Roles of acidic residues in the hydrophilic loop regions of metal-tetracycline/H^+ antiporter Tet(K) of *Staphylococcus aureus*

Erika Fujihira[a,b], Tomomi Kimura[a], Akihito Yamaguchi[a,b,*]

[a]*Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki-shi, Osaka 567, Japan*
[b]*Faculty of Pharmaceutical Sciences, Osaka University, Suita-shi, Osaka 565, Japan*

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Abstract Three transmembrane glutamic acid residues play essential roles in the metal-tetracycline/H^+ antiporter Tet(K) of *Staphylococcus aureus* [Fujihira et al., FEBS Lett. 391 (1996) 243–246]. In the putative hydrophilic loop region of the Tet(K) and Tet(L) proteins, six acidic residues are conserved. Asp^{74}, Asp^{200}, Asp^{318} and Glu^{381} are located on the putative cytoplasmic side, and Asp^{39} and Glu^{345} on the putative periplasmic side. These residues were replaced by a neutral amino acid residue or a charge-conserved one. In contrast to the transmembrane glutamic acid residues, the replacement of the two glutamic acid residues (Glu^{345} and Glu^{381}) did not affect the tetracycline resistance level. Out of the other four aspartic acid residues, the only essential residue is Asp^{318}, any replacement of which resulted in complete loss of the tetracycline resistance and transport activity. Asp^{318} is located in cytoplasmic loop 10–11 in the putative 14-transmembrane-segment topology of Tet(K). In the case of the tetracycline exporters of Gram-negative bacteria, the only essential acidic residue in the cytoplasmic loop region is located in loop 2–3 [Yamaguchi et al., Biochemistry 31 (1992) 8344–8348]. It may be a general role for tetracycline efflux proteins that three transmembrane and one cytoplasmic acidic residues are mandatory for the tetracycline transport function.

Key words: Tetracycline resistance; Site-directed mutagenesis; Acidic residue; Efflux protein; Antibiotic resistance

1. Introduction

Most of the tetracycline-resistant determinants of enteric bacteria are based on the drug efflux mechanism, whereas the resistance determinants of Gram-positive bacteria are based on the ribosomal protection mechanism [1,2] as well as the drug efflux mechanism [3,4]. Tetracycline exporters of Gram-positive bacteria are classified into Tet(K), from *Staphylococcus aureus* [5], and Tet(L), from *Bacillus subtilis* [6], whose amino acid sequences show about 65% similarity [7]. The tetracycline exporter genes of Gram-positive bacteria are phylogenetically distant from those of enteric tetracycline exporters such as Tet(B) and Tet(C) [8]. Their molecular sizes are about 50 kDa, which is larger than the 43 kDa of the enteric tetracycline exporters. Hydropathic analysis of Tet(K) and Tet(L) revealed that their membrane topologies contain 14 membrane-spanning segments [8], whereas Tet(B) and Tet(C) were found to have a 12 membrane-spanning structure, like major facilitator family transporters [9].

Although there are large differences in their molecular sizes and membrane topologies, Tet(K) mediates active tetracycline efflux on the basis of the same molecular mechanism as in the case of Tet(B) [10], that is, it exports a monocationic metal-tetracycline complex through antiport with a proton [11]. The antiport seems to be electrically neutral [12], similar to that in the case of Tet(B) [13], while Tet(L) of *B. subtilis* has been reported to mediate the electrical exchange of metal-tetracycline and protons [14].

There are three transmembrane aspartic acid residues conserved in the enteric tetracycline exporters which are essential for the tetracycline transport function [15]. On the other hand, Tet(K) and Tet(L) do not contain any aspartic acids in their putative transmembrane regions, while three glutamic acids are conserved [16,17]. We found that these transmembrane glutamic acid residues play similar roles to the transmembrane aspartic acid residues of Tet(B) and Tet(C) in site-directed mutagenesis [17]. In particular, Glu^{397} in transmembrane helix XIII, which is the only transmembrane acidic residue in the C-terminal half of Tet(K), showed very similar behavior on mutation to Asp^{285} in helix IX of Tet(B), which is also the only acidic residue in the C-terminal half of Tet(B) [15]. On the basis of the symmetrical distribution of essential acidic residues in the N- and C-terminal halves, we proposed a 12 membrane-spanning structure for Tet(K) in our previous paper [17]. However, a 14 membrane-spanning structure of Tet(K) has been experimentally indicated by PhoA fusion experiments [18].

In this article, we report the roles of acidic residues located in the hydrophilic loop regions of Tet(K). Tet(K) and Tet(L) contain greatly modified forms of the conserved sequence motifs of major facilitator family transporters like Tet(B) and Tet(C). The first motif, GKXSDRXGRR, in loop 2–3 of Tet(B), is modified to GKXSDXXXXKK in loop 2–3 of Tet(K), and the second motif, GRXXXKXGEK, in Tet(B) is modified to GXXXDRKG in loop 10–11 in Tet(K). Two (Asp^{74} and Asp^{318}) of the six conserved acidic residues in the hydrophilic region are located in these conserved motifs. Out of the other four residues, two (Asp^{200} and Glu^{381}) are on the cytoplasmic side and two (Asp^{39} and Glu^{345}) on the periplasmic side. In this study, Tet(K) and the mutant genes were constitutively expressed in *Escherichia coli*, and the tetracycline transport in everted membrane vesicles prepared from *E. coli* cells was measured.

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strains and plasmids

E. coli W3104 [19] was used as the host strain for expression of the

*Corresponding author.

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tet(K) gene and for the preparation of everted membrane vesicles. pTZ1252, which was a gift from N. Noguchi, Tokyo College of Pharmacy, was constructed by insertion of a 2.3 kbp *Hin*dIII fragment of pNS1, which carries the *tet*(K) gene of *S. aureus* [4], 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 [20]. pTKN1 and pTKC3 are plasmids constructed by subcloning of the 5′- and 3′-halves of the *tet*(K) gene, respectively, into pUC118 [21] and pUC119, respectively. pTKN1 was constructed by insertion of the *Eco*RI-*Kpn*I fragment of pTZ1252 into the corresponding cloning site of pUC118. pTKC3 was constructed by insertion of the *Hin*dIII-*Kpn*I fragment of pTZ1252 into the corresponding cloning site of pUC119.

2.3. Site-directed mutagenesis

Mutagenesis at codons 39 and 74, and at codons 200, 318, 345 and 381 in the *tet*(K) gene was performed using pTKN1 and pTKC3, respectively, as templates. The method employed for mutagenesis was mainly that of Kunkel [22]. The mutagenic primers used are listed in Table 1. A mutation was first detected by restriction analysis and then confirmed by DNA sequencing. The mutant *tet*(K) gene was reconstructed from mutant pTKN1 or pTKC3 by corresponding fragment exchange with pTZ1252.

2.4. Preparation of inverted membrane vesicles and transport assaying

Everted membrane vesicles were prepared from *E. coli* W3104 cells harboring pTZ1252 or a mutant plasmid, as described in our previous paper [11]. Respiration-driven [^{3}H]tetracycline uptake by everted membrane vesicles was measured in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$, as described previously [11].

3. Results and discussion

3.1. Distribution of conserved acidic residues in the putative hydrophilic region of Tet(K)

The 14 hydrophobic segments, whose length was enough to traverse the membrane once, were estimated by hydropathy analysis of the Tet(K) protein [8]. In our previous paper, the two central hydrophobic segments were postulated to be in the large cytoplasmic loop because of the theoretical symmetry of the distribution of essential transmembrane acidic residues in the N- and C-terminal halves of Tet(K) [17]. However, after that, since we obtained experimental evidence that the region between central helices VII and VIII is located on the periplasmic side in PhoA fusion experiments (unpublished observation), the 14 membrane-spanning structure seems to be correct. Ginn et al. also proposed a 14 membrane-spanning structure on the basis of the results of a PhoA fusion experiment [18], as well as QacA/B [23]. Our proposed structure (Fig. 1) is almost the same as that proposed by Ginn et al. [18], except for a minor discrepancy as to the boundary between the loop and membrane-embedded regions. Out of nine conserved acidic residues, six including Asp^{200} are located in the hydrophilic region in our topology, while Asp^{200} is located in the N-terminal region of transmembrane helix VII in the topology of Ginn et al. [18].

Table 1
Mutagenic primers used for site-directed mutagenesis

primer	nucleotide sequence	codon change	amino acid substitution
D39N	5′-TCTTTACCTAATATTGCAAATC-3′ *SspI*	GAT→AAT	Asp→Asn
D74N	5′-TGGAAAGCTTTCTAATTATA-3′ *HindIII*	GAT→AAT	Asp→Asn
D74E	5′-ATGGAAAGCTTTCTGAGTATATA-3′ *HindIII*	GAT→GAG	Asp→Glu
D200N	5′-ATACATTAAATATTGTAGGTAT-3′ *SspI*	GAT→AAT	Asp→Asn
D318N	5′-GGTTTTTTAGTTAACAGAAAAGG-3′ *HpaI*	GAT→AAC	Asp→Asn
D318E	5′-AGTGGAGAGAAAAGGGTCCTTAT-3′ *AvaII*	GAT→GAG	Asp→Glu
E345Q	5′-TTGTTCAGTTTAGCATGTGGTTG-3′ *NspI*	GAG→CAG	Glu→Gln
E381Q	5′-AAGAACAAGTTGCTAGCGGAATG-3′ *NheI*	GAA→CAA	Glu→Gln

Asterisks indicate mismatched bases which generate an amino acid replacement and a new restriction site. The underlined letters indicate the new restriction site.

3.2. Effect of replacement of conserved acidic residues in the hydrophilic region of Tet(K)

Table 2 shows the tetracycline resistance level of *E. coli* W3104 cells harboring plasmid pTZ1252 or its derivative encoding a mutant *tet*(K) gene. When the two glutamic acids,

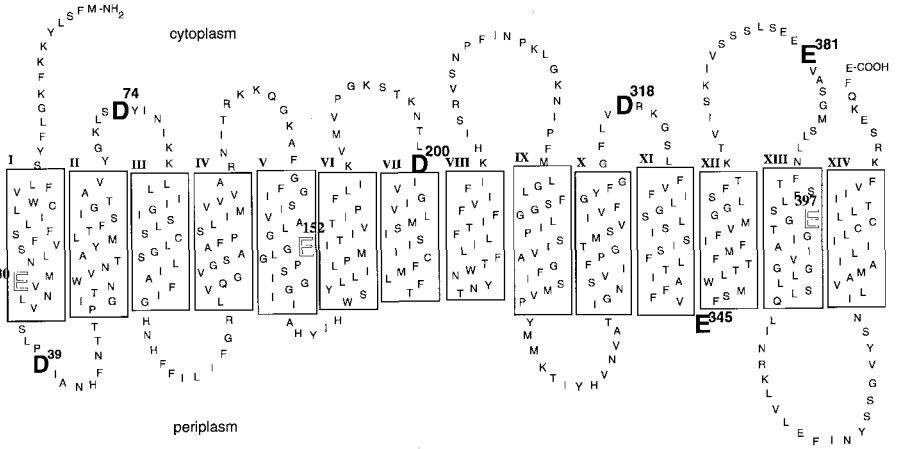

Fig. 1. Putative 14 membrane-spanning structure of the Tet(K) protein on the basis of hydropathy analysis. Conserved acidic residues in the hydrophilic loop and transmembrane regions are indicated by bold and outlined letters, respectively.

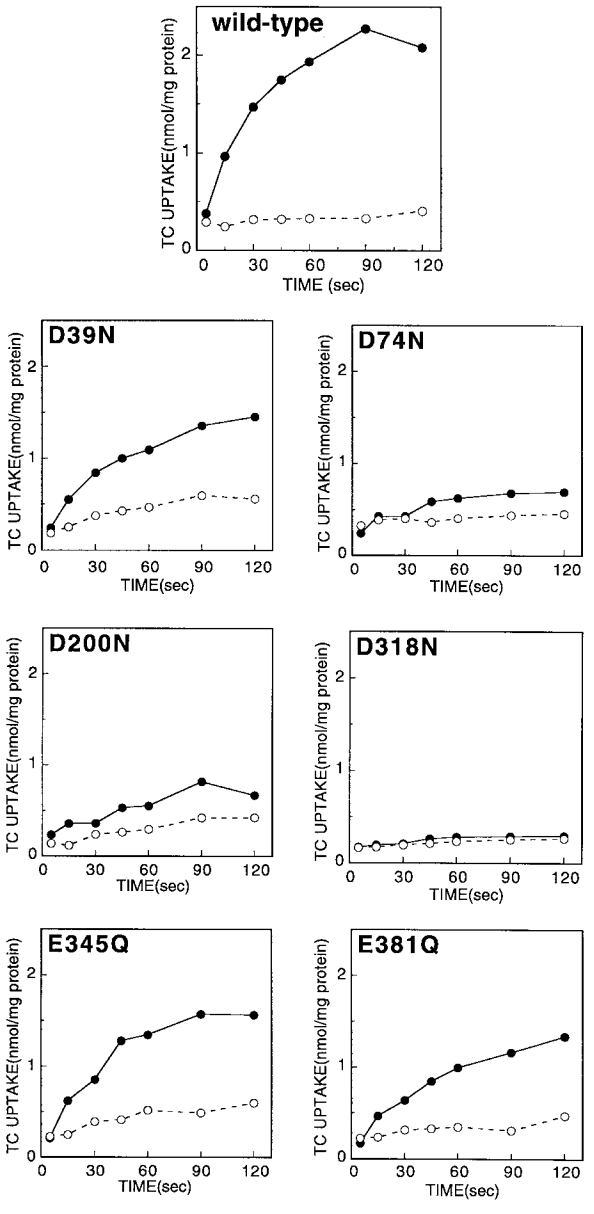

Fig. 2. Tetracycline uptake by everted membrane vesicles prepared from *E. coli* W3104 cells harboring pTZ1252 or its derivatives encoding mutant Tet(K) proteins whose conserved acidic residue was replaced by a neutral amino acid. Closed and open symbols indicate the uptake by vesicles in the presence and absence of NADH, respectively, as an energy source.

Glu^{345} and Glu^{381}, were replaced by glutamine, the resulting mutants conferred the same level of tetracycline resistance as the wild type (Table 2). On the other hand, when four conserved aspartic acid residues were replaced by asparagine, the level of resistance was more or less reduced. These results indicated that, in contrast to in the transmembrane region, aspartic acid residues play a more important role in the hydrophilic region of Tet(K) than glutamic acid residues. The replacement of Asp^{39}, Asp^{74} and Asp^{200} with neutral residues resulted in a moderate level of resistance (Table 2). Only the replacement of Asp^{318} with a neutral residue caused complete loss of the resistance. With respect to Asp^{74} and Asp^{318}, these residues were each replaced with a charge-conserved glutamic acid residue. The resistance levels of the mutants with glutamic acid replacement were significantly higher than those of ones with asparagine replacement (Table 2).

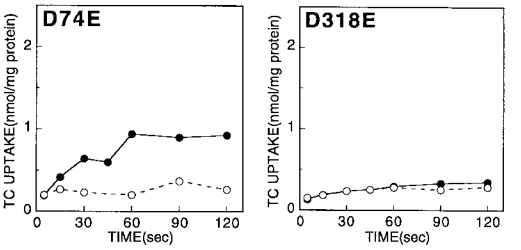

Fig. 3. Tetracycline uptake by everted membrane vesicles prepared from *E. coli* W3104 cells harboring a plasmid encoding a mutant Tet(K) protein whose Asp^{74} or Asp^{318} is replaced by a glutamic acid residue. Open and closed symbols indicate the uptake in the presence and absence of an energy source, respectively.

Tetracycline transport activity was measured in everted membrane vesicles containing these mutant Tet(K)s (Figs. 2 and 3), as described in Section 2. As expected, the two glutamic acid replacement mutants (E345Q and E381Q) showed high transport activity, i.e. about 35–60% of the wild-type level. With respect to the three aspartic acid mutants, D39N, D74N and D200N, the first (D39N) showed the highest residual activity, while its resistance level was the lowest of these three mutants. The D74N mutant showed very low activity, i.e. about 5% of the wild-type level. Only the D318N mutant completely lost its transport activity. As to the charge-conserved mutants, the D318E mutant showed no transport activity, while the D74E mutant retained high activity, i.e. about 35% of the wild-type level.

On the basis of these results, it can be concluded that the only essential residue in the hydrophilic region of Tet(K) is Asp^{318}. As judged from the resistance level, a negative charge at this position plays an essential role in the transport function. Asp^{318} is located in the putative cytoplasmic loop between helices X and XI of the C-terminal half of Tet(K) (Fig. 1). It is the only negatively charged residue in the second conserved motif. In contrast, Asp^{74}, which is the only negatively charged residue in the first conserved motif, plays some role in the transport process but is not mandatory. In the case of Tet(B), there is no acidic residue at the corresponding position of the second motif [24]. Only the acidic residue in the second motif, Glu^{274}, of Tet(B) is not essential [24]. The only essential acidic residue in the hydrophilic region of Tet(B) is Asp^{66} in the first motif [25], which corresponds to Asp^{74}. It is not yet obvious why the position of the essential acidic residue

Table 2
Tetracycline resistance (shown as the minimum inhibitory concentration (MIC)) of *E. coli* W3104 cells harboring plasmids encoding mutant Tet(K) proteins

Mutant	MIC (μg/ml)
No plasmid	0.8
pTZ1252	25
D39N	4.7
D74N	9.5
D200N	9.5
D318N	0.8
E345Q	25
E381Q	25
D74E	12.5
D318E	4.7

is in the C-terminal half in Tet(K), although the most essential transmembrane acidic residue, Glu^{397}, is in the C-terminal half of Tet(K), similar to Asp^{285} in Tet(B). It may be a general rule for tetracycline transporters that three transmembrane residues and one cytoplasmic acidic residue are mandatory for the tetracycline transport function.

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References

[1] Burdett, V. (1986) J. Bacteriol. 165, 564–569.
[2] Manavathu, E.K., Fernandez, C.L., Cooperman, B.S. and Taylor, D.E. (1990) Antimicrob. Agents Chemother. 34, 71–77.
[3] Kahn, S.A. and Novick, R.P. (1983) Plasmid 10, 251–259.
[4] Noguchi, N., Aoki, T., Sasatsu, M., Kono, M., Shishido, K. and Ando, T. (1986) FEMS Microbiol. Lett. 37, 283–288.
[5] Mojumdar, M. and Khan, S.A. (1988) J. Bacteriol. 170, 5522–5528.
[6] McMurry, L.M., Park, B.H., Burdett, V. and Levy, S.B. (1987) Antimicrob. Agents Chemother. 31, 1648–1650.
[7] Hoshino, T., Ikeda, T., Tomizuka, N. and Furukawa, K. (1985) Gene 37, 131–138.
[8] Levy, S.B. (1992) Antimicrob. Agents Chemother. 36, 695–703.
[9] Henderson, P.J.F. (1990) J. Bioenerg. Biomemb. 22, 525–569.
[10] Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) J. Biol. Chem. 265, 4809–4813.
[11] Yamaguchi, A., Shiina, Y., Fujihira, E., Sawai, T., Noguchi, N. and Sasatsu, M. (1995) FEBS Lett. 365, 193–197.
[12] Hirata, T., Wakatabe, R., Nielsen, J., Someya, Y., Fujihira, E., Kimura, T. and Yamaguchi, A. (1997) FEBS Lett. 412, 337–340.
[13] Yamaguchi, A., Iwasaki-Ohba, Y., Ono, N., Kaneko-Ohdera, M. and Sawai, T. (1991) FEBS Lett. 282, 415–418.
[14] Guffanti, A.A. and Krulwich, T.A. (1995) J. Bacteriol. 177, 4557–4561.
[15] Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M. and Sawai, T. (1992) J. Biol. Chem. 267, 7490–7498.
[16] Schwarz, S., Cardoso, M. and Wegener, H.C. (1992) Antimicrob. Agents Chemother. 36, 580–588.
[17] Fujihira, E., Kimura, T., Shiina, Y. and Yamaguchi, A. (1996) FEBS Lett. 391, 243–246.
[18] Ginn, S.L., Brown, M.H. and Skurray, R.A. (1997) J. Bacteriol. 179, 3786–3789.
[19] Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y. and Yamagishi, S. (1981) J. Bacteriol. 145, 808–813.
[20] Noguchi, N., Emura, A., Sasatsu, M. and Kono, M. (1994) Biol. Pharm. Bull. 17, 352–355.
[21] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[22] Kunkel, T.A. (1987) Proc. Natl. Acad. Sci. USA 82, 488–492.
[23] Paulsen, I.T., Brown, M.H., Littlejohn, T.G., Mitchell, B.A. and Skurray, R.A. (1996) Proc. Natl. Acad. Sci. USA 93, 3630–3635.
[24] Yamaguchi, A., Kimura, T., Someya, Y. and Sawai, T. (1993) J. Biol. Chem. 268, 6496–6504.
[25] Yamaguchi, A., Nakatani, M. and Sawai, T. (1992) Biochemistry 31, 8344–8348.