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The in vivo assembly and function of the N- and C-terminal halves of the Tn10-encoded TetA protein in *Escherichia coli*

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The *tet*A gene was cut into its N- and C-terminal halves at the central *Eco*RI site and the two halves were subcloned individually or together under a separate *lac* promoter/operator. The expression of the C-terminal half was detected with a C-terminal-specific antibody. The amount of the N-terminal half in the cytoplasmic membrane was not affected by the presence of the C-terminal half. In contrast, the amount of the C-terminal half in the membrane was increased in the presence of the N-terminal half, indicating that the N-terminal half helps the stable folding of the C-terminal half in the membrane. Each half individually showed no tetracycline transport activity, however, when both halves were expressed together, the resultant complex showed about 40% of the tetracycline transport activity of the wild-type per number of the C-terminals of TetA protein in the membrane.

Tetracycline; Antiporter; Gene separation; Tetracycline/H^+ antiporter; In vivo assembly

1. INTRODUCTION

The transposon Tn10-encoded tetracycline resistance protein (TetA) is a metal-tetracycline/H^+ antiporter [1]. The TetA protein comprises 401 amino acid residues [2,3] and is predicted to be composed of 12 transmembrane segments connected by hydrophilic loops [4,5]. Both the N- and C-termini face the cytoplasmic surface [4,6]. According to the tandem duplication hypothesis [7], the TetA protein can be separated into two halves at the central large hydrophilic loop, each half containing 6 transmembrane segments. The two halves showed functional complementarity [8,9] and were predicted to be involved in the different processes in the antiport function [10]. Through site-directed mutagenesis studies, it was revealed that functionally essential residues are distributed in both halves [5]. If functional reassembly can be performed in vivo or in vitro after the TetA protein has been cut into the two halves, the resulting TetA protein complex should be very useful for determining the individual roles of the two halves in the transport function, and for revealing the functional and structural interaction between essential residues in the two halves.

For *lac* permease, Bibi and Kaback [11] performed separation and in vivo functional assembly. The resulting *lac* permease showed 30% of the wild-type activity. In spite of the differences between the directions of the substrate–proton coupling in *lac* permease and TetA, that is, the former is a symporter while the latter is an antiporter, their putative secondary structures are fundamentally similar to each other [12], indicating that the coupling mechanisms are the same and that the coupling directions are determined by the distribution of functional residues. In *lac* permease, functionally essential residues are mainly distributed in the C-terminal portion [13], whereas functionally essential residues especially related to the substrate translocation function are located in the N-terminal portion (α-region), as well as the C-terminal portion (β-region), of the TetA protein [5], suggesting a difference in the roles of the N-terminal portions in the two transporters. In this study, at first, we constructed a *tet*A gene subcloned under the control of the *lac* promoter/operator in a plasmid, pUC118. The resulting plasmids caused over-production of the TetA protein when the expression was induced by IPTG. Then, the *tet*A gene was cut into two halves at the *Eco*RI site. The two halves were expressed individually or together, and the assembly of the TetA protein fragments into the cytoplasmic membrane, and the transport function, were measured. The resulting complex composed of separate N- and C-halves showed active tetracycline transport activity.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E coli CJ236 [14], BMH71-18 *mut*S [14], JM109 [15], and TG1 [16], were used for oligonucleotide-directed mutagenesis by the Kunkel method [14]. *E coli* RB791 [17] was used for expression of the *tet*A gene from the *lac* promoter and preparation of inverted membrane vesicles. pCT1182 [5] is a plasmid containing the *tet*A and *tet*R genes as a 2.45-kb *Bgl*II–*Bam*HI fragment subcloned into pUC118.

pLG339BII [5] is a plasmid constructed from pLG339 [16] by insertion of a *Bgl*II linker into its *Eco*RI site.

2.2. *Site-directed mutagenesis*

Site-directed mutagenesis was performed by the method of Kunkel [14] with some modification using mutagenic primers corresponding to the antisense strand. The mutagenic primers listed in Table I were synthesized with a Cyclone Plus DNA/RNA Synthesizer (MilliGen Bioscarch Co.). Mutations were first detected as the appearance or disappearance of restriction sites and then confirmed by DNA sequencing.

2 3. *Growth of cells and preparation of inverted membrane vesicles*

E coli RB791 cells harboring plasmids encoding *tet*A or its derivatives under the control of the *lac* promoter/operator were grown on minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. The expression of *tet*A was induced by the addition of 0 5 mM IPTG to late log-phase cells, followed by 2 h incubation. Inverted membrane vesicles were prepared from the induced cells as described previously [1].

2.4. *SDS-gel electrophoresis and immunoblot analysis*

SDS-PAGE of the inverted vesicles was followed by electroblotting of the proteins. The intact TetA protein and N-terminal half of the TetA protein were detected by Coomassie brilliant blue staining. The C-terminal half, as well as TetA proteins, was detected by immunoblotting using a TetA C-terminal peptide antibody and [^{35}S]protein A. The quantitation of the radioactive bands was performed with a Bioimaging Analyzer BAS 2000 (Fuji Film Co., Tokyo).

2.5. *Transport assay*

[^{3}H]Tetracycline uptake by inverted membrane vesicles was assayed as described previously [1], in the presence of 50 μM $CoCl_2$, 10 μM [^{3}H]tetracycline and 2 5 mM NADH in 50 mM MOPS-KOH buffer (pH 7.0).

3. RESULTS

3.1. *Construction of mutant plasmids*

First of all, the *tet*A gene was separated from the *tet*R gene and placed under the control of the *lac* promoter/operator. For this purpose, the *Eco*RI site at the middle of *tet*A was removed and a new *Eco*RI site was introduced at the initiation codon of *tet*A simultaneously, using mutagenic primers EDR and EGR (Table I), and pCT1182 [5] as a template, by site-directed mutagenesis. The resulting plasmid, pCT1184, which had two *Eco*RI sites, was digested with *Eco*RI, and then the larger DNA fragment was isolated. pSYTET was obtained by self-ligation of the fragment. In this plasmid, the *tet*A gene was fused in-frame downstream of the initial five codons of *lac*Z (Fig. 1). As a result, the N-terminal Met residue of *tet*A was replaced by the N-terminal five residues of *lac*Z, MetThrMetIleThr-. *E. coli* strains carrying no *lac*I^q could not be transformed with pSYTET, probably because the over-production of TetA was lethal [18].

In order to subclone the 5′ portion of *tet*A, a stop codon was introduced at the 201st codon of *tet*A encoding Ser-201, in addition to new restriction sites, *Bam*HI and *Stu*I, downstream from the stop codon by site-directed mutagenesis using mutagenic primer SBGR and pCT1184 as a template. Then, the small *Eco*RI fragment (containing *tet*R) and the small *Bam*HI fragment (containing the 3′ portion of *tet*A) were removed from the mutant plasmid by digestion with *Eco*RI and *Bam*HI, followed by isolation of the two large *Eco*RI–*Bam*HI fragments (one contained most of pUC118 and the other contained the 5′ portion of *tet*A) and self-ligation of them. The resulting plasmid, named pSYTETα, encoded the N-terminal 199 residues of the TetA protein with the N-terminal five residues of LacZ (Fig. 1).

Prior to subcloning of the 3′ portion of *tet*A, a *Bgl*II site was introduced between the *ori* gene and *lac* p/o in pCT1182 by site-directed mutagenesis using mutagenic

Table I

Mutagenic primers used for the creation or deletion of restriction site(s)

Primer	Sequence	Corresponding positions in the *tet*A gene
EDR	5′-GTATACCGAGTTCGATTGC-3′ (*Eco*RI)	669–651
EGR	5′-TTTGTCGAAGAATTCATTTC-3′ *Eco*RI	77– 58
SBGR	5′-TTTAAATAAAGGGATCCAGGCCTAGTTCGATTG-3′ *Bam*HI stop *Stu*I (*Eco*RI)	684–652
B2GR	5′-TCCAGTCGGGAGATCTGTCGTGCC-3′ *Bgl*II	1081–1104 (positions in pUC118 sequence)

Primers, EDR, EGR and SBGR, correspond to the complementary sequence of *tet*A, and primer B2GR corresponds to the sequence between *ori* and *lac* p/o of pUC118 upstream from the cloning site of *tet*A. SBGR introduces a stop codon, which is present as a complementary sequence (CTA) of a stop codon in this primer, into *tet*A, in addition to the creation and deletion of the restriction sites. The restriction sites in parentheses have been deleted. Asterisks indicate the mismatches.

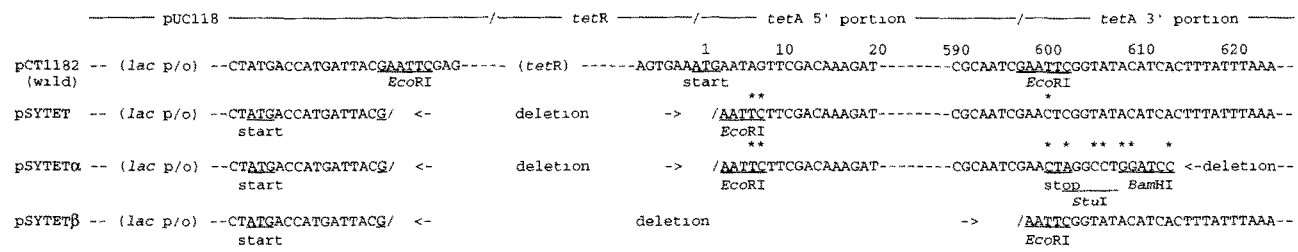

Fig. 1. Sequences around the region of the *lacZ–tetA* connection. The 5′-terminal portion of *lacZ* is ligated to the beginning of *tetα* (pSYTET, pSYTETα and pSYTETα*β*) or *tetβ* (pSYTET*β* and pSYTETα*β*) at the *Eco*RI site. In this in-frame ligation, the N-terminal five residues of LacZ were connected with Asn-2 in TetA and Tetα, or with Asn-200 in Tet*β*.

primer B2GR (Table I). The resulting plasmid was digested with *Eco*RI and the resulting large fragment was ligated to itself. The resulting plasmid, pSYTET*β*, encoded a protein in which the C-terminal 201 residues of the TetA protein were fused downstream with the N-terminal five residues of LacZ (Fig. 1).

In order to construct a plasmid containing both the 5′ and 3′ portions of *tetA* separately under the independent control of *lac* p/o, the *Bgl*II–*Bam*HI fragment, which contained the 3′ portion of *tetA*, was isolated from pSYTET*β* and then ligated to the large *Bgl*II–*Bam*HI fragment of pLG339BII. The resulting plasmid, pLGTET*β*, contained the 3′ portion of *tetA* and a Km^r marker. Then, the large *Stu*I–*Bam*HI fragment of pSYTETα, which contained the 5′ portion of *tetA*, an Amp^r marker and the *ori* gene, was ligated to the large *Pvu*II–*Bam*HI fragment of pLGTET*β*, from which the *ori* gene was lost. The resulting plasmid, pSYTETα*β*, was selected with the dual resistant markers, Km^r and Amp^r. The putative secondary structures of the separate polypeptides derived from TetA are shown in Fig. 2.

3.2. *Expression of* tet*A and its derivatives*

The expression of *tet*A and its derivatives was induced by the addition of 0.5 mM IPTG in the case of cells harboring pSYTETα, pSYTET*β* or pSYTETα*β*. In the case of cells harboring pSYTET, the expression was induced by the addition of 0.1 mM IPTG. After 2 h of induction, the cells were harvested and inverted vesicles were prepared as described previously [1]. The SDS-PAGE patterns of the inverted vesicles were visualized by Coomassie brilliant blue staining (Fig. 3A). When *tet*A was expressed under the control of *lac* p/o, the band observed at 36 kDa was very dense, indicating the over-production of the TetA protein. In addition to the 36 kDa band, a new band at about 15 kDa was also observed in the case of pSYTET (Fig. 3A, lane 2), probably due to the partial proteolysis of TetA.

When the separated *tet*A genes were expressed individually, the dense band corresponding to the N-terminal half of TetA was observed at about 20 kDa (Fig. 3A, lane 3), whereas the band corresponding to the C-terminal half was not detected (Fig. 3A, lane 4). When they

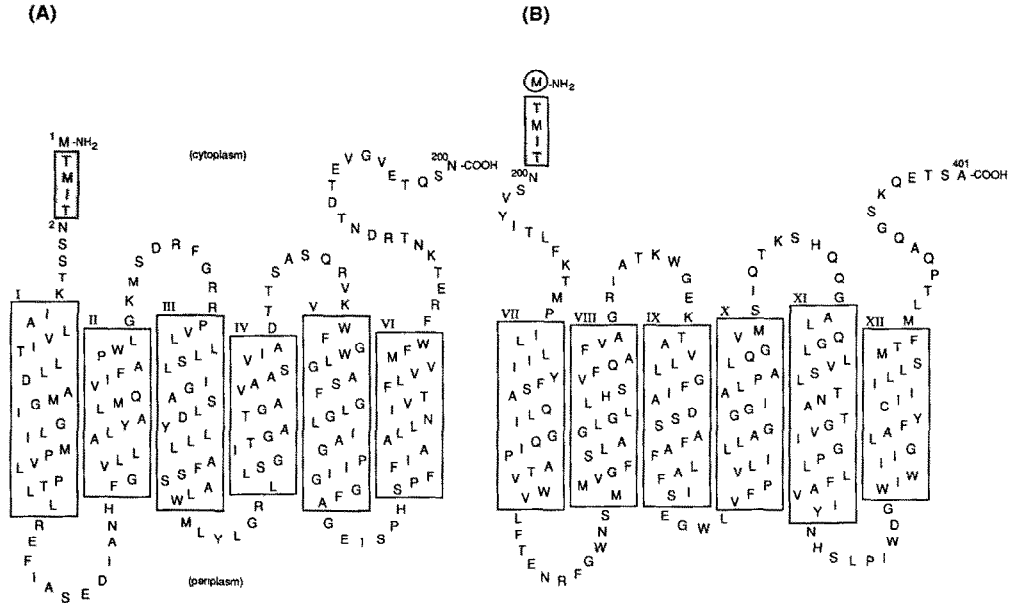

Fig. 2. Putative secondary structures of Tetα (A) and Tet*β* (B). Hydrophobic transmembrane regions are shown in boxes, and the amino acid residues from LacZ are shown in shaded boxes.

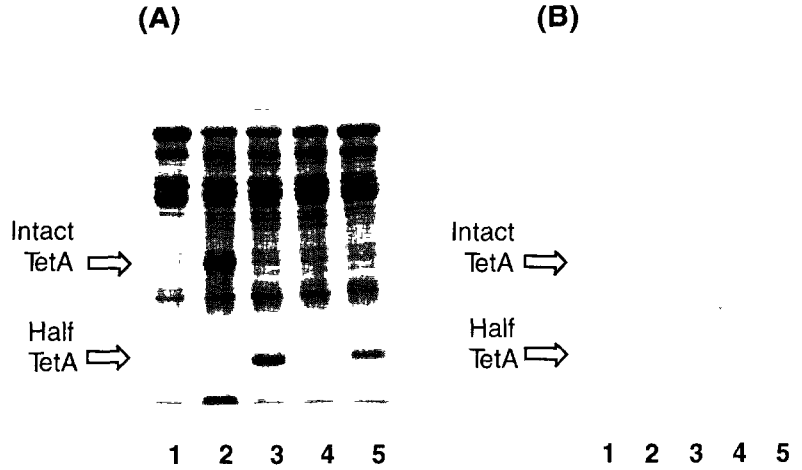

Fig. 3. (A) SDS-PAGE of inverted membrane vesicles (10 μg membrane protein per lane) prepared from *E coli* RB791 harboring pSYTET or its derivatives The bands were visualized by Coomassie brilliant blue staining. Lane 1, vesicles from host cells without a plasmid; lane 2, pSYTET, lane 3, pSYTETα, lane 4, pSYTETβ; lane 5, pSYTET$\alpha\beta$. (B) Western blotting of the inverted membrane vesicles (0.2 μg membrane protein per lane). The [^{35}S]protein A-labeled bands were visualized by autoradiography. The SDS-PAGE conditions were the same as in A except for the amount of membrane proteins. Lane 1, vesicles from host cells without a plasmid, lane 2, pSYTET, lane 3, pSYTETα, lane 4, pSYTETβ, lane 5, pSYTET$\alpha\beta$.

were expressed together, one band at 20 kDa was observed (Fig. 3A, lane 5). Since the molecular weights of the two halves were the same, they might not be separated in the SDS-PAGE pattern. Therefore, the C-terminals were detected by immunoblotting using C-terminal-specific antiserum and [^{35}S]protein A [6]. As shown in Fig. 3B, when the intact *tet*A gene was expressed under the control of *lac* p/o, a dense TetA band at 36 kDa was visualized on immunoblotting. The vesicles containing the N-terminal half showed, of course, no detectable band (Fig. 3B, lane 3) since there was no C-terminal end. When the 3′ portion of *tet*A was individually expressed, a thin band at 20 kDa, corresponding to the C-terminal half of TetA, was detected (Fig. 3B, lane 4). On the other hand, when they were expressed together, the density of the band corresponding to the C-terminal half significantly increased (Fig. 3B, lane 5). The relative density of the radioactive band was quantitated by a Bioimaging Analyzer BAS 2000 (Fuji Film Co., Tokyo). The amount of the C-terminals expressed from pSYTET$\alpha\beta$ was about 1.5-fold more than that from pSYTETβ, although the former was only about 40% of that expressed from pSYTET.

These observations indicated that (i) the assembly of the C-terminal half in the membrane is far less efficient than that of the N-terminal half, and (ii) the presence of the N-terminal half helps the insertion and stability of the C-terminal half in the membrane. In contrast, the presence of the C-terminal half did not affect the assembly of the N-terminal half in the membrane. It is not clear whether the insertion or the stability of the C-terminal half was affected by the presence of the N-terminal one, however, since the thin band of the C-terminal half could be detected on immunoblotting even in the absence of the N-terminal one, it is clear that the insertion of the C-terminal one was not prohibited. Roepe et al. [19] reported that some truncated mutants of *lac* premeases are instable in membranes and rapidly digested by proteases. It is very likely that the C-terminal half cannot undergo stable folding in the membrane without the N-terminal one.

3.3. *Transport activity*

[^{3}H]Tetracycline uptake by inverted membrane vesicles was measured in the presence of 50 μM $CoCl_2$ and 10 μM [^{3}H]tetracycline. The vesicles containing the N-terminal half and the C-terminal half of the TetA protein, individually, showed almost the same tetracycline uptake as the control vesicles in the absence of NADH (Fig. 4), indicating that they had no active transport activity. On the other hand, the vesicles containing both the N-terminal half and the C-terminal half showed significant transport activity, which was about 18% of the vesicles containing intact TetA protein (Fig. 4). Considering the fact that the amount of the C-terminals in the vesicles prepared from cells harboring pSYTET$\alpha\beta$ was only 40% of that expressed from pSYTET-containing cells, the specific transport activity per number of the C-terminals of the complex of the two terminal fragments was about 40% of the intact TetA protein.

4. DISCUSSION

Rubin and Levy introduced a 4 bp frame shift at the *Eco*RI site in the middle of the *tet*A gene [20]. The resultant plasmid conferred about 20% the tetracycline resistance of the wild-type. They anticipated that the resistance shown by the frame-shift plasmid was due to the expression of the two domains of the *tet*A gene as

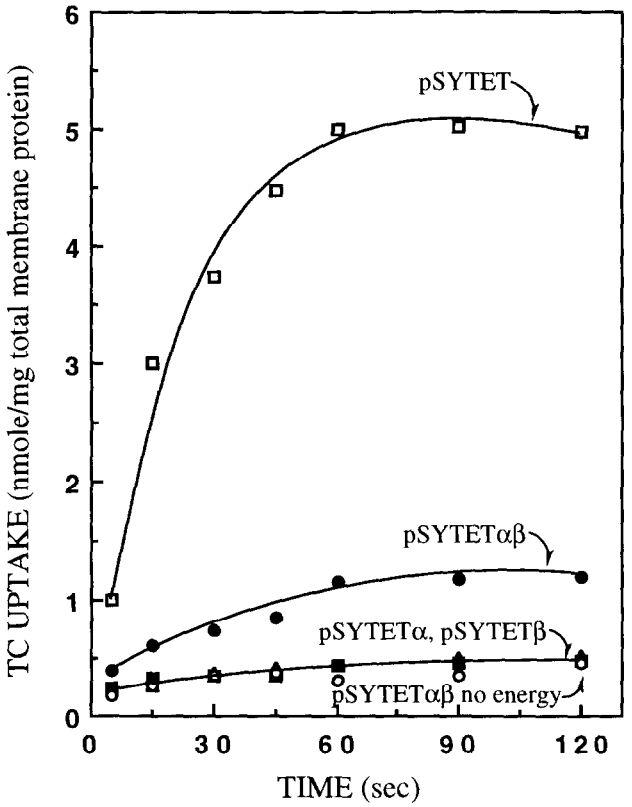

Fig. 4. Tetracycline uptake by inverted membrane vesicles prepared from *E coli* RB791 cells harboring pSYTET or its derivatives. The uptake was driven by the addition of NADH. □, pSYTET; ▲, pSYTETα; ■, pSYTETβ; ●, pSYTETαβ; ○, pSYTETαβ without NADH.

separate polypeptides, however, the actual starting position of the gene encoding the C-terminal domain was unclear in the plasmid, and they could not detect the C-terminal polypeptide in the membrane.

In this study, we constructed plasmids which express the exact two halves of *tet*A, individually or together, as separate polypeptides under the control of a separate *lac* p/o. As a result, it was revealed that the N-terminal half was inserted into the membrane independently to the C-terminal one. In contrast, the degree of the insertion of the C-terminal half into the membrane depended on the presence of the N-terminal one. In the case of the separation of *lac* permease [11], when the gene fragments are expressed individually, the C-terminal half was not observed and observation of the N-terminal half was inconsistent. The N-terminal half of the TetA protein in our experiment seems to be more stable than that of *lac* permease, however, the instability of the separate C-terminal portion is fundamentally the same.

The complex of the N- and C-terminal halves showed compatible tetracycline transport activity per number of the C-terminals. This observation was consistent with the results for *lac* permease [11] and for the frame shift mutant of *tet*A [20]. The TetA protein composed of the two separate polypeptides may be a useful tool for determination of the transmembrane protein structure involving disulfide cross-linking [21] using cysteine-substituted TetA proteins.

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