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Substitutions in the interdomain loop of the Tn*10* TetA efflux transporter alter tetracycline resistance and substrate specificity

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Cysteine replacement of Asp190, Glu192 and Ser201 residues in the cytoplasmic interdomain loop of the TetA(B) tetracycline efflux antiporter from Tn*10* reduces tetracycline resistance [Tamura, N., Konishi, S., Iwaki, S., Kimura-Someya, T., Nada, S. & Yamaguchi, A. (2001). *J Biol Chem* **276**, 20330–20339]. It was found that these Cys substitutions altered the substrate specificity of TetA(B), increasing the relative resistance to doxycycline and minocycline over that to tetracycline by three- to sixfold. Substitutions of Asp190 and Glu192 by Ala, Asn and Gln also impaired the ability of TetA(B) to mediate tetracycline resistance while Ser201Ala and Ser201Thr substitutions did not. A Leu9Phe substitution in the first transmembrane helix of TetA(B) suppressed the Ser201Cys mutation, undoing the alterations in resistance and specificity. That the interdomain loop might contact substrate during transport, as is suggested from its role in substrate specificity, is unexpected considering that the primary sequence in the loop is not conserved among a group of otherwise homologous TetA proteins. However, in the interdomain loop of 11 of 14 homologous TetA efflux proteins, computational analysis revealed a short α-helix, which includes some residues affecting activity and substrate specificity. Perhaps this conserved secondary structure accounts for the role of the non-conserved interdomain loop in TetA function.

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INTRODUCTION

The tetracyclines are a family of broad-spectrum antibiotics, first introduced in 1948, which inhibit protein synthesis by binding to the 30S ribosomal subunit (Brodersen *et al.*, 2000; Chopra & Roberts, 2001). TetA(B) is a 401 aa cytoplasmic membrane protein belonging to the major facilitator superfamily (Pao *et al.*, 1998; Saier *et al.*, 1999). It maintains intracellular tetracycline concentrations below growth-inhibitory levels via the energy-dependent efflux of tetracyclines from bacterial cells, mediated by the transmembrane (TM) proton motive force (McMurry *et al.*, 1980). The membrane topology of TetA(B) has been studied by proteolysis (Eckert & Beck, 1989), chemical labelling (Kimura *et al.*, 1996; Yamaguchi *et al.*, 1992a), gene fusion (Hickman & Levy, 1988) and its percentage α-helix was calculated from circular dichroism spectral analysis (Aldema *et al.*, 1996). Based on these studies and hydropathy analysis, the protein is predicted to have 12 TM α-helices and is divided into two halves, α and *β*, by a large putative cytoplasmic loop designated the interdomain region (Fig. 1). The α and *β* domains of the protein (N-terminal and C-terminal halves, respectively) have presumably evolved from a duplication of a single domain (Rubin *et al.*, 1990). The group 1 set of related classes of tetracycline resistance determinants, A-E, G, H, J, Y, Z, 30, 31 and 33, all have an efflux pump, TetA, and a divergently transcribed repressor, TetR (Guillaume *et al.*, 2004; McMurry & Levy, 2000). These homologous TetA proteins have 45–75 % identity. Hybrid interclass Tet protein constructions (Rubin & Levy, 1990), complementation studies (Curiale *et al.*, 1984) and second-site suppressor studies (Saraceni-Richards & Levy, 2000a, b) showed that interactions between both domains are required for TetA function. Complete cysteine-scanning mutagenesis of the class B TetA(B) (Tamura *et al.*, 2001) has revealed that a total of 58 (14 %) of the 401 aa contribute to its structure and/or function. Among them, 17 residues, mostly glycine and proline, are considered to be essential, whereas the other 41 affect the tetracycline resistance phenotype mediated by TetA(B) only moderately. In addition, the cysteine-scanning data indicated the presence of a putative TM water-filled channel in the transporter. Of the 12 TMs, four (TM3, TM6, TM9 and TM12) do not face the channel, four (TM2, TM5, TM8 and TM11) face it along their full-length and the four remaining (TM1, TM4, TM7 and TM10) contribute by only half of their length to the side of it while their second half is embedded in a hydrophobic region (Tamura *et al.*, 2001).

Abbreviation: TM, transmembrane.

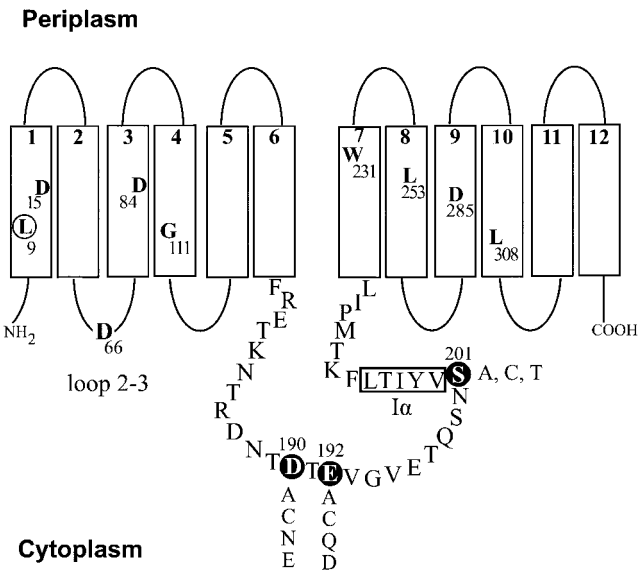

Fig. 1. Diagram of TetA(B) tetracycline/H^+ antiporter. TM α-helices are indicated by rectangles numbered 1–12. Amino acid residues of the interdomain loop are shown. Black circles, residues studied with list of substitutions introduced; white circles, secondary-site suppressor of Ser201Cys mutant; bold type, selected residues important for tetracycline resistance and substrate specificity; Iα, α-helix in the interdomain loop (boxed).

The interdomain loop is not conserved among the different classes of tetracycline efflux proteins, but appears nevertheless to be somehow involved in TetA function. Four mutations, Asp190Cys, Glu192Cys, Ser201Cys and Met210Cys, within the interdomain loop of the class B TetA, caused greatly reduced (8- to 32-fold) tetracycline resistance levels without altering the amount of TetA protein (Kimura *et al.*, 1997; Tamura *et al.*, 2001). In TetA(A), alteration of three adjacent amino acids (residues 201–203) in the interdomain loop changed the substrate specificity, increasing resistance to minocycline and glycylcyclines (Tuckman *et al.*, 2000). The insertion of five residues (between Arg190 and Pro191) which lengthened the TetA(C) interdomain loop increased tetracycline susceptibility (Jewell *et al.*, 1999). In TetA(C), the interdomain mutation Ser202Phe led to a 12-fold decrease in activity (Saraceni-Richards & Levy, 2000a); second-site suppressor mutations which restored resistance were found in TM1, in the interdomain loop and in the cytoplasmic loop connecting TM8 and TM9 (Sapunaric & Levy, 2003). To examine further the role of the interdomain loop of TetA(B) in substrate specificity, we performed site-directed mutagenesis at Asp190, Glu192 and Ser201, and assayed resistance to tetracycline and tetracycline analogues. We also applied computational analysis to the interdomain loop to understand why that non-conserved region might be involved in TetA function.

METHODS

Chemicals. Restriction enzymes were obtained from New England Biolabs. *Pfu* DNA polymerase and T4 DNA ligase were purchased from Stratagene and Invitrogen, respectively. Antibiotics were obtained from Sigma. 9-(*t*-butylglycylamido)-minocycline (a glycylcycline) and 5α,6-anhydrotetracycline were gifts from Paratek Pharmaceuticals. All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and plasmids. *Escherichia coli* DH5α (Woodcock *et al.*, 1989) was the host strain used for all cloning experiments and propagation of plasmid DNA. Cell cultures were grown at 37 °C in Luria–Bertani broth (Seoane *et al.*, 1992) supplemented with 20 μg chloramphenicol ml^{-1} and 30 μg kanamycin ml^{-1} as needed. Fifteen nanograms of 5α,6-anhydrotetracycline ml^{-1} was used as a gratuitous inducer of Tet protein where applicable (Moyed *et al.*, 1983).

Low-copy-number plasmids pLGT2 [wild-type *tetA*(*B*), origin of replication pSC101] (Yamaguchi *et al.*, 1990c) and its derivative pLGTS201C [Ser201Cys mutant of TetA(B)] (Kimura *et al.*, 1997) were kindly provided by A. Yamaguchi as were other plasmids (see text). All plasmids used during this work possess the TetB determinant comprising both TetR (repressor) and TetA (efflux pump). The TetB determinant isolated from pLGT2 by *Bgl*II/*Bam*HI enzymic restriction was cloned into the medium-copy-number plasmid pMCL210, origin of replication p15A (Nakano *et al.*, 1995), to produce transitional plasmid pMCL-WT-TetB, ready to use as template for site-directed mutagenesis.

Site-directed mutagenesis and nucleotide sequencing. Site-directed mutagenesis of *tetA*(*B*) on plasmid pMCL-WT-TetB was performed by adaptation of a PCR overlap method (Deng & Nickoloff, 1992; Wang & Malcom, 1999). Mutagenic PCR primers were designed to incorporate a restriction endonuclease site along with the desired mutation where possible (Table 1). Once the mutation was confirmed by restriction enzyme analysis and sequencing, the fragment was exchanged from the mutagenized plasmid into the low-copy-number unmutagenized pLGT2 plasmid (Yamaguchi *et al.*, 1990c). The restriction enzymes used to exchange the fragments were (i) *Eco*RV/*Eco*RI for Asp190 and Glu192 substitutions, (ii) *Eco*RV/*Bam*HI for Ser201 substitutions, and (iii) *Eco*RV/*Xba*I for the Leu9Phe substitution.

DNA sequencing was performed at the Tufts University Core Facility using a ABI3100 Genetic Analyser.

Determination of susceptibility to tetracycline and tetracycline analogues. *E. coli* DH5α cells with and without plasmids bearing wild-type and *tetA*(*B*) mutated genes were grown overnight in the presence of 25 μg kanamycin ml^{-1} on Mueller–Hinton agar plates. Cells were swabbed for confluent growth onto a Mueller–Hinton agar before the application of doxycycline, minocycline and tetracycline E-test strips (gift from AB Biodisk). The minimum inhibitory concentration (MIC) was that amount of the tetracycline showing an inhibition growth zone with the E-test after 24 h of incubation at 37 °C.

Western blot analysis. DH5α cells expressing various plasmid-specified TetA proteins were grown in the presence of 15 ng 5α,6-anhydrotetracycline ml^{-1} and rapidly chilled when they reached the late exponential growth phase (OD_{550} = 0·8). Cells were resuspended at an OD_{550} of 80 in 20 mM Tris/HCl (pH 8), 2 mM $MgCl_2$, 1 mM EDTA and 30 μg lysozyme ml^{-1} and disrupted by sonication (Branson Sonifier 250; Branson Ultrasonics). Total-cell lysate was used for immunological detection. Proteins were incubated in reducing sample buffer (Sambrook & Russell, 2001) for 20 min at room temperature, then a volume of extract corresponding to 0·1 OD_{550} units was separated by electrophoresis in a 12 % SDS-polyacrylamide

Table 1. Primers used for site-directed mutagenesis

Two primers, corresponding to the sense and antisense sequence of the *tetA*(*B*) gene, were used to create each mutation. The second mutagenic primer (not shown) was the reverse complement of the first. The mutated codon is in lower case. Mismatches are indicated in bold letters and newly created restriction sites are underlined.

Mutation	Sequence (5′→3′)	Restriction site
Asp190Ala	GTGATAATACAg**cc**ACCGAAGT**G**GGGGTTGAGAC	*Ale*I
Asp190Asn	GTGATAATACA**aac**ACCGAAGT**G**GGGGTTGAGAC	*Ale*I
Asp190Cys	AATACACGTGATAATACA**tg**tACCGAAGTAGGGGTTGAG	*Nsp*I
Asp190Glu	GTGATAATACAga**g**ACCGAAGTAGGGGTTGAGAC	None
Glu192Ala	GATAATACAGA**C**ACCg**c**aGT**G**GGGGTTGAGACG	*Ale*I
Glu192Gln	GATAATACAGA**C**ACC**c**aaGT**G**GGGGTTGAGACG	*Ale*I
Glu192Cys	CGTGATAATACAGATAC**Atgt**GTAGGGGTTGAGACGCAA	*Nsp*I
Glu192Asp	GATAATACAGA**C**ACCga**c**GT**G**GGGGTTGAGACG	*Ale*I
Ser201Ala	GAGACGCAATCGAAT**g**cgGTATACATCACTTTA	Loss of *Eco*RI
Ser201Thr	GAGACGCAATCGAAT**a**cgGTATACATCACTTTA	Loss of *Eco*RI
Leu9Phe	ACAAAGATCGCAtt**t**GT**G**AT**C**ACGTTACTCGATGC	*Bcl*I

gel (Sambrook & Russell, 2001) using a Miniprotein II gel apparatus (Bio-Rad), and the proteins were then transferred electrophoretically to a PolyScreen polyvinylidene difluoride membrane (Perkin Elmer Life Sciences) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) according to the manufacturer's recommendations. Western blot analysis was performed according to Sambrook & Russell (2001) with blocking by 0·5 % bovine serum albumin, with the exception that the detergent Tween 80 was used instead of Tween 20. Immunological detection was carried out with polyclonal antibodies directed against the 14 carboxyl-terminal (Ct) amino acids of TetA(B) (anti-Ct antibody, kindly provided by A. Yamaguchi) (Yamaguchi *et al.*, 1990a). The antigen–antibody complexes were detected with horseradish peroxidase coupled to anti-rabbit IgG (New England Biolabs). Blots were developed with the Western Lighting Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences) and exposed to Kodak BioMax Light Film.

RESULTS AND DISCUSSION

Effect on tetracycline resistance of substitution at Asp190 with Glu, Asn, Ala or Cys

The cytoplasmic interdomain loop of TetA(B) extends from aa 179 to 213 and connects TM6 to TM7 (Fig. 1). It possesses two aspartate residues which are located in the first third of the loop. By cysteine-scanning mutagenesis of the loop, Yamaguchi and his coworkers (Tamura *et al.*, 2001) showed that a cysteine substitution for aspartate at position 190 reduces tetracycline resistance. To evaluate the amino acid requirements for normal function at this position, we replaced this negatively charged aspartate residue with three other amino acids: Glu (negatively charged), Asn (uncharged and similar size) and Ala (small and hydrophobic). We also independently recreated the Asp190Cys mutation. All four site-directed substitutions were introduced into *tetA*(*B*) by a PCR overlap method using two primers (see Methods). The levels of tetracycline resistance conferred by the single substitutions are presented in Table 2 (column 3). The Asp190Glu substitution (which retained a negative charge) showed only a slight reduction of tetracycline resistance, whereas the replacement of Asp190 with Asn, Ala or Cys decreased the tetracycline resistance by 3·4-, 4·4- and 12-fold, respectively. The results indicate that the negative charge allows near wild-type expression of resistance, while other residues are only partially functional. The level of protein production (Fig. 2a) showed that the changes in resistance were not attributable to a variation in the expression of the protein. Therefore, the TetA(B) protein can accommodate various amino acids at Asp190 since in every mutant studied some activity was still remaining; however, retention of the negative charge provided the best tetracycline resistance activity.

Effect of substitution on tetracycline resistance at Glu192 with Asp, Gln, Ala or Cys

TetA(B) possesses 10 glutamate residues which are equally distributed in loops protruding in the cytoplasmic and periplasmic space. Three of them, Glu181, Glu192 and Glu206, are located in the interdomain loop; of these three only Glu192 showed some role in tetracycline resistance as demonstrated by cysteine-scanning mutagenesis (Tamura *et al.*, 2001). The same mutagenic method used above was applied to construct four single substitutions (Asp, Gln, Ala and Cys) at Glu192. The Glu192Asp substitution had little effect on tetracycline resistance; thus a good level of activity could be maintained when the negative charge was retained. The replacement of Glu192 with Gln, Ala or Cys decreased tetracycline resistance by 3-, 4·2- and 8·7-fold, respectively. The reduced tetracycline MICs were not due to a lower expression of the protein as confirmed by Western-immunoblot analysis (Fig. 2b).

The pattern of MIC values for the different Glu192 substitutions was identical to that for the Asp190 replacements. In both cases, retention of negative charge was optimal, while the other changes similarly reduced, but never completely eliminated activity, with Cys giving the most drastic reduction.

Table 2. Susceptibility to tetracycline and tetracycline analogues by wild-type and mutant TetA(B) proteins

WT-TetB, No mutation; Dox, doxycycline; Min, minocycline; Tet, tetracycline. Numbers in bold differ by more than 1·5-fold from the control strain WT-TetB.

Mutation	Mutation location	MIC (μg ml^{-1})*			Ratio Dox/Tet†	Ratio Min/Tet†
		Tet	Dox	Min		
WT-TetB		128	32	16	1	1
Asp190Cys	Interdomain loop	11	14	8	**5·3**	**6·2**
Asp190Asn	Interdomain loop	37	28	16	**3·0**	**3·5**
Asp190Ala	Interdomain loop	29	20	12	**2·7**	**3·3**
Asp190Glu	Interdomain loop	117	32	16	1·1	1·1
Glu192Cys	Interdomain loop	15	16	10	**4·4**	**5·6**
Glu192Gln	Interdomain loop	43	32	16	**3·0**	**3·0**
Glu192Ala	Interdomain loop	31	14	12	**1·8**	**3·1**
Glu192Asp	Interdomain loop	117	28	16	0·9	1·1
Ser201Cys	Interdomain loop	27	28	16	**4·3**	**4·9**
Ser201Ala	Interdomain loop	96	32	14	1·3	1·2
Ser201Thr	Interdomain loop	96	32	14	1·3	1·2
Leu9Phe	TM1	75	24	12	1·3	1·3
Leu9Phe/Ser201Cys	TM1 and interdomain loop	96	24	12	1·0	1·0
Gly20Cys	TM1	32	12	3	1·4	0·6
Asn184Cys	Interdomain loop	96	24	14	1·0	1·2
Thr191Cys	Interdomain loop	96	24	12	1·0	1·0
Val339Cys	TM11	80	16	7	0·8	0·7
Gly366Cys	Periplasmic loop 11–12	80	24	12	1·2	1·2
No plasmid	–	0·75	1·25	0·63	–	–

*Mean MIC values of three separate experiments obtained by the E-test method (see Methods).
†MIC of control cells was subtracted before calculation of ratios. Ratios are normalized to WT-TetB = 1.

Effect of substitution on tetracycline resistance at Ser201 with Thr, Ala or Cys

The plasmid bearing the Ser201Cys mutation (Kimura *et al.*, 1997) was obtained from A. Yamaguchi. The two additional substitutions chosen were Ala (small and hydrophobic) and Thr (conservative mutation that maintains the hydroxyl group of Ser). The Ala and Thr substitutions of Ser201 each lowered the tetracycline MIC by only 1·3-fold (Table 2). Therefore, a hydroxyl group at position 201 is not obligatory for tetracycline resistance function, nor does the residue need be hydrophilic. As expected, the Cys mutation reduced tetracycline resistance by 4·8-fold (Table 2).

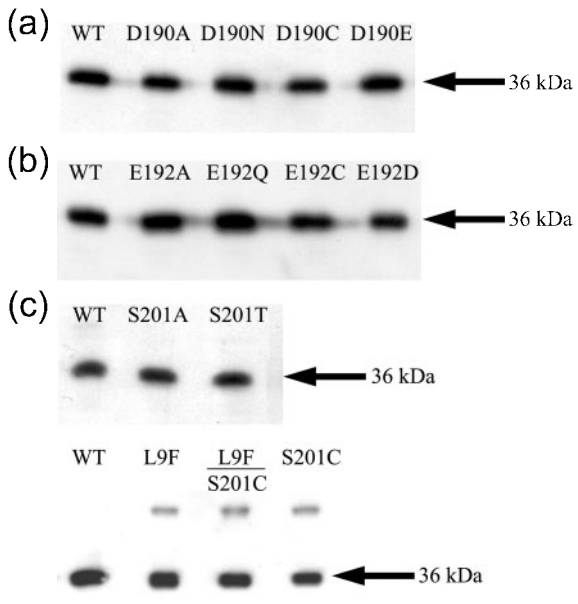

Fig. 2. Western blot analysis of wild-type and mutated TetA(B) proteins. Proteins from total-cell lysates were separated by SDS-PAGE, electroblotted and probed with anti-Ct antibody. (a) Asp190 substitutions; (b) Glu192 substitutions; (c) Ser201 substitutions and second-site suppressor of Ser201Cys. Arrows indicate the position of the 36 kDa Tet protein. WT, Wild-type.

Effect of various substitutions at Asp190, Glu192 and Ser201 and a second-site Ser201Cys suppressor mutation on substrate specificity

To assay whether a region of TetA(B) is involved in tetracycline binding, one may ask if amino acid substitutions in that region change the specificity of the pump for

different tetracycline analogues. For the wild-type and mutant proteins, we therefore compared the resistance to tetracycline with that to two other analogues, doxycycline and minocycline (Table 2). We noted that no resistance was offered by any of the mutants to 9-(*t*-butylglycylamido)-minocycline (data not shown), a glycylcycline used in previous studies (Tuckman *et al.*, 2000). The data are presented as the ratio of the MIC for the analogue to the MIC for tetracycline, which was then normalized to that for the wild-type.

The substitutions at Asp190 and Glu192 with Cys, Ala and Asn or Gln primarily altered tetracycline resistance without affecting analogue resistance (except Asp190Cys). The effect led to a 1·8- to 6·2-fold preference for doxycycline/minocycline over tetracycline relative to the wild-type pump (Table 2) and so altered substrate specificity. Only those substitutions that have greater than a 1·5-fold effect were considered as having altered the substrate specificity. Of note, retention of the negative charge at Asp190 and Glu192 restored tetracycline resistance, removing the preference for doxycycline and minocycline over tetracycline.

At position Ser201, substitution with Ala or Thr had no effect on specificity. However, substitution with Cys led to a 4·3- to 4·9-fold relative preference for doxycycline/minocycline. Again, this altered specificity can be seen as a loss in tetracycline resistance and not in analogue resistance, when compared to wild-type (Table 2).

Since the inactivating Ser201Cys substitution of class B is at a position similar to that of the inactivating Ser202Phe mutation of class C (Saraceni-Richards & Levy, 2000a), we examined whether the former would be suppressed by Leu9Phe, which corresponds to the Leu11Phe in TM1 which suppresses the class C mutation (Sapunaric & Levy, 2003). The Leu9Phe replacement indeed suppressed the Ser201Cys mutation, restoring a nearly wild-type level of tetracycline resistance; the Leu9Phe replacement by itself had little effect on TetA(B) function (Table 2). It is therefore possible that this secondary suppressor mutation modifies the structure of TM1, which in turn corrects the interdomain region that had been inactivated by the Ser201Cys mutation. The substrate specificity effect of 201Cys is suppressed by Leu9Phe, by restoring the tetracycline resistance (Table 2).

We conclude that retention of the negative charge at residues Asp190 and Glu192 did not affect substrate specificity. If the replacement at residues 190 and 192 was uncharged, but retained the same size, the specificity was somewhat altered. If the replacement was smaller (Ala or Cys), a more dramatic specificity shift occurred. At position Ser201, substitutions of a similar size had no effect, except for Cys. At all three positions, replacement by Cys caused the most drastic shift in specificity towards the analogues. Since doxycycline and minocycline are both more lipophilic than tetracycline (Barza *et al.*, 1975), all of the specificity-altering substitutions may somehow reduce the ability of the protein to handle hydrophilic substrates.

To further demonstrate that the substrate specificity effects attributed to the mutations at Asp190, Glu192 and Ser201 of TetA(B) were meaningful, we chose as 'controls' four cysteine mutants which significantly lowered activity of TetA(B) according to Tamura *et al.* (2001): Gly20Cys, Val339Cys, Gly366Cys and Asn184Cys. We also intentionally chose the Thr191Cys mutant for its strategical position between Asp190 and Glu192 as another 'control' (Tamura *et al.*, 2001). The single cysteine mutations on a low-copy-number plasmid were provided by A. Yamaguchi and their tetracycline MICs were consistent with those reported previously (Kimura-Someya *et al.*, 2000; Konishi *et al.*, 1999; Tamura *et al.*, 2001). For each single cysteine mutant, the MICs and the ratio of the MIC for the two analogues to the MIC for tetracycline showed a proportional decrease in resistance to both tetracycline and to the analogues in all cases (see Table 2). None of the five cysteine 'control' mutations provided a change of substrate specificity towards the tetracycline analogues tested. We conclude that Asp190, Glu192 and Ser201 in the interdomain cytoplasmic loop all appear to be involved in substrate specificity and therefore are likely to interact with the substrate.

The substrate for TetA is a tetracycline–divalent metal cation complex with a net charge of +1 (Yamaguchi *et al.*, 1990b, 1991). That the negative charge of Asp190 and Glu192 is not essential (Table 2) suggests that these residues may not interact directly with the Mg^{2+} region of the substrate. Moreover, substitutions at the three positions cause a greater loss in activity towards tetracycline than towards the lipophilic analogues, which also utilize Mg^{2+}.

Other negative residues located in TMs and loops of TetA(B) that are important for activity have been identified previously. The negative charge held by Asp15 (TM1) (McMurry *et al.*, 1992; Yamaguchi *et al.*, 1992b), Asp66 (loop 2-3) (Yamaguchi *et al.*, 1992b), Asp84 (TM3) (Yamaguchi *et al.*, 1992b) and Asp285 (Yamaguchi *et al.*, 1992b), were shown to be important for transport activity. For Asp15 and Asp84, helical projections and cysteine-scanning mutagenesis demonstrated that both residues are oriented towards the putative central water-filled channel and may be a part of the translocation pathway of tetracycline (Tamura *et al.*, 2001). Asp66 is located in the conserved sequence motif, GXXXXRXGRR, of cytoplasmic loop 2-3, which was postulated to be a part of the entrance gate of the tetracycline divalent cation complex (Yamaguchi *et al.*, 1992a, 1990c). In contrast with the previous negatively charges residues, Asp285, located in the TM9, is essential for TetA(B) activity (Yamaguchi *et al.*, 1992b) and cannot be replaced even with glutamate (Yamaguchi *et al.*, 1992b).

Other residues involved in substrate specificity in TetA(B) are Gly111Glu (TM4), Trp231Cys and Trp231Gly (TM7), Leu253Phe (TM8) and Leu308Ser (TM10) (Fig. 1) all of which increase the resistance to 9-(dimethylglycylamido)-minocycline and tigecycline GAR936 (Guay *et al.*, 1994; Tuckman *et al.*, 2000). Since these mutations lie in or near regions which probably face the putative water-filled

channel (see Tamura *et al.*, 2001), the different substitutions may introduce a conformational change in their respective TM so as to preferentially affect the binding and/or translocation pathway of the tetracycline analogues.

In LacY, another member of major facilitator superfamily, the interdomain loop affects the insertion and stability of the protein into the membrane, but has not been implicated in substrate binding (Weinglass & Kaback, 2000). Hydrophilic residues within the interdomain loop of LacY are required to permit a temporal delay for the insertion of both domains. In TetA(B), the cytoplasmic interdomain loop is more than a linker between the α and β domains since mutations in it affect resistance (Sapunaric & Levy, 2003; Saraceni-Richards & Levy, 2000a). We have shown here that this loop also possesses at least three residues that appear to be involved in interacting with the tetracycline/Mg^{2+} substrate.

Structural analysis of the interdomain loop of tetracycline efflux pumps

The crystal structure of two transporters belonging to the major facilitator superfamily, the *E. coli* lactose permease (LacY) and the glycerol 3-phosphate transporter GlpT were solved at 3·5 and 3·3 Å respectively (Abramson *et al.*, 2003; Huang *et al.*, 2003). In addition to their 12 TM segments, secondary structures were revealed in both interdomain loops. No function has been attributed to it, but stability of the protein appears linked to the interdomain loop of LacY (Weinglass & Kaback, 2000). Since the interdomain loop has no homology among the related TetA proteins, it is surprising that it appears to play a part in binding to substrate. We reasoned that secondary structure might be present in the loop region of all TetA proteins.

Using PSIPred, a computational tool from University College London, that predicts secondary structure (Jones, 1999; McGuffin *et al.*, 2000), we analysed the secondary structure of the interdomain loop of 14 TetA efflux pump homologues. We used the 3D structure of LacY and GlpT (Abramson *et al.*, 2003; Huang *et al.*, 2003) as controls to test the validity of the prediction. The prediction from PSIPred showed two short helical segments in the interdomain cytoplasmic loop of LacY, in agreement with the resolved structure (Fig. 3a). The degree of confidence for each of the two helices is different, with a very high score for the second structure of eight residues. The shorter one was

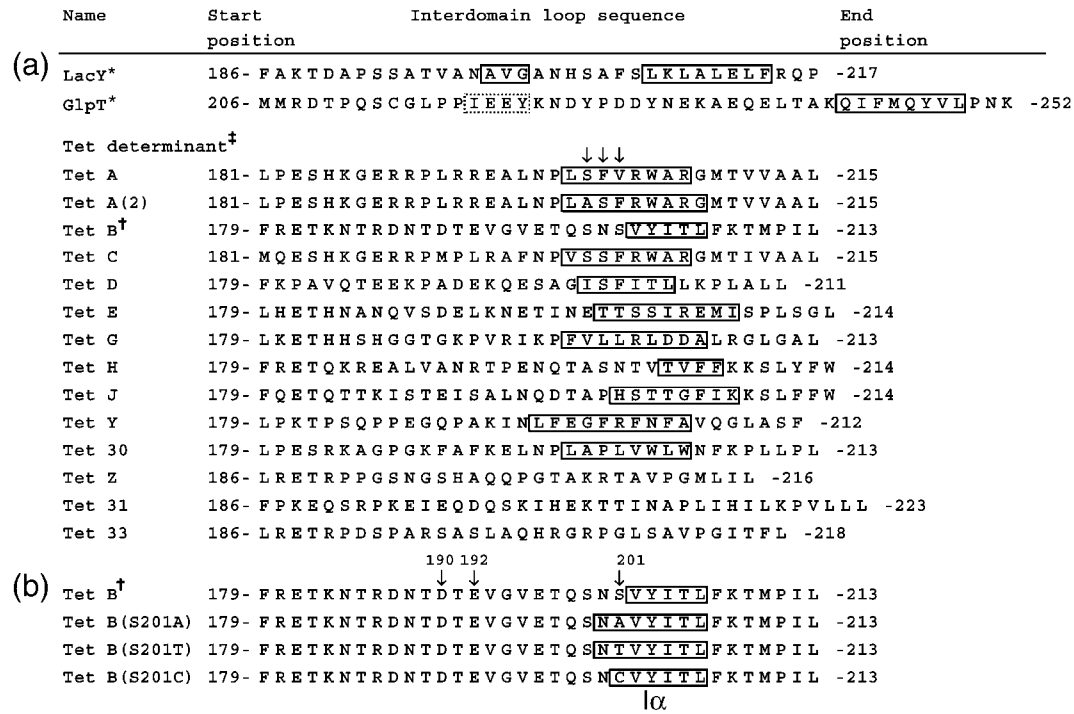

Fig. 3. Predicted secondary structure of the interdomain loop of tetracycline efflux TetA proteins from 14 different tetracycline resistance determinants compared to known LacY and GlpT structures. (a) Secondary structure in LacY and GlpT and predicted secondary structure in TetA efflux proteins. (b) Prediction of secondary structure in wild-type and in serine 201 substitution TetA(B) mutants. Iα, α-helix in the interdomain loop of TetA efflux protein (boxed). Arrows indicate positions affecting substrate specificity in TetA(A) and TetA(B). *, Coordinates of start and end residues of the interdomain loop of LacY and GlpT were determined in their 3D structure (Abramson *et al.*, 2003; Huang *et al.*, 2003); †, coordinates of the interdomain loop of TetA(B) are based on cysteine-scanning analysis (Tamura *et al.*, 2001); ‡, coordinates of the interdomain residues of the TetA efflux determinants are derived from TetA(B) coordinates after ClustalW alignment of all the Tet efflux proteins.

misinterpreted by one residue. The secondary structure prediction for GlpT also proposes two short helices, one of four amino acids, 219IEEY223, and one of seven residues, 242QIFMQYV248. The actual structure of GlpT has only the second helix (Huang *et al.*, 2003). The predicted first short helix might be missing since it is adjacent to a disordered region from Asn232 to Leu239. Based on comparison of these predictions with the known structures, we accepted a PSIPred value of $\geqslant 4$ as indicating a good level of confidence in predicting a secondary structure and, moreover, that a difference between two successive values equal or greater than 2 would put an end to the predicted secondary structure. As can be noted, in almost all of the tetracycline efflux pumps examined, a short α-helical segment was predicted (Fig. 3a). The length of this secondary structure varied from four to ten residues. This new putative structured element of the interdomain loop of tetracycline efflux proteins was named Iα (for interdomain α-helix). In only three tetracycline transporters [Tet(Z), Tet(31) and Tet(33)] were no helix or other secondary structures predicted in the interdomain loop. We note that Tet(Z) and Tet(33) are closely related and, unlike the other classes, come from Gram-positive organisms. For TetA(A), two different sequences are shown in Fig. 3(a). The first corresponds to the wild-type TetA(A) (GenBank accession no. P02982) and the second sequence, TetA(A)(2), is from a veterinary *Salmonella* minocycline-resistant isolate which diverges from the wild-type by three amino acid changes in the interdomain loop (Tuckman *et al.*, 2000). This modification is accompanied by a change in substrate specificity of the pump (Tuckman *et al.*, 2000). The mutations in TetA(A)(2) increase the length of the predicted Iα by one residue and raise the level of confidence for the entire segment.

For TetA of class A and C, the level of confidence is high for the predicted Iα. These two proteins share high identity (78 %) over their full lengths. Moreover, they are the only two classes that have extensive identity (68 %) over their interdomain loop. For class B, D and H, shorter secondary structures were predicted with a lower level of confidence [residues 202–206 for TetA(B)]. The sequences in helix Iα are different for each of the TetA proteins.

We also analysed the effect of the different substitutions at Ser201 in TetA(B), since this serine is at the border of the predicted Iα. As can be seen in Fig. 3(b), each of the three substitutions increased the length of the predicted Iα structure by one or two residues. Another factor is that residues 201, 203, 204, (and 211) of TetA(B), when changed to Cys, were the only residues in the interdomain loop inaccessible to a sulfhydral reagent (Tamura *et al.*, 2001). Thus, these four residues might be 'occluded', further supporting the idea of an ordered secondary or tertiary structure covering this region. In the structures of both LacY and GlpT, the longer Iα is at a similar position in the interdomain loop, although no function has been attributed to it (Abramson *et al.*, 2003; Huang *et al.*, 2003). According to the structural data of LacY (Abramson *et al.*, 2003) and of GlpT (Huang *et al.*, 2003), this helix lies parallel to the membrane surface near the cytoplasmic end of TM11.

Concluding remarks

Our data suggest that residues Asp190, Glu192 and Ser201 of the interdomain loop of TetA(B) interact with substrate. The change of the substrate specificity caused by single mutations depended on the nature of the substitution itself and was associated with an increased length of a putative helical secondary structure Iα in the interdomain loop of TetA(A) and TetA(B).

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