The tetracycline efflux protein encoded by the *tet*(K) gene from *Staphylococcus aureus* is a metal-tetracycline/H^+ antiporter

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Abstract **The *tet*(K) gene from *Staphylococcus aureus* was highly expressed in *Escherichia coli* by an alteration of its initiation codon from TTG to ATG and its ribosome-binding sequence from GAGG to GGAGG [Noguchi, N. et al. (1994) Biol. Pharm. Bull. 17, 352–355]. The inverted membrane vesicles prepared from the *tet*(K)-expressing cells showed respiration-dependent [^{3}H]tetracycline transport comparable to the vesicles from the *tet*(B)-expressing cells. The affinity of Tet(K) vesicles to tetracycline was the same as that of Tet(B) vesicles, whereas the former V_{max} value was about 60% of the latter one. Contrary to Tet(B) vesicles, Tet(K) vesicles showed no significant minocycline uptake, which was consistent with the low minocycline resistance of the Tet(K)-producing cells. The tetracycline transport mediated by Tet(K) vesicles was coupled with proton transport and the translocation of $^{60}Co^{2+}$ ions as well as in Tet(B) vesicles. This observation indicates that the class K tetracycline resistance determinant from Gram-positive bacteria also encodes a metal-tetracycline/H^+ antiporter that is functionally similar to that encoded by *tet*(B), although there is a considerable difference in the primary sequences and the putative topologies of these Tet proteins.**

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

1. Introduction

In Gram-negative bacteria, the mechanism for tetracycline resistance is mainly based on the active efflux of the drug out of the cells [2]. The active efflux is mediated by a metal-tetracycline/H^+ antiporter (Tet(B)), which exports a tetracycline-divalent cation complex by 1:1 antiport with a proton [3]. In order for tetracycline to be transported by the Tet(B) protein, the antibiotic must be bound to a divalent cation such as Co^{2+}, Mn^{2+} or Mg^{2+} [3]. These divalent cations are transported with the antibiotic by TetA [3]. The TetA protein in Gram-negative bacteria has 12 membrane-spanning segments [4,5] and belongs to the major facilitator family [6].

On the other hand, in Gram-positive bacteria, there are several types of tetracycline resistance determinants [2]. Among them, the Tet(M) and Tet(O) proteins confer tetracycline resistance through ribosomal protection [7,8]. There are two related classes of efflux proteins among Gram-positive bacteria which bear little homology to the efflux proteins of Gram-negative bacteria: Tet(L) from *Bacillus, Staphylococcus*, and *Streptococcus* species [9,10] and Tet(K) from *Staphylococcus aureus* [11,12]. The amino acid sequence of Tet(L) shows 65% identity with Tet(K) [13]. Althoguh Tet(K) and Tet(L) have a detectable degree of similarity in the N-terminal sequences with that of the TetA proteins of Gram-negative bacteria [14], the C-termini have little homology with one another. In addition, the putative topologies of Tet(K) and Tet(L), based on their hydropathy profile, contain 14 membrane-spanning segments [2] similar to QacA/B [15] and Mmr [16]. Thus, the latter is postulated to belong to a drug-exporter family different from that of Tet(B) of Gram-negative bacteria, Bmr [17], and NorA [18], which have 12 membrane-spanning segments. Some of the cloned genes of Tet(L) have been expressed in *E. coli*, resulting in tetracycline resistance [19,20] due to active efflux [19]. In the case of Tet(K), two independent groups succeeded in expressing the genes in *E. coli* in different ways [1,21]. Guay and Rothstein [21] expressed the *tet*(K) gene under the control of the *lac* repressor. On the other hand, Noguchi et al. [1] succeeded in obtaining high level expression via its own promotor by some alterations in the initiation codon and the ribosome binding sequence.

In this study, we report on the first direct detection of active tetracycline transport and tetracycline-dependent proton and divalent cation transport mediated by Tet(K) proteins by using inverted membrane vesicles prepared from *E. coli* cells expressing the Tet(K) protein. The expression vector used in this study is pTZ1252, which was constructed by Npguchi etal. [1]. As a result, Tet(K) was proved to be a metal-tetracycline/H^+ antiporter similar to Tet(B) of Gram-negative bacteria.

2. Materials and methods

2.1. Materials

[7-^{3}H]Tetracycline and $^{60}CoCl_2$ were purchased from DuPont-New England Nuclear. [^{14}C]Minocycline was a gift from Lederle Japan Co., Saitama, Japan. All other chemicals were of reagent grade and from commercial sources.

2.2. Bacterial strains and plasmids

E. coli W3104 [22] was used as the host strain expressing the *tet*(K) gene and for preparation of the inverted membrane vesicles. pTZ1252 was constructed by insertion of a 2.3 kb *Hin*dIII fragment of pNS1, which carries the *tet*(K) gene of *Staphylococcus aureus* [12], 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 [1].

2.3. Preparation of inverted membrane vesicles

E. coli W3104/pTZ1252 cells were grown in 1 liter of minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. At the

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Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; TC, tetracycline; MINO, minocycline; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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middle of the logarithmic phase, *tet*A gene expression was induced for 2 h by incubation with 1 μg/ml heat-inactivated chlortetracycline (HCTC). Inverted vesicles were prepared by disruption of the cells with a French press in 50 mM MOPS-KOH buffer (pH 6.6) containing 0.1 M KCl and 10 mM EDTA. Then the vesicles were washed once with 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl.

2.4. Transport assaying of inverted vesicles

A mixture of 10 μl of the vesicle suspension (3.5 mg of protein/ml) and 0.5 μl of 250 mM NADH was preincubated at 30°C for 1 min. The uptake of tetracycline and minocycline was initiated by the addition of [^{3}H]tetracycline and [^{14}C]minocycline (final conc., 10 μM), respectively, in the presence of 40 μl of MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and $CoCl_2$ (final conc., 50 μM), unless otherwise stated. After incubation at 30°C for the indicated periods, 2 ml of 5 mM MOPS-KOH (pH 7.0) containing 0.15 M LiCl was added, and then the mixture was immediately filtered through a Millipore filter (pore size, 0.45 μm) and washed twice, and then the radioactivity of the filter was measured.

For the $^{60}Co^{2+}$ uptake assay, a mixture containing 10 μM unlabeled tetracycline and 50 μM $^{60}CoCl_2$ was used. The other procedures were the same as in assaying [^{3}H]tetracycline uptake.

2.5. Measurement of proton transport across the membrane by the fluorescence change of quinacrine

Proton translocation across inverted vesicles was measured as the change in the fluorescence of quinacrine [23]. A suspension (25 μl) of inverted vesicles (2 mg protein/ml) was diluted with 1 ml of 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl and 10 mM $MgSO_4$. After the addition of 5 μl of a quinacrine solution (final, 160 μM) and 2.5 μl of 250 mM β-NADH (final, 625 μM), the emission (500 nm)/excitation (440 nm) ratio was monitored. Then, 10 μl of 2 mM tetracycline solution (final, 20μM) was added at the indicated period. Finally 2.5 μl of 10 mM CCCP (final, 25 μM) was added to destroy ΔpH.

3. Results

3.1. Tetracycline transport activity of the Tet(K) protein in inverted membrane vesicles prepared from E. coli *cells*

Inverted membrane vesicles were prepared from *E. coli* W3104/pTZ1252 cells in which the expression of Tet(K) was at first induced by HCTC. As shown in Fig. 1A, the initial rate of [^{3}H]tetracycline uptake by inverted vesicles of the induced cells was 1.26 nmol/mg protein/30 s. This was the same level as that of the vesicles prepared from Tet(B)-induced *E. coli* W3104/pLGT2 cells (1.37 nmol/mg prtoein/30 s). Thus, it is clear that Tet(K) can mediate tetracycline transport in *E. coli* cells. The vesicles prepared from the uninduced cells showed almost the same level of tetracycline transport activity as that of induced cells (Fig. 1B). The expression of Tet(K) in *Staphylococcus aureus* is inducible, however, the mechanism of induction is different from Tet(B) [12]. The *tet*(K) operon carries no gene for the reppressor protein. Instead, it contains a short ORF-D encoding a leader peptide, which acts as a control region in the upstream region of the Tet(K) structural gene [12]. In the plasmid, pTZ1252, the control region might not work due to the alteration in the ribosome binding sequence (RBS) and the distance from the RBS to the initiation codon [1]. When *tet*(K) gene expression was induced by 1 μg/ml tetracycline, the uptake by the vesicles was almost the same as in Fig. 1A and B (data not shown).

Table 1
Kinetic constants for tetracycline uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells carrying pTZ1252 and pLGT2, which encode Tet(K) and Tet(B) proteins, respectively

Plasmid	K_m (μM)	V_{max} (nmol/mg protein/30 s)
pTZ1252	35.6 (± 4.0)	9.4 (± 0.9)
pLGT2	36.2 (± 11.6)	14.9 (± 4.0)

These constants were calculated by non-linear regression. The values in parentheses indicate a standard error.

Table 1 shows the kinetic constants of tetracycline uptake by inverted vesicles prepared from the *E. coli* W3104 cells carrying pTZ1252 or pLGT2. It is noteworthy that the K_m values of the two vesicles were almost the same in each other, indicating the similarity of the substrate affinity of Tet(K) and Tet(B). On the

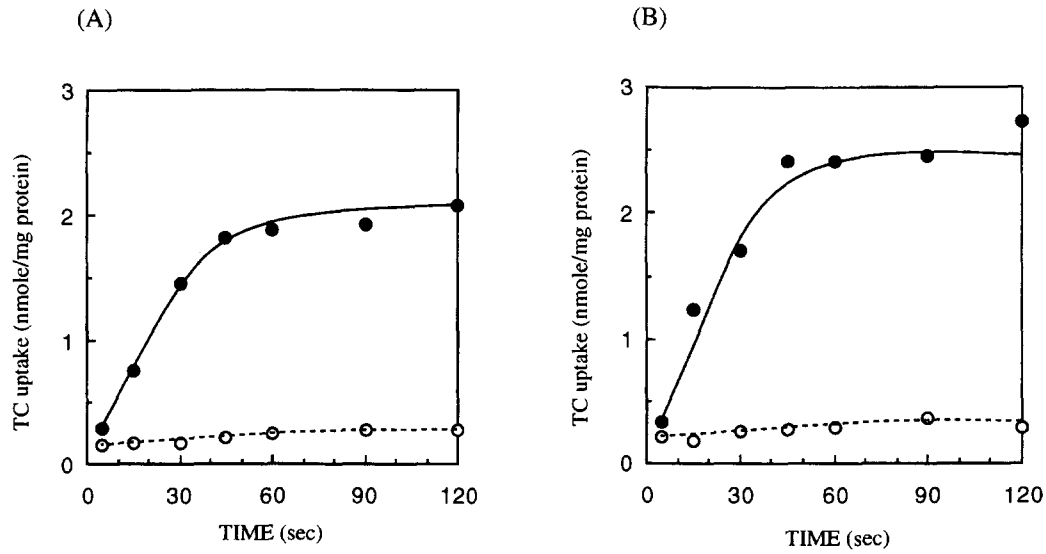

Fig. 1. Tetracycline (TC) uptake by inverted membrane vesicles prepared from *E. coli* W3104/pTZ1252 cells, which carry *tet*(K) gene. In panel (A), the expression of *tet*(K) gene was induced for 2 h by 1 μg/ml heat-inactivated chlortetracycline (HCTC) at the mid-log phase. In panel (B), cells were grown without induction. The uptake assay was carried out in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. Closed symbols indicate the uptake when the vesicles were energized by 2.5 mM NADH. Open symbols indicate the uptake without energization of the vesicles.

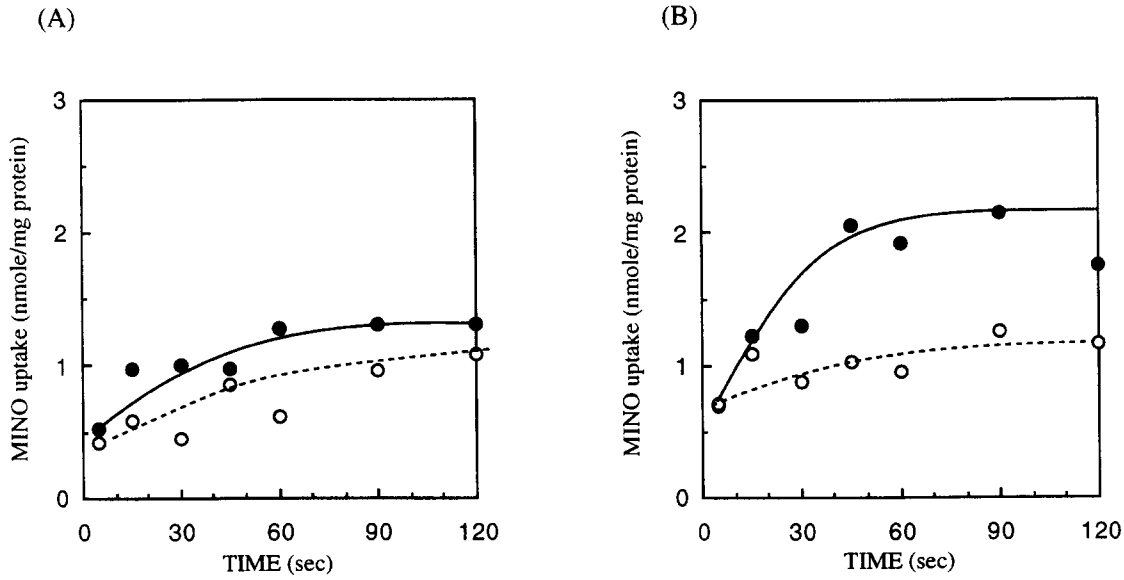

Fig. 2. Minocycline (MINO) uptake by inverted membrane vesicles prepared from *E. coli* W3104/pTZ1252 cells, which carry *tet*(K) gene (A), and *E. coli* W3104/pLGT2 cells, which carry *tet*(B) gene (B). The uptake assay was carried out in the presence of 10 μM [^{14}C]minocycline and 50 μM $CoCl_2$. Closed and open circles represent the uptake in the presence and absence of 2.5 mM NADH, respectively.

other hand, the V_{max} value of the Tet(K) vesicles was about 60% of that of the Tet(B) vesicles.

3.2. *Minocycline transport mediated by the vesicles containing Tet(K) or Tet(B)*

Minocycline is a hydrophobic derivative of tetracycline [24], which is more efficient antibiotic against tetracycline resistant organisms than tetracycline and the other derivatives. The *tet*(B) gene confers moderate resistance to minocycline, whereas *tet*(K) gene confers only very low resistance [21]. Minocycline uptake by inverted membrane vesicles was measured under the same condition as that of tetracycline uptake except for using 10 μM [^{14}C]minocycline. The active minocycline uptake, which is represented by the difference between the uptake in the presence and absence of NADH, by Tet(B) vesicles was 0.78 nmol/mg protein/30 s (Fig. 2B), whereas the active uptake by Tet(K) vesicles was not significant (Fig. 2A). The high background uptake of minocycline by both vesicles was due to the simple diffusion via the lipid bilayer region.

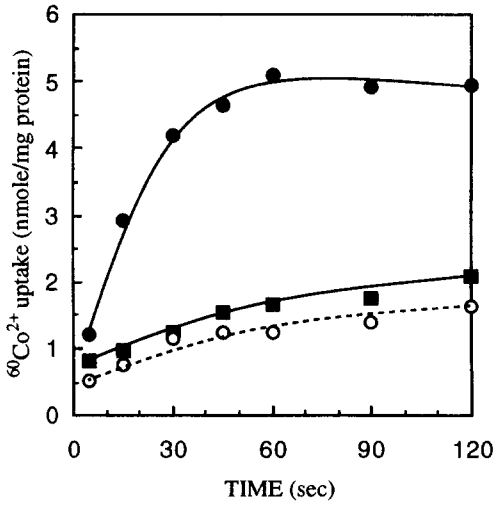

Fig. 3. The uptake of Co^{2+} by inverted vesicles prepared from *E. coli* W3104/pTZ1252 cells. The uptake was measured in the presence of 10 μM tetracycline, 50 μM $^{60}CoCl_2$, and 2.5 mM NADH (closed circles). Open circles indicate the uptake without energization by NADH. Closed squares indicate the uptake in the absence of tetracycline.

3.3. *Tetracycline-dependent $^{60}Co^{2+}$ transport mediated by Tet(K)*

As shown in Fig. 3, inverted membrane vesicles containing Tet(K) showed respiration-driven tetracycline-dependent $^{60}Co^{2+}$ uptake. In the absence of tetracycline or NADH, the uptake was only at the background level. The initial rate of $^{60}Co^{2+}$ was about 2.9 nmol/mg protein/30 s, which was comparable to that mediated by Tet(B) [3]. The net rate of $^{60}Co^{2+}$ uptake was about twice the rate of [^{3}H]tetracycline uptake under the same condition, probably due to leakage of free tetracycline through the lipid bilayer region of the membrane [25].

3.4. *Tetracycline-dependent proton transport in inverted membrane vesicles containing Tet(K)*

When NADH was added to the suspension of the inverted membrane vesicles mixed with quinacrine, the fluorescence of quinacrine was quenched depending upon the pumping of protons into the vesicles via the respiratory chain (Fig. 4). The addition of tetracycline to the suspension of vesicles prepared from the Tet(K)-producing cells (Fig. 4A) and Tet(B)-producing cells (Fig. 4B) caused a restoration of the flourescence, indicating that proton efflux from the vesicles is coupled with Tet(K) or Tet(B) mediated tetracycline uptake. In contrast, when tetracycline was added to the vesicles from the host cells carrying no plasmid, no fluorescence change was observed (Fig. 4C), clearly indicating that the tetracycline-dependent fluores-

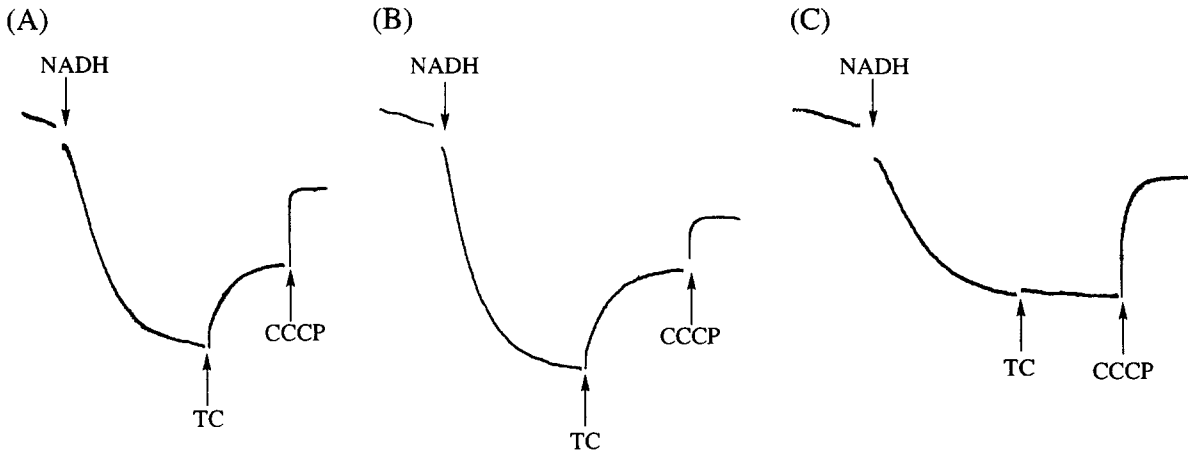

Fig. 4. Proton translocation across inverted membrane vesicles containing Tet(K) (A), Tet(B) (B), and no Tet protein (C). The vesicles were prepared from *E. coli* W3104 cells carrying pTZ1252, pLGT2 and no plasmid, respectively. The fluorescence of quinacrine (160 μM) was monitored. At indicated periods, 625 μM NADH, 20 μM tetracycline, and 25 μM CCCP were added in this order.

cence change was mediated by Tet(K) and Tet(B). The degree of tetracycline-dependent fluorescence change in the Tet(K) vesicles was comparable to that in the Tet(B) vesicles.

4. Discussion

In this study, we first proved that the tetracycline efflux protein of Gram-positive bacteria is an antiporter of a tetracycline-divalent cation complex with proton similar to the TetA of Gram-negative bacteria. The sequence homology of Tet(K) and Tet(L) with the efflux proteins of Gram-negative bacteria was less than that with QacA/B and Mmr [2], whereas the latter two were transporters of completely different substrates. Likewise, the homology between Tet(B), Bmr, and NorA is higher than that between Tet(B) and Tet(K) [2], whereas Bmr and NorA are multidrug exporters [26]. Therefore, according to the amino acid sequence, Tet(K) and Tet(L) belong to a different family of drug exporters to the TetA of Gram-negative bacteria in spite of the similarity of their substrate specificity. However, the tetracycline export mechanism of Tet(K) is the same as that of Tet(B) as shown in this study.

It should be noted that Tet(K) showed tetracycline transport activity comparable to Tet(B) in *E. coli* cells as shown in this study. Tet(B) is known to be the most efficient exporter of teracycline among the drug exporters of Gram-negative bacteria [27]. The detectable similarity in the amino acid sequence of Tet(K) and Tet(B) is only detected in their N-terminal sequence [2]. These observation may suggest that the N-terminal region contributes not only to substrate recognition but also to substrate/proton coupling.

The Tet(B) protein contains four essential Asp residues, Asp^{15}, Asp^{66}, Asp^{84}, and Asp^{285}. Three of them are located in the N-terminal region. Among them, only Asp^{66} is located in the hydrophilic loop region [28], whereas the other three aspartic acid residues are located in the hydrophobic transmembrane region [29]. Tet(K) also contains Asp^{74} corresponding to Asp^{66} of Tet(B) in the widely-conserved sequence motif. However, Tet(K) has no Asp residue in the putative transmembrane region. Instead of Asp residues, there are two Glu residues, Glu^{152} and Glu^{397}, in the transmembrane region of Tet(K). The positions of these residues are different from the Asp residues of Tet(B). It is surprising that such different proteins can mediate the same mechanism of metal-tetracycline/H^+ antiport. The comparative studies of the site-directed mutants of Tet(K) and Tet(B) are now in progress.

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