Identification of structural and functional domains of the tetracycline efflux protein TetA(P) from *Clostridium perfringens*

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The *Clostridium perfringens* tetracycline-resistance protein, TetA(P), is an integral inner-membrane protein that mediates the active efflux of tetracycline from the cell. TetA(P) acts as an antiporter, presumably transporting a divalent cation–tetracycline complex in exchange for a proton, and is predicted to have 12 transmembrane domains (TMDs). Two glutamate residues that are located in predicted TMD 2 were previously shown to be required for the active efflux of tetracycline by TetA(P). To identify additional residues that are required for the structure or function of TetA(P), a random mutagenesis approach was used. Of the 61 tetracycline-susceptible mutants that were obtained in *Escherichia coli*, 31 different derivatives were shown to contain a single amino acid change that resulted in reduced tetracycline resistance. The stability of the mutant TetA(P) proteins was examined by immunoblotting and 19 of these strains were found to produce a detectable TetA(P) protein. The MIC of these derivatives ranged from 2 to 15 µg tetracycline ml⁻¹, compared to 30 µg tetracycline ml⁻¹ for the wild-type. The majority of these mutants clustered into three potential loop regions of the TetA(P) protein, namely the cytoplasmic loops 2–3 and 4–5, and loop 7–8, which is predicted to be located in the periplasm in *E. coli*. It is concluded that these regions are of functional significance in the TetA(P)-mediated efflux of tetracycline from the bacterial cell.

Keywords: tetracycline, resistance, efflux, transmembrane domains, *Clostridium perfringens*

INTRODUCTION

Tetracycline antibiotics inhibit bacterial protein synthesis by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Schnappinger & Hillen, 1996). Bacterial resistance to tetracycline is generally mediated by active efflux of tetracycline from the cell or by the protection of ribosomes against the action of tetracycline (Speer *et al.*, 1992). Active efflux is mediated by multidrug resistance proteins (Paulsen *et al.*, 1996) or proteins that specifically efflux tetracycline (Levy, 1992). The latter have been identified in both Gram-positive [TetA(K), TetA(L), TetA(P) and OtrB] and Gram-negative [TetA(A–E), TetA(G) and TetA(H)] bacteria (Roberts, 1996). These tetracycline efflux

proteins are generally regarded as members of the major facilitator superfamily (MFS), which includes uniporters and symporters as well as antiporters (Pao *et al.*, 1998). Members of this family have been either shown or predicted to be integral membrane proteins that utilize the proton motive force for transport functions. Within this family, groups have been differentiated by their function and by the number of transmembrane domains (TMDs) contained within the protein (Pao *et al.*, 1998).

The prototype tetracycline efflux protein, TetA(B), is encoded on the transposable element Tn10 from *Escherichia coli*. It is an integral membrane protein which catalyses the antiport of a divalent cation–tetracycline complex and a proton (Yamaguchi *et al.*, 1990b). It has been experimentally shown to have 12 TMDs (Allard & Bertrand, 1992; Eckert & Beck, 1989; Kimura *et al.*, 1997) and has a very high level of amino acid sequence identity to a range of tetracycline efflux

Abbreviations: MFS, major facilitator superfamily; TMD, transmembrane domain.

proteins from other Gram-negative bacteria. Many residues that are required for TetA(B) activity have been identified (Kimura *et al.*, 1998; Kimura & Yamaguchi, 1996; Yamaguchi *et al.*, 1990a, 1992b, 1993a, 1996). In particular, three negatively charged amino acids, D15, D84 and D285, which are situated within TMDs, are essential for tetracycline transport function, probably by forming a transmembrane efflux pathway for the tetracycline complex (Yamaguchi *et al.*, 1992a). Other conserved negatively charged residues located within predicted loop regions of tetracycline efflux proteins from Gram-negative bacteria are also required for efficient tetracycline efflux (Yamaguchi *et al.*, 1992c).

Two tetracycline efflux proteins from Gram-positive organisms, TetA(K) (Mojumdar & Khan, 1988) and TetA(L) (McMurry *et al.*, 1987), differ from TetA(B). Both are larger proteins, with TetA(K) shown to consist of 14 TMDs (Ginn *et al.*, 1997). The TetA(K) protein contains three functional glutamate residues that are located within TMDs (Fujihara *et al.*, 1996). These residues appear to play a similar role to the transmembrane-located aspartate residues of TetA(B). In addition, several aspartate residues that are conserved in TetA(K) and TetA(L), and are located in hydrophilic loops, are required for the function of TetA(K) (Fujihara *et al.*, 1997). Within hydrophilic loop 2–3, the TetA(K) and TetA(B) proteins contain a conserved motif (GxxxxRxGRR) that is common to this family of MFS transport proteins (Henderson, 1990; Paulsen *et al.*, 1996). Site-directed mutagenesis has shown that this motif plays a role in the translocation of tetracycline across the membrane (Fujihara *et al.*, 1997; Yamaguchi *et al.*, 1992d). The same motif is also important for other transporters such as α -ketoglutarate permease (Seol & Shatkin, 1993) and lactose permease (Jessen-Marshall *et al.*, 1995).

Tetracycline resistance in the Gram-positive anaerobe *Clostridium perfringens* is primarily due to the presence of the Tet(P) determinant. This novel determinant has two overlapping tetracycline-resistance genes. The *tetA(P)* gene encodes an integral membrane protein responsible for the active efflux of tetracycline from the cell, whereas the product of the *tetB(P)* gene has very strong similarity to members of the Tet(M) family of ribosomal-protection tetracycline-resistance proteins (Sloan *et al.*, 1994). The efflux protein TetA(P) comprises 420 amino acids and is predicted to have 12 TMDs, but it does not have significant amino acid sequence identity to other tetracycline efflux proteins. As for most other tetracycline efflux proteins, acidic residues located within TMDs have been shown to be required for the efflux of tetracycline. Like TetA(K), TetA(P) contains two TMD-located glutamate residues that are required for function, although unlike TetA(K) these residues are both situated within putative TMD 2 (Kennan *et al.*, 1997).

Since TetA(P) has very little similarity to other tetracycline efflux proteins and does not contain the conserved MFS motifs, we decided to carry out additional

studies on this unusual efflux protein. In this paper we report the results of random mutagenesis experiments that were successfully used to identify TetA(P) residues of structural or functional significance.

METHODS

Bacterial strains and culture methods. The *E. coli* host strain used for most of these experiments was DH5 α (Life Technologies). Bacterial strains were grown in 2 $\times$ YT media (Miller, 1972) supplemented with 100 μ g ampicillin ml⁻¹ and various concentrations of tetracycline, where appropriate.

Random mutagenesis. Random mutants of the *tetA(P)* gene were isolated by passing the plasmid pJIR71 (Abraham *et al.*, 1988), a pUC18 derivative which encodes the complete *tetA(P)* gene including the upstream regulatory region, through the DNA-repair-deficient strain XL1-Red. The mutagenesis procedure was as described in the Epicurian Coli XL1-Red Competent Cells instruction manual (Stratagene). Three independently derived samples of pJIR71 DNA were isolated after passage through XL1-Red and were used to transform competent *E. coli* DH5 α cells. The resultant ampicillin-resistant single colonies were screened for their ability to grow in the presence of 10 μ g tetracycline ml⁻¹ and susceptible colonies were selected and retested. The *tetA(P)* genes from all of the tetracycline-sensitive derivatives that had a missense mutation were then completely sequenced with the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI373A automated fluorescent sequencing apparatus (Applied Biosystems). Other mutants were partially sequenced in a manner sufficient to identify the nature of the mutation.

Tetracycline MIC. For each of the *tetA(P)* mutants, the tetracycline MIC was determined at 37 °C as described previously (Kennan *et al.*, 1997). Each strain was tested for growth inhibition at the concentrations ranging from 1 to 30 μ g tetracycline ml⁻¹.

Immunoblot analysis. The TetA(P) protein was detected by immunoblot analysis as described previously (Kennan *et al.*, 1997), with the following modifications. *E. coli* cells were lysed by sonication (4 $\times$ 30 s) and whole cells were removed by centrifugation at 12000 *g* for 2 min. Samples of the resultant cell lysates were mixed with gel loading buffer at room temperature and were subjected to SDS-PAGE (Laemmli, 1970). Samples of known protein concentrations were loaded onto the gel and, following ECL Western blotting detection (Amersham Life Sciences), the amount of each of the TetA(P)-derived bands detectable with the anti-TetA(P) antiserum (Kennan *et al.*, 1997) was determined using a 2022 Ultrascan laser densitometer (LKB Produkter).

RESULTS AND DISCUSSION

Isolation of random tetracycline-sensitive TetA(P) mutants

The TetA(P) protein removes tetracycline from the cell by an active efflux process (Sloan *et al.*, 1994) that is dependent upon three glutamate residues, E52, E59 and E89, two of which are proposed to be located within TMD 2 (Kennan *et al.*, 1997). To identify additional structural or functional residues of TetA(P), a random mutagenesis approach was used. A plasmid, pJIR71 (Abraham *et al.*, 1988), that contained the entire *tetA(P)*

Fig. 1. Schematic representation of the proposed membrane structure of the TetA(P) protein from *C. perfringens*. This model was generated with the TopPred II algorithm (Claros & von Heijne, 1994) and the hydrophobicity scale of Kyte & Doolittle (1982). The TMDs are shown as rectangles and are numbered in a square in the top-left corner of each TMD. The non-enclosed numbers correspond to the amino acid residue present at the beginning and end of each TMD. Residues of TetA(P) that were changed and resulted in reduced resistance to tetracycline have been circled. The resultant residue at these positions is enclosed based on the following criteria: squares, immunoreactive TetA(P) protein detectable at $\geq 40\%$ of wild-type; diamonds, TetA(P) detectable at 5–40% of wild-type; uneven shape, TetA(P) levels at $< 1\%$ of wild-type; white on black, TetA(P) mutants with a tetracycline MIC < 4 ; black on white, TetA(P) mutants with a tetracycline MIC of 7.5–15. Note that the wild-type MIC is $30 \mu\text{g}$ tetracycline ml^{-1} . In addition, the previously identified (Kennan *et al.*, 1997) functional glutamate residues (E52, E59 and E89) have been outlined.

gene was passed through the DNA-repair-deficient strain XL1-Red. After a second cycle of transformation into the *E. coli* strain DH5 α , 5700 ampicillin-resistant colonies were screened for their ability to grow in the presence of $10 \mu\text{g}$ tetracycline ml^{-1} . Sixty-one tetracycline-sensitive mutants were isolated, which corresponds to a mutant detection frequency of 1.1%. After confirming that the mutants all carried plasmids that were indistinguishable from pJIR71 by restriction analysis, the *tetA(P)* structural gene and promoter region of all of these mutants was partially sequenced to identify the mutated nucleotide. Subsequently, every derivative which contained a mutation that resulted in a unique single amino acid substitution was completely sequenced to confirm that only one mutation was present.

The current model of the TetA(P) protein (Fig. 1) was derived using the TopPred II algorithm (Claros & von

Heijne, 1994), in combination with the positive-inside rule for integral membrane proteins (von Heijne, 1992). Although the membrane structure of the TetA(P) protein is yet to be experimentally determined, previous analyses of integral membrane proteins using this or similar algorithms have shown that the computer-generated structures are usually close representations of the expected protein structure (Boyd *et al.*, 1993; Paulsen *et al.*, 1995; Roy & Isberg, 1997). Accordingly, the predicted single amino acid changes in the mutant TetA(P) proteins were analysed in the context of the TetA(P) transmembrane model.

Several categories of tetracycline-sensitive mutants were identified. Half of the mutants were found to contain a single mutation in the *tetA(P)* structural gene (Table 1; Fig. 1). These 31 mutations were scattered throughout the whole of the TetA(P) protein, with the majority occurring in the N-terminal half of the molecule. This

Table 1. Characteristics of tetracycline-sensitive TetA(P) derivatives containing missense mutations

Plasmid	Nucleotide mutation*	TetA(P) derivative†	Tetracycline MIC ($\mu\text{g ml}^{-1}$)	TetA(P) protein (% WT)‡
pJIR71	—	Wild-type	30.0	100
pJIR1694	525AA	Promoter mutant	15.0	40
pJIR1693	T1241C	I60T	10.0	60
pJIR1686	C1243T	P61S	2.0	6
pJIR1651	A1246G	T62A	4.0	16
pJIR1668	C1259T	A66V	2.0	<1
pJIR1670	C1273T	R71C	2.0	<1
pJIR1663	G1294A	G78R	2.0	<1
pJIR1655	G1295A	G78E	2.0	<1
pJIR1707	G1310A	G83E	2.0	<1
pJIR1698	C1366A	Q102K	7.5	62
pJIR1699	G1385A	G108E	2.0	<1
pJIR1673	G1403A	G114D	7.5	132
pJIR1677	C1415T	A118V	2.0	8
pJIR1689	C1424A	A121E	2.0	37
pJIR1662	G1429A	E123K	2.0	<1
pJIR1665	A1430G	E123G	4.0	<1
pJIR1679	G1468A	G136R	2.0	20
pJIR1681	G1489A	G143R	2.0	<1
pJIR1683	G1562A	G167E	2.0	<1
pJIR1678	C1625T	P188L	10.0	5
pJIR1702	G1630A	A190T	15.0	159
pJIR1658	C1634T	P191L	7.5	27
pJIR1672	G1756A	E232K	2.0	59
pJIR1701	G1765A	D235N	7.5	134
pJIR1695	A1766G	D235G	10.0	13
pJIR1659	G1844A	G261E	4.0	55
pJIR1675	G2107A	A349T	15.0	172
pJIR1654	C2141T	S360F	2.0	100
pJIR1680	C2165T	P368L	7.5	13
pJIR1674	G2207A	G382D	2.0	<1
pJIR1692	T2221C	S387P	10.0	<1
pJIR1661	C2236T	P392S	20.0	<1

* Nucleotide positions refer to the previously published sequence of the *tet(P)* gene region (Sloan *et al.*, 1994) (GenBank accession no. L20800). Nucleotide position 1063 is the first base of the *tetA(P)* structural gene.

† Original TetA(P) residue, number of the residue, mutant residue.

‡ Mean of at least two independent experiments.

result suggests that the N-terminal domain is most important for the structure or function of TetA(P) since it is unlikely that this distribution reflects any inherent bias in the mutagenesis technique. Surprisingly, no derivatives were detected that contained mutations in the previously identified (Kennan *et al.*, 1997) functional amino acids of TetA(P). One additional plasmid had a single mutation in the promoter region (Table 1). Of the remaining mutants, 13 plasmids encoded truncated TetA(P) proteins that were derived from either the introduction of a stop codon or frameshift mutations that resulted from the insertion or deletion of a single base (Table 2). The final 16 mutants were not studied further: they included five mutants which had multiple

mutations and 11 derivatives that were identical to mutants already isolated.

To examine the quantitative effect of the mutations on tetracycline resistance, we determined the tetracycline MIC of *E. coli* DH5 α derivatives carrying the mutated pJIR71-derived plasmids. Note that the level of tetracycline efflux precluded a more detailed kinetic analysis of the individual mutant proteins (Sloan *et al.*, 1994; Kennan *et al.*, 1997).

The wild-type derivative DH5 α (pJIR71) had an MIC of 30 $\mu\text{g ml}^{-1}$, whereas the negative control DH5 α (pUC18) had an MIC of 2 $\mu\text{g ml}^{-1}$. Each of the mutated plasmids tested had an MIC of between 2 and 15 $\mu\text{g ml}^{-1}$ (Table

Table 2. Characteristics of tetracycline-sensitive TetA(P) truncation derivatives

Plasmid	Nucleotide mutation*	TetA(P) derivative†	Tetracycline MIC ($\mu\text{g ml}^{-1}$)
pJIR1704	1298 + G	TetA(P)79 + 14	2
pJIR1706	C1474T	TetA(P)138	2
pJIR1660	G1595A	TetA(P)178	2
pJIR1687	1636 + T	TetA(P)192 + 13	10
pJIR1708	1658 + A	TetA(P)199 + 5	2
pJIR1671	1699 Δ A	TetA(P)212 + 3	2
pJIR1653	1790 + T	TetA(P)242 + 7	2
pJIR1664	1790 Δ T	TetA(P)242 + 14	2
pJIR1652	1839 Δ G	TetA(P)258 + 7	2
pJIR1666	1851 + T	TetA(P)263 + 19	2
pJIR1667	G2075A	TetA(P)337	ND
pJIR1656	2084 Δ G	TetA(P)341 + 1	2
pJIR1700	C2131T	TetA(P)357	2

ND, Not determined.

* Nucleotide positions refer to the previously published sequence of the *tet(P)* operon (Sloan *et al.*, 1994). Nucleotide position 1063 is the first base of the TetA(P) structural gene. +, Insertion of a nucleotide at that position; Δ , deletion of a nucleotide.

† Mutations have introduced either stop codons, e.g. TetA(P)138, or a frameshift which leads to a certain number of normal TetA(P)-encoded residues plus residues following the frameshift up to the next stop codon, e.g. TetA(P)79 + 14.

1). Each of the truncated TetA(P) mutant proteins tested did not confer resistance to tetracycline (MIC $2 \mu\text{g ml}^{-1}$). The one exception was pJIR1687, which harboured a TetA(P) derivative truncated at amino acid 192, which includes the first six of the proposed 12 TMDs of TetA(P) (Fig. 1). Strains carrying pJIR1687 had an MIC of $10 \mu\text{g ml}^{-1}$. It is possible that the low level tetracycline resistance observed with this mutant is due to multiple 6-TMD monomers forming a partially active TetA(P) derivative. A variation of this result was observed with the *E. coli* TetA(B) protein. When expressed independently, the first half of TetA(B) was not able to efflux tetracycline, but when the N-terminal and C-terminal 6 TMD regions of TetA(B) were expressed together within a cell, 40% of wild-type tetracycline efflux was observed (Yamaguchi *et al.*, 1993b).

Detection of mutant proteins by immunoblotting

To examine the level of expression of the mutant TetA(P) proteins, cell extracts were prepared from the 31 DH5 α derivatives carrying plasmids that were predicted, from DNA sequence analysis, to produce TetA(P) proteins that contained single amino acid changes. These extracts were examined by immunoblotting with a TetA(P)-specific polyclonal antiserum that was raised by use of a synthetic peptide which consisted of the last 17 amino acids of TetA(P) (Kennan *et al.*, 1997). In these experiments, 18 of the 31 mutants produced detectable TetA(P) protein (Fig. 2), with TetA(P) expression levels varying from 5 to 172% when compared to the wild-type (Table 1). Control experi-

ments that utilized different amounts of wild-type cell lysates showed that TetA(P) protein levels could be reliably titrated using this procedure (data not shown). Similar analysis showed that DH5 α (pJIR1694), which carries the only promoter mutation detected in this study, produced slightly reduced levels of TetA(P) (P. Johanesen, D. Lyras & J. Rood, unpublished results).

The remaining 13 TetA(P) mutants did not produce any detectable TetA(P) protein. Failure to produce wild-type levels of TetA(P) could be due to the mutations resulting in conformational changes that decreased the stability of these TetA(P) proteins. Alternatively, these derivatives may be producing a less immunoreactive protein.

Mutants that did not produce detectable TetA(P) protein

Within the group of mutant TetA(P) proteins analysed, a wide range of protein expression levels was observed. In addition, we note that this random mutagenesis approach produced an unexpectedly high percentage (42%) of mutant TetA(P) derivatives from which no detectable TetA(P) protein was observed, each resulting in tetracycline sensitivity (MIC $2 \mu\text{g ml}^{-1}$). Previous studies of the TetA(B) protein, which also involved the examination of large numbers of mutant derivatives, reported that very few mutant proteins were unable to be detected with anti-TetA(B) protein antiserum (Kimura *et al.*, 1998; Yamaguchi *et al.*, 1992c, d).

Of the 13 mutants that did not produce detectable TetA(P) protein, six mutations involved glycine residues that were located within proposed TMDs, with each of

Fig. 2. Western blot analysis of mutant TetA(P) derivatives. Cell lysates (30 µg) were subjected to SDS-PAGE, transferred to nitrocellulose, reacted with polyclonal rabbit anti-TetA(P) antibodies, and analysed with the ECL method (Boehringer Mannheim). In both (a) and (b), the lanes are labelled with the relevant TetA(P) derivative. The corresponding tetracycline MIC is included as is the relative TetA(P) expression level, shown as the percentage of wild-type TetA(P). Lane WT, cell lysates from the wild-type DH5α(pJIR71); lane C, cell lysates from the vector control strain DH5α(pUC18).

these changes involving replacement with a charged residue, i.e. the mutants G78E, G78R, G83E, G108E, G143R and G167E. The addition of these charged residues appears to have produced unstable TetA(P) derivatives, which is consistent with the proposed location of these residues within a hydrophobic environment. It is likely that the introduction of a charged residue into a TMD has destabilized the integral transmembrane structure of the TetA(P) protein. A similar hydroxylamine-based random mutagenesis approach, when used to locate functional residues of the TetA(C) protein, also led to the identification of a high proportion of mutants containing glycine to charged amino acid changes (McNicholas *et al.*, 1992).

Other pJIR71 mutants that were producing undetectable levels of TetA(P) were derivatives carrying the mutations A66V, R71C, E123G, E123K, G382D, S387P and P392S. It is not known why these changes destabilized the TetA(P) protein although it is possible that the R71 and E123 residues form salt-bridges that may be required for the correct TetA(P) structure. Note that the failure to detect the S387P and P392S proteins may reflect their inability to be recognized by the C-terminal-specific TetA(P) antibodies.

Mutants in loop 2–3 of TetA(P)

Of greatest interest were the mutants that harboured stably produced TetA(P) proteins with lower tetracycline MIC values. These mutants represent functional rather than purely structural changes in the protein. Most of these mutants were clustered into putative cytoplasmic or periplasmic loop regions of the TetA(P) protein. Located within or very near to the proposed loop 2–3 were three TetA(P) mutants, I60T, P61S and T62A (Fig. 1). Each of the mutants produced detectable TetA(P) protein and had a lower tetracycline MIC, suggesting that these residues may play a role in the function of TetA(P). In particular, the TetA(P)-I60T protein was expressed at a high level and had an intermediate resistance phenotype (Table 1). By contrast, mutants P61S and T62A consistently produced

detectable, but much lower, levels of immunoreactive TetA(P) protein. In fact, every proline mutant detected in this study produced detectable but lower amounts of the TetA(P) protein. This result was consistent with the likely structural role of proline residues within the TetA(P) protein.

The E52 and E59 residues were previously shown to be essential for tetracycline efflux (Kennan *et al.*, 1997). We have now shown that the mutation of several amino acids located in close proximity to these residues also leads to loss of tetracycline resistance. These studies provide strong evidence that regions close to the proposed cytoplasmic loop 2–3 are important for the structure and function of TetA(P). In this region, the A66V and R71C mutations also resulted in loss of tetracycline resistance although they led to the production of either an unstable protein or a protein no longer recognized by the antiserum. Similar results were observed when the nearby residue D67 was targeted previously by site-directed mutagenesis (Kennan *et al.*, 1997). The equivalent loop in other tetracycline efflux proteins contains the functionally essential conserved motif (GxxxxRxGRR) (Yamaguchi *et al.*, 1992d). Although the TetA(P) protein does not have this exact motif it is clear that the same region of the protein is important for tetracycline efflux.

Conserved efflux protein motif

Comparison of TetA(P) with the databases only identified proteins with relatively low levels of similarity. The highest level of amino acid sequence identity observed was approximately 20%, occurring mainly with putative transport proteins whose functions are yet to be characterized. These include a hypothetical multi-drug efflux transporter, BBI26, from *Borrelia burgdorferi* (Fraser *et al.*, 1997), a hypothetical tetracycline-resistance protein, HP1165, from *Helicobacter pylori* (Tomb *et al.*, 1997) and a hypothetical 44.7 kDa protein, YxaM, from *Bacillus subtilis* (Yoshida *et al.*, 1995). Alignment of these proteins with TetA(P) identified a consensus motif, ExPxxxxxDxxxRK, where

TetA(P) Motif		E X P X X X X X D X X X R K																						
TetA(P)	<i>Clostridium perfringens</i>	...56	F	I	F	E	I	P	T	A	I	V	A	D	V	Y	S	R	K	L	S	I	75...	420aa
HP1165	<i>Helicobacter pylori</i>	...53	L	V	A	E	Y	P	S	G	V	L	A	D	L	M	S	R	K	N	L	F	72...	386aa
BBI26	<i>Borrelia burgdorferi</i>	...54	I	I	F	E	F	P	S	G	V	I	S	D	I	F	D	R	K	I	V	Y	73...	392aa
YxaM	<i>Bacillus subtilis</i>	...48	L	I	F	E	V	P	S	G	V	F	A	D	R	Y	G	I	K	I	S	I	67...	399aa
TetA(B)	<i>Escherichia coli</i>	...55	V	I	F	A	P	W	L	G	K	M	S	D	R	F	G	R	R	P	V	L	74...	401aa
TetA(B) Motif		G X X X X R X G R R																						

Fig. 3. Alignment of the regions containing the TetA(P) motif. The first column lists the names of the proteins included, followed by their host organism. The entire TetA(P) protein (GenPept accession no. 456034) has similarity to the hypothetical HP1165 (GenPept 2314322), BBI26 (GenPept 2690088) and YxaM (GenPept 2280498) proteins (19–23 % identity). The numbers of the first and last residues included are given, as well as the total length of the protein. Identical residues are shown in bold. The prototype tetracycline efflux protein TetA(B) (GenPept P02980) as well as the previously identified TetA(B) motif (Yamaguchi *et al.*, 1992d) have been included for comparison.

x corresponds to any amino acid residue (Fig. 3). This motif overlaps the putative TMD 2-loop 2,3-TMD 3 region of TetA(P). It is of particular interest as the mutations which affect the function or protein stability of TetA(P) have been observed in the first four of the five conserved residues of this motif, specifically E59Q (Kennan *et al.*, 1997), P61S, D67N (Kennan *et al.*, 1997) and R71C. This observation suggests that these residues may be important for the function of each of these proteins. The DxxxRK portion of this TetA(P) motif is found in the majority of 12 TMD transport proteins, and actually forms part of the major identifying motif (Motif A) of proteins within MFS family 3 (Pao *et al.*, 1998; Paulsen *et al.*, 1996). In addition, this DxxxRK sequence is found within the consensus sequence previously identified as being conserved within tetracycline efflux proteins identified from Gram-negative species (Fig. 3) (Yamaguchi *et al.*, 1992d). Functional studies have shown that the aspartate and arginine residues are required for the efflux of tetracycline by TetA(B) (Yamaguchi *et al.*, 1992d).

Mutants in or near loop 4–5 of TetA(P)

Moving along the TetA(P) protein, the next stable mutant was Q102K, which is located in the proposed central domain of TMD 4. Based on its intermediate tetracycline-resistance phenotype, and its high TetA(P) expression levels, it appears that the polarity of the side chain provided by the glutamine residue at this position is required for TetA(P) function. This mutation was somewhat unusual since the introduced change generated a charged residue in a proposed hydrophobic environment yet the mutant derivative was stably expressed.

Mutations were detected in five of the residues of loop 4–5. Both the G114D and A121E proteins were expressed at relatively high levels (Table 1), considering the change to charged residues, indicating that these residues are functionally important. By comparison, the A118V mutation, which would normally be considered as a relatively conservative change, led to a greater than 10-fold reduction in stable TetA(P) expression. The mutant

G136R, which introduced a charged residue into putative loop 4–5, was producing TetA(P) protein at 20 % of wild-type levels but had an MIC of only 2 and therefore also appears to be functionally important.

The proposed cytoplasmic loop 4–5 is of particular interest due to its high percentage of charged residues. On the basis of their low tetracycline MIC and relatively high TetA(P) levels the most interesting mutants in this region are G114D and G136R. The presence of charged residues at these positions disrupts the function of TetA(P). The role of these glycine residues is not clear but may be structural in nature. Glycine residues have previously been reported as being important for the structure of the TetA(B) protein (Yamaguchi *et al.*, 1992d).

TetA(P) derivatives with changes in loops 6–7 and 7–8

Each of the derivatives with alterations in the putative cytoplasmic loop 6–7, P188L, A190T and P191L, were stably produced at various levels. However, since with the P188L and P191L TetA(P) proteins the reduction in tetracycline MIC was related to the reduction in TetA(P) expression, it was concluded that these changes were likely to represent structural rather than functional effects. This conclusion is supported by the fact that these mutations involved proline residues, which are known to introduce conformational bends into proteins.

The next set of mutations involved the largest proposed periplasmic loop 7–8. E232 was the second mutagenized glutamate residue to be identified in this random screen. The E232K derivative did not confer tetracycline resistance but was expressed at 60 % of wild-type TetA(P), which suggests that this residue plays an important functional role in TetA(P)-mediated tetracycline efflux. Due to the proposed location of E232 it is possible that this residue plays a gating role in the movement of tetracycline on the periplasmic membrane of the cell, in a similar way that D66 is proposed to play a gating role for tetracycline on the cytoplasmic side of TetA(B) (Yamaguchi *et al.*, 1992c).

Two independent variants were identified in the nearby D235 residue. Both derivatives had reduced tetracycline MIC levels but there was a large variation in the observed level of immunoreactive TetA(P), with D235N being expressed at slightly higher than wild-type levels, whereas D235G was expressed at much less than wild-type levels. Although the reduced level of resistance conferred by TetA(P)-D235G can be explained by the lower level of stability or expression, the D235N data clearly suggest that this residue is functionally important. A similar functional requirement has been observed with aspartate residues from TetA(K) (Fujihara *et al.*, 1997).

Finally, G261 is predicted to be situated close to loop 7–8 but within TMD 8. The mutant identified had a charged glutamic acid residue at this position and was shown to confer only low level tetracycline resistance. The TetA(P)-G261E protein was stably produced, unlike other glycine to charged amino acid changes. Therefore, it appears that the presence of a charged residue at this position, although still allowing a stable protein to be produced, alters the structure of the protein in such a way that TetA(P) function is disrupted. Based on the hypothesis that the introduction of a charged residue into a hydrophobic region of the protein leads to instability, it is possible that this residue may be situated outside the TMD.

The TetA(P)-S360F derivative

Two mutations were identified within or very near to the proposed cytoplasmic loop 10–11. Both mutants, A349T and S360F, were expressed at high levels. The latter derivative was of particular interest as the change from serine to phenylalanine resulted in a greatly reduced MIC, which suggests that this serine residue may play an important structural or functional role in TetA(P)-mediated tetracycline efflux. Although several serine residues have been identified as being part of the tetracycline translocation channel of TetA(B) (Yamaguchi *et al.*, 1992b), this serine residue is in a different relative position in TetA(P) and its putative role therefore remains unknown.

Functional amino acids are located throughout TetA(P)

Previous studies on TetA(P) identified three glutamic acid residues which are required for function (Kennan *et al.*, 1997). These residues, E52, E59 and E89, are located within TMD 2 and close to TMD 3. We have now extended these studies by using random mutagenesis and have shown that there are potentially functional residues located over a much wider portion of the protein. In particular, we have shown that the putative cytoplasmic loops 2–3 and 4–5, and the periplasmic loop 7–8 are important functional domains in the efflux of tetracycline from the cell. In this study several residues of putative functional importance were identified, specifically P61, T62, A118, A121, G136, E232, D235 and S360. Analysis of the precise physical requirements

at these positions using site-directed mutagenesis will aid in the further understanding of their role in tetracycline efflux by the TetA(P) protein.

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REFERENCES

- Abraham, L. J., Berryman, D. I. & Rood, J. I. (1988). Hybridization analysis of the class P tetracycline resistance determinant from the *Clostridium perfringens* R-plasmid, pCW3. *Plasmid* **19**, 113–120.
- Allard, J. D. & Bertrand, K. P. (1992). Membrane topology of the pBR322 tetracycline resistance protein. TetA-PhoA gene fusions and implications for the mechanism of TetA membrane insertion. *J Biol Chem* **267**, 17809–17819.
- Boyd, D., Traxler, B. & Beckwith, J. (1993). Analysis of the topology of a membrane protein by using a minimum number of alkaline phosphatase fusions. *J Bacteriol* **175**, 553–556.
- Claros, M. G. & von Heijne, G. (1994). TopPred II: an improved software for membrane protein structure predictions. *Comput Appl Biosci* **10**, 685–686.
- Eckert, B. & Beck, C. F. (1989). Topology of the transposon Tn10-encoded tetracycline resistance protein within the inner membrane of *Escherichia coli*. *J Biol Chem* **264**, 11663–11670.
- Fraser, C. M., Casjens, S., Huang, W. M. & 35 other authors (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390**, 580–586.
- Fujihira, E., Kimura, T., Shiina, Y. & Yamaguchi, A. (1996). Transmembrane glutamic acid residues play essential roles in the metal-tetracycline/H⁺ antiporter of *Staphylococcus aureus*. *FEBS Lett* **391**, 243–246.
- Fujihira, E., Kimura, T. & Yamaguchi, A. (1997). Roles of acidic residues in the hydrophilic loop regions of metal-tetracycline/H⁺ antiporter Tet(K) of *Staphylococcus aureus*. *FEBS Lett* **419**, 211–214.
- Ginn, S. L., Brown, M. H. & Skurray, R. A. (1997). Membrane topology of the metal-tetracycline/H⁺ antiporter TetA(K) from *Staphylococcus aureus*. *J Bacteriol* **179**, 3786–3789.
- von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**, 487–494.
- Henderson, P. J. (1990). Proton-linked sugar transport systems in bacteria. *J Bioenerg Biomembr* **22**, 525–569.
- Jessen-Marshall, A. E., Paul, N. J. & Brooker, R. J. (1995). The conserved motif, GXXX(D/E)(R/K)XG[X](R/K)(R/K), in hydrophilic loop 2/3 of the lactose permease. *J Biol Chem* **270**, 16251–16257.
- Kennan, R. M., McMurry, L. M., Levy, S. B. & Rood, J. I. (1997). Glutamate residues located within putative transmembrane helices are essential for TetA(P)-mediated tetracycline efflux. *J Bacteriol* **179**, 7011–7015.
- Kimura, T. & Yamaguchi, A. (1996). Asp-285 of the metal-tetracycline/H⁺ antiporter of *Escherichia coli* is essential for substrate binding. *FEBS Lett* **388**, 50–52.
- Kimura, T., Ohnuma, M., Sawai, T. & Yamaguchi, A. (1997). Membrane topology of the transposon 10-encoded metal-tetracycline/H⁺ antiporter as studied by site-directed chemical labeling. *J Biol Chem* **272**, 580–585.

- Kimura, T., Nakatani, M., Kawabe, T. & Yamaguchi, A. (1998). Roles of conserved arginine residues in the metal-tetracycline/H⁺ antiporter of *Escherichia coli*. *Biochemistry* **37**, 5475–5480.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Levy, S. B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrob Agents Chemother* **36**, 695–703.
- McMurry, L. M., Park, B. H., Burdett, V. & Levy, S. B. (1987). Energy-dependent efflux mediated by class L (tetL) tetracycline resistance determinant from streptococci. *Antimicrob Agents Chemother* **31**, 1648–1650.
- McNicholas, P., Chopra, I. & Rothstein, D. M. (1992). Genetic analysis of the *tetA*(C) gene on plasmid pBR322. *J Bacteriol* **174**, 7926–7933.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mojumdar, M. & Khan, S. A. (1988). Characterization of the tetracycline resistance gene of plasmid pT181 of *Staphylococcus aureus*. *J Bacteriol* **170**, 5522–5528.
- Pao, S. S., Paulsen, I. T. & Saier, M. H., Jr (1998). Major facilitator superfamily. *Microbiol Mol Biol Rev* **62**, 1–34.
- Paulsen, I. T., Brown, M. H., Dunstan, S. J. & Skurray, R. A. (1995). Molecular characterization of the staphylococcal multidrug resistance export protein QacC. *J Bacteriol* **177**, 2827–2833.
- Paulsen, I. T., Brown, M. H. & Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev* **60**, 575–608.
- Roberts, M. C. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol Rev* **19**, 1–24.
- Roy, C. R. & Isberg, R. R. (1997). Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. *Infect Immun* **65**, 571–578.
- Schnappinger, D. & Hillen, W. (1996). Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Arch Microbiol* **165**, 359–369.
- Seol, W. & Shatkin, A. J. (1993). Membrane topology model of *Escherichia coli* alpha-ketoglutarate permease by PhoA fusion analysis. *J Bacteriol* **175**, 565–567.
- Sloan, J., McMurry, L. M., Lyras, D., Levy, S. B. & Rood, J. I. (1994). The *Clostridium perfringens* Tet P determinant comprises two overlapping genes: *tetA*(P), which mediates active tetracycline efflux, and *tetB*(P), which is related to the ribosomal protection family of tetracycline-resistance determinants. *Mol Microbiol* **11**, 403–415.
- Spee, B. S., Shoemaker, N. B. & Salyers, A. A. (1992). Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin Microbiol Rev* **5**, 387–399.
- Tomb, J. F., White, O., Kerlavage, A. R. & 39 other authors (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547.
- Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T. & Sawai, T. (1990a). 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**, 15525–15530.
- Yamaguchi, A., Udagawa, T. & Sawai, T. (1990b). Transport of divalent cations with tetracycline as mediated by the transposon Tn10-encoded tetracycline resistance protein. *J Biol Chem* **265**, 4809–4813.
- Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M. & Sawai, T. (1992a). Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by transposon Tn10. Roles of the aspartyl residues located in the putative transmembrane helices. *J Biol Chem* **267**, 7490–7498.
- Yamaguchi, A., Ono, N., Akasaka, T. & Sawai, T. (1992b). Serine residues responsible for tetracycline transport are on a vertical stripe including Asp-84 on one side of transmembrane helix 3 in transposon Tn10-encoded tetracycline/H⁺ antiporter of *Escherichia coli*. *FEBS Lett* **307**, 229–232.
- Yamaguchi, A., Nakatani, M. & Sawai, T. (1992c). Aspartic acid-66 is the only essential negatively charged residue in the putative hydrophilic loop region of the metal-tetracycline/H⁺ antiporter encoded by transposon Tn10 of *Escherichia coli*. *Biochemistry* **31**, 8344–8348.
- Yamaguchi, A., Someya, Y. & Sawai, T. (1992d). Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by transposon Tn10. The role of a conserved sequence motif, GXXXXRXGRR, in a putative cytoplasmic loop between helices 2 and 3. *J Biol Chem* **267**, 19155–19162.
- Yamaguchi, A., Akasaka, T., Kimura, T., Sakai, T., Adachi, Y. & Sawai, T. (1993a). Role of the conserved quartets of residues located in the N- and C-terminal halves of the transposon Tn10-encoded metal-tetracycline/H⁺ antiporter of *Escherichia coli*. *Biochemistry* **32**, 5698–5704.
- Yamaguchi, A., Someya, Y. & Sawai, T. (1993b). The in vivo assembly and function of the N- and C-terminal halves of the Tn10-encoded TetA protein in *Escherichia coli*. *FEBS Lett* **324**, 131–135.
- Yamaguchi, A., Samejima, T., Kimura, T. & Sawai, T. (1996). His257 is a uniquely important histidine residue for tetracycline H⁺ antiport function but not mandatory for full activity of the transposon Tn10-encoded metal-tetracycline H⁺ antiporter. *Biochemistry* **35**, 4359–4364.
- Yoshida, K., Seki, S., Fujimura, M., Miwa, Y. & Fujita, Y. (1995). Cloning and sequencing of a 36-kb region of the *Bacillus subtilis* genome between the *gnt* and *iol* operons. *DNA Res* **2**, 61–69.

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