Second-Site Suppressor Mutations for the Asp-66 → Cys Mutant of the Transposon Tn10-Encoded Metal-Tetracycline/H^+ Antiporter of *Escherichia coli*†

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ABSTRACT: Asp66 is the only essential acidic residue in the putative hydrophilic $loop_{2-3}$ region of the transposon Tn10-encoded metal-tetracycline/H^+ antiporter [TetA(B)] [Yamaguchi, A., Nakatani, M., & Sawai, T. (1992a) *Biochemistry 31*, 8344−8348]. *Escherichia coli* cells producing a D66C mutant of TetA(B) showed no tetracycline resistance. A spontaneous second-site revertant was isolated from the cells carrying the D66C mutant gene, which showed moderate resistance to tetracycline [minimum inhibitory concentration (MIC), 50 μg/mL]. The entire sequencing of the revertant genes revealed two secondary mutations, i.e., the codon 40 of GCT (Ala) → GAT (Asp) and T → G at 17 bases upstream from the initiation codon of the *tet*A gene. There was a T → G mutation at position −17, which was a mutation in the *tet* promoter/operator region, which caused a decrease in TetA(B) production. The full expression of the A40D/D66C double and the A40D single mutants, which were constructed by site-directed mutagenesis, was deleterious for cell growth. The −17T → G mutation mitigated the deleterious effect of these mutants through reduction of expression. The −17T → G single mutation introduced into the wild-type *tet* gene did not affect the level of resistance, although the expression was significantly reduced. Intact cells carrying the A40D/D66C and −17T → G/A40D/D66C mutant plasmids showed a reduced level of tetracycline accumulation due to active efflux, whereas no significant tetracycline uptake was observed in inverted membrane vesicles prepared from these mutant-producing cells. The A40D mutation is located at opposite side of the membrane to the D66C mutation in the putative secondary structure of TetA(B). The second-site mutation might mediate its effects through a structural perturbation propagated along the polypeptide backbone.

The analyses of second-site suppressor mutations isolated from mutants of essential residues are very useful for elucidating the molecular mechanisms of membrane transporters. In the lactose permease, it is known that the effect of the mutation of some of the basic or acidic residues in the putative transmembrane region is suppressed by the mutation of other oppositely charged residues (King et al., 1991; Lee et al., 1992). This observation indicates that (1) these charged residues are stabilized in the hydrophobic environment by forming a charge-neutralizing pair and (2) these residues are sterically close to each other. However, it is noted that the charge-pair neutralization approach will not reveal charge-paired residues if they are essential for activity (Sahin-Toth et al., 1992).

The bacterial tetracycline resistance protein [TetA(B)] is a metal-tetracycline/H^+ antiporter (Yamaguchi et al., 1990c). It has many advantages for studying the molecular mechanism of antiporters as a model system, i.e., the phenotype can be assayed in any host cell because TetA(B) is an exogenous protein for bacterial cells (Levy, 1992) and the mutants are easily selected by the change in drug resistance (Guay et al., 1994). Among a large class of tetracycline efflux proteins, the TetA(B) protein encoded by transposon Tn10 (class B) confers the highest level of resistance as well as TetA(D) (Levy & McMurry, 1974) and tetracycline transport activity can be easily determined using inverted membrane vesicles (McMurry et al., 1980).

In our previous paper (Yamaguchi et al., 1993b), we reported on a spontaneous second-site suppressor mutant isolated from an Asp285 → Asn mutant TetA(B). Asp285 is one of the three conserved Asp residues located in the transmembrane region and is essential for tetracycline transport (Yamaguchi et al., 1990b). The D285N mutant did not confer drug resistance; however, the spontaneous revertant could be isolated, which showed a resistance level similar to that of the wild-type TetA(B). The second-site suppressor mutation was revealed to be Ala220 → Glu. The residues Asp285 and Ala220 are located in the middle of the putative transmembrane helices 9 and 7, respectively. Since the generation of a net negative charge by the Ala → Glu single mutation did not result in the loss of activity (Yamaguchi et al., 1993b), the revertant was not due to the reconstruction of a charge-neutralizing pair. Site-directed mutagenesis of Ala220 and its neighbors revealed that (1) an acidic residue is required at position 220 for suppression, (2) position 220 is specific for the suppression, and (3) the Lys285 mutation is not suppressed by Glu220 or Asp220 (Someya et al., 1995). These findings indicated that an acidic residue introduced at position 220 can substitute the function of Asp285 and both residues are close to each other in the active site.

Asp66 is the only essential acidic residue out of the 8 conserved acidic residues located in the hydrophilic loop region (Yamaguchi et al., 1992a). It is located in the putative cytoplasmic $loop_{2-3}$ (Yamaguchi et al., 1990b) and estimated to play a role in the initial interaction with the substrate

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(Yamaguchi et al., 1993a). $Loop_{2-3}$ also contains a widely conserved sequence motif, GXXXDRXGRR, common not only to drug efflux proteins (Yoshida et al., 1990; Neyfakh et al., 1991; Neyfakh, 1992) but also to solute/H^+ symporters and sugar uniporters (Maiden et al., 1987). Asp66 is the only one negatively charged residue in this polycationic loop. Replacement of Asp66 with a neutral residue caused complete loss of resistance, while the conserved replacement to Glu maintained very low resistance (Yamaguchi et al., 1990b). In this paper, the spontaneous second-site revertants were isolated from the Asp66 → Cys mutant TetA(B). The suppression was apparently due to the regain of a new acidic residue at another position, similar to the case of the suppression of the Asp285 → Asn mutant. However, the nature of the second-site suppression for Cys66 mutant was significantly different from the nature of the suppression for the Asn285 mutant with Glu220 mutation.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Tetracycline was purchased from Du Pont−New England Nuclear. [^{32}P]dCTP was purchased from Amersham. The DNA ligation kit was purchased from Takara (Kyoto, Japan). The DNA Sequenase kit (7-deaza-dGTP kit) was purchased from U.S. Biochemical Corp. (Cleveland, OH). The Lambda-Lift expression detection kit for immunoblotting was purchased from Bio-Rad. All other reagents used were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. Escherichia coli TG1(Taylor et al., 1985) and W3104 (Yamamoto et al., 1981) were used for transformation and expression of *tet* genes, respectively. *E. coli* JM109 (Yanisch-Perron et al., 1985), BMH71-18 mutants (Kunkel, 1985), and CJ236 (Kunkel, 1985) were used for site-directed mutagenesis by the method of Kunkel (1985). pLGT2 (Yamaguchi et al., 1992b) and pCT1183 are low- and high-copy plasmids, respectively, which carry 2.45-kb Tn10-*tet* gene. The plasmid pCT1183 was constructed by deleting the second *Eco*RI site near the *Bgl*II site from pCT1182 (Yamaguchi et al., 1992b). pLGT2 and pCT1183 were mainly used for *tet* gene expression and mutagenesis, respectively.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by the method of Kunkel (1985) using the synthetic mutagenic primers and pCT1183 as a template. Mutagenic primers were synthesized with a Cyclone Plus DNA/RNA synthesizer (MilliGen Biosearch Co.). Mutagenic primers used in this experiment contained the silent mismatch generating the new restriction site in addition to the mismatch for the codon change. Mutant plasmids were at first selected by the appearance of the restriction site and then the sequences were confirmed by DNA sequencing. Mutant *tetA* gene was then subcloned into the low-copy plasmid, pLGT2 (Yamaguchi et al., 1992b), by recombination of the 485-bp *Eco*RV−*Eco*RI fragment of the mutant gene, which contains the mutation site, with the corresponding fragment of pLGT2. The recombination was verified by restriction analysis.

Isolation of Spontaneous Revertants. E. coli W3104/pLGD66C, which carries the Asp66 → Cys mutant *tetA(B)* gene, was grown at 37 °C in 2 × YT medium (1.6% Bactotryptone, 1% yeast extract, and 0.5% NaCl) containing 50 μg/mL kanamycin until OD_{610nm} = 1.0. The cells were harvested from 1 mL of the medium and the concentrated suspension was poured onto a YT agar plate (0.8% Bactotryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) containing 50 μg/mL tetracycline. The plate was incubated at 37 °C for 5 days and then the colonies were isolated.

Measurement of Bacterial Resistance to Tetracycline. Bacterial resistance to tetracycline was measured by the agar dilution method (Yamaguchi et al., 1990b) and expressed as the minimum inhibitory concentration. IC_{50} of tetracycline against cell growth was measured in the penassay broth as drug concentrations in which the cell growth rate at the logarithmic phase decreased to half that in the absence of the drug.

Preparation of Inverted Membrane Vesicles. Cells were grown in 1 L of the minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. At the middle of the logarithmic phase, *tetA* gene expression was induced for 2 h by incubation with 0.25 μg/mL heat-inactivated chlortetracycline. 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 (Yamaguchi et al., 1990c).

Immunoblot Analysis. SDS−polyacrylamide gel electrophoresis of the inverted vesicles was followed by electroblotting of the proteins. The TetA(B) protein was detected by means of an enzyme-linked immunosorbent assay (ELISA) using an anticarboxyl-terminal peptide antibody and an Express blot assay kit (Bio-Rad), as described in a previous paper (Yamaguchi et al., 1991). The density of the ELISA bands was measured with a dual-wavelength TLC scanner, Model CS-910 (Shimazu, Kyoto, Japan).

Tetracycline 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 tetracycline uptake was initiated by the addition of 40 μL of MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, $CoCl_2$ (final concentration 50 μM), and [^{3}H]tetracycline (final concentration 10 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.

Measurement of Tetracycline Accumulation in Intact E. coli Cells. Cells were grown on 10 mL of minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. At the middle of the logarithmic phase, *tetA* gene expression was induced for 2 h by incubation with 0.25 μg/mL heat-inactivated chlortetracycline. Cells were harvested and washed once with 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and 1 mM $MgSO_4$, followed by suspension in the same buffer and adjusting the OD_{530nm} to 1.0. The cell suspension (500 μL) was preincubated with 10 mM glucose at 30 °C for 10 min, and then the uptake of tetracycline was started by addition of 2 μM [^{3}H]tetracycline. At the indicated time intervals, aliquots of the sample were withdrawn and diluted 40-fold in 5 mM MOPS-KOH buffer (pH 7.0) containing 0.15 M LiCl, followed by rapid filtration through a Millipore filter (pore size, 0.45 μm) and washed

Table 1: Tetracycline Resistance of *E. Coli* W3104 Cells Harboring the Plasmids Encoding Asp66 Mutant TetA(B)

plasmid	MIC (μg/mL)
no plasmid	0.8
pLGT2 (wild)	200
pLGD66C (Asp66 → Cys)	0.8
pLGD66A (Asp66 → Ala)	0.4
pLGD66N (Asp66 → Asn)	0.8
pLGD66E (Asp66 → Glu)	4.7

twice with the same buffer. The radioactivity of the filter was counted.

RESULTS

Effect of the Mutations of Asp66. Asp66 → Asn (D66N) and Asp66 → Glu (D66E) mutants of TetA(B) were constructed in our previous paper (Yamaguchi et al., 1990b). In addition, Asp66 → Cys (D66C) and Asp66 → Ala (D66A) mutants were constructed by site-directed mutagenesis in this work using mutagenic primers of 5′-ACCAAATC**TGCAG**-GACATTTT-3′ for D66C and 5′-GACCAAATCG**AGCGCT**-CATTTTTCC-3′ for D66A, which are complementary sequences of the coding strand; the underlined bold letters represent the mismatch with the wild type sequence. These mutant *tet* genes were subcloned into the low-copy plasmid (pLGT2) by fragment exchange. The tetracycline resistance of *E. coli* W3104 cells carrying these mutant plasmids is shown in Table 1. The mutants in which Asp66 was replaced by a neutral amino acid residue completely lost drug resistance, i.e., their MIC values were the same as, or rather lower than, that of the host cells without plasmid. On the other hand, the conserved replacement of Asp66 to Glu maintained very low but significant resistance (MIC 4.7 μg/mL), indicating that the carboxyl group is essential for the function at position 66. These findings confirmed the conclusion of our previous paper (Yamaguchi et al., 1990b).

Isolation of Spontaneous Revertants from the Asp66 → Cys Mutant. Since the first and second bases of the wild-type codon 66 (GAC) were replaced in the codon of the D66C mutant (TGC), the possibility of the spontaneous back mutation from Cys66 to Asp66 was hardly expected. When a concentrated cell suspension of *E. coli* W3104/pLGD66C was cultured on nutrient agar plates containing 50 μg/mL tetracycline at 37 °C for 5 days, 4 colonies (R1−R4) were obtained. Plasmids were prepared from these revertant strains and transfered into *E. coli* W3104 cells. Among them, only the cells transfered by pLGR2 showed moderate tetracycline resistance (MIC 50 μg/mL). In contrast, the resistance level of the *E. coli* cells transfered by pLGR1, pLGR3, or pLGR4 was very low (1.6 μg/mL), indicating that the restoration of the moderate resistance of these revertants might be due to a chromosomal mutation.

Location of the Second-Site Mutation in the tet Gene Sequence. In order to limit the range of the location of the second-site mutation, the *tet* gene of pLGR2 was separated into three fragments (Figure 1). The first 1.1-kb *Bgl*II−*Eco*RV fragment (named *PO-tet*R*) contains the promoter/operator of the *tet* operon and the whole *tetR* gene in addition to a small part of the 5′-terminal region of the *tetA* structural gene. The second 485-bp *Eco*RV−*Eco*RI fragment (named *tetA*α*) contains most of the 5′-half of the *tetA* structural gene including codon 66. The third 868-bp *Eco*RI−*Bam*HI

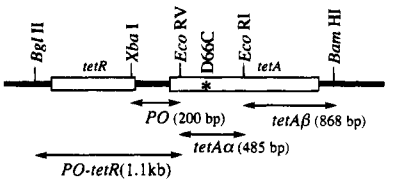

FIGURE 1: Map of the restriction sites on the *tetA(B)* genes and the fragments exchanged between pLGD66C and the revertant plasmid.

Table 2: Tetracycline Resistance of *E. coli* W3104 Cells Harboring the Recombinant Plasmids

recombinant plasmid	MIC (μg/mL)
pLGT2 (wild)	200
pLGD66C	0.8
pLGR2 (spontaneous revertant)	50
pLGD66C/*tet*Aα* [a]	0.8 ~ 25
pLGD66C/*tetAβ** [a]	0.8
pLGD66C/*tet*Aα*/*tetAβ** [a]	0.8 ~ 25
pLGD66C/*PO-tet*R* [a]	1.6
pLGD66C/*PO-tet*R*/*tet*Aα* [a]	50
pLGD66C/*PO**/*tet*Aα* [a]	50

[a] These recombinant plasmids were constructed with the exchange of the fragments of pLGD66C or pLGT2 for the corresponding fragments of pLGR2.

fragment (named *tetAβ**) contains the 3′-half of the *tetA* structural gene and the sequence downstream of the *tet* operon.

These fragments were subcloned by recombination with the corresponding fragment of pLGD66C, which encodes the site-directed Asp66 → Cys mutant TetA(B). The MIC values of *E. coli* W3104 cells transfered by these plasmids are shown in Table 2. It is clear that the second-site mutation is not localized in the *tetAβ* region because the plasmid pLGD66C/*tetAβ** was as sensitive as the host cells against tetracycline. Cells transfered by pLGD66C/*tetA*α* showed a unique phenotype, that is, although the growth was once inhibited in the presence of 0.8 μg/mL tetracycline, there was significant growth up to a tetracycline concentration of 25 μg/mL. Such a leaky growth phenotype suggests the possibility that the expression of the *tetA*α** gene hinders the cell growth, while low-level expression of the gene is enough to confer moderate drug resistance. On the other hand, the resistance level of pLGD66C/*PO-tet*R* was similar to that of pLGD66C.

The two combinations of the fragments from pLGR2 were then subcloned into pLGD66C by recombination. The resulting pLGD66C/*PO-tet*R*/*tet*Aα* plasmid showed the same moderate resistance to tetracycline as pLGR2 without a leaky growth phenotype, whereas the *tet*Aα*/*tetAβ** plasmid showed the same leaky phenotype as pLGD66C/*tet*Aα* (Table 2). Thus, the mutations contributing to the moderate resistance of the revertant seem to be located in both the *PO-tet*R* and *tet*Aα* regions. In order to restrict the possible region in the *PO-tet*R*, the 256-bp *Xba*I−*Eco*RV fragment was isolated. The fragment named *PO** contains the whole promoter/operater region of the *tet* gene, which includes the two operators, O_L and O_R, and the promoters for *tet*A and *tet*R, i.e., P_A, P_{R1}, and P_{R2} (Daniels & Bertrand, 1985), with short fragments of the 3′-termini of the *tetA* and *tet*R genes. The combination of *PO**/*tet*Aα* showed the same phenotype as the revertant, pLGR2, and

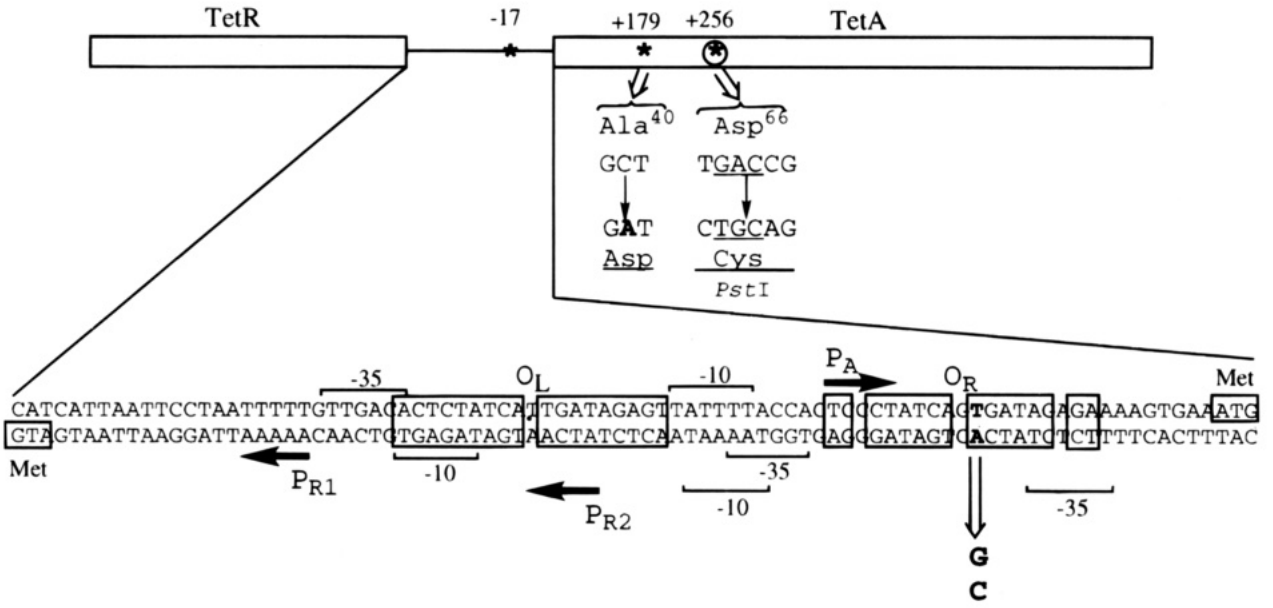

FIGURE 2: Location of the mutations on the sequence of the *tetA(B)* genes.

Table 3: Tetracycline Resistance of *E. coli* W3104 Cells Harboring the Site-Directed Second-Site Mutant Plasmids

site-directed mutant plasmid[a]	MIC[b] (μg/mL)	IC_{50}[c] (μg/mL)
D66C	0.8	0.6
A40D	3.1 ~ 25	29
A40D/D66C	0.8 ~ 25	3.7
PO*	200	25
PO*/D66C	1.6	0.5
PO*/A40D	200	24
PO*/A40D/D66C	50	4.0

[a] These plasmids were subcloned into a low copy plasmid pLGT2 by the exchange of the *Xba*I-*Eco*RI fragment of pLGT2 for the fragment of the mutant *tet* gene. [b] Minimum inhibitory concentration was obtained on agar plate. [c] IC_{50} indicates the drug concentration in which the cell growth rate decreased to half that in the absence of the drug when cells were grown in liquid medium.

*PO-tet*R*/*tetA*α* (Table 2). Therefore, the location of the third mutation must be in the *PO** region.

Determination of the DNA Sequence of the tet Gene of the Revertant. The *tet* gene of pLGR2 was subcloned into pUC119 and its DNA sequence was determined. As expected, there was no mutation in the *tetAβ** region. The sequence of the codon 66 was TGC (Cys), which was the same as pLGD66C, indicating that the revertant was not a simple back mutant to the wild-type codon 66 of GAC (Asp). The second-site mutation was found to be a change of GCT to GAT at codon 40 (Figure 2). By this single base change, Ala40 was replaced by Asp. That is, the loss of Asp at position 66 was compensated by the new gain of Asp at position 40. There was no other mutation in the *tetA*α** region.

In the *PO** region, there was only one base change from T to G at the position 17 bases upstream from the initiation codon of *tetA*. This position is located in the palindromic sequence of O_R (Figure 2). Wissmann et al. (1988) reported that a similar mutation from T to G in the corresponding position of O_L caused a decrease in the expression of the *tetA* gene.

Site-Directed Mutation of Ala40. In order to determine the role of the mutation of Ala40, site-directed mutagenesis of Ala40 → Asp was performed in both the D66C *tetA* and the wild-type *tetA* using the mutagenic primer 5′-AAGTGGTTATCGATATCTT-3′ (the underlined bold letter represents the mismatch with the wild-type sequence). The resulting mutant *tetA* genes were combined with *PO** by recombination. As shown in Table 3, the tetracycline resistance phenotype of the A40D/D66C double mutant and the *PO**/A40D/D66C triple mutant were the same as pLGD66C/*tetA*α* and pLGD66C/*PO**/*tetA*α*, respectively. Therefore, it was confirmed that the mutations suppressing the D66C mutant were a combination of the two second-site mutations of Ala40 → Asp and −17T → G.

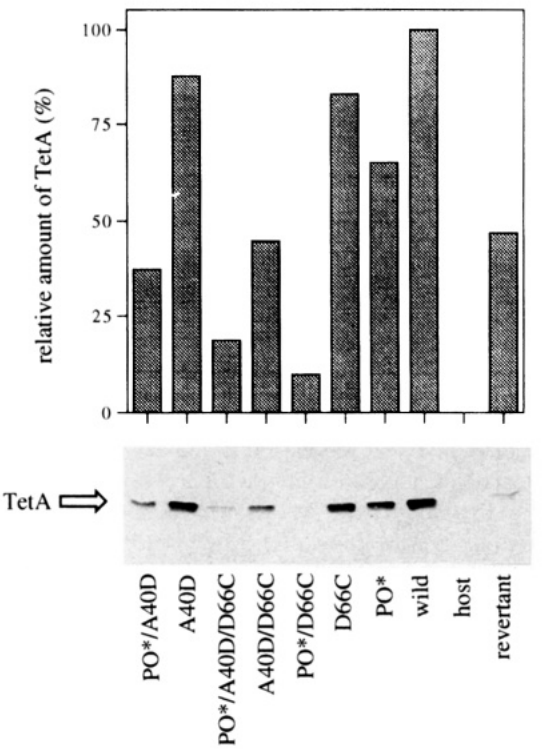

FIGURE 3: Expression of *tetA(B)* mutant gene in *Escherichia coli* W3104. The TetA protein was detected by Western blotting using anti-TetA-C-terminal-peptide antiserum. The amount of TetA in each track was calculated from a densitometer scan of the ELISA band and depicted as a percentage of the wild-type level.

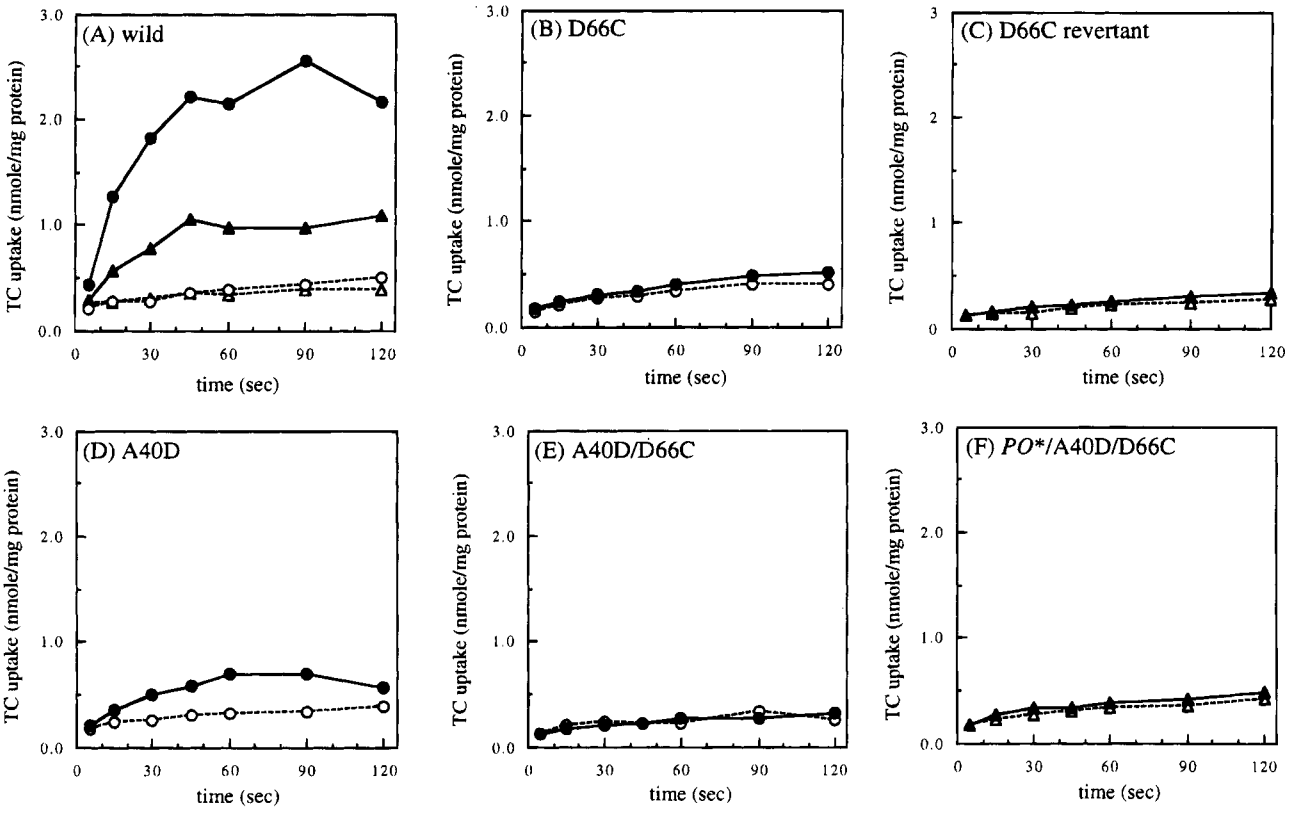

FIGURE 4: [^{3}H]Tetracycline uptake by inverted membrane vesicles containing the wild-type or mutant TetA proteins. Inverted membrane vesicles were energized with NADH and then mixed with the assay solution containing 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. Vesicles prepared from *E. coli* W3104/pLGT2 (A, circles), which carries the wild-type *tet* gene, and *E. coli* W3104/pLG*PO** (A, triangles), which carries a *tet* gene with a −17T → G mutation; *E. coli* W3104/pLGD66C (B); *E. coli* W3104/pLGR2 (C), which is a spontaneous revertant from pLGD66C; *E. coli* W3104/pLGA40D (D); *E. coli* W3104/pLGA40D/D66C (E); and *E. coli* W3104/pLG*PO**/A40D/D66C (F). Triangles indicate the *tet* genes comprising the −17T → G mutation. Closed and open symbols indicate uptake in the presence and absence of NADH, respectively.

IC_{50} values clearly showed that A40D mutation partly suppresses the effect of the D66C mutation (Table 3). It was noted that the A40D single mutant showed a leaky resistance phenotype similar to that of the A40D/D66C double mutant (Table 3). However, the IC_{50} value is approximately similar to that of the wild type. Thus, the A40D mutant is active as a resistance protein but the expression is somewhat deleterious for cell growth. The *PO** mutation caused the restoration of MIC value of the A40D mutant through reduction of expression.

Tetracycline-Induced TetA(B) Production. TetA(B) product was induced by a 2 h incubation of the mid-log-phase cells carrying the mutant plasmids with 0.25 μg/mL chlortetracycline. Then, inverted membrane vesicles were prepared from these cells. SDS-polyacrylamide gel electrophoresis of 1 μg of the total membrane protein in each lane was performed, followed by electroblotting, and then the TetA(B) proteins were detected by Western blotting using TetA(B)-C-terminal-specific antiserum (Yamaguchi et al., 1990a) (Figure 3). The amount of TetA(B) protein was calculated from the density of the ELISA band (Figure 3).

As shown in Figure 3, the *PO** mutation caused a significant decrease in the amount of TetA(B). The wild-type TetA(B) content was reduced by about 35% by the *PO** mutation. Although the contents of the D66C, A40D/D66C, and A40D mutants were originally less than the wild type, the contents of these mutants were largely decreased by the *PO** mutation to about 12%, 43%, and 42% of each original level, respectively. Therefore, it can be concluded that the effect of the −17T → G mutation was due to the reduction of *tetA* gene expression.

Tetracycline Transport in the Inverted Membrane Vesicles. [^{3}H]Tetracycline uptake by inverted membrane vesicles was measured in the presence or absence of NADH (Figure 4). The inverted vesicles prepared not only from the cells carrying pLGD66C but also from those carrying pLGR2, pLGA40D/D66C, or pLG*PO**/A40D/D66C showed no significant active uptake of tetracycline. Even if the [^{3}H]-tetracycline concentration was increased to 100 μM, the uptake was not observed (data not shown). Thus, we were unable to ascertain whether the revertants were low-affinity mutants for tetracycline. On the other hand, the A40D single mutant vesicles retained about 15% of the wild-type activity in the presence of 10 μM [^{3}H]tetracycline, indicating that the A40D mutation itself caused a significant reduction in the transport activity.

The *PO** mutation introduced into the wild-type *tet* gene also caused about a 3-fold decrease in the transport activity (Figure 4), in spite of the fact that the drug resistance level of the *PO** mutant cells was the same as that of the wild type (Table 3). The tetracycline transport activity of the *PO** mutant vesicles corresponds to the level of expression of the *tetA* gene in this mutant, which was about 40% of the wild-type level (Figure 3). These observations indicate that the resistance level is not proportional to the tetracycline transport activity, although the transport activity is propor-

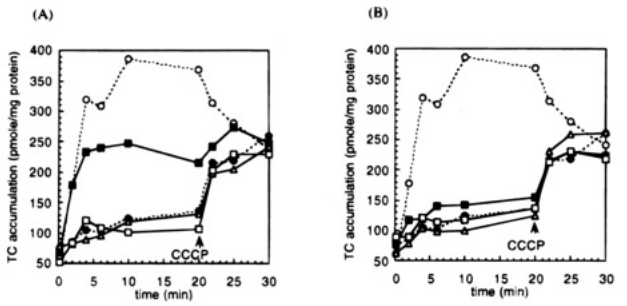

FIGURE 5: Tetracycline accumulation in intact *E. coli* cells expressing wild-type or mutant TetA proteins. Cells were energized by glucose and the tetracycline uptake was measured in the presence of 2 μM [^{3}H]tetracycline and 1 mM $MgCl_2$. Closed and open circles with dashed lines indicate that the uptake by cells producing the wild-type TetA and host cells carrying no plasmid, respectively. (A) Closed squares, *E. coli* W3104/pLGD66C; open triangles, *E. coli* W3104/pLGA40D; open squares, *E. coli* W3104/pLGA40D/D66C. (B): Closed squares, *E. coli* W3104/pLGR2; open triangles, *E. coli* W3104/pLG*PO**/A40D; open squares, *E. coli* W3104/pLG*PO**/A40D/D66C. The arrows indicate the addition of 200 μM CCCP.

tional to the amount of TetA(B) protein. This may be due to the well-known "negative gene-dosage effect" of tetracycline resistance determinants (Moyed et al., 1983).

Tetracycline Accumulation in Intact cells. [^{3}H]Tetracycline accumulation in intact cells was measured as described under Experimental Procedures. In the host cells carrying no plasmid, [^{3}H]tetracycline was accumulated up to about 400 pmol/mg of cell protein. Since the accumulated [^{3}H]-tetracycline in the host cells leaked out when an uncoupler, CCCP, was added (Figure 5), this indicates that the accumulation was dependent on the ΔpH. In contrast, for the cells carrying the plasmids encoding wild-type TetA-(B), the accumulation settled down to about a 4-fold lower level than the host cells. The settling down of the accumulation was due to the active efflux of tetracycline because the addition of CCCP caused an increase in the accumulation, contrary to the case of the host cells.

The D66C mutant cells showed an intermediate level of tetracycline accumulation between host cells and that of the wild-type TetA(B) cells, and the addition of CCCP caused no significant effect (Fig. 5), indicating that the concentration of tetracycline in the cell interior of these mutant cells was in equilibrium with the concentration in the medium. On the other hand, A40D/D66C mutant cells clearly showed an actively lowered level of accumulation similar to that of the wild-type TetA(B) cells. The revertant cells and the cells producing *PO**/A40D/D66C mutant TetA(B) also showed the same results as the ones producing the A40D/D66C mutant TetA(B). The addition of CCCP caused an increase in the tetracycline accumulation in these double mutant cells. These observations indicate that the active efflux of tetracycline out of the intact cells was surely restored by A40D mutation in the D66C mutant.

DISCUSSION

The defective drug resistance by the loss of an acidic residue at position 66 was partially restored by the gain of the same acidic residue at position 40 with the help of a mutation in the operator region. Figure 6 shows the putative secondary structure of TetA(B), which contains 12 transmembrane segments connected by hydrophilic loops (Eckert & Beck, 1989b). Asp66 is located in cytoplasmic $loop_{2-3}$ and postulated to play a role in the initial interaction with the monocationic substrate, metal-tetracycline chelation complex (Yamaguchi et al., 1993a). Surprisingly, the

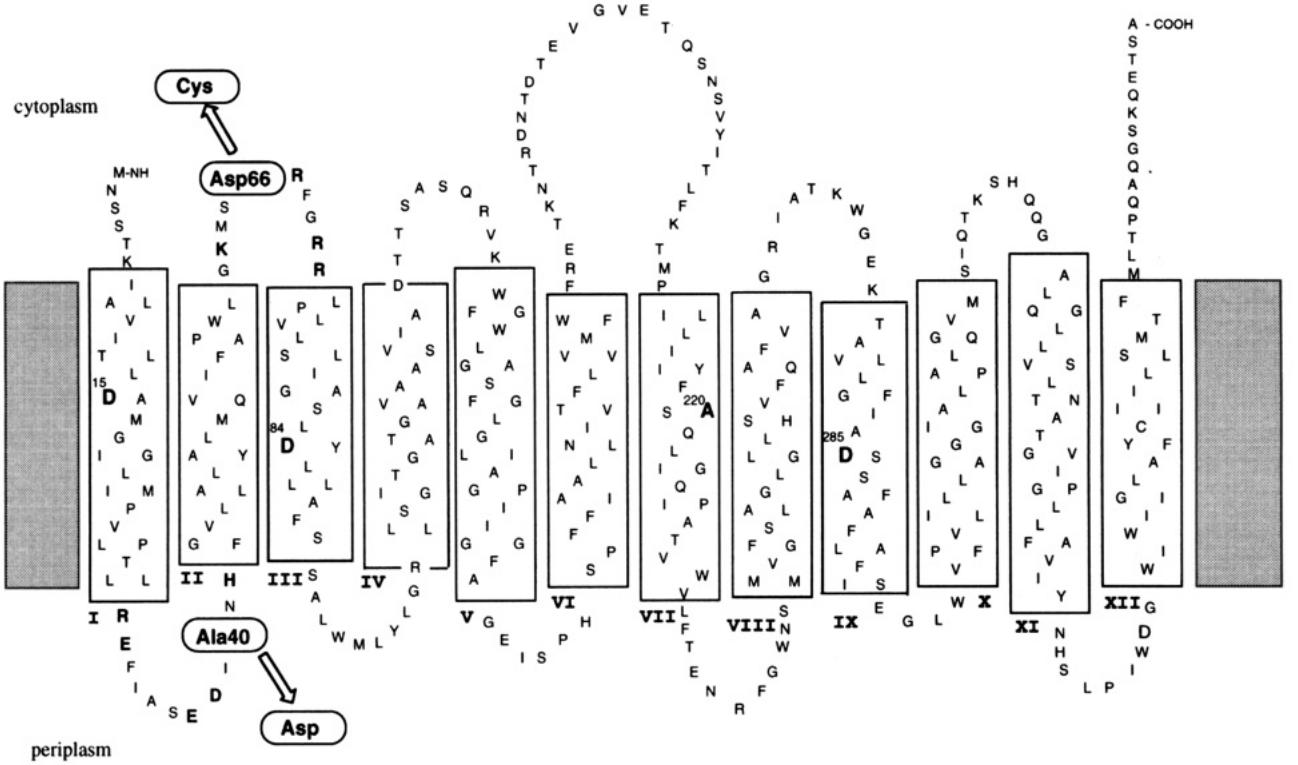

FIGURE 6: Putative secondary structure of TetA(B). Functionally essential Asp residues in the putative transmembrane region and Ala220, which is a position of the second-site mutation suppressing the Asp285 → Asn mutation, are depicted by bold letters with numbers representing their positions. Charged residues located in $loop_{1-2}$ and $loop_{2-3}$ are depicted with bold letters without numbers. The mutation sites in this study are enclosed.

second-site suppressor mutation, Ala40 → Asp, was located in the putative periplasmic $loop_{1-2}$ (Figure 6). Since the putative topology shown in Figure 6 is supported by a lot of experimental evidence, such as protease digestion (Eckert & Beck, 1989b), antibody binding (Eckert & Beck, 1989b; Yamaguchi et al., 1990a), alkaline phosphatase fusion experiments (Allerd & Bertrand, 1992), and *N*-ethylmaleimide binding to the Cys-scanning mutants of TetA(B) (our unpublished observation), it is very unlikely that Ala40 is placed sterically close to Asp66. Therefore, it would seem impossible for Asp40 to directly substitute for the Asp66 function.

The A40D/D66C mutant conferred a moderate level of resistance; however, the expression was deleterious for cell growth. The mutation of −17T → G in the O_R region helped in the restoration of the resistance through the reduction of the expression to a low but sufficient level for moderate resistance. The expression of the A40D single mutant was also deleterious for cell growth and the mutation in O_R region caused the restoration of the wild-type resistance through a reduction in the A40D expression. It is well-known that the overexpression of the *tetA(B)* gene is lethal for bacterial cells due to the loss of membrane potential (Eckert & Beck, 1989a) and that the *tetA(B)* gene shows a negative gene dosage effect (Coleman & Foster, 1981; Moyed et al., 1983); that is, the *tetA(B)* gene encoded in the low-copy plasmid confers higher drug resistance than that encoded in the multicopy plasmid. Thus, the expression of the wild-type TetA(B) was originally somewhat deleterious for cell growth and the expression for the low-copy plasmid is enough to provide maximal activity. It seems that the A40D mutation strengthened such a characteristic of TetA(B).

In our previous paper (Yamaguchi et al., 1993b), we reported that the Asp285 → Asn mutation of TetA(B) was suppressed by the second-site Ala220 → Glu mutation. In that case, both positions are located in the middle of the putative transmembrane helices. Although Asp285 and Ala220 are placed in different helices, 9 and 7, respectively, it is possible to postulate that both helices are sterically close to each other and compose the substrate-translocation pathway. Since the A220E/D285N double mutant showed a similar phenotype to the wild-type TetA(B), Glu220 simply substituted for the Asp285 function. In contrast, as described above, the role of Asp40 was unlikely to be a simple substitution for the Asp66 function. The Ala40 → Asp mutation probably mediates its effects through a structural perturbation propagated along the polypeptide backbone. Such a perturbation may allow TetA(B) to translocate tetracycline without the initial substrate-carrier interaction residue.

$Loop_{1-2}$ is rich in acidic residues. It contains three acidic residues, Glu32, Glu37, and Asp38. None of these acidic residues was functionally essential because single mutants of each of these residues to a neutral residue caused no effect on transport activity (Yamaguchi et al., 1992a). Thus, it is surprising that the Ala40 → Asp mutation caused such a large effect on TetA(B) function, especially when one considers that Asp38 is very close to Ala40. However, Cys-scanning mutation experiments have revealed that each position in a loop region is generally unique. For example, the Ser65 → Cys mutant was completely inactivated by NEM but no other Cys mutants around Ser65 were affected by NEM (Yamaguchi et al., 1992c).

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