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Genetic Analysis Suggests Functional Interactions between the N- and C-Terminal Domains of the TetA(C) Efflux Pump Encoded by pBR322

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Genetic analysis of the *tetA*(C) gene of pBR322 indicates the importance of two cytoplasmic loops in the TetA(C) protein (P. McNicholas, I. Chopra, and D. M. Rothstein, J. Bacteriol. 174:7926–7933, 1992). In this study, we characterized second-site suppressor mutations that suggest a functional interaction between these two cytoplasmic regions of the protein.

Among enteric bacteria, the most clinically prevalent mechanism of tetracycline resistance is an active efflux system mediated by a very hydrophobic membrane-associated protein (3, 19) which expels tetracycline against a concentration gradient by coupling efflux of the drug with the influx of a proton (8, 11, 23, 24, 26). Five related tetracycline efflux proteins, designated TetA(A) to TetA(E), are encoded by determinants found in gram-negative enteric bacteria (2, 10, 14–16, 20). Computer modeling suggests that they have a common secondary structure, consisting of six α-helices that span the inner membrane, a central hydrophilic region of some 40 amino acids, and six more membrane-spanning α-helices. Biochemical (6, 22) and genetic (1) evidence has verified some features of this model.

To identify critical regions of these transport proteins, we conducted extensive and systematic mutagenesis of the efflux pump gene of plasmid pBR322 which revealed the importance of two cytoplasmic loop regions, connecting transmembrane helices 2 and 3 (designated loop A) within the N-terminal half and helices 10 and 11 (loop B) in the C-terminal half of the TetA(C) protein (13; Fig. 1). Other genetic experiments have also indicated the importance of residues in loop A in the related TetA(B) protein (23, 25), a region which is very strongly conserved among bacterial transport proteins that utilize the proton-pumping mechanism (3), as well as residues in transmembrane regions of TetA(B) (12, 21, 23) and TetA(C) (1).

This work focused on mutations at codon 71 in loop A of the *tetA*(C) gene and at codon 322 in loop B (Fig. 1). The mutated alleles are of an unusual class of pH-dependent lesions which result in cells with increased tetracycline resistance in LB medium buffered at pH 5.8 with 0.1 M sodium phosphate compared with resistance in LB, at pH 7.0 buffered (13). The pH dependence of proteins suggests that these lesions specifically affect the energetics of the TetA(C) efflux pump (13). The growth of cells at lower pH may compensate for this defect by increasing the ΔpH component of the electrochemical gradient, the source of energy for tetracycline efflux pumps (3). To identify other residues that may interact in a common function, we isolated second-site suppressor mutations that alleviate the effect of the primary mutations. Our results suggest an interaction between loop A in the N-terminal half of TetA(C) and loop B in the C-terminal half of the protein.

It is possible that the mutations at codons 71 and 322 cause protein instability and/or proteins that fail to assemble properly. However, genes carrying the primary mutations in codon 71 or 322 retain the capacity to complement for a second transport function, i.e., the ability to mediate potassium transport in an *Escherichia coli* mutant which is defective in potassium transport. Previous studies have shown that the wild-type TetA(C) pump has the capacity to mediate potassium uptake in strains containing mutations in the Kdp and Trk transport systems. Furthermore, promoter mutations decreasing the level of tetracycline resistance slightly (MIC of 100 μg/ml reduced to an MIC of 75 μg/ml), and presumably decreasing the number of tetracycline efflux pumps expressed, resulted in partial loss of the ability to complement this potassium uptake defect (5a). We found previously that plasmids carrying the primary mutations at codons 71 and 322 of *tetA*(C) fully complement potassium uptake defects and mediate growth at the same rate in low-potassium medium as does the wild-type plasmid (13), consistent with the idea that the TetA(C) protein altered at either codon 71 or 322 is stable and assembles properly in the membrane.

Suppressor mutations of primary lesions in codon 71. It was reported previously (13) that two changes at position 71 within loop A resulted in the loss of tetracycline resistance: Gly→Asp and Gly→Ser. Plasmid pBR322N is a pBR322 derivative containing an *Nde*I site at the start of the *tetA*(C) structural gene (13). Plasmid derivatives of pBR322N containing the primary mutations at codon 71 were mutagenized with hydroxylamine and transformed into *E. coli* DH5α (Bethesda Research Laboratories) as previously described (13), and transformants were selected on LB agar containing tetracycline at a concentration of 1.5 times the original MIC. The MICs of tetracycline for the transformants were measured by testing their growth on buffered LB agar containing 2, 4, 6, 8, 12, 16, 20, 25, 30, 35, 40, 50, 60, 75, 85, 100, 125, or 150 μg of tetracycline per ml as previously described (13). MIC measurements were repeated at least three times. The wild-type *tetA*(C) gene on plasmid pBR322N conferred tetracycline resistance levels of 100 μg/ml at pH 7 and 125 μg/ml at pH 5.8.

Suppressor mutations were shown to reside in the *tetA*(C) structural gene as follows. Plasmids were digested with *Nde*I and *Ava*I, each 1.3-kb fragment containing the entire structural gene was transferred to an unmutagenized vector, and tetra-

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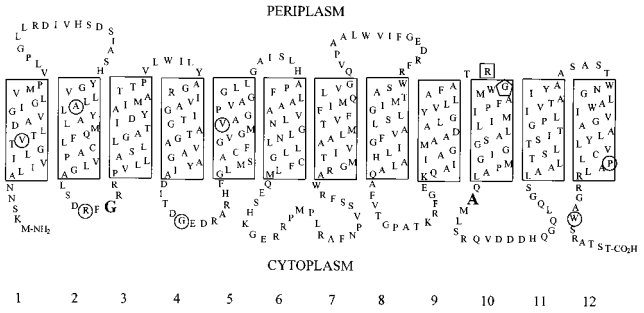

FIG. 1. Model of the two-dimensional topology of the TetA(C) efflux protein based on the TopPred program developed by von Heijne (19a). Transmembrane-spanning regions of the protein are indicated by rectangles numbered 1 to 12. The original mutations, which result in a pH-dependent defect in tetracycline resistance, are in boldface type. Second-site suppressor mutations are indicated by the following symbols: □, suppressor of primary mutations in codon 71; ○, suppressors of primary mutations in codon 322; ⬠, suppressor of primary mutations in both codons 71 and 322.

cycline resistance levels were confirmed. To facilitate the verification process, a new wild-type plasmid, pBR322N-K, was constructed which contains the kanamycin resistance gene from Tn*903* in place of the ampicillin resistance gene. Plasmid pBR322N was digested with endonucleases *Ssp*I and *Pst*I, and the ends were made blunt by treatment with T4 polymerase (9). The large fragment was ligated to the 1.2-kb fragment obtained by digesting plasmid pUC4K (Pharmacia) with endonuclease *Pst*I and blunting the ends as already described. Plasmid pBR322N-K, containing the kanamycin resistance gene in the opposite orientation relative to *tetA*(C), was isolated following transformation with kanamycin selection (9). Plasmid pBR322N-K conferred the same level of resistance to tetracycline as pBR322N for cells grown at either pH 7 or 5.8. By transferring the mutagenized *tetA*(C) gene into the pBR322N-K backbone and selecting for kanamycin-resistant transformants, we were assured that the backbone was derived from a wild-type plasmid. The DNA sequences of the structural gene were determined by Sanger DNA sequencing with double-stranded templates isolated and prepared in accordance with standard protocols (9).

Cells grown at pH 7.0 containing the plasmids with the position 71 Gly→Asp mutation in loop A had an MIC of just 4 μg of tetracycline per ml (Table 1). When a suppressor mutation was present in addition to the primary mutation, then the MIC was increased to 12 μg of tetracycline per ml (either by a codon 299 Arg→Gln change or a codon 300 Gly→Asp change). These two suppressor mutations were the only ones isolated for the loop A primary mutations. They lie in adjacent codons and affect the protein at or near the periplasmic region connecting transmembrane helices 9 and 10 (Fig. 1). Cells grown at pH 7.0 containing plasmids with the codon 71 Gly→Ser modification had an MIC of 8 μg of tetracycline per ml. However, when the 299 codon Arg→Gln suppressor mutation was selected, the MIC increased to 20 μg of tetracycline per ml (Table 1). Since all 11 independently isolated second-site mutations were in either codon 299 or 300, it is possible that suppressor mutations for the loop A mutations are confined to this one region of the gene.

Suppressor mutations of primary lesions in codon 322. Cells grown at pH 7.0 containing plasmids with the codon 322 Ala→Thr mutation in loop B had an MIC of 16 μg of tetracycline per ml (Table 1). Second-site mutations suppressing this loop B primary lesion were isolated and verified as described above. Plasmids encoding any of seven different amino acid changes throughout the gene (Fig. 1) increased the MIC

TABLE 1. Effect of a primary mutation and a second-site suppressor mutation on the resistance to tetracycline mediated by the *tetA*(C) gene within plasmid pBR322N and pBR322N-K derivatives

Primary mutation		Second-site mutation		MICs (μg/ml) at pH 7/5.8
Codon	Base change	Codon	Base change	
Gly-71→Asp	GGC→GAC	None	None	4/20
Gly-71→Asp	GGC→GAC	Arg-299→Gln	CGA→CAA[a]	12/50
Gly-71→Asp	GGC→GAC	Gly-300→Asp	GGC→GAC	12/30
Gly-71→Ser	GGC→AGC	None	None	8/30
Gly-71→Ser	GGC→AGC	Arg-299→Gln	CGA→CAA[b]	20/50
Ala-322→Thr	GCC→ACC	None	None	16/50
Ala-322→Thr	GCC→ACC	Val-19→Ile	GTA→ATA	35/75
Ala-322→Thr	GCC→ACC	Ala-50→Val	GCG→GTG	30/60
Ala-322→Thr	GCC→ACC	Arg-69→Cys	CGC→TGC[a]	50/75
Ala-322→Thr	GCC→ACC	Gly-126→Lys	GGG→AAG	35/60
Ala-322→Thr	GCC→ACC	Val-145→Met	GTG→ATG	40/75
Ala-322→Thr	GCC→ACC	Gly-300→Asp	GGC→GAC	40/75
Ala-322→Thr	GCC→ACC	Pro-383→Leu	CCC→CTC	35/60
Ala-322→Thr	GCC→ACC	Trp-390→stop	TGG→TAG	35/60
Ala-322→Thr	GCC→ACC	Arg-69→His	CGC→CAC	20/60[c]
Ala-322→Val	GCC→GTC	None	None	4/40
Ala-322→Val	GCC→GTC	Arg-69→His	CGC→CAC	25/75
Ala-322→Val	GCC→GTC	Arg-69→Cys	CGC→TGC	50/75[c]
None	None	Ala-50→Val	GCG→GTG	30/60
None	None	Arg-69→Cys	CGC→TGC	50/60
None	None	Arg-69→His	CGC→CAC	25/60
None	None	Arg-299→Gln	CGA→CAA	100/125
None	None	Gly-300→Asp	GGC→GAC	60/75
None	None	None	None	100/125

[a] Isolated two independent times.
[b] Isolated eight independent times.
[c] The suppressor mutation was combined with the primary mutation located on pBR322N-K by standard cloning techniques.

to 30 to 50 μg of tetracycline per ml (Table 1). Suppression also occurred when the last 10 amino acids of the TetA(C) protein were deleted (codon 390 Trp→stop mutation).

A codon 69 Arg→Cys change in loop A suppressed the codon 322 Ala→Thr primary mutation. A suppressor mutant was also isolated by starting with the strain carrying the primary mutation encoding the codon 322 Ala→Val change, which was also found to encode a change within codon 69, but this time causing a codon 69 Arg→His alteration (Table 1). To determine if suppressor mutations at codon 69 were allele specific, the unique *Nde*I-*Sal*I sites were used to purify the fragments bearing the first half of *tetA*(C), containing the modified codon 69, and ligating into plasmid pBR322N-K derivatives containing the primary lesion at codon 322 (9). These new plasmids expressed approximately the same resistance to tetracycline as the original suppressor-primary mutation combination, indicating that suppression was not allele specific (Table 1).

The suppressor mutations at position 69 were isolated from their primary mutation at position 322 by transferring the *Nde*I-*Sal*I fragment into plasmid pBR322N-K which had been digested with the two enzymes. Plasmids bearing the position 69 suppressor mutations and the wild-type allele at codon 322 were found to mediate resistance to only low concentrations of tetracycline (Table 1), indicating that a single modification at position 69 in loop A impaired the function of TetA(C).

One suppressor mutation of the loop B primary mutation, codon 300 Gly→Asp, also suppressed the loop A codon 71 Gly→Asp primary mutation. The common suppression of two primary mutations, one located in loop A and another located in loop B, suggests a commonality of function of residues Gly-71, Gly-300, and Ala-322. The second-site mutation at position 299 or 300 was isolated from the codon 71 Gly→Asp primary mutation by using the *Sal*I and *Ava*I restriction sites. Whereas the codon 299 Arg→Gln single mutation had no apparent effect on tetracycline resistance, the codon 300 Gly→Asp lesion resulted in slightly reduced resistance compared with the wild-type plasmid (Table 1).

A network of residues may have a common functional interaction. The work of Levy and coworkers suggests that tetracycline efflux pumps are composed of two functional domains, on the basis of (i) intragenic complementation between mutations in the first and second halves of the *tetA*(B) gene (4, 5, 7), (ii) the abilities of hybrid tetracycline efflux pumps to confer resistance (17), and (iii) the ability of efflux pump fragments to interact (18). Our work supports the idea that the C- and N-terminal halves of the TetA(C) efflux pump interact in at least one common function. This functional interaction may explain why it is crucial in hybrid proteins for the N- and C-terminal halves of Tet to be encoded by closely related determinants in order to form a working complex in conferring tetracycline resistance (17).

The loop A region is thought to be critical for the proton-pumping ability of the protein, on the basis of the universal homology within this region for all bacterial proton pumps (3). The fact that a pH-dependent mutation is located in a site encoding a loop A residue is in keeping with this idea; the lower pH of the medium should increase the ΔpH component of the electrochemical gradient, which drives the tetracycline efflux pump (3). The location of pH-dependent mutations in the region encoding loop B (13), as well as suppression of mutations in loop B by second-site mutations in loop A, suggests a physical and/or functional interaction between these loops. The common suppression by mutations at or near the periplasmic region connecting transmembrane helices 9 and 10 suggests an interaction between residues in cytoplasmic loops A and B and at least this one periplasmic region. It is very improbable that cytoplasmic and periplasmic residues could physically interact. It therefore seems likely that these residues interact functionally, and we suggest that the common function is the proton pump.

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