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Second-site Suppressor Mutations for the Serine 202 to Phenylalanine Substitution within the Interdomain Loop of the Tetracycline Efflux Protein Tet(C)*

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Frederic M. Sapunaric and Stuart B. Levy‡

From the Center for Adaptation Genetics and Drug Resistance and the Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

The serine 202 to phenylalanine substitution within the cytoplasmic interdomain loop of Tet(C) greatly reduces tetracycline resistance and efflux activity (Saraceni-Richards, C. A., and Levy, S. B. (2000) *J. Biol. Chem.* 275, 6101–6106). Second-site suppressor mutations were identified following hydroxylamine and nitrosoguanidine mutagenesis. Three mutations, L11F in transmembrane 1 (TM1), A213T in the central interdomain loop, and A270V in cytoplasmic loop 8–9, restored a wild type level of resistance and an active efflux activity in *Escherichia coli* cells bearing the mutant *tet*(C) gene. The Tet S202F protein with the additional A270V mutation was expressed in amounts comparable with the original mutant, whereas L11F and A213T Tet(C) protein mutants were overexpressed. Introduction of each single mutation into the wild type *tet*(C) gene by site-directed mutagenesis did not alter tetracycline resistance or efflux activity. These secondary mutations may restore resistance by promoting a conformational change in the protein to accommodate the S202F mutation. The data demonstrate an interaction of the interdomain loop with other distant regions of the protein and support a role of the interdomain loop in mediating tetracycline resistance.

One of the major bacterial protections against the growth inhibitory action of tetracycline is exporting the drug out of the cytoplasm, thus preventing its reaching the ribosome target (2–4). Tet(B) and Tet(C) are cytoplasmic membrane proteins belonging to the major facilitator superfamily (MFS) (5) that maintain intracellular tetracycline concentrations below inhibitory levels via the energy-dependent antiport of H^+ and a divalent metal ion-tetracycline complex (6–8). Members of this group, which includes uniporters, symporters, and antiporters, share a common topology as well as regions of amino acid sequence identity (9). The membrane topologies of Tet(B) and Tet(C) have been studied by proteolysis (10, 11), chemical labeling (12–14), circular dichroism spectral analysis (15), and gene fusion (16). Based on these studies and hydropathy analysis, the proteins are predicted to have 12 transmembrane (TM)[1] α-helices divided into two equal intermembrane domains, α and β, by a large putative cytoplasmic loop designated the interdomain region (Fig. 1) (11, 16, 17). Some resistance can be obtained by cloning the two domains of the Tet protein separately in the cell without the interdomain loop (18, 19). Hybrid interclass Tet protein constructions and second-site suppressor studies revealed that interactions of both domains are required for Tet function (20, 21). Complete cysteine-scanning mutagenesis of Tet(B) (22) has revealed that a total of 58 (14%) of the 401 amino acids contribute to the structure and/or the function. Among them, 17, mostly glycine and proline, are considered to be essential, whereas the other 41 affect the Tc resistance phenotype mediated by Tet(B) only slightly. Four mutations, D190C, E192C, S201C, and M210C, located within the interdomain loop, caused greatly reduced tetracycline resistance levels (8–32-fold) (22). A double frameshift within the interdomain of Tet(A) changed its substrate specificity and increased the efflux of minocycline and glycylcyclines (23). In Tet(C), the interdomain mutation S202F leads to a 12-fold increase in tetracycline susceptibility of the *Escherichia coli* cells bearing the *tet* gene on a low copy number plasmid (1). Moreover, the insertion of four residues increasing the length of the Tet(C) interdomain loop increased Tc susceptibility (14). Despite the poor conservation of the amino acid sequence, the interdomain loop of tetracycline efflux proteins appears to be important in the mediation of tetracycline resistance. This study was designed to look for amino acid changes that restored tetracycline efflux activity to a mutant strain having a deleterious first mutation in the Tet(C) interdomain loop.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Tc (0.93 Ci/mmol) was purchased from PerkinElmer Life Sciences. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase, T4 DNA polymerase, and T4 DNA ligase were purchased from Stratagene and Invitrogen. Antibiotics were obtained from Sigma, except for AHTc, which was prepared in this laboratory. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains, Plasmids, and Medium—Table I lists the bacterial strains and plasmids used in this study. *Escherichia coli* cell cultures were grown at 37 °C in Luria-Bertani (LB) broth (24) supplemented with chloramphenicol (20 μg/ml) and tetracycline (15 μg/ml) as needed. AHTc (15 ng/ml) was used as a gratuitous inducer of the Tet protein where applicable (25).

Mutagenesis of Plasmid DNA—Mutagenesis *in vitro* was performed with hydroxylamine, which specifically induces GC → AT transitions by deaminating the cytosine residues (26, 27). Approximately 3 μg of plasmid p202FCH prepared using the Qiagen Spin Mini preparation kit

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‡ To whom correspondence should be addressed: Center for Adaptation Genetics and Drug Resistance and Dept. of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel.: 617-636-6764; Fax: 617-636-0458; E-mail: stuart.levy@tufts.edu.

[1] The abbreviations used are: TM, transmembrane domain; AHTc, 5α,6-anhydrotetracycline; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; Tc, tetracycline; [^{3}H]Tc, [7-^{3}H(N)]Tc.

TABLE I
Bacterial strains and plasmids

	Relevant genotype	Ref.
Strain		
DH5α	*supE44 ΔlacU169(ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	40
AG100A	Δ*acrAB*::Tn*903 kan*^{r} *argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44*	41
Plasmid		
pCR2	Cm^r, P15A *ori*, wild type Tet (C) determinant[a]	1
p202FCH	pCR2 carrying mutant S202F-Tet (C) determinant[a]	1
pMCL210	Cm^r, P15A *ori, lacZ′* cloning vector	42
pFS1	pMCL210 carrying the 3.2kb Tet (C) determinant[a] from pCR2	This study

[a] Tet (C) determinant was genetically constructed to express the last 14 amino acids of Tet(B) (instead of its last six ones). This change allowed detection of the class C protein with antibody raised against the region of Tet(B) (1).

were incubated in 400 mM hydroxylamine, 50 mM potassium phosphate buffer (pH6), and 100 mM EDTA in a 100-μl volume for 40 min at 68 °C. The entire volume was drop-dialyzed against sterile water for 1 h 30 min at room temperature on a type VS filter (0.025-μm pore size) (Millipore, Bedford, MA). The treated DNA was ethanol precipitated and washed with 70% ethanol before being resuspended in sterile water. Mutagenesis of p202FCH with MNNG was performed as described previously in DH5α cells (28).

After both mutagenic events, 40 ng of plasmid DNA were introduced by electroporation into DH5α electro-competent cells (24) using 0.2-cm cuvettes and a Gene Pulser (Bio-Rad) at 200 ohms, 25 microfarads, and 25 kV/cm. To maximize the isolation of independent mutants, the entire transformation mix (1 ml) was divided into 10 equal portions prior to incubation and plating. Subsequently, transformants were selected on plates containing chloramphenicol (20 μg/ml) (for plasmid maintenance) and tetracycline (15 μg/ml). Tc-resistant mutants appeared usually after 24 h of incubation at 37 °C. Only one single colony per plate was chosen for analysis to ensure that the mutants isolated were generated in separate mutagenic events. Each colony was checked for its tetracycline susceptibility by overnight liquid growth in LB broth containing chloramphenicol and tetracycline (15 μg/ml). The plasmids from the Tc-resistant clones were extracted, and their copy numbers were estimated by plasmid preparation on gels. Those plasmids whose copy numbers were not increased were chosen to retransform DH5α to assure that the Tc resistance observed was a plasmid-mediated mutation in the *tet* gene.

Site-directed Mutagenesis—Site-directed mutagenesis of *tet*(C) on plasmid pFS1 was performed by a two-stage PCR method adapted from a PCR overlap method (29, 30). Two primers corresponding to the sense and antisense sequence of the *tet*(C) gene were designed to incorporate a restriction endonuclease site along with the desired mutation where possible. The following sense primers were used: primer L11F, 5′-CAATGCGCTCATCGTAATATTCGGCACCGTC-3′; primer A213T, 5′-GGGGCATGACTATCGTCACCGCACTTATGACTGTC-3′; and primer A270V, 5′-GCCTTCGTCACTGGTCCGGTCACCAAACGTTTCGGCGAG-3′. Restriction endonuclease sites (*Ssp*I and *Bst*EII in primers L11F and A270V respectively), which were introduced to facilitate identification of the desired mutants, are underlined.

Once the mutation was confirmed by sequencing and restriction enzyme analysis, the 3.2-kb *Xho*I-*Xba*I-mutated Tet(C) determinant was exchanged by cloning into the *Xho*I-*Bgl*II restriction sites of the parental unmutagenized pCR2 plasmid. To facilitate the cloning of the determinants, compatible cohesive ends were produced by blunt-ended *Xba*I and *Bgl*II restriction sites with T4 DNA polymerase.

Nucleotide Sequencing—DNA sequencing was performed at the Tufts University Core Facility using a ABI3100 Genetic Analyzer.

Determination of Tetracycline Susceptibility—AG100A cells harboring plasmids bearing wild type and *tet*(C)-mutated genes were grown in the presence of chloramphenicol and AHTc to an A_{530} of 0.8. Cells were swabbed for confluent growth onto a LB agar plate containing AHTc (15 ng/ml) before the application of the tetracycline E-test strips (gift from AB Biodisk, Solna, Sweden). The minimum inhibitory concentration was that amount of tetracycline showing an inhibition growth zone with the E-test after 24 h of incubation at 37 °C.

Membrane Isolation and Western Blot Analysis—AG100A cells expressing various plasmid-specified Tet proteins were grown in the presence of AHTc (15 ng/ml) and rapidly chilled when they reached the late logarithmic growth phase (A_{530} = 0.8). Following centrifugation, cells were resuspended in 20 mM Tris-HCl (pH8), 2 mM $MgCl_2$, 1 mM EDTA, and 30 μg/ml lysozyme (A_{530} of 100) prior to sonication (Branson Sonifier 250, Branson Ultrasonics Corporations, Danbury, CT). Membranes were collected by centrifugation at 60 000 × *g* for 1 h at 4 °C. Tet proteins were solubilized by incubating the membranes in 20 mM Tris-HCl (pH8), 150 mM NaCl, 10% glycerol, and 1.5% dodecylmaltoside at 4 °C for 1 h (A_{530} = 250). Membranes were removed by sedimentation for 30 min at 15,000 × *g* in 1.5-ml Eppendorf tubes, and extracts were stored at −80 °C. Before electrophoresis, extracted proteins were incubated in reducing sample buffer (24) for 20 min at room temperature. Proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel (24) using a Miniprotein II gel apparatus (Bio-Rad) and then transferred to a PolyScreen polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences) per the manufacturer's recommendations. Immunological detection was carried out with polyclonal antibodies directed against the 14 carboxyl-terminal (Ct) amino acids of Tet(B) (anti-Ct antibody, kindly provided by A. Yamaguchi) (31). The antigen-antibody complexes were detected with horseradish peroxidase coupled to the anti-rabbit IgG (New England Biolabs). Blots were developed with the Renaissance Western blot chemiluminescence reagent plus kit (PerkinElmer Life Sciences). The band intensities of each Tet(C) derivative were determined using NIH Image 1.6.2 free software (www.scioncorp.com).

Tetracycline Accumulation Assays—The measurement of [^{3}H]Tc uptake by intact AG100A cells containing mutant plasmids was adapted from previous works (1, 4). Bacteria grown to exponential phase in LB broth containing AHTc were pelleted by centrifugation (15,000 × *g* for 5 min), washed in 10 mM Tris-HCl (pH8) buffer, and suspended in 50 mM potassium phosphate buffer (pH 6.6), 10 mM $MgSO_4$, and 0.2% glucose (A_{530} = 4). After 3 min of preincubation, [^{3}H]Tc was added to 270 μl of cell suspension with shaking at 30 °C in a water bath, yielding a final tetracycline concentration of 1 μM. At various intervals, 50 μl of the suspension were removed, mixed with 10 ml of 0.1 M potassium phosphate buffer (pH6.6) and 0.1 M LiCl at 20 °C, and filtered through a Metricel® membrane filter (pore size, 0.45 μm). The filters were washed with 4 ml of the same buffer and dried before the radioactivity was measured with a liquid scintillation counter. The protonophore CCCP was added to a final concentration of 100 μM at 18 min to deenergize the cells. Each strain was assayed in triplicate in three separate experiments. The value at each time point deviated ≤8%.

RESULTS

Characterization of Tc-resistant Mutants—Following chemical treatments with hydroxylamine or nitrosoguanidine, a total of nine individual Tc-resistant mutants were isolated. The sequencing of the promoter and coding region of *tet*(C) confirmed a single base change in each clone leading to three different types of amino acid substitution (Fig. 1). Among all the clones identified, no reversion to the wild type codon was seen. In six different clones obtained with both methods (five hydroxylamine and one MNNG), a CTC → TTC mutation was observed at position 11, which led to the substitution of the leucine by a phenylalanine residue. The leucine 11 is conserved among all classes and is predicted to be within the TM1 (32). In two additional MNNG mutants, a GCC → ACC transition resulted in a change from alanine 213 to a threonine residue. Based on the current topological model of Tet(C) developed by Varela *et al.* (33) and alignment with the Tet(B) model (17), the location of alanine 213 is predicted to be within the central interdomain loop connecting the α and β domains. This alanine residue is also conserved in the class D Tet protein that shares 78% homology with the class C protein. Finally, one last mutant, resulting from mutagenesis with MNNG, contained a GCC to GTC change that resulted in the substitution of valine for the alanine at position 270. The alanine was predicted to be in the cytoplasmic loop linking TM8 and TM9 (13, 34).

AG100A cells devoid of the AcrAB pump were highly susceptible to tetracycline (minimum inhibitory concentration = 0.3 μg/ml) (Table II). When complemented with a low copy number plasmid bearing the wild type *tet*(C), the minimum inhibitory

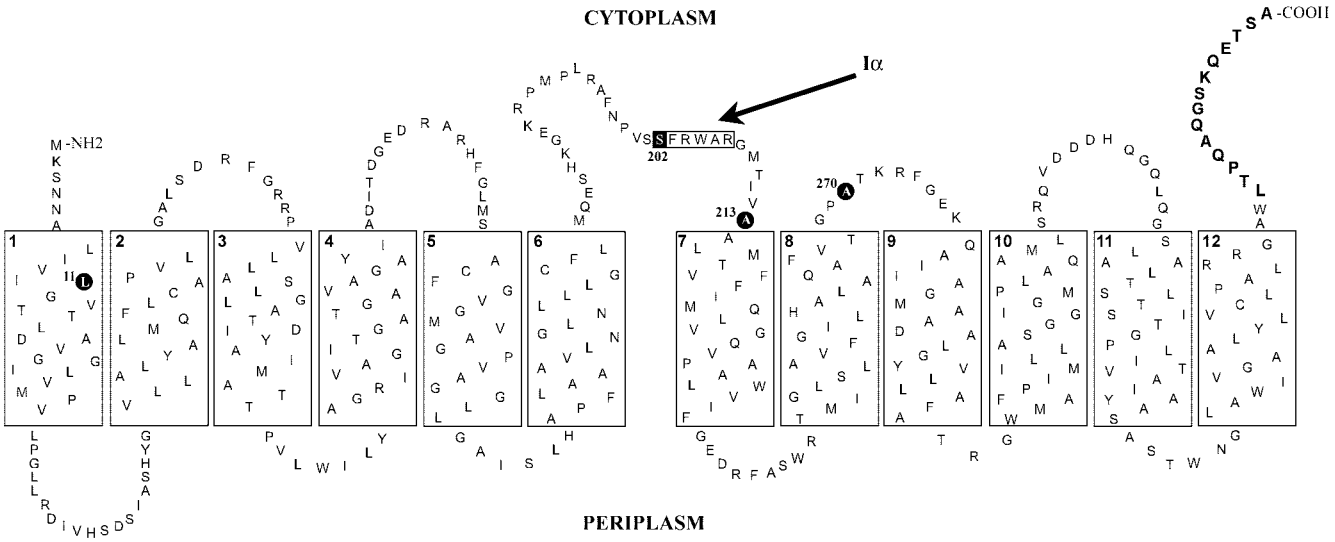

FIG. 1. **Membrane topology of the Tet(C) protein.** Transmembrane α-helices of the protein are indicated in the *rectangles* numbered *1–12*. Residues recognized by an antibody raised against the class B Tet protein are shown in *boldface* at the carboxyl-terminal end. Serine 202 is within the interdomain cytoplasmic loop and is designated by a *black square*. *Circled letters* locate secondary suppressor mutations of S202F. The Iα helix is *boxed* and indicated by an *arrow*.

TABLE II
Tetracycline susceptibility of AG100A cells bearing plasmids expressing the wild type or mutant Tet(C) proteins

The susceptibilities are expressed as minimal inhibitory concentration revealed by E-test on AHTc-induced AG100A cells. Representative of experiments performed three times.

Plasmid-specified Tet(C)	Minimum inhibitory concentrations of Tc
	μg/ml
None	0.3
Wild type	16
S202F	4
L11F/S202F	24
A213T/S202F	16
A270V/S202F	16
L11F	16
A213T	16
A270V	16

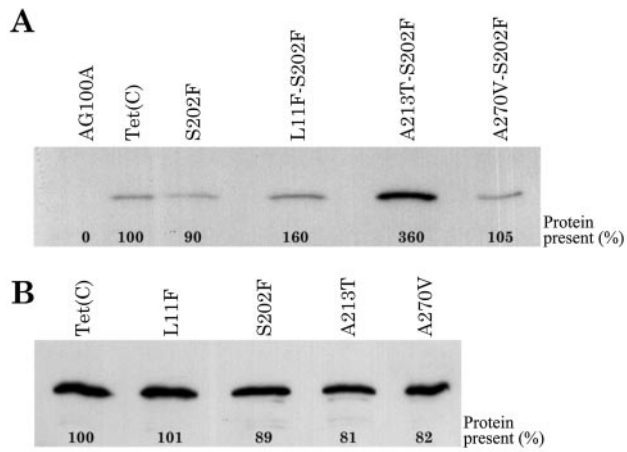

FIG. 2. **Western blot analysis of wild type and mutated Tet(C) proteins.** Extracted membrane proteins (2.5 μg) of AG100A cells expressing various Tet proteins were transferred and probed with anti-Ct antibody. *A*, wild type Tet(C) and secondary suppressor mutations of S202F. *B*, wild type Tet(C) and single mutations. Quantities of mutant Tet(C) proteins are represented as a percentage of the wild type protein assigned a value of 100% within the same blot. Representative of experiments performed in triplicate.

concentration in AG100A rose to 16 μg/ml. The presence of the single mutation S202F in Tet(C) decreased the tetracycline resistance by 4-fold to 4 μg/ml. Wild type Tc resistance was restored by each of the secondary mutations, A213T/S202F and A270V/S202F, and an even higher level of resistance was provided with the L11F/S202F mutation (24 μg/ml). Subsequently, each mutation was introduced by site-directed mutagenesis into Tet(C) specified by the plasmid pFS1. These mutations alone had no effect on the level of Tc resistance and could, therefore, be considered as not essential for Tet(C) activity (Table II). Thus, the secondary suppressor mutations are necessary to suppress the effect of the first mutation but are not by themselves critical for Tet protein function.

Western Blot Analysis of Tet Protein Expression—Western blot analysis of the Tet protein was performed using the anti-Ct antibody. The inactivating mutation S202F slightly reduced (10%) the level of protein production (Fig. 2) but confirmed the belief that the low level of resistance was not attributable to a poor expression of the protein. The double mutant protein A270V/S202F was expressed in quantities comparable with the parental S202F mutant (Fig. 2*A*). On the other hand, the protein with the secondary L11F or A213T mutations showed increased amounts in the cells in which they were expressed (Fig. 2*A*). The introduction of each single suppressor mutation into the wild type determinant did not change protein expression or mobility in a detectable manner (Fig. 2*B*).

Analysis of Tetracycline Efflux Activity—Tc resistance is characterized by a reduction in the cellular accumulation of Tc brought about by a proton motive force-dependent efflux (4, 35). We measured the energy-dependent Tc efflux as the relative uptake of [^{3}H]Tc before and after deenergization of the cells with the protonophore CCCP. AG100A, devoid of the AcrAB pump and any Tet protein, accumulated 38 pmol of [^{3}H]Tc in 18 min (Fig. 3*A*). The addition of 100 μM CCCP resulted in a loss of Tc from the cell, which was attributed to the dissipation of the proton gradient across the membrane upon which Tc uptake is dependent (7). However, when AG100A expressed the wild type Tet(C), it showed a lower uptake of [^{3}H]Tc (13.9 pmol) (Fig. 3*A*), which increased when cells were treated with CCCP. The strain carrying the S202F mutation accumulated nearly 21.9 pmol of [^{3}H]Tc in 18 min but was unaffected by CCCP addition (Fig. 3*A*). This lack of effect of CCCP was observed previously for some low level Tet protein mutants (36).

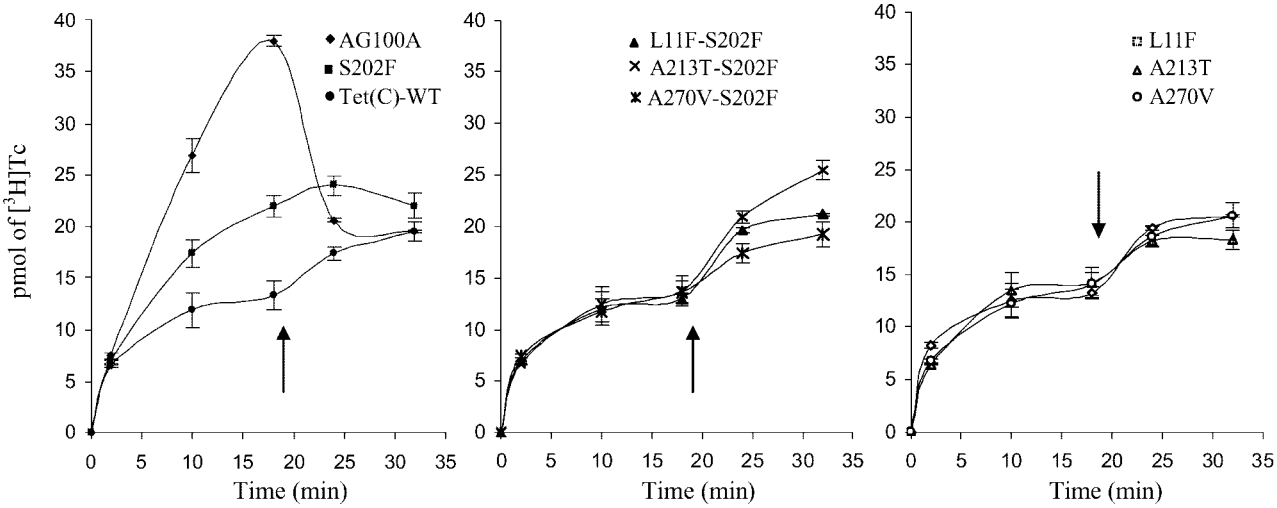

FIG. 3. **Accumulation of [^{3}H]Tc by *E. coli* AG100A cells expressing wild type and mutant Tet proteins.** Accumulation was measured as pmol of [^{3}H]Tc in intact exponential phase cells. The *arrows* indicate CCCP (100 μM) addition. Representative of experiments performed three times.

All three suppressor mutations restored an active Tc efflux comparable with the wild type (Fig. 3*B*). Modification of the wild-type Tet(C) protein with each single suppressor mutation revealed no distinguishable change in the efflux activity as compared with the wild type Tet protein (Fig. 3*C*).

DISCUSSION

The cytoplasmic interdomain loop of Tet proteins has been considered to be only a means of connecting two active domain halves, because the loop is so divergent in its sequence among various tetracycline resistance determinants whose two α and β domains are much more similar (37). Moreover, some resistance can be obtained by cloning the two domains of the Tet protein separately in the cell without the interdomain loop (18, 19). However, previous studies pointed out that a double frameshift mutation in the interdomain loop of TetA modified the substrate specificity of the protein (23) and that local mutations within the loop greatly reduced the Tc resistance phenotype (1, 17, 22).

We have isolated and characterized three different types of amino acid substitution suppressor mutations arising in nine independently isolated mutants that restore the tetracycline efflux function in Tet(C) with the S202F mutation in the interdomain loop. One secondary suppressor mutation, L11F, would increase the bulkiness of the side chain at this position. Leucine 11, predicted to be relatively close to the cytoplasmic side of the membrane within TM1, is conserved among all of the classes of Gram-negative Tet proteins that have been identified to date. Its change suggests an interaction of TM1 with the interdomain loop. According to Tamura *et al.* (22), TM1 is a partly amphiphilic helix with several residues facing the water channel built by portions of TM2, TM4, TM5, TM7, TM8, TM10, and TM11. It is therefore possible that this secondary suppressor mutation modifies the structure of TM1 and restores a wild type tetracycline resistance phenotype by correcting the active site altered by the S202F mutation.

A second suppressor mutation, A213T in the cytoplasmic loop (Fig. 1), slightly increases the size of the side chain while introducing a hydrophilic residue. Prediction deduced from hydropathy plots has shown an additional cytoplasmic α helix-(Iα) located within the interdomain loop (Fig. 1), between serine 202 and arginine 207. A similar feature of 13 amino acids has been pointed out in the three-dimensional crystallographic structure of the efflux transporter AcrB, and it has been postulated that the Iα helix is attached to the cytoplasmic membrane surface (38). This feature may also be true for Tet(C). The predicted position of A213T near the cytoplasmic surface of the protein could improve binding of the mutated putative Iα structure to the membrane. Of note, the mutation led to a 3–4-fold higher amount of the protein without showing a greater efflux activity, suggesting that the protein itself is still not completely wild type in function. Although the reason for the increased protein is not yet clear, the single A213T change in Tet(C) did not alter protein amount or function (Figs. 2 and 3). Thus, it appears that the combined interdomain mutations (S202F + A213T) add stability to the protein embedded in the membrane. Analogously, the length and nature of the residues of the interdomain loop of LacY, a member of the major facilitator superfamily, affected the insertion and stability of the protein in the membrane (39). Hydrophilic residues within the interdomain loop of LacY are required to permit a temporal delay for the insertion of both domains.

A third suppressor mutation, A270V, predicted to be within the cytoplasmic loop between TM8 and TM9, restored an efficient tetracycline resistance phenotype and efflux activity without modifying the level of Tet production. The position on the same side of the protein suggests that the loop 8-9 interacts somehow with the interdomain loop and that the A270V mutation improves the interaction in the presence of the S202F mutation.

The loss of tetracycline efflux activity of the mutant S202F Tet(C) could be interpreted as a modification of the conformation of the Iα helix that could perturb the correct positioning of the active site involving the interaction between α and β domains. In Tet(B), substitution of the homologous serine 202 by a cysteine residue also led to an increase in tetracycline susceptibility (17). Interestingly, cysteine-scanning mutagenesis analysis revealed no labeling of S201C with [^{14}C]*N*-ethylmaleimide. This finding suggests that the serine, while in the water-exposed loop, is oriented into the protein interior as a membrane-interactive residue (17).

The introduction of the single mutations L11F, A213T, and A270V into Tet(C) did not produce any detectable change in the wild type Tet(C) activity or expression. This finding suggests that serine 202 plays a capital but not essential role in the functional activity of Tet(C). In this case, any conformational modification brought by each mutation is not sufficient to de-

stroy the interaction of serine 202 with other specific portion(s) of the protein. Although the precise role of the cytoplasmic interdomain loop in tetracycline resistance is not clear, it should be reevaluated as being more than a simple linker between the α and β domains because it affects Tet protein-specified tetracycline resistance.

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