(K) and Tet(B) suggest that the roles of the N- and C-terminal halves of Tet(K) in the transport function are in consistent with those of the N- and C-terminal halves of Tet(B), respectively.

Our current observation revealed that the transmembrane glutamic acid residues in Tet(K) play roles similar to those of the transmembrane aspartic acid residues in Tet(B). The mu-

tational correspondence shown in this manuscript confirms the fundamental similarity in the molecular mechanisms between Tet(K) and Tet(B) as described in our previous paper [9].

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