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Membrane Topology of the *Staphylococcal* Tetracycline Efflux Protein Tet(K) Determined by Antibacterial Resistance Gene Fusion

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To determine the membrane topology of Tet(K), conventional antibiotic resistance genes were used as reporter molecules. A series of fusion genes comprising a resistance gene —β-lactamase (*amp*) or chloramphenicol acetyl transferase (*cat*)—and one loop of the 14 putative transmembrane segments of Tet(K) was constructed. *Escherichia coli* TG1 with the *tet*(K)-*amp* fusion gene at the site of the putative periplasmic loop showed resistance to ampicillin, but the bacterium with the *tet*(K)-*cat* fusion gene at the site of the putative cytoplasmic loop showed resistance to chloramphenicol. These findings supported a topology of 14 membrane-spanning segments for Tet(K). Three exceptional cases were observed, which were apparently due to the presence of an acidic residue, Glu, in the preceding transmembrane segment. Mutants in which these acidic residues was substituted for alanine were also constructed, and the effect of glutamic acid in the transmembrane segment on the topology of the fusion proteins was examined.

Key words: chloramphenicol acetyltransferase, β-lactamase, Tet(K), tetracycline, topology.

The efflux of tetracycline is one of the major mechanisms of resistance against tetracyclines (*1*). Several tetracycline efflux proteins are known, which can be classified into two families (*2, 3*). One family consists of several classes of Tet proteins—including Tet(A), (B), and (C)—found in Gram-negative bacteria such as *Escherichia coli* (*4*). The Tet(K) and Tet(L) classes comprise the other family, and these are found in Gram-positive bacteria; Tet(K) in *Staphylococcus aureus*, and Tet(L) in *Bacillus, Staphylococcus*, and *Streptococcus* species (*4*).

Tet transporters belong to the major facilitator superfamily (MFS) of transporters, which includes uniporters, symporters, and antiporters (*5*). Most of these major facilitator transporters have topologies incorporating 12 membrane-spanning segments (*e.g.* lactose permease and glucose transporters) and all of the Tet proteins from Gram-negative bacteria also have 12 transmembrane domains. The 12 membrane-spanning segment topology of Tet(B) and (C) has been established by a number of workers using several techniques (*6-10*), as has that of other MFS transporters (*5, 11, 12*). However, exceptions to this highly-conserved 12-segment topology have been predicted for some drug exporters, including Tet(K) and Tet(L), on the basis of their hydropathy profiles (*5*).

The tetracycline-effluxing mechanisms of Tet(B), and Tet(K), and (L) have common features, that is (i) they mediate the efflux of a tetracycline as a chelation complex with a divalent cation, and (ii) the efflux is driven by an antiport with a proton (*13-15*). However, there is little sequence homology between Tet(B), and Tet(K), and (L) (*2, 3*), the only detectable degree of similarity being observed in the N-terminal half of each family (*16*). It is thus of interest to determine why tetracycline is transported by such diverse transporters using the same transport mechanism.

Only one group (Ginn, Brown, and Skurray, *17*) has reported biochemical evidence for Tet(K) having 14 transmembrane segments, using fusions to the proteins, alkaline phosphatase and β-galactosidase. However, their data indicated no positive reporter activity for some loop regions of Tet(K) (*17*). Using the same alkaline phosphatase as a reporter enzyme, we obtained different results indicating Tet(K) has a topology of 12 membrane-spanning segments without considering the so-called "stop-transfer effect" (*8*) of a transmembrane charged residue in a reporter fusion protein (E. Fujihira, unpublished observation). To clarify this discrepancy, we examined the membrane topology of Tet(K) using the gene fusion technique with a different set of antibacterial resistance reporter proteins, β-lactamase, and chloramphenicol acetyltransferase.

Ampicillin resistance can be expected only when β-lactamase is fused to the periplasmic loop of a membrane protein due to the location of the target protein, a penicillin-binding protein, which is on the periplasmic side of the inner membrane (*18*). On the other hand, Zelazny and Bibi (*11*) reported that bacteria show resistance to chloram-

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phenicol when chloramphenicol acetyltransferase is fused to the cytoplasmic loop of a membrane protein because acetyl-CoA, as an acetyl donor, is only present in the cytoplasm. Hence, using these reporters to determine antibiotic resistance, it is sufficient to test only the antibacterial activity of each antibiotic. This is thus a simple and useful technique for topological determination.

MATERIALS AND METHODS

Bacteria and Plasmids—*Escherichia coli* TG1 was used as a host strain (*19*). pTZ1252, a tet(K)-encoded plasmid, was described previously (*20*). pTZ1252PX1 was used as a vector to construct the Cat-fusion protein. pTZ1252PX1 is a derivative of pTZ1252 containing a *Pst*I restriction site at the C-terminal of *tet*K and a *Xho*I site about 635 bp downstream of the *tet*K terminal codon. pUC118, pACYC184, and pHSG398 were purchased from Takara. pHSGamp1, 2, and 3 were used as vectors for the Amp-fusion protein, the β-lactamase gene being cloned into the multiple cloning site of pHSG398 as described below.

Chemicals and Media—Ampicillin and chloramphenicol were purchased from Wako. Taq DNA polymerase was purchased from Pharmacia. Primer DNA was synthesized by Sawady Technology (Tokyo). Yeast extract, Bact-tryptton, and Bact-agar were from Difco. A medium of 2×YT broth or YT agar were used for bacterial growth (*21*). All other chemicals were of reagent grade.

Molecular Biological Techniques and Other Methods—The technique used for genetic engineering was essentially that of Sambrook *et al.* (*21*) with slight modification.

Construction of tet(K)-cat Gene Fusion Plasmids—A *Pst*I restriction site was introduced at the site of interest to fuse the *cat* gene in the *tet*(K) sequence in pTZ1252pX1 by the Kunkel method (*22*). The chloramphenicol acetyltransferase gene (*cat*) was amplified by the PCR method from pACYC 184 with a primer, introducing a *Pst*I site at the 5′ terminal and a *Xho*I site at the 3′ end, shifting three reading frames. The *cat* gene was cloned between the *Pst*I and *Xho*I sites of each *Pst*I introduced into pTZ1252pX1 with a matching reading frame. The resultant plasmids were designated as pTKXXC, in which XX is the number of amino acids in Tet(K) directly connected with the Cat protein (*e.g.* pTK82C).

Construction of tet(K)-amp Gene Fusion Plasmids—The β-lactamase gene (*amp*) was amplified from pUC118 by removing the signal peptide sequence with a primer, introducing a *Pst*I site at the 5′ terminal and an *Eco*RI site at the 3′ end, shifting three reading frames. The *amp* gene was cloned into the *Pst*I-*Eco*RI site in the multiple cloning site of pHSG398, and the resultant plasmid was designated as pHSGamp1, 2, or 3 with three reading frames, respectively. The fragment between the *Sph*I site, 324 bp upstream of the tet(K) open reading frame, and the *Pst*I site of the corresponding pTKXXC was cloned into the *Sph*I-*Pst*I cloning site of pHSGamp1, 2, or 3, located just before the *amp* gene, with a matching reading frame. The resultant plasmids were designated as pTKXXA (*e.g.* pTK82A).

Construction of E152A and E397A Mutants of the Fusion Plasmids—After site-directed mutagenesis of E152 or E397 to alanine in the pTZ1252 plasmid by the method of Kunkel *et al.* (*22*), the 1,680-bp *Eco*O109I-*Eco*O109I or 1,074-bp *Hpa*I-*Spe*I fragment containing the mutation site was cut out and cloned into the intermediate vector, and then the appropriate restriction fragment of the mutation was swapped with that of pTK169C, pTK169A, pTK433C, or pTK433A.

Measurement of Resistance to Antibiotics—The constructed plasmids were transformed into *E. coli* TG1 and the resistance to ampicillin or chloramphenicol was then measured. An overnight culture of bacteria grown in 2×YT broth with appropriate antibiotics to ensure plasmid maintenance was diluted ten-thousand times with the same medium without antibiotics and then spotted onto YT agar plates (approximately 5×10^3 cells per spot) containing serial dilutions of ampicillin (80, 40, 20, 10, 8, 6, 4, 2, and 1 μg/ml) or chloramphenicol (20,12,10, 8, 6, 4, 2, and 1 μg/ml). After incubation at 37°C for 17 h, the minimum inhibitory concentration (MIC) was determined.

Kinetic Measurement of Bacterial Growth—A biophotometric recorder (Advantec Toyo TN-2612) was used for time-course measurements of bacterial growth. An optical density (OD) of more than 660 nm can be measured with this instrument. Ten microliters of an overnight culture was inoculated into 10 ml of 2×YT broth medium containing 4 μg of chloramphenicol/ml. Growth was monitored at 37°C with shaking.

RESULTS

Construction of tet(K)-Reporter Gene Fusions—The *tet*(K) gene from *Staphylococcus aureus* was cloned previously and was expressed in *E. coli* by Noguchi *et al.* (*20*). In order to determine the membrane topology of Tet(K), we constructed a series of *tet*(K)-*amp* or *tet*(K)-*cat* fusion genes in which the *amp* or *cat* gene was fused at various sites of *tet*(K). The connected sites and mutation sites were determined by DNA sequence analysis. The expression of each protein in *E. coli* TG1 was analyzed by immunoblotting of a membrane fraction using rabbit anti-β-lactamase or anti-chloramphenicol acetyltransferase IgG (5 prime, 3 prime, Inc.). All the β-lactamase fusion proteins were significantly expressed. In the case of the Cat-fusion proteins, some were only detected as faint bands, but these were sufficient for antibiotic resistance (data not shown).

Antibiotic Resistance of E. coli Tansformed with the Fusion Genes—The antibiotic resistance of *E. coli* TG1 carrying the *tet*(K)-*amp* or *tet*(K)-*cat* fusion gene is shown in Table I. Ampicillin resistance was significant for the six strains carrying pTK48A, pTK106A, pTK218A, pTK-224A, pTK292A, and pTK346A. Their MIC values were in the range of 40-80 μg/ml, which is 10 to 20 times higher than that of the vector control (4 μg/ml). *E. coli* TG1/pTK201A showed moderate ampicillin resistance (MIC= 10 μg/ml), and the remaining nine strains (pTK82A, pTK111A, pTK140A, pTK169A, pTK255A, pTK323A, pTK383A , pTK433A, and pTK459A) were as sensitive as the vector control.

In the case of the *tet*(K)-*cat* fusion plasmids, the ten strains carrying pTK82C, pTK111C, pTK140C, pTK169C, pTK201C, pTK255C, pTK323C, pTK383C, pTK433C, and pTK459C showed resistance to chloramphenicol. The degree of the resistance was low (6-12 μg/ml)—less than 3 times that of the vector control (4 μg/ml). However, these increases in the MIC values were highly reproducible. The six strains carrying pTK48C, pTK106C, pTK218C, pTK-

224C, pTK292C, and pTK346C were sensitive to chloramphenicol to the same degree as the vector control. To confirm the resistance to chloramphenicol, the growth inhibition of cells carrying these plasmids in the presence of chloramphenicol was measured with a biophotometric instrument. The growth in liquid medium supplemented with 4 μg of chloramphenicol/ml is shown in Table I and Fig. 1. No growth of the sensitive strains was observed up to 14 h, which was similar to the vector control (MIC=4 μg/ml), shown by the open symbols. In contrast, significant growth of the resistant strains was measured, even those with MIC=6 μg/ml. It was therefore confirmed that strains with MIC values of 6-12 μg of chloramphenicol/ml are drug-resistant.

Judging from these results, positions 48, 106, 218, 224, 292, and 346 are located on the periplasmic side while positions 82, 111, 140, 169, 255, 323, 383, 433, and 459 are on the cytoplasmic side. Position 201 may be in the middle of a transmembrane segment. As to position 111, although the data indicated cytoplasmic localization, its location is too close to position 106 for the peptide chain to cross the membrane. Hence, position 111 may also be in the middle of a transmembrane segment. These findings indicated a structure of 10 membrane-spanning segments for Tet(K) (Fig. 2A).

Effect of Acidic Residue Substitution on the Orientation of the Reporter Enzyme—Allard and Bertrand (*8*) found, using Tet(C), that alkaline phosphatase connected to the predicted periplasmic loop directly following the transmembrane domain containing a charged amino acid residue did not exhibit activity, probably because it was not correctly oriented in respect to the periplasmic surface. Since alanine mutants of these charged amino acids exhibited PhoA activity, they argued that charged amino acids in the transmembrane domain would act as a stop transfer signal (*8*). Prinz and Beckwith (*23*) also observed the same phenomenon for lactose permease in both β-lactamase and alkaline phosphatase fusion. Therefore, we examined the case of Tet(K). We replaced E152 in helix 5 or E397 in helix 13 with alanine, and observed the effects. As shown in

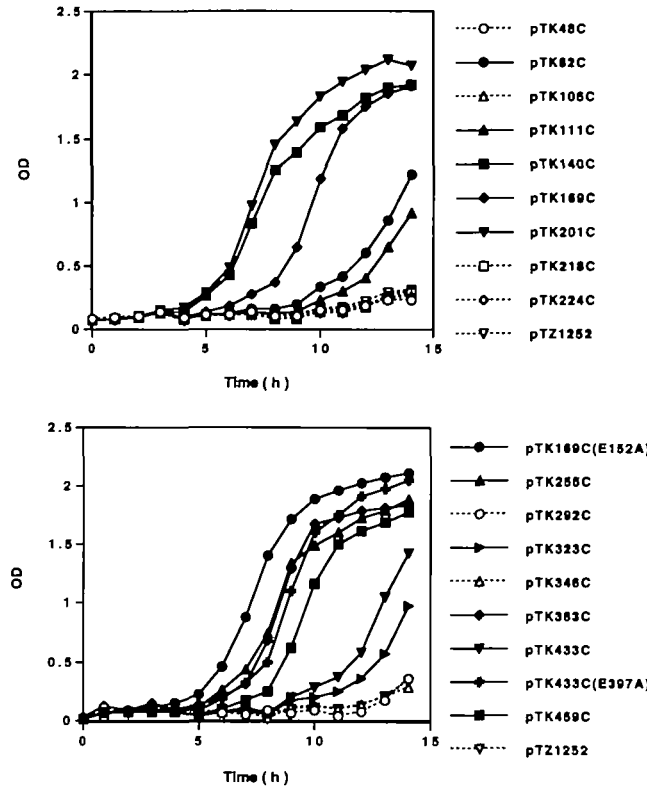

Fig. 1. **Growth rates of *E. coli* TG1 transformed with *cat*-fusion plasmids, pTKXXC, in the presence of 4 μg of chloramphenicol/ml.** The symbols and plasmids are as indicated in the figure. Sensitive and resistant strains are shown by open and closed symbols, respectively. *E. coli* TG1/pTZ1252 is a sensitive strain and served as the vector control. See "MATERIALS AND METHODS" for the experimental details.

TABLE I. **Resistance of *Escherichia coli* TG1 transformed with the *tet*(K)-*amp* or *tet*(K)-*cat* fusion gene to ampicillin or chloramphenicol.**

Plasmids[a] *tet*(K)-*amp* fusion/*tet*(K)-*cat* fusion	Fusion site[b] in Tet(K)	MIC (μg/ml)[c] Ampicillin (*amp* fusion)	MIC (μg/ml)[c] Chloramphenicol (*cat* fusion)	Growth in 4 μg/ml chloramphenicol[d]
pTK48A/pTK48C	Pro-48	80	4	−
pTK82A/pTK82C	Leu-82	4	6	+
pTK106A/pTK106C	Leu-106	40	4	−
pTK111A/pTK111C	Leu-111	4	6	+
pTK140A/pTK140C	Ala-140	4	8	++
pTK169A/pTK169C	Ser-169	4	6	++
pTK169A(E152A)/pTK169C(E152A)	Ser-169/E152A	40	8	++
pTK201A/pTK201C	Ile-201	10	12	++
pTK218A/pTK218C	Thr-218	40	4	−
pTK224A/pTK224C	Thr-224	40	4	−
pTK255A/pTK255C	Asn-255	4	10	++
pTK292A/pTK292C	Ala-292	80	4	−
pTK323A/pTK323C	Leu-323	4	6	+
pTK346A/pTK346C	Phe-346	40	4	−
pTK383A/pTK383C	Ala-383	4	8	++
pTK433A/pTK433C	Leu-433	4	6	+
pTK433A(E397A)/pTK433C(E397A)	Leu-433/E397A	10	6	++
pTK459A/pTK459C	Glu-459	4	10	++
pHSGamp1(*amp* fusion vector control)		4		
pTZ1252 (*cat* fusion vector control)			4	−

[a]Plasmid name of β-lactamase (*amp*) or chloramphenicol acetyl transferase (*cat*) fusion to *tet*(K); [b]fusion site of the amino acid, which is also indicated in Fig. 2; [c]minimum inhibitory concentration; [d]see Fig. 1.

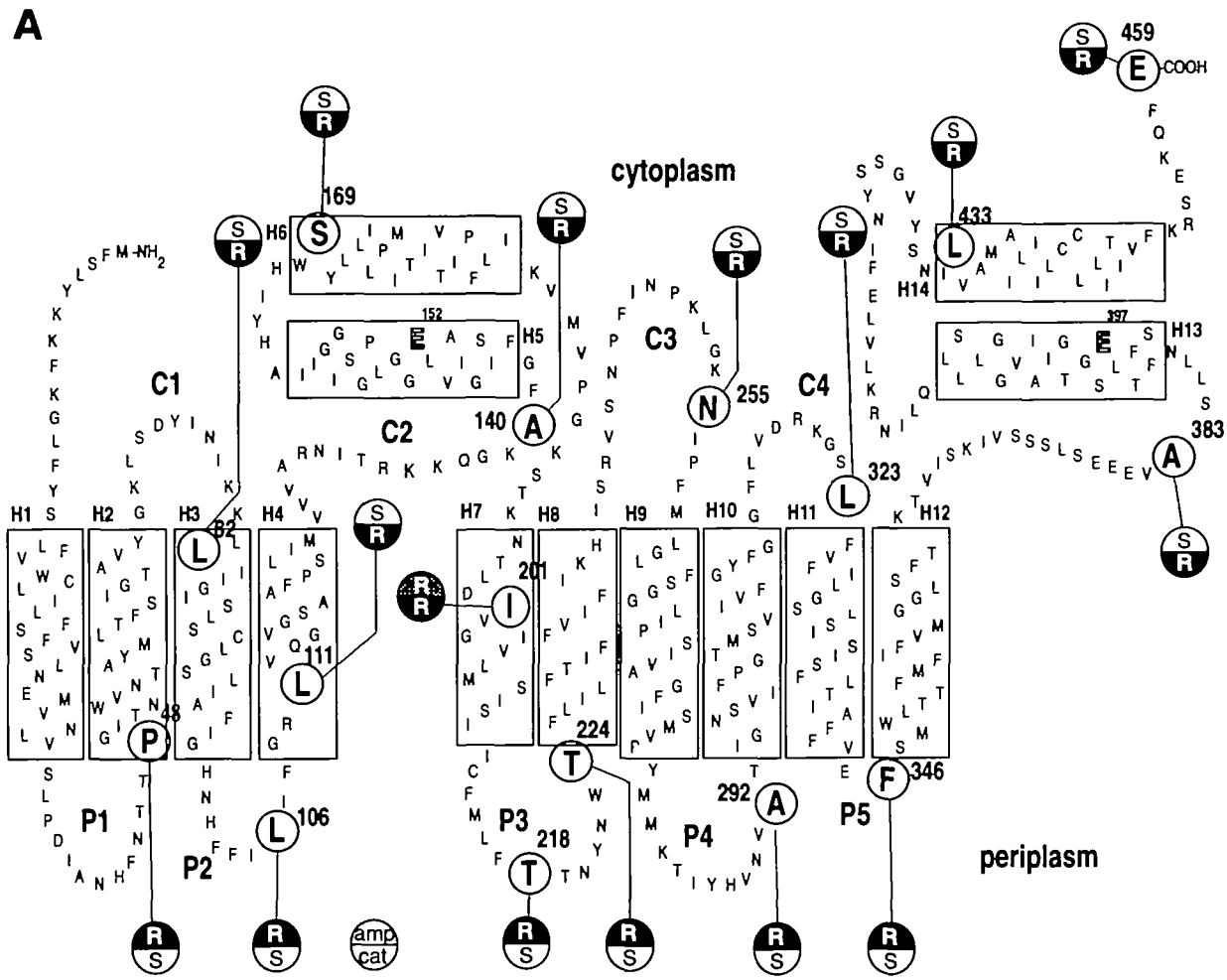

Fig. 2A

Table I, the MICs for ampicillin of pTK169A (E152A) and pTK433A (E397A) transformed cells were increased by 10 and 2.5 times, respectively, compared to those of the corresponding parents, pTK169A and pTK433A. This indicated that β-lactamases fused at positions 169 and 433 are on the periplasmic side when a transmembrane acidic amino acid in the preceding transmembrane region is replaced with a neutral one. As to the *cat*-fusion protein, the MICs for chloramphenicol of cells carrying pTK-169C(E152A) and pTK433C(E397A) were not decreased, and slightly increased in the case of pTK169C(E152A), compared to those of the parents, pTK169C and pTK433C. The effect of a stop transfer signal has so far been reported for alkaline phosphatase and β-lactamase (*8, 23*), which may depend on the nature of the reporter enzyme used. Judging from the data on ampicillin resistance obtained taking consideration of the stop-transfer signaling effect of a transmembrane acidic residue, we obtained a structure of 14 transmembrane segments for Tet(K) (Fig. 2B),which is in agreement with the prediction based on hydropathy analysis.

DISCUSSION

Using a set of β-lactamase and chloramphenicol acetyltransferase as antibacterial reporters, the membrane-spanning topology of Tet(K) appears to consist of 14 segments when the effect of the stop transfer signal is taken into consideration (Fig. 2B). Among the 16 fusion sites, ampicillin and chloramphenicol resistance were clearly opposite in 13 cases—resistance to ampicillin was observed in five putative periplasmic loops (six fusion cases because two fusions were made in the P4 loop) and resistance to chloramphenicol in five putative cytoplasmic loops, one transmembrane domain (L111), and the C-terminal (E459). These orientations of the P and C loops of 1, 2, 4, 5, and 6, and the C-terminal were clearly supported by the activities of both reporters. Although the resistance data suggested L111 is located close to the cytoplasmic side, this is impossible in the topology because position 111 is too close to periplasmic position 106. Thus, in our topology, position 111 is placed in transmembrane segment 4 (H4, Fig. 2B). In the three cases of fusions to S169 (E152A), I201, and L433 (E397A), resistance was observed in both reporter fusions in alanine mutants. In the cases of S169 (E152A) and L433 (E397A), the effect of an alanine mutation of the stop transfer signal on β-lactamase fusion was clearly observed (Table I), indicating that these loops (P3 and P7) are likely to be located in the periplasm. With regards to the fusion to I201, moderate ampicillin resistance was observed for cells carrying pTK201A. PhoA fusion to I201 also gave PhoA activity, which was lost when

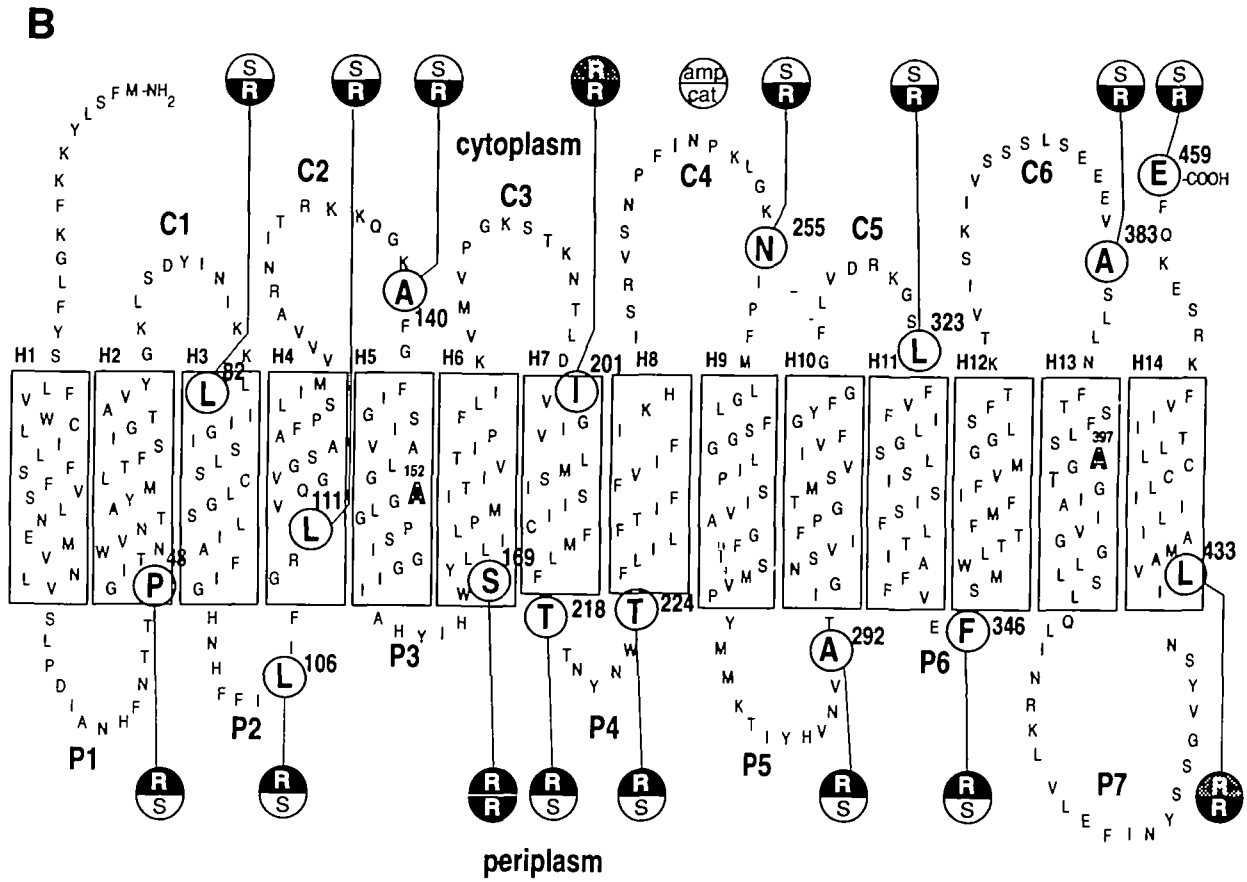

Fig. 2. **Summary of antibiotic resistance and topology of Tet(K) without (A) or with (B) consideration of the stop transfer effect of a transmembrane acidic residue.** The hydrophobic segments are enclosed in boxes. Cytoplasmic loops, periplasmic loops, and helices are denoted as C, P, and H, respectively. Amino acids fused with the reporter enzymes are indicated by bold letters and are circled. The antibiotic resistance of *E. coli* TG1, which was transformed with the fusion plasmids, is schematically indicated in half-circles by S or R. The upper half of each circle shows the ampicillin susceptibility of the strains transformed with *amp*-fusion plasmids and the lower half the chloramphenicol susceptibility of those transformed with *cat*-fusion plasmids: S in a white half-circle, and R in a black, or gray half-circle indicate sensitive, resistant, and moderately resistant, respectively. See Table I for the minimum inhibitory concentrations. (A) The resistances of the parental strains are indicated. The two glutamic acids located in the transmembrane segment are indicated by outlined letter E. (B) The resistances of the alanine mutant strains are indicated for the S169 and L433 fusions [pTK169 A/C (E152A) and pTK433 A/C (E152A)]. The two substituted alanines located in the transmembrane segments are indicated by outlined letter A.

E152 was mutated to alanine (E. Fujihira, unpublished observation). There is a possibility that the stop transfer signal in helix 5 remotely affected the orientation of the β-lactamase fused to the C3 loop to some extent. Considering the highest resistance to chloramphenicol (MIC=12 μg/ml) of cells carrying pTK201C, the C3 loop must be located in the cytoplasm.

Ginn *et al.* reported a topology of 14 membrane-spanning segments for Tet(K) using phoA fusions, but they did not consider the effect of the stop transfer signal (*17*). In contrast, without consideration of the stop transfer signal, we obtained a topology of 10 membrane-spanning segments for Tet(K) in the present study (Fig. 2A). We do not know why Ginn *et al.* obtained the 14 membrane-spanning segments without considering the stop transfer effect, which was also clearly observed in PhoA-fusions of Tet(K) (Fujihira *et al.*, unpublished observation). The effect of the stop transfer signal has also been validated in Tet(C) and lactose permease (*8, 23*), whose topologies have already been established by several other techniques. Although, a final decision on whether or not the stop transfer effect should be considered must await topology determination based on active Tet(K) by means of site-directed chemical modification of cysteine-scanning mutants (*9, 10*), a 14-segment topology for Tet(K) appears reasonable to the best of our knowledge in the light of current techniques of topological determination. It must be noted that the conventional technique using protease digestion cannot be applied to determine the periplasmic localization of a loop region of Tet proteins because these proteins are not digested from outside (*6*).

Although the molecular mechanisms of tetracycline efflux mediated by Tet(B) and Tet(K) are similar to each other, the different topologies of these Tet proteins—12 and 14 membrane-spanning segments, respectively—were confirmed in this study. It is intriguing that common substrate recognition and substrate transfer mechanisms are possible in such diversely structured Tet proteins. Corresponding functional negatively-charged amino acid residues were found in the transmembrane segments of Tet(B) and Tet(K) (*24*). The three-dimensional structures of Tet(B) and Tet(K) may thus be similar, at least in their

functional domains.

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