Remote Conformational Effects of the Gly-62 → Leu Mutation of the Tn*10*-Encoded Metal-Tetracycline/H^+ Antiporter of *Escherichia coli* and Its Second-Site Suppressor Mutation†

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ABSTRACT: Substitution of Gly-62 of the Tn*10*-encoded metal-tetracycline/H^+ antiporter caused a functional defect corresponding to the volume of the substituent [Yamaguchi, A., Someya, Y., and Sawai, T. (1992) *J. Biol. Chem. 267*, 19155−19162]. A spontaneous revertant exhibiting tetracycline resistance was isolated from *Escherichia coli* cells carrying the *tetA*(B) gene encoding the G62L mutation. The revertant showed a second-site mutation at codon 30 of TTA (Leu) to TCA (Ser). Site-directed mutagenesis studies revealed that the L30S mutation could suppress the effects of the G62A, G62C, G62N, and G62V mutations as well as the G62L mutation. Positions 62 and 30 are located in hydrophilic loops estimated to be in the cytoplasm and periplasm, respectively. Their sidedness was confirmed by the fact that, in intact cells, the [^{14}C]*N*-ethylmaleimide (NEM) binding to the G62C mutant was not affected by preincubation with a membrane-impermeant sulfhydryl reagent, whereas the binding to the L30C mutant was blocked by the reagent. The reactivity of the L30C and L29C mutants with [^{14}C]NEM was drastically decreased when Gly-62 was replaced by Leu, indicating that the residues around position 30 became embedded in the intramembrane region due to the remote conformational effect of the G62L mutation across the membrane. Moreover, the reactivity of the L29C/G62L mutant with [^{14}C]NEM was restored with the L30S mutation. These results clearly indicate that the second-site suppression by the L30S mutation was based on the blocking of the remote conformational change around position 30 across the cell membrane caused by the G62L mutation.

The metal-tetracycline/H^+ antiporter [Tet(B)][1] is an intrinsic inner membrane protein of *Escherichia coli* encoded by Tn*10* (*1*−*3*). It actively effluxes tetracyclines using the H^+ concentration gradient across the membrane as an energy source (*4*−*6*). This transporter is thought to be composed of 12 transmembrane segments as well as pBR322-encoded Tet(C), the topology being confirmed by partial protease digestion (*7*), an alkaline phosphatase fusion experiment (*8*), and site-directed chemical labeling of cysteine-introduced mutants (*9*). The tetracycline/H^+ antiporters belong to the major facilitator superfamily that has putative 12 transmembrane domains in common. The transporters in this family have a conserved sequence motif, GXXXDRXGRR, in the first cytoplasmic loop, loop2−3 (*10*). We first investigated the role of this sequence motif by the combination of localized random and site-directed mutageneses in order to replace each amino acid residue in loop2−3 (*11*). Among the 10 residues, the important ones, of which substitution resulted in significant reduction or complete loss of the transport activity, were Gly-62, Asp-66, Gly-69, and Arg-70. With respect to Asp-66 and Arg-70, the negatively and positively charged side chains, respectively, were essential for the transport function because none of these mutants except for the charge-conserved ones showed significant activity (*11*, *12*). Our findings were confirmed by similar observations for α-ketoglutarate permease (*13*) and Tet(C) protein (*14*). In the case of lactose permease (*15*), the first position Gly and fifth position Asp were important but none of the basic residues were individually important for transport. On the other hand, when Gly-69 was substituted by several other amino acid residues, the residual activity decreased in inverse proportion to the β-turn propensity of the introduced amino acid residues (*11*), indicating that Gly-69 may contribute to the β-turn structure of the peptide backbone of loop2−3. As to Gly-62, which is located at the boundary between transmembrane segment 2 and loop2−3 (Figure 1), no mutants showed transport activity except for the Gly-62 → Ala one, which retained only less than 5% of the wild-type activity, suggesting that there is little room for the bulky side chain around position 62.

In this study, we found that the effect of a mutation at position 62 was suppressed by a second-site mutation at position 30, which is on the opposite side to position 62 across the membrane. The direct interaction between the side chains at positions 62 and 30 is impossible because the separation of these two positions by the cytoplasmic membrane was verified by the difference in the reactivity of the cysteine mutants as to these positions to membrane-impermeant and membrane-permeant sulfhydryl reagents. In this

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[1] Abbreviations: AMS, 4-acetamido-4′-dimaleimidylstilbene-2,2′-disulfonic acid; MIC, minimum inhibitory concentration; MOPS, 3-(morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; SDS, sodium dodecylsulfate.

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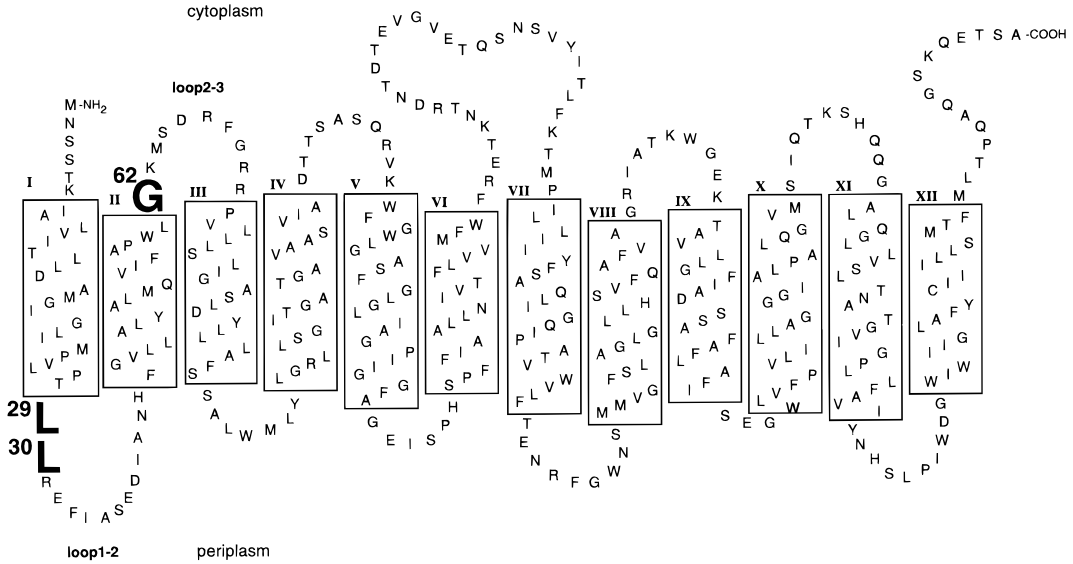

FIGURE 1: Putative secondary structure of the Tet(B) protein. The secondary structure was deduced by hydropathic analysis and from the results of limited proteolysis (*7*), immunological examinations (*24*), analysis of the reactivity of cysteine-scanning mutants with [^{14}C]NEM (*27*), and analysis of cysteine-introduced mutants with a membrane-impermeant sulfhydryl reagent (*9*). Bold letters indicate the mutation sites detected or introduced in this study.

study, we investigated the cause of the second-site suppressor mutation by the site-specific chemical labeling of cysteine-introduced mutants of Tet(B).

EXPERIMENTAL PROCEDURES

Materials. *N*-[*Ethyl*-1-^{14}C]maleimide (1.5 GBq/mmol) was purchased from DuPont-New England Nuclear. 4-Acetamido-4′-dimaleimidylstilbene-2,2′-disulfonic acid (AMS) and Amplify were from Molecular Probes, Inc. (Oregon), and Amersham (Buckinghamshire, U.K.), respectively. All other materials were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* TG1 (*16*) was used for plasmid isolation and W3104 (*17*) for the determination of tetracycline resistance and preparation of everted membrane vesicles. pCT1183 (*18*) and pLGT2 (*19*) are high-copy-number and low-copy-number plasmids, respectively, which carry the 2.45 kb *Tn10-tetA* and *tetR* gene fragments. pCT1183 was used as a template for site-directed mutagenesis. pLGT2 was used for expression of the *tetA*(B) gene. pLGG62L, pLGG62A, pLGG62N, and pLGG62V are derivatives of pLGT2, which were constructed by site-directed mutagenesis in our previous study (*11*).

Isolation of Revertants of the G62L Mutant. *E. coli* TG1 cells harboring plasmid pLGG62L, which exhibited no tetracycline resistance, were grown in 2×YT (1% yeast extract, 1.6% tryptone, 0.5% NaCl) broth to the late logarithmic phase and then a 1.5 mL portion was spread on a YT agar plate containing 10 μg/mL of tetracycline. After 4 days of incubation at 37 °C, colonies were isolated.

Determination of Tetracycline Resistance. Tetracycline resistance were determined by the agar dilution method and expressed as the minimum inhibitory concentration (MIC) as described previously (*20*) at least twice for each mutant to make sure the reproducibility of the MIC values.

Site-Directed Mutagenesis. Site-directed mutants were constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (*21*) using mutagenic primers having a complementary sequence and single-stranded DNA of pCT1183 as a template. The sequences of the primers were 5′-ATTTTGCAAAGCCATGGAGC-3′, 5′-TAAATTCGCGACATAACGTT-3′, 5′-TAAATTCGCGATTTAACGTT-3′, 5′-ATAAATTCGCGAAGGCACGTTGGCA-3′, and 5′-GCAATAAATTCTCTCGAGCACGTTGGCA-3′, which were used for creating the G62C, L30C, L30N, L29C, and L29C/L30S mutants, respectively. The underlining indicates mismatches. Mutations were detected as the appearance of a newly introduced restriction site and then verified by DNA sequencing with a Shimadzu DNA sequencer DSQ-1000. The mutations of the *tetA*(B) gene were then transferred to pLGT2 or a derivative of it, pLGG62L or pLGG62L-R1, which carry a G62L or G62L/L30S mutation(s), respectively, by *Bgl*II−*Eco*RV or *Eco*RV−*Eco*RI DNA fragment exchange in order to construct single, double, or triple mutants. The entire DNA sequences within the region of these fragments were confirmed by DNA sequencing.

Preparation of Everted Membrane Vesicles. Everted membrane vesicles were prepared from *E. coli* W3104 cells carrying a low-copy-number plasmid, pLGT2 or one of its derivatives encoding mutations, as described previously (*19*).

Assaying of the Reaction of [^{14}C]N-Ethylmaleimide with Tet(B) Proteins. The [^{14}C]NEM-binding experiment was performed as described previously (*22*, *23*). Briefly, everted membrane vesicles were incubated with 0.5 mM *N*-[1-^{14}C]-ethylmaleimide for 5 min at 30 °C, and then the reaction mixture was diluted with the same buffer containing 5 mM unlabeled NEM, followed by ultracentrifugation. The resultant pellet was solubilized with 1% Triton X-100 and 0.1% SDS. Tet(B) proteins were immunoprecipitated with anti-Tet(B)-carboxyl-terminal peptide antiserum (*24*) and PANSORBIN (*25*). The immunoprecipitates were subjected to SDS−polyacrylamide gel electrophoresis. Then gels were soaked in Amplify prior to being dried. The dried gels were exposed to imaging plates for 2 days and then examined with a BAS-1000 Bio-Imaging Analyzer (Fuji Film Co., Tokyo, Japan).

Table 1: Tetracycline Resistance Levels of *E. coli* Cells Harboring the Plasmid Encoding the Wild-Type, the G62L Mutant, or a Revertant of It

plasmid	minimum inhibitory concentration (MIC) of tetracycline (μg/mL)
pLGT2 (wild-type)	200
no plasmid	0.8
pLGG62L	1.6
pLGG62L-R1	100

Assaying of Protection of the [^{14}C]NEM Binding of the Cysteine Mutants by AMS in Intact Cells. The L30C and G62C mutants were examined as to whether or not the labeling of cysteine residues with [^{14}C]NEM is protected by AMS or not, as described previously (*9*). *E. coli* W3104 cells harboring a plasmid, pLGL30C or pLGG62C, were grown in the minimal medium and then *tetA*(B) gene expression was induced with 0.25 μg/mL of heat-inactivated chlortetracycline (*26*) for 2 h. The cells were harvested and washed with 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, followed by suspension in the same buffer. The cell suspension was incubated with 5 mM AMS for 30 min at 30 °C. Then 0.5 mM [^{14}C]NEM was subsequently added to the reaction mixture, followed by incubation for another 5 min. After dilution with buffer containing an excess amount of unlabeled NEM, the cells were collected and washed with the same buffer. Then the cells were disrupted by brief sonication. After the removal of unbroken cells, the membrane fraction was collected by ultracentrifugation. The resultant precipitate was solubilized and Tet(B) proteins were immunoprecipitated as described above.

RESULTS

Isolation of Second-Site Revertants. Although the Gly-62 → Leu (G62L) mutant Tet(B) did not contribute to the tetracycline resistance at all (*11*), seven colonies (R1−R7) were isolated when *E. coli* cells carrying plasmid pLGG62L were grown on agar plates containing 10 μg/mL of tetracycline for 4 days as described under Experimental Procedures. Among them, five clones (R1−R5) showed resistance to 50 μg/mL of tetracycline, while the other two clones (R6 and R7) could not grow on agar plates containing 50 μg/mL of tetracycline. The plasmid DNAs of these clones were isolated and transferred to *E. coli* W3104 cells. The tetracycline resistance of the transformant with the plasmid isolated from each clone was examined again. Among these transformants, only R1 showed significant tetracycline resistance (100 μg/mL of MIC; Table 1), the other transformants showing very low resistance, between 0.2 and 6.3 μg/mL. It is likely that the restoration of the tetracycline resistance of the clones except for R1 was due to the chromosomal mutation because the resistance could not be transferred by the plasmids. Hence, pLGG62L-R1 was used for the following analysis.

In order to determine the position of the mutation site causing the restoration of the drug resistance of the G62L mutant, the *Eco*RV−*Eco*RI (485 bp), *Xba*I−*Bam*HI (1580 bp), and *Bgl*II−*Eco*RI (1580 bp) DNA restriction fragments containing various parts of the *tetA*(B) and/or *tetR* gene(s) of pLGG62L-R1 were exchanged with the corresponding fragments of the parent plasmid, pLGG62L (Figure 2A). The transformants with the resultant recombinant plasmids showed resistance to 0.8, 50, and 100 μg/mL of tetracycline,

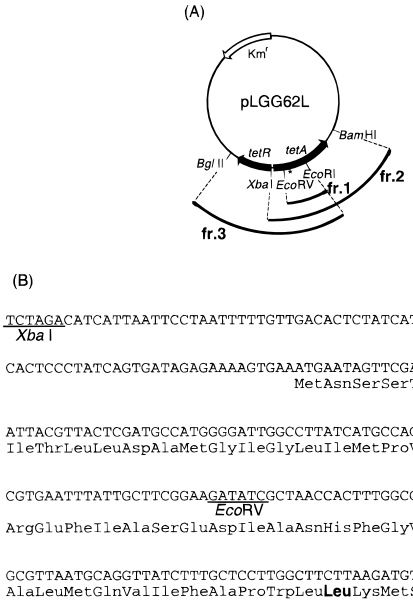

FIGURE 2: (A) Restriction map of pLGG62L containing the G62L mutant *tet* genes. The DNA restriction fragments used for the fragment exchange were the *Eco*RV-*Eco*RI (485 bp), *Xba*I−*Bam*HI (1580 bp), and *Bgl*II−*Eco*RI (1580 bp) fragments, which are designated as fr. 1, fr. 2, and fr. 3, respectively. (B) The entire DNA sequence between the *Xba*I and *Eco*RV restriction sites with a short downstream sequence including codon-62 of the *tet* gene encoded in pLGG62L-R1.

respectively. Therefore, it is clear that the revertants were due to the second-site suppressor mutation because the second mutation was not localized between the *Eco*RV and *Eco*RI sites, in which the Gly-62 → Leu mutation was encoded. In addition, the region containing the second-site suppressor mutation could be restricted between *Xba*I and *Eco*RV sites because only this region overlapped the *Xba*I−*Bam*HI and *Bgl*II−*Eco*RI fragments and did not contain the *Eco*RV−*Eco*RI fragment (Figure 2A). The *Xba*I−*Eco*RV region contains the N-terminals of the TetR and Tet(B) protein-coding regions and their promotor/operator regions. The DNA sequence around this region was determined. The second-site mutation was found at codon 30 of the *tetA*(B) gene, in which one base change from T to C was generated. As a result, codon 30 was changed from TTA (Leu) to TCA (Ser), while the G62L mutation was not changed (Figure 2B). No other mutations were found in this region. The amino acid residue at position 30 is located at the boundary between helix I and periplasmic loop1−2 in the putative secondary structure (Figure 1). It may be surprising that the first G62L mutation and the second L30S suppressor mutation were localized on opposite sides of the membrane.

Sidedness of Gly-62 and Leu-30. Two different possibilities for the second-site suppressor mutation could be considered. (1) The predicted topology is incorrect, and positions 30 and 62 are close to each other on the same side of the membrane, and (2) the mutations at positions 30 and 62 had a mutual effect through the remote conformational change across the membrane. In order to determine which possibility is the case, a cysteine residue was introduced at position 30 or 62. Then, it was examined whether or not the binding of [^{14}C]NEM to Cys-30 and Cys-62 was prevented or not by preincubation of intact cells expressing the mutant *tetA*(B) genes with a membrane-impermeant maleimide, AMS. As shown in Figure 3, preincubation with AMS prevented the binding of [^{14}C]NEM to the L30C

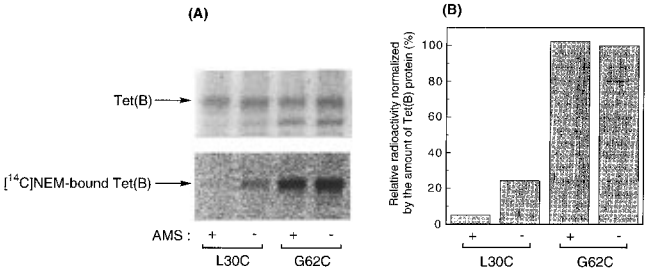

FIGURE 3: Effect of a membrane-impermeant maleimide, AMS, on the binding of [^{14}C]NEM with Cys-30 and Cys-62. *E. coli* cells harboring plasmid pLGL30C or pLGG62C were incubated with 5 mM AMS for 30 min at 30 °C, followed by incubation with 0.5 mM [^{14}C]NEM for 5 min. Then the cells were solubilized, and the radiolabeled Tet(B) proteins were immunoprecipitated as described under Experimental Procedures. After electrophoresis, the radioactive bands were visualized and quantitated with a Bio-Imaging Analyzer BAS-1000. (A) Upper panel, Coomassie Brilliant Blue staining of the SDS−polyacrylamide gel; lower panel, radioactive bands on the gel. (B) The amount of bound [^{14}C]NEM normalized by the amount of Tet(B) protein. The values are indicated as percentages relative to the value of G62C in the absence of AMS. The amount of Tet(B) protein was determined from the densitiy of Coomassie Brilliant Blue-stained bands in the upper panel of (A).

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

minimum inhibitory concentration of tetracycline (μg/mL)	Leu-30 (wild-type)	Ser-30	Cys-30	Asn-30
Gly-62 (wild-type)	200	200	100	50
Leu-62	1.6	100	12.5	6.3
Ala-62	25	100	nc[a]	nc
Cys-62	12.5	100	nc	nc
Asn-62	1.6	6.3	nc	nc
Val-62	0.2	100	nc	nc

[a] nc, not constructed.

mutant, whereas it did not affect the binding to the G62C mutant, indicating that position 30 is located on the outside surface of the cytoplasmic membrane, while position 62 is on the cytoplasmic side. This confirmed the predicted structure of the Tet(B) protein. Thus, the mutual effect of mutations at positions 62 and 30 should be a remote conformational one.

Site-Directed Mutagenesis of Gly-62 and Leu-30. Gly-62 was replaced by Ala, Asn, Val, and Leu in our previous study (*11*). Among them, the G62A mutant showed very low but significant tetracycline transport activity, while no other mutants showed transport activity. As shown in Table 2, mutations at position 62 to amino acid residues having a bulky side chain, such as Leu, Val, or Asn, caused complete loss of the drug resistance, while with mutations to small ones such as Ala or Cys, moderate resistance was retained. In order to determine whether or not the suppression of the G62L mutation by the remote Leu-30 → Ser (L30S) mutation depends on the characteristics of the side chain at position 62, various Gly-62/Leu-30 double mutants were constructed on the basis of the L30S mutant through exchange of the *Eco*RV−*Eco*RI DNA fragment of the *tetA*(B) gene encoding the G62A, G62C, G62N, or G62V mutation with the corresponding fragment of pLGG62L-R1. All the resultant double mutants, except for the G62N/L30S one, showed high level resistance (MIC, 100 μg/mL) comparable to that of the wild-type (Table 2). The G62N/L30S mutant showed only low level resistance (MIC, 6.3 μg/mL), but the level

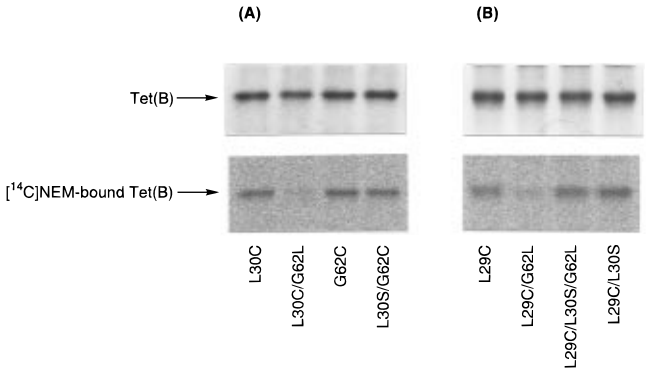

FIGURE 4: (A) Binding of [^{14}C]*N*-ethylmaleimide to cysteine residues introduced at positions 30 and 62. Everted membrane vesicles were prepared from cells harboring pLGL30C, pLGL30C/G62L, pLGG62C, or pLGL30S/G62C. The vesicles were incubated with 0.5 mM [^{14}C]NEM for 5 min at 30 °C. Radioactive bands were visualized with a Bio-Imaging Analyzer BAS-1000. (B) Binding of [^{14}C]NEM to cysteine residues introduced at position 29 of Tet(B). Everted membrane vesicles were prepared from cells harboring pLGL29C, pLGL29C/G62L, pLGL29C/L30S/G62L, or pLGL29C/L30S. Upper panel, Coomassie Brilliant Blue-staining of the SDS-polyacrylamide gel; lower panel, radioactive bands on the gel.

was higher than that of the G62N single mutant (MIC, 1.6 μg/mL; Table 2). These results indicate that the L30S mutation could suppress any mutations at position 62 almost independently of the characteristics of the side chain introduced at position 62.

Next, in order to determine what characteristics of a side chain introduced at position 30 contribute to the suppression of the G62L mutation, L30C and L30N mutants were constructed by site-directed mutagenesis. L30C/G62L and L30N/G62L double mutants were constructed by replacing the *Bgl*II−*Eco*RV DNA fragment of pLGG62L with the corresponding fragments of the L30C and L30N mutant *tetA*(B) genes. The resulting L30C/G62L and L30N/G62L mutants exhibited only low levels of tetracycline resistance (MIC, 12.5 and 6.3 μg/mL, respectively), suggesting that replacement with a residue having a small and hydrophilic side chain is needed for full level suppression of the G62L mutation.

Remote Conformational Change around Position 30 Caused by the G62L Mutation. We have reported that the reactivity of [^{14}C]NEM to a cysteine residue introduced into the Tet(B) protein is a good measure of the degree of exposure of the side chains to the medium (*27*). A cysteine residue embedded in the hydrophobic region hardly reacts with NEM, while one in the water-exposed region shows high reactivity with NEM, since the reactivity of maleimides with sulfhydryl groups depends on the degree of deprotonation of the sulfhydryl groups. In order to determine whether or not the G62L and L30S mutations mutually affected the local conformation around these positions, the reactivity of [^{14}C]NEM with the L30C, L30C/G62L, G62C, and L30S/G62C mutants was examined by autoradiography of the immunoprecipitated mutant Tet(B) proteins after incubation with [^{14}C]NEM for 5 min as described under Experimental Procedures. Since the L30C single mutant conferred tetracycline resistance comparable to that of the wild-type (Table 2), it is likely to retain the wild-type conformation. The L30C mutant was easily labeled with [^{14}C]NEM (Figure 4A, lane 1), indicating that position 30 is exposed to the aqueous phase. Interestingly, the G62L

Table 3. Tetracycline Resistance Levels of *E. coli* Cells Harboring the Plasmids Encoding the L29C Mutant Tet(B) or Derivatives of It

mutant	MIC of tetracycline (μg/mL)
L29C	300
L29C/G62L	6.3
L29C/L30S/G62L	50
L29C/L30S	25

mutation caused a drastic decrease in the reactivity of Cys-30 with [^{14}C]NEM (Figure 4A, lane 2), suggesting that the residue at position 30 was embedded in the membrane, probably due to the remote conformational effect of the G62L mutation. The reactivity of Cys-62 with NEM was not affected by the Leu-30 → Ser mutation (Figure 4A, lanes 3 and 4), although the possibility of a local conformational change without embedding of residue-62 caused by the L30S mutation could not be excluded.

In order to determine whether or not the conformational change caused by the G62L mutation was restricted to position 30, L29C and L29C/G62L mutants were also constructed. The L29C mutant retained the wild-type tetracycline resistance, similar to the L30C mutant (Table 3). The residue at position 29 is also exposed to the aqueous phase because the reactivity of Cys-29 with [^{14}C]NEM was high like that of Cys-30 (Figure 4B, lane 1). The G62L mutation also caused a drastic decrease in the reactivity of Cys-29 with NEM (Figure 4B, lane 2), suggesting a remote conformational change due to the G62L mutation, which also caused the embedding of the residue at position 29 in the membrane interior.

Blocking of the Remote Conformational Change by the Second-Site Suppressor Mutation. The effect of the L30S mutation on the local conformation around position 30 was examined using the reactivity of Cys-29 with [^{14}C]NEM as a conformational indicator. As shown in Figure 4B, the reactivity of Cys-29 in the L29C/G62L double mutant was restored by the L30S mutation (Figure 4B, lane 3). The L30S mutation did not affect the degree of the reactivity of Cys-29 in the L29C/L30S double mutant (Figure 4B, lane 4). Out of the four mutants listed in Figure 4B, only the L29C/G62L double one almost completely lost the resistance, while the L29C/L30S/G62L and L29C/L30S mutants retained moderate resistance (Table 3). Therefore, it seems that the residual resistance level corresponds to the degree of exposure of the residues at positions 29 and 30. These results indicate that the second-site suppression caused by the mutation at position 30 is based on the effect of blocking of the deleterious remote conformational change caused by the G62L mutation.

DISCUSSION

In this study, we found that the Gly62 → Leu mutation, which was introduced at the boundary between cytoplasmic loop2−3 and transmembrane helix II of Tet(B), caused a remote conformational effect across the membrane resulting in a shift of the residues around position 30, which is predicted to be localized at the boundary between periplasmic loop1−2 and helix I, from the water-extruding region to the membrane-embedded region. The second-site suppressor mutation, Leu30 → Ser, blocked the shift caused by the remote conformational effect of the G62L mutation and the conformation remained normal in the L30S/G62L double mutant.

Second-site suppressor mutants are possibly very useful for elucidation of the functional role and the mutual interaction between separate residues if a reasonable mechanism for the second-site suppression could be determined. However, experimental verification of the mechanism of second-site suppression is sometimes very difficult. For example, a mutant introduced into the putative loop region on the matrix side of a yeast mitochondrial ADP/ATP carrier could be suppressed by second-site mutations occurring on the opposite side across the membrane (*28*). We found that the Asp-66 → Cys mutation of Tet(B) was suppressed by the mutation of Ala-40 to Asp (*29*). Asp-66 is located in cytoplasmic loop2−3 like Gly-62, and the negative charge of the side chain is essential for the tetracycline transport function, probably through the initial interaction with a positively charged substrate (*20, 30*). On the other hand, Ala-40 is located in periplasmic loop1−2. Therefore, it seems to be a general rule that a mutation introduced into cytoplasmic loop2−3 is suppressed by a second-site mutation occurring in periplasmic loop1−2, indicating the close relationship between loop2−3 and loop1−2 of Tet(B) across the membrane. Recently, Jessen-Marshall and Brooker (*31*) reported another example of transmembrane second-site suppression in lactose permease of *E. coli*. They found that one of the second-site suppressor mutations against the mutation at Asp-68, which corresponds to Asp-66 of Tet(B), was located in loop 1−2. They discussed the possibility that the movement of the helix connected with the loops was caused by the mutations. However, there has been no experimental verification of transmembrane second-site suppression hitherto. This manuscript is the first case of clear experimental verification for the cause of a transmembrane second-site suppression to the best of our knowledge.

Gly-62 is highly conserved not only in bacterial drug exporters but also symporters and antiporters belonging to a major facilitator family (*10*). When Gly-62 was replaced by any other amino acid except for Ala, the transport activity was completely lost (*11*), suggesting that the space for the side chain at position 62 is very small. Thus, a bulky side chain introduced at position 62 causes rearrangement of the surrounding side chains and probably also the main chain, resulting in the twisting and/or shifting of connecting helix II and adjacent helices I and III and finally causes a shift of residues around the boundary between the periplasmic loops and the transmembrane helices toward the membrane-embedded region. Helices I and III contain functionally important aspartic residues, Asp-15 and Asp-84, respectively (*19*). Helix II contains two important residues, Tyr-50 and Gln-54, composing the conserved quartet related to substrate recognition (*32*). Therefore, the rearrangement or shifting of the relative positions of these residues caused by the remote conformational effect of the G62L mutation resulted in a defect of transport activity. The second-site mutation at position 30 from Leu to Ser acts as "a conformational hook" to block a series of such deleterious remote conformational changes.

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