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Letters to the Editor

Interdomain Loop Mutation Asp190Cys of the Tetracycline Efflux Transporter TetA(B) Decreases Affinity for Substrate▽

TetA(B), the tetracycline efflux protein from Tn*10*, has 12 predicted α-helices that span the *Escherichia coli* cytoplasmic membrane (10) and is a member of the major facilitator superfamily (4). TetA(B) and related tetracycline antiporters use the bacterial transmembrane proton motive force to export one metal-tetracycline complex in exchange for one H^+ (12), thereby reducing the intracellular tetracycline concentration.

In our recent studies of the cytoplasmic interdomain loop connecting transmembrane helices 6 and 7 of TetA(B) (7) and TetA(C) (6), we showed that this loop contains residues implicated in tetracycline efflux activity. Furthermore, we established that residues Asp190, Glu192, and Ser201 of TetA(B) are involved in substrate specificity of the pump (7). Mutations of three adjacent amino acids in the interdomain loop of TetA(A) in two veterinary *Salmonella* isolates showed an altered substrate specificity with reduced susceptibility to minocycline and glycylcyclines (11). In the present work, we investigated the basis for the effect of the Asp190Cys mutation on tetracycline efflux mediated by TetA(B) in everted membrane vesicles using [H^3]tetracycline.

The low-copy plasmids (origin of replication pSC101) that specified the Asp190Cys mutation (7) and wild-type TetB (13) were transformed into *Escherichia coli* DH5α; everted membrane vesicles were prepared as described previously (3). The protein content was quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and vesicle aliquots were stored in 50 mM MOPS (morpholinepropanesulfonic acid), pH 6.6, at −80°C. The antiport activity of vesicles was verified by an acridine orange fluorescence method. The amount of TetA(B) protein in the membranes was also determined by Western immunoblotting using anti-Ct antibody as described previously (7) and showed the same amount in each strain.

Graphpad prism 4 software was used to determine the K_m and V_{max} values by fitting to the Michaelis-Menton equation. The cysteine substitution for aspartate at position 190 showed an average K_m value 3.8 times that of the wild type but did not produce any modification in V_{max} (Table 1). The K_m and V_{max} values obtained for the wild type are in agreement with those reported in the literature (3, 8, 9, 14). The lower affinity for tetracycline of the mutant Asp190Cys suggests that the aspartate residue is involved in the substrate interaction. Possibly the negatively charged aspartate interacts with the positively charged divalent metal cation-tetracycline complex which is the substrate. That a substitution causing a change in K_m of a protein indicates a position involved in substrate binding has been shown with the β-lactamase TEM1 (1, 2).

Although Asp190 is not an essential residue for tetracycline efflux (10), we show clearly that changing it to Cys lowered the affinity (higher K_m) of the pump for its substrate. The sequence of the approximately 30 amino acids comprising the cytoplasmic interdomain loop is not conserved among the dozen or so related tetracycline efflux pumps (5). This loop has been assumed until recently to be simply a tether holding the two halves of the protein together. Our biochemical results now support previous data in vivo that this loop of TetA(B) has an unexpected role in tetracycline transport.

TABLE 1. Tetracycline resistance levels of *E. coli* DH5α cells with and without plasmid-borne wild-type and mutant TetA(B) and K_m and V_{max} for tetracycline uptake by everted membrane vesicles

TetA(B) protein expressed in DH5α cells	MIC (μg/ml) in intact cells[a]		Normalized net doxycycline/tetracycline ratio[b]	Tetracycline uptake by membrane vesicles[c]	
	Tetracycline	Doxycycline		V_{max} (pmol/mg protein/min)	K_m (μM)
Wild type	128	32	1.0	1,274 ± 106	22 ± 6
Asp190Cys	11	14	5.2	1,327 ± 187	83 ± 24
None	0.75	1.25			

[a] See reference 7 for details.
[b] The MIC of control (none) cells was subtracted before calculation of ratios, which were then normalized to the wild-type ratio.
[c] Average of three experiments.

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Frédéric M. Sapunaric†
Stuart B. Levy*
Center for Adaptation Genetics and Drug Resistance
Tufts University School of Medicine
136 Harrison Ave.
Boston, MA 02111

*Phone: (617) 636-6764
Fax: (617) 636-0458
E-mail: stuart.levy@tufts.edu

† Present address: Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B-4000 Liège, Belgium.

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