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Twelve-Transmembrane-Segment (TMS) Version (ΔTMS VII-VIII) of the 14-TMS Tet(L) Antibiotic Resistance Protein Retains Monovalent Cation Transport Modes but Lacks Tetracycline Efflux Capacity

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A "Tet(L)-12" version of Tet(L), a tetracycline efflux protein with 14 transmembrane segments (TMS), was constructed by deletion of two central TMS. Tet(L)-12 catalyzed Na^+/H^+ antiport and antiport with K^+ as a coupling ion as well as or better than wild-type Tet(L) but exhibited no tetracycline-Me^{2+}/H^+ antiport in *Escherichia coli* vesicles.

The majority of the prokaryotic tetracycline (Tet) efflux proteins fall within structurally related families of the major facilitator superfamily (MFS) of transporters that have either 12 transmembrane segments (TMS) or 14 TMS (21, 24). The 12-TMS Tet proteins include most of the tetracycline efflux proteins found in gram-negative bacteria, while the 14-TMS tetracycline efflux proteins are predominantly found in gram-positive bacteria (17). These two groups of Tet proteins are structurally similar to a larger group of drug and multidrug efflux proteins (DHAs) that also function by a secondary antiporter mechanism (16, 21, 22). The major and closely related examples of the 14-TMS Tet proteins are Tet(L), including the chromosomally encoded Tet(L) of *Bacillus subtilis*, and Tet(K), including that encoded in *Staphylococcus aureus* plasmids (17). Both Tet(L) and Tet(K) catalyze the exchange of extracellular H^+ for a cytoplasmic complex of Tet and a divalent metal ion that is optimally Co^{2+} (10, 27), as do 12-TMS Tet proteins (28). Because the reactions catalyzed by the 12-TMS and 14-TMS tetracycline efflux proteins are so similar, it has been suggested that the three-dimensional structures of the catalytically active "cores" of these two types of Tet proteins will also be similar (12). Moreover, there is symmetry within structures of individual 12-TMS tetracycline efflux proteins, and complementation between mutants in different halves of the molecule has been shown (23). This led to the proposal that the 12-TMS tetracycline efflux proteins arose from gene duplication of an ancestral 6-TMS-encoding gene (7, 16, 22, 23). It was further suggested that the origin of the 14-TMS DHA family was the incorporation of two TMS from another source into the middle of a 12-TMS precursor (7). In possible support of this proposal, it is interesting to note the displacement in 14-TMS proteins of a large, central cytoplasmic loop that is typically found in 12-TMS MFS proteins (Fig. 1). This loop has recently been demonstrated to promote membrane insertion of 12-TMS LacY (26).

From a functional point of view, there are reasons to hypothesize that, as with eukaryotic multidrug efflux proteins, Tet proteins and other prokaryotic drug exporters possess (or evolved from proteins that possessed) physiological functions that are unrelated to their current drug substrates. First, chromosomes of several prokaryotes contain numerous genes with significant sequence similarity to established DHA-12 or DHA-14 members whose products do not show comparable activities (19). Also, the chromosomally encoded DHA-12 or DHA-14 proteins are often expressed at levels that would confer little if any drug resistance, suggesting that they may have other roles that are well served by that expression level (24). Studies from our laboratory have shown that Tet(L) and Tet(K) are indeed multifunctional, having the capacity to confer resistance to low levels of tetracycline (Tc^r) and also having two other modes of antiport activity (1–4, 11, 25). They catalyze electrogenic $Na^+(K^+)/H^+$ antiport (with H^+/Na^+ or K^+ of >1), which is physiologically important for Na^+ resistance and for Na^+- and K^+-dependent pH homeostasis in *B. subtilis* (1–4, 25). Tet(L) and Tet(K) also catalyze an electrogenic antiport of the cytoplasmic solutes in exchange for K^+ rather than H^+, such that net K^+ uptake occurs (11, 14). This mode has a physiological role in K^+ acquisition in *B. subtilis* (14, 25). These findings raise the possibility that some tetracycline efflux proteins may have evolved from housekeeping antiporters that catalyze monovalent cation/$H^+(K^+)$ antiport. It was thus of interest to take advantage of a pair of strategically located restriction sites that facilitated removal, from the 14-TMS Tet(L) protein, of the middle two TMS, VII and VIII: i.e., the ones proposed to have been inserted late in the evolution of the Tet(L) and Tet(K) family.

Figure 1 provides a diagrammatic representation of the Tet(L) protein from *B. subtilis* drawn by using topological data that were obtained for Tet(K) by others (6, 12). The diagram shows the cleavage sites, a pair of *Msl*I sites, that were used to cleave and religate to form the Tet(L)-12 construct. Shaded in the diagram are the two charged amino acids, D200 and R222, that are in TMS VII and VIII but are deleted in the Tet(L)-12 construct. D200, but not R222, is conserved among the 14-TMS Tet proteins. The deletion construct was fully sequenced to confirm that it was correctly constructed and contained no additional mutations. Separate individual site-directed mutations were made in *tet*(L) to change the two charged residues D200 and R222 to cysteines. This was achieved by the method

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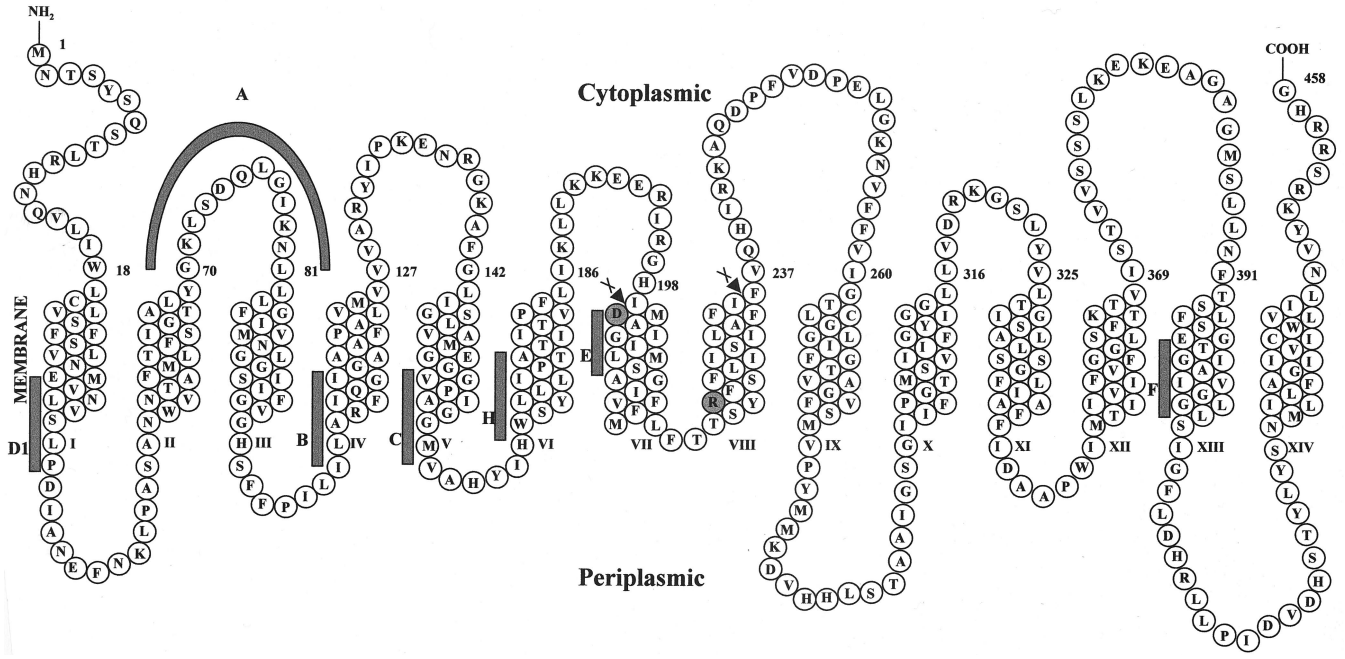

FIG. 1. Topological diagram of Tet(L) highlighting the region deleted in construction of Tet(L)-12. The gray bars show the positions of motifs that were noted by Paulsen et al. (21) in 12- and/or 14-TMS drug/H^+ antiporters. The two *Msl*I sites used in construction of Tet(L)-12 are indicated by the scissors arrowheads.

described by Kunkel et al. (15), followed by complete sequencing. The three mutated *tet*(L) forms, *tet*(L)-12, the D200C mutant, and the R222C mutant, were each cloned into pGEM3Zf(+) (Promega) under control of the T7 promoter; this gives low-level expression that is optimal for Na^+-related assays that were carried out with transformants of the antiporter-deficient *Escherichia coli* NM81 strain (Δ*nhaA*) (20). The three *tet*(L) forms were also cloned into the shuttle vector pBK15 (obtained from K. Zen) under the control of the *ermC* promoter; this gives a higher level of expression that is optimal for tetracycline-related assays carried out in transformants of *E. coli* DH5α and K^+-related assays that were carried out in an *E. coli* mutant, TK2420, that has mutations in each of three K^+ uptake systems (5). Tetracycline-Co^{2+}/H^+ antiport and Na^+/H^+ antiport activities were assessed by energy (Tris D-lactate)-dependent uptake of the radioactive solute into everted membrane vesicles of the appropriate *E. coli* transformant. Everted vesicles were prepared and assayed as described previously (10), except that 5 mM dithiothreitol was used during preparation, and instead of a potassium phosphate buffer, the preparation and assay buffer was 10 mM BTP {1, 3-*bis* [*tris* (hydroxymethyl) methylamino] propane}. Data are corrected for a control experiment in which the uncoupler carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) was present at 10 μM. The net K^+ uptake mode was assayed in right-side-out membrane vesicles that were prepared by the method of Kaback (13). The assay, described previously (11), compared energy (Tris D-lactate)-dependent, net $^{86}Rb^+$ uptake by vesicles loaded with 100 μM KCl, as opposed to choline Cl, under conditions in which there was no chemical gradient of $K^+(Rb^+)$ across the membrane. The data presented are averages of assays conducted in duplicate in at least two independent experiments. For determinations of the amounts of different mutant Tet(L) proteins that were incorporated into the membrane relative to wild-type levels, Western analyses were conducted with membrane vesicles from *E. coli* DH5α transformants with an antibody that had been raised to a peptide corresponding to the Tet(L) N terminus (4). Detection by chemiluminescence was accomplished with the Amersham ECL (enhanced chemiluminescence) kit (Amersham), and quantification was carried out with ImageQuant software (Molecular Dynamics). As shown in Fig. 2, Tet(L)-12 and the R222C mutant protein were incorporated into the membrane at approximately one-third the level of the wild-type Tet(L),

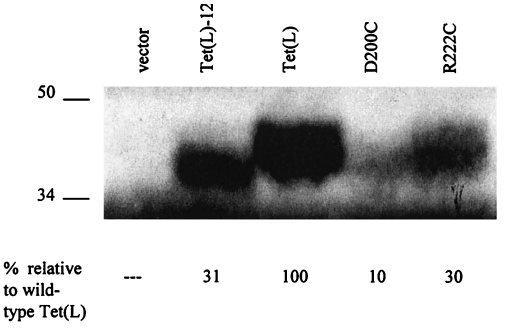

FIG. 2. Western analyses of everted membrane vesicles from *E. coli* DH5α transformed with either a vector control or recombinant pBK15 plasmids bearing genes encoding the indicated forms of Tet(L). M_r values are indicated to the left, and the percentage of protein incorporation relative to wild-type Tet(L) is shown for Tet(L)-12 and the two site-directed mutant forms at the bottom of the figure.

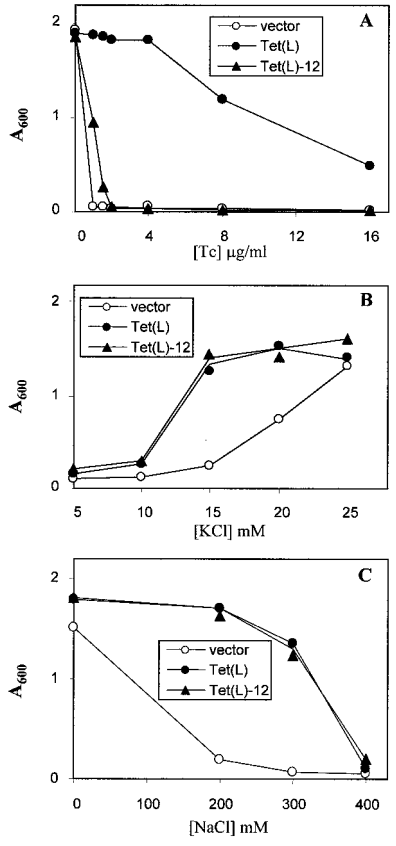

FIG. 3. Complementation capacities of Tet(L)-12 compared to that of wild-type Tet(L). The growth of the indicated transformants of *E. coli* DH5α on different concentrations of tetracycline (A), *E. coli* TK2420 on different concentrations of added KCl (B), and *E. coli* NM81 on different concentrations of added NaCl (C) are shown as the A_{600} measured after 15 h of growth. The results are the mean values of at least three independent experiments conducted in duplicate.

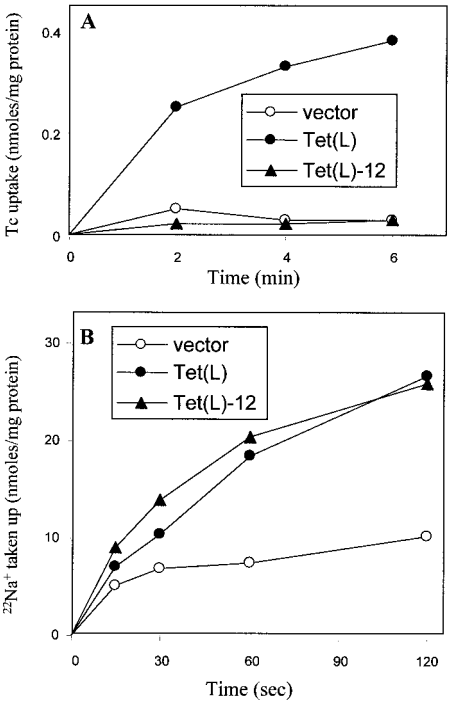

FIG. 4. Transport of tetracycline (Tc) and Na^+ by everted vesicles of Tet(L)-12 compared with that of wild-type Tet(L). (A) Everted vesicles from the indicated transformants of *E. coli* DH5α were assayed for tetracycline-Co^{2+}/H^+ antiport via the energy-dependent accumulation of radiolabeled tetracycline (25 μM) in the presence of 100 μM $CoCl_2$. (B) Everted vesicles from the indicated transformants of *E. coli* NM81 were assayed for energy-dependent Na^+/H^+ antiport via $^{22}Na^+$ uptake.

whereas the incorporation of the D200C mutant was significant but lower.

As shown in Fig. 3, Tet(L)-12 supported almost no Tc^r in a direct comparison with wild-type Tet(L) and a vector control (Fig. 3A) but exhibited a wild-type capacity to complement the K^+ uptake-deficient *E. coli* strain TK2420 (Fig. 3B) and to restore Na^+ resistance to Na^+-sensitive *E. coli* NM81. Transport data correlated with these findings. As shown in Fig. 4, everted vesicles from Tet(L)-12 exhibited no energy-dependent Tet uptake (Fig. 4A), whereas it exhibited wild-type levels of Na^+ uptake (Fig. 4B). Presumably the modest Tc^r conferred by Tet(L)-12 results from retention of some tetracycline binding capacity. Net K^+ uptake activity was assayed in a protocol in which energy-dependent Rb^+ uptake was monitored in right-side-out membrane vesicles; net K^+ accumulation was dependent upon the presence of intravesicular K^+ (as opposed to choline). As shown in Fig. 5, Tet(L)-12 exhibits more rapid and extensive Rb^+ uptake. Since the results are normalized to total vesicle protein and the Tet(L)-12 vesicles contain less Tet protein than found in the wild-type vesicles, the difference is even greater than drawn had it been it normalized to Tet content.

The small periplasmic loop between TMS VII and VIII that is deleted does not contain any residues of known importance in the transport mechanism. Therefore, in identifying candidate residues whose loss in Tet(L)-12 might relate to the loss of tetracycline transport capacity, we focused upon the two charged amino acids that are predicted by topology studies to be in TMS regions, D200 and R222. As shown in Fig. 6, the R222C mutant Tet(L) exhibited tetracycline transport activity that was less than that of the wild type but significant, whereas the D200C mutant protein exhibited no tetracycline transport activity. Even though less of the Tet(L) D200C protein was incorporated into the membrane, activity would have been detected were the protein active. Indeed, the D200C mutant protein was found to bind tetracycline well, as evidenced by much higher tetracycline binding controls than were observed with wild-type Tet(L) or R222C. Transport assays were consistent with the growth experiments, with the R222C mutant exhibiting all three transport modes but with D200C exhibiting only Na^+/H^+ antiport and net K^+ (Rb^+) uptake capacity. It is possible that D200C is an essential residue for tetracycline-Co^{2+}/H^+ antiport but not for any of the other activities of Tet(L). Either its alteration or its deletion together with the central two TMS might then account for the absence of that activity in Tet(L)-12. However, the excision of two entire TMS is likely to have had global effects on the protein, and even the single mutagenic change of D200 might affect activity indirectly. The basis for the change in the activity spectrum upon

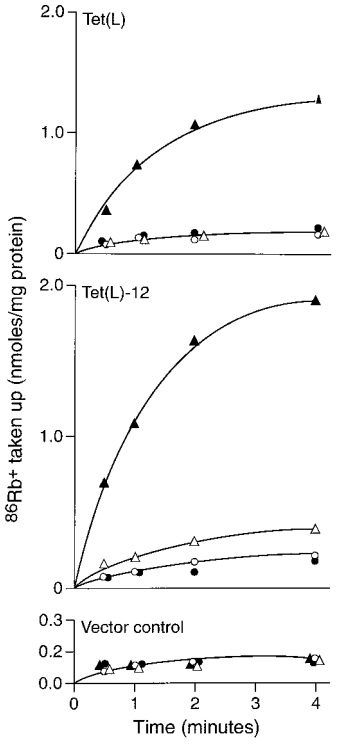

FIG. 5. Capacity of Tet(L)-12 and wild-type Tet(L) to support energy-dependent accumulation of Rb^+ by right-side-out vesicles of TK2420. Vesicles loaded with either choline Cl (circles) or KCl (triangles) were diluted into buffer containing the labeled Rb^+ alone (open symbols) or together with the electron donor, D-lactate (closed symbols). The uptake observed in the vector controls under all conditions (lower panel) represents the level of equilibration of Rb^+ as opposed to accumulation.

formation of Tet(L)-12 is thus likely to be complex. Whatever the details of how the change develops, the retention of robust Na^+/H^+ activity and net K^+ uptake activity by Tet(L)-12 resonates with suggestions that ancestral forms of current antibiotic efflux proteins may have had different topologies and may

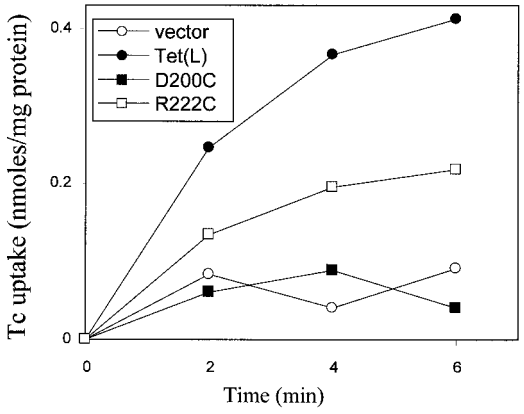

FIG. 6. Tetracycline-Co^{2+}/H^+ antiport activity of D200C and R222C mutants of Tet(L) assayed in everted vesicles via energy-dependent uptake of tetracycline. The uptake activity of wild-type Tet(L) vesicles of *E. coli* DH5α is shown in comparison with that for vesicles from cells with the vector control or the D200C or R222C mutant.

have had a different spectrum of activities that included physiologically important transport capacities.

Finally, two truncated forms of the wild-type Tet(L) were prepared as part of this study in view of the finding of others that approximately the N-terminal one-quarter to one-half of the Tet(B) and Tet(K) proteins, respectively, was sufficient to complement K^+-deficient *E. coli* strains (8, 9,18). An earlier construct that we had prepared to resemble the reported truncated Tet(K) had not shown K^+ uptake activity, but we had not assessed membrane incorporation of that protein (11). New constructs were made of wild-type Tet(L) that retained either six or eight full N-terminal TMS; incorporation of these proteins into the membrane was shown to be close to wild-type levels, but neither Tc^r nor complementation of *E. coli* K^+ uptake was observed. Thus, Tet(L)-12 is the only smaller Tet(L) form for which we have so far been able to confirm activity.

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