

Interdomain loop mutation Asp190Cys of the tetracycline efflux transporter TetA(B) decreases affinity for substrate

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TetA(B), the tetracycline efflux protein from *Tn10*, 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 helix (TM) 6 and TM7 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 glycylicyclines (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 membranes vesicles using [H³]tetracycline.

The low copy plasmids (origin of replication pSC101) which specified the Asp190Cys mutation (7) and wild-type TetB (13) were transformed into *E. coli* DH5 α ; everted

membrane vesicles were prepared as described (3). The protein content was quantified using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) and vesicle aliquots were stored in 50 mM MOPS, pH 6.6 at -80°C . The antiport activity of vesicles was verified using 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 (7) and showed the same amount in each strain.

The 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} . 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 DH5 α *E. coli* cells with and without plasmid-borne wild type and mutant TetA(B) and *Km* and *Vmax* for tetracycline uptake by everted membrane vesicles

TetA(B) protein expressed in DH5 α cells	<u>Intact cells</u>		<u>Membrane vesicles</u>		
	Minimal Inhibitory Concentration ($\mu\text{g/ml}$) (7)		Normalized ratio* net Dx/Tc	<i>Vmax</i> ** (pmol Tc / mg protein / min)	<i>Km</i> ** (μM)
	Tc	Dx			
wild-type	128	32	1.0	1274 \pm 106	22 \pm 6
Asp190Cys	11	14	5.2	1327 \pm 187	83 \pm 24
none	0.75	1.25	-	-	-

Tc: tetracycline; Dx: doxycycline.

*MIC of control (none) cells was subtracted before calculation of ratios, which were then normalized to the wild-type ratio.

**Average of three experiments.

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1. **Dubus, A., J. M. Wilkin, X. Raquet, S. Normark, and J. M. Frere.** 1994. Catalytic mechanism of active-site serine beta-lactamases: role of the conserved hydroxy group of the Lys-Thr(Ser)-Gly triad. *Biochem. J.* **301** (Pt 2):485-94.
2. **Fonze, E., P. Charlier, Y. To'th, M. Vermeire, X. Raquet, A. Dubus, and J. M. Frere.** 1995. TEM1 beta-lactamase structure solved by molecular replacement and refined structure of the S235A mutant. *Acta Crystallogr. D Biol. Crystallogr.* **51**:682-94.
3. **McMurry, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **77**:3974-7.
4. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**:575-608.
5. **Sapunaric, F. M., M. L. Aldema-Ramos, and L. M. McMurry.** 2005. Chapter 1. Tetracycline Resistance: Efflux, Mutation, and Other Mechanisms, p. 3-18. *In* D. G. White, M. N. Alekshun, and P. F. McDermott (ed.), *Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy*. ASM Press, Washington D. C..
6. **Sapunaric, F. M., and S. B. Levy.** 2003. Second-site suppressor mutations for the serine 202 to phenylalanine substitution within the interdomain loop of the tetracycline efflux protein Tet(C). *J. Biol. Chem.* **278**:28588-92.
7. **Sapunaric, F. M., and S. B. Levy.** 2005. Substitutions in the interdomain loop of the Tn10 TetA efflux transporter alter tetracycline resistance and substrate specificity. *Microbiology* **151**:2315-22.
8. **Someya, Y., T. Kimura-Someya, and A. Yamaguchi.** 2000. Role of the charge interaction between Arg(70) and Asp(120) in the Tn10-encoded metal-tetracycline/H⁽⁺⁾ antiporter of *Escherichia coli*. *J. Biol. Chem.* **275**:210-4.
9. **Someya, Y., and A. Yamaguchi.** 1996. Mercaptide formed between the residue Cys70 and Hg²⁺ or Co²⁺ behaves as a functional positively charged side chain operative in the Arg70-->Cys mutant of the metal-tetracycline/H⁺ antiporter of *Escherichia coli*. *Biochemistry* **35**:9385-91.
10. **Tamura, N., S. Konishi, S. Iwaki, T. Kimura-Someya, S. Nada, and A. Yamaguchi.** 2001. Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J. Biol. Chem.* **276**:20330-9.

11. **Tuckman, M., P. J. Petersen, and S. J. Projan.** 2000. Mutations in the interdomain loop region of the tetA(A) tetracycline resistance gene increase efflux of minocycline and glycylcyclines. *Microb Drug Resist* **6**:277-82.
12. **Yamaguchi, A., Y. Iwasaki-Ohba, N. Ono, M. Kaneko-Ohdera, and T. Sawai.** 1991. Stoichiometry of metal-tetracycline/H⁺ antiport mediated by transposon Tn10-encoded tetracycline resistance protein in *Escherichia coli*. *FEBS Lett.* **282**:415-8.
13. **Yamaguchi, A., N. Ono, T. Akasaka, T. Noumi, and T. Sawai.** 1990. Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by a transposon, Tn10. The role of the conserved dipeptide, Ser65-Asp66, in tetracycline transport. *J. Biol. Chem.* **265**:15525-30.
14. **Yamaguchi, A., R. O'Yauchi, Y. Someya, T. Akasaka, and T. Sawai.** 1993. Second-site mutation of Ala-220 to Glu or Asp suppresses the mutation of Asp-285 to Asn in the transposon Tn10-encoded metal-tetracycline/H⁺ antiporter of *Escherichia coli*. *J. Biol. Chem.* **268**:26990-5.

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