Glutamate Residues Located within Putative Transmembrane Helices Are Essential for TetA(P)-Mediated Tetracycline Efflux

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The *tetA(P)* gene from *Clostridium perfringens* encodes a unique membrane protein that is responsible for the active efflux of tetracycline from resistant cells. The novel TetA(P) protein has neither the typical structure nor the conserved motifs that are found in tetracycline efflux proteins from classes A through H or classes K and L. Site-directed mutagenesis of selected residues within TetA(P) was performed to elucidate their role in tetracycline efflux. Glutamate residues 52 and 59, negatively charged residues located within putative transmembrane helix 2, could not be replaced by either glutamine or aspartate and so were essential for tetracycline efflux. Replacement of Glu89, which was located at the end of helix 3, by aspartate but not by glutamine allowed TetA(P) function, indicating the importance of a carboxyl group at this position. After mutation of the Asp67 residue, located within cytoplasmic loop 1, no immunoreactive protein was detected. It is concluded that negatively charged residues that appear to be located within or near the membrane are important for the function of TetA(P).

Tetracycline resistance is the most common antibiotic resistance phenotype found in *Clostridium perfringens* and can be either chromosomally or plasmid encoded (18, 19). The conjugative plasmid pCW3 from *C. perfringens* carries the Tet(P) determinant, which consists of an operon containing two distinct overlapping tetracycline resistance genes, *tetA(P)* and *tetB(P)* (21). The *tetA(P)* gene encodes a unique tetracycline efflux protein, while the *tetB(P)* gene encodes a protein related to the ribosomal modification proteins of the TetM family (21).

Tetracycline efflux proteins from gram-negative bacteria usually have 12 transmembrane helices, comprising two related 6-transmembrane domain segments separated by a large central hydrophilic domain, while those from gram-positive organisms may have 14 helices (10, 12). Yamaguchi et al. (26) showed that for the proteins from gram-negative bacteria there was structural, although not functional, symmetry between helix 2–loop 2–helix 3 and helix 8–loop 8–helix 9, that is, the corresponding regions on either side of the central hydrophilic region. While the transmembrane model of TetA(P) indicates that it has 12 transmembrane helices, they do not fall into two related domains (21). TetA(P) also does not have the conserved sequence motif, GXXXXRXGRR, which is located within a cytoplasmic loop between transmembrane helices 2 and 3 of tetracycline efflux proteins of gram-negative bacteria and in proton-linked sugar transporters (see reference 30). The tetracycline efflux proteins of gram-negative bacteria all contain conserved aspartate residues within transmembrane domains, but aspartate residues are absent from the transmembrane domains of TetA(P). Instead, glutamate residues are present, making TetA(P) similar in this respect to the tetracycline efflux proteins Tet(K) and Tet(L) of other gram-positive bacteria (9).

The substrate for tetracycline efflux proteins is believed to be a positively charged metal-tetracycline complex which is exchanged for a proton (25, 29, 31). The Asp15, Asp84, and

Asp285 residues within the Tn10-encoded TetA(B) protein have been shown to be important in tetracycline efflux (15, 24). Analogous aspartate residues located within the TetA(C) protein from pBR322 have also been shown to be important (2, 16), while the three glutamate residues located within transmembrane helices of the Tet(K) protein are important in tetracycline efflux, with Glu397 being essential for activity (9).

In the transmembrane model of TetA(P) there is only one charged residue, Glu52, which is located deep within a transmembrane helix. It was predicted that this residue would play a role in tetracycline efflux (21). Other charged residues predicted to be located near the boundaries of transmembrane helices, namely Glu59 and Glu89, could also have a functional role in tetracycline efflux. The role of these acidic amino acids within the TetA(P) protein therefore was investigated by using site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5 α (Bethesda Research Laboratories) was used as the host strain for all except the uptake studies, which utilized strain DL-54 (20). Site-directed mutagenesis was done on plasmid pJIR71 (1), which carries the *tetA(P)* gene from pCW3 on a 2.9-kb *Pst*I fragment cloned into pUC18. Strains were grown on 2YT (17), unless otherwise noted, supplemented with ampicillin (100 μ g/ml) and various concentrations of tetracycline where appropriate.

Site-directed mutagenesis. Site-directed mutagenesis was performed by the method of Deng and Nickoloff (8) by using the U.S.E. Mutagenesis Kit (Pharmacia Biotech) according to the manufacturer's instructions. Oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer. The selection primer eliminated the single *Aar*II site within pJIR71, while the mutagenic primers changed one or two bases within the selected codon. Mutants were first detected by failure of the plasmid to be digested by *Aar*II and confirmed by sequencing with the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI373A automated fluorescent sequencing apparatus (Applied Biosystems).

Determination of tetracycline resistance. For each of the mutants, the MIC of tetracycline was determined in one of two ways. For strains based on DH5 α , overnight broth cultures were diluted 1:25 in 2YT broth containing ampicillin, grown to a turbidity at 550 nm of 0.7 to 0.8, and diluted 1:100, after which 10- μ l volumes were placed onto 2YT agar plates containing various concentrations of tetracycline, as described elsewhere (2). After incubation for 18 to 20 h at 37°C, the MIC was determined as the lowest concentration of tetracycline that completely inhibited growth. For strains based on DL-54, susceptibility was measured by using gradient plates (6), with Luria-Bertani broth and agar.

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FIG. 1. Putative-transmembrane model of TetA(P). This model is based on analysis of the TetA(P) protein done by using the TopPred II software package (4) and varies slightly from the previous model (21). The potential transmembrane domains are indicated by the shaded regions, and the numbers indicate relevant amino acid residues. Positively charged amino acids are shown by dark circles, and negatively charged residues are represented by open circles.

Immunoblot analysis. Detection of the TetA(P) protein in cell membrane fractions was performed by immunoblot analysis with an antibody to the TetA(P) carboxy terminus [anti-TetA(P)-CT]. A peptide corresponding to the 17 carboxy-terminal amino acids of the TetA(P) protein was synthesized and conjugated to diphtheria toxin by Chiron Mimotopes (Australia). The conjugated peptide was emulsified with Freund's complete adjuvant (Difco Laboratories) and injected into New Zealand White rabbits. The resultant antiserum was allowed to absorb against DH5 α (pUC18) cells to remove nonspecific antibodies.

To carry out immunoblot analyses, inner membrane proteins were prepared as described elsewhere (5). Cells from overnight cultures of DH5 α strains carrying pJIR71, pUC18, or a plasmid bearing a mutated gene were harvested and lysed by sonication, and the cellular debris was removed by centrifugation at $4,000 \times g$ for 10 min at 4°C. Membrane proteins were subsequently sedimented by centrifugation of the cell lysate at $353,000 \times g$ for 15 min at 4°C. Membranes were separated into inner and outer fractions by the addition of 1% Sarkosyl and centrifugation as described above to pellet the outer membrane proteins. The solubilized inner membrane fractions (10 μ g) were mixed with gel loading buffer at room temperature and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (11). Gels were electroblotted onto nitrocellulose sheets (22), and blots were developed according to the manufacturer's instructions by using the absorbed anti-TetA(P)-CT serum, goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate, and the ECL Western blotting detection system in which peroxidase produces a chemiluminescent product (Amersham Life Sciences, Little Chalfont, England).

RNA dot blots. RNA was prepared from DH5 α strains containing pJIR71, pUC18, or selected mutated plasmids by a single-step method using TRISOL reagent (Gibco BRL). Equivalent amounts of total RNA were then applied in duplicate to a nylon membrane (Amersham) by using a dot blot apparatus. Blots were analyzed according to the manufacturer's instructions by using a DIG Labelling and Detection Kit (Boehringer Mannheim), in which digoxigenin-UTP is incorporated into the probe and detected immunologically. Two probes were used, a *tetA*(P)-specific fragment (13) and a 364-bp *Bgl*I/*Sca*I fragment internal to the *bla* gene of pUC18 as a control. Hybridization was overnight at 68°C in 7% (wt/vol) SDS–50 mM sodium phosphate buffer, pH 7.0–50% formamide–2% digoxigenin blocking reagent–5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.1% Sarkosyl. After hybridization, membranes were washed twice in 2 $\times$ SSC–0.1% (wt/vol) SDS at room temperature and twice in 0.1 $\times$ SSC–0.1% (wt/vol) SDS at 65°C. Blots were developed by using standard procedures for digoxigenin-labelled probes and CPD Star, a chemiluminescent substrate for alkaline phosphatase (Boehringer Mannheim).

Accumulation of [³H]tetracycline by cells. The non-K-12 *E. coli* unc strain DL-54 (20) was transformed with pJIR71 or its mutant derivatives. Cells were grown at 37°C in Luria-Bertani broth (100 μ g of ampicillin per ml) to a turbidity at 530 nm of approximately 0.6, harvested at room temperature, washed four times with an equal volume of minimal medium A (14) without glucose, and resuspended in the same medium to a turbidity at 530 nm of 2.0. Following equilibration for 4 min at 37°C, [³H]tetracycline (1.0 Ci/mmol; New England Nuclear) was added to 1 μ M. A 50- μ l sample was taken at both 10 and 18 min, mixed rapidly into 10 ml of minimal medium A containing 0.1 M LiCl, filtered through a Gelman Metrical GN-6 0.45- μ m-pore-size filter, and washed with 4 ml of medium A–LiCl. At 18.5 min glucose was added to 0.2%, and further samples were taken at 26 and 34 min. The filters were dried and counted in EcosintO (National Diagnostics) in a scintillation counter. The uptake curves showed that

equilibrium accumulation before and after glucose addition was achieved by 18 and 34 min, respectively.

RESULTS

Effect of specific mutations upon function of Tet(P). The transmembrane model of the TetA(P) protein (21) predicts that Glu52, Glu59, and Glu89 are the only acidic residues located within or adjacent to putative transmembrane helices (Fig. 1). To determine if these residues had a functional role in TetA(P)-mediated tetracycline resistance, site-directed mutagenesis of pJIR71 was used to systematically change these residues to similarly sized amino acids of the same, opposite, and neutral charges (Table 1). All of the sequence alterations were confirmed by nucleotide sequence analysis. In addition, for each of the mutants the entire *tetA*(P) gene was completely resequenced to confirm that no other sequence alterations were introduced. Determination of the MIC of tetracycline for DH5 α derivatives containing the mutated plasmids revealed that changes at all three of the Glu residues caused a marked reduction in tetracycline resistance (Table 1). Alteration of Glu52 and Glu59 to Gln, Asp, or Lys virtually abolished tetracycline resistance, indicating the functional importance of the Glu residue at these positions. Similar results were obtained with alteration at Glu89, except that the Asp89 mutant was still tetracycline resistant; a carboxyl group at residue 89 therefore appears to be sufficient for resistance. Similar mutations were also introduced at Lys214 or Arg328 (Fig. 1), but these caused only a minor reduction in the MIC, the resultant derivatives still conferring tetracycline resistance. These basic residues therefore do not appear to play a major functional role in the resistance phenotype.

Since the three Glu residues all were located within or near the putative transmembrane helices 2 and 3 (Fig. 1), we examined whether acidic residues in the putative cytoplasmic and periplasmic loops adjoining these helices may also be of functional significance in tetracycline efflux. The Glu35 residue predicted to be located in the periplasmic loop between transmembrane helices 1 and 2 and the Asp67 residue predicted to be located in the cytoplasmic loop between transmembrane helices 2 and 3 (Fig. 1) both were mutagenized as described before. The results (Table 1) revealed that alteration of Glu35 to Gln and Lys only marginally affected the resultant level of

TABLE 1. Effect of site-directed mutagenesis on tetracycline resistance

Plasmid	TetA(P) designation	Mutation	Tetracycline MIC ($\mu\text{g/ml}$)
pUC18	Control		1.0
pJIR71	Wild type		27.5
pJIR1330	E52K	GAA→AAA	1.0
pJIR1356	E52Q	GAA→CAA	1.5
pJIR1338	E52D	GAA→GAC	1.5
pJIR1395	E59K	GAA→AAA	1.0
pJIR1349	E59Q	GAA→CAA	3.0
pJIR1350	E59D	GAA→GAC	3.0
pJIR1354	E89K	GAA→AAA	5.0
pJIR1353	E89Q	GAA→CAA	5.0
pJIR1355	E89D	GAA→GAC	15.0
pJIR1374	K214E	AAA→GAA	15.0
pJIR1375	K214Q	AAA→CAA	15.0
pJIR1376	K214R	AAA→AGA	17.5
pJIR1381	R328K	AGA→AAA	17.5
pJIR1382	R328Q	AGA→GAA	15.0
pJIR1383	R328E	AGA→CAA	15.0
pJIR1403	D67K	GAT→AAG	1.0
pJIR1404	D67E	GAT→GAA	1.0
pJIR1418	D67N	GAT→AAT	1.0
pJIR1407	E35D	GAA→GAC	25.0
pJIR1408	E35K	GAA→AAA	17.5
pJIR1409	E35Q	GAA→CAA	17.5
pJIR1388	C55S	TGC→AGC	17.5

tetracycline resistance conferred by the plasmid; alteration of Glu35 to Asp had virtually no phenotypic effect. By contrast, all three changes at the Asp67 residue completely abolished tetracycline resistance (Table 1). However, since these changes resulted in the loss of the immunoreactive TetA(P) protein (see below), no conclusions about the functional significance of this residue could be drawn.

Finally, the Cys55 residue, which was also located in putative transmembrane helix 2, was changed to Ser to determine if disulfide bonds were important in the structure and function of TetA(P). The tetracycline MIC for the resultant Ser55 mutant was only marginally lower than the MIC for the wild type (Table 1), indicating that the Cys55 residue was not critical for tetracycline efflux.

Detection of mutant proteins. Immunoblotting was carried out to determine if the TetA(P) protein was produced in DH5 α derivatives carrying the mutated plasmids. To facilitate these studies, rabbit antibodies were raised against a conjugated synthetic peptide consisting of the last 17 amino acids of TetA(P). The analysis of cell membrane preparations showed that in all of the mutant derivatives the TetA(P) protein was produced in quantities similar to those produced in the wild type (Fig. 2), except in the case of TetA(P)-E59K and the three Asp67 mutants, each of which produced little or no detectable TetA(P) protein. Immunoreactive protein was not detected in any cellular fractions, cellular debris, total soluble proteins, inner membrane proteins, or outer membrane proteins from the three Asp67 mutants. RNA dot blots carried out by using a *tetA*(P)-specific probe showed that equivalent amounts of *tetA*(P)-specific mRNA were produced by the three Asp67 mutants, the E59K mutant, and the wild type (data not shown).

Tetracycline uptake. To examine the effect of selected mutations on tetracycline accumulation, tetracycline uptake experiments were performed. As expected, cells containing the wild-type TetA(P) protein showed a decrease in accumulation of tetracycline upon energization with glucose, indicative of

active efflux; conversely, the vector-only control showed an increase in tetracycline accumulation, indicating active uptake (Fig. 3, far left versus far right bars). The somewhat lower values for wild-type TetA(P) than for the vector alone, each in the absence of glucose, probably reflect endogenous energization by both cell types. On the addition of glucose, the tetracycline-sensitive mutants with mutations at Glu52 and Glu59 and the E89Q mutant all showed increased tetracycline uptake, indicating that the active efflux mechanism in these mutants had been disrupted. By contrast, the E35D and E89D mutants showed a reduction in tetracycline accumulation after the addition of glucose, indicating that these mutants were still able to actively effect efflux of tetracycline, although not as efficiently as the wild type (Fig. 3). The efflux data were consonant with the susceptibilities of cells to tetracycline (Fig. 3).

DISCUSSION

Site-directed mutagenesis of the charged amino acids located within predicted transmembrane helices of the TetA(P) protein, coupled with the subsequent determination of tetracycline MICs and tetracycline transport studies, revealed that Glu52 and Glu59 were essential for tetracycline efflux since Asp residues at these positions did not permit tetracycline resistance. By contrast, at Glu89 the presence of a negative charge in the form of an Asp residue still mediated some tetracycline resistance, although at a slightly reduced level (Table 1). This mutant also conferred reduced tetracycline accumulation upon the addition of glucose (Fig. 3), confirming that strains carrying the mutated plasmid were still able to actively effect efflux of tetracycline, although not as efficiently as the wild type.

These results are consistent with the hypothesis that these Glu residues function as substrate binding sites for the passage of a positively charged metal-tetracycline complex through the membrane. A similar role has been attributed to Asp residues within transmembrane helices of the Tn10-encoded TetA(B) protein (24) and the Glu residues of Tet(K) (9). The cytoplasmic loop between helices 2 and 3 in Tet proteins from gram-negative organisms so far analyzed bears the conserved motif GXXXXRXGRR (30). Extensive mutagenesis within this region of the Tn10-encoded TetA(B) protein has shown that a negatively charged Asp66 and a positively charged Arg70 are the only charged residues within this loop that are essential for tetracycline efflux, although mutations in other residues were proposed to destabilize or distort the peptide backbone structure (27, 30). It has been proposed that Asp66 may play a gating role in tetracycline efflux (28). Similarly, the cytoplasmic loop between transmembrane helices 2 and 3 of the TetA(C) protein has been shown to be crucial for tetracycline efflux (16), while Asp74 is conserved in the equivalent loops of the Tet(K) and Tet(L) proteins (9). However, TetA(P) is a unique tetracycline efflux protein in that it does not have this conserved motif, although it does have Asp67 as the only charged residue within the equivalent cytoplasmic loop. The results

FIG. 2. Immunoblot of inner membrane proteins prepared from *E. coli* DH5 α cells harboring pJIR71 or its mutant derivatives. Each lane contained approximately 10 μg of total protein. Lane 1, pJIR71; lane 2, pUC18; lane 3, E35D mutant; lane 4, E52Q; lane 5, E52D; lane 6, E59D; lane 7, E89K; lane 8, E89D; lane 9, E59K; lane 10, D67K; lane 11, D67E; lane 12, D67N. Only the relevant portion of the blot is shown.

FIG. 3. Effect of energization by glucose upon accumulation of tetracycline by DL-54 cells containing cloned wild-type or mutant TetA(P) proteins. The equilibrium incorporation of [3 H]tetracycline (added at 1 μ M) was measured in minimal medium A before (black bars) and after (white bars) the addition of glucose, as described in Materials and Methods. The MICs of tetracycline (in micrograms per milliliter) for the different strains, as determined by using gradient plates (6), are shown above the bars. The strain represented by the bars on the far right contained only the cloning vector, pUC18.

showed that mutation of Asp67 caused loss of function, apparently via protein instability since the protein was not detected in any cellular fraction by immunoblotting, although transcription appeared to be normal. Mutagenesis of the Glu35 codon, which could possibly play a gating role on the external side of the membrane, showed that Glu35 was not essential since substitution with Gln or Lys at this position still led to tetracycline resistance, although at a slightly reduced level. It therefore appears more likely that Glu89, not Glu35, plays a gating role in tetracycline efflux.

Yamaguchi et al. (23) suggested that a substrate/ H^+ antiporter requires at least three features: a substrate binding site(s); at least two gating sites, one on either side of the membrane; and an H^+ transfer site(s). The results presented in this study suggest that in the TetA(P) protein the three Glu residues (Glu52, Glu59, and Glu89) may be involved in these roles. It is now clear that in three quite different types of tetracycline efflux proteins [Tet(A) to Tet(H), Tet(K) and Tet(L), and TetA(P)], carboxylic acid side chains within the membrane are important for activity. The Tet(A) to Tet(E) efflux proteins all have Asp residues located deep within transmembrane helices 1, 3, and 9 and have 12 transmembrane helices (3). TetK and TetL have Glu residues within transmembrane helices 1, 5, and 13 of their proposed 14 transmembrane helices (10). The Asp residues of TetA(B) and TetA(C) have been shown to be important in tetracycline efflux, as have the Glu residues of TetK (2, 9, 15, 16, 24). In this work, we have shown that Glu residues within transmembrane helix 2 of the proposed 12-transmembrane-domain TetA(P) protein are important for tetracycline efflux. We also note from our own analysis that the tetracycline efflux protein TcrC from *Streptomyces aureofaciens* (7) has 14 predicted transmembrane heli-

ces, with an Asp residue within the first helix and two Glu residues near the end of the seventh helix. Thus, there is variability in the helices in which these acidic residues are located in tetracycline efflux proteins; only for TetA(P) are they located in or adjacent to the second putative transmembrane helix. Therefore, different helices in different Tet proteins may serve homologous functions.

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