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MINI-REVIEW

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# Tetracyclines: antibiotic action, uptake, and resistance mechanisms

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**Abstract** Tetracyclines probably penetrate bacterial cells by passive diffusion and inhibit bacterial growth by interfering with protein synthesis or by destroying the membrane. A growing number of various bacterial species acquire resistance to the bacteriostatic activity of tetracycline. The two widespread mechanisms of bacterial resistance do not destroy tetracycline: one is mediated by efflux pumps, the other involves an EF-G-like protein that confers ribosome protection. Oxidative destruction of tetracycline has been found in a few species. Several efflux transporters, including multidrug-resistance pumps and tetracycline-specific exporters, confer bacterial resistance against tetracycline. Single amino acids of these carrier proteins important for tetracycline transport and substrate specificity have been identified, allowing the mechanism of tetracycline transport to begin to emerge.

**Key words** **A**ntibiotic resistance · Tetracycline resistance · Ribosomal protection · Efflux pumps · Multidrug resistance · Tetracycline-proton antiporters

**Abbreviations** $M^{2+}$ Divalent metal ions · $[M\text{-}tc]^{+}$ Metal tetracycline chelate complex · $Tc^{R}$ Tetracycline resistance

Several properties make tetracyclines (Fig. 1) nearly perfect therapeutic agents: (1) they are active against most common pathogens, (2) they show good oral absorption, (3) they exhibit low toxicity, (4) they cause only few allergic reactions, and (5) they are relatively inexpensive (Moellering 1990; Standiford 1990). This has led to an intensive use of tetracyclines in therapy and prophylactic control of bacterial infections in humans and animals over the last 48 years. Subtherapeutic levels of oxytetracycline

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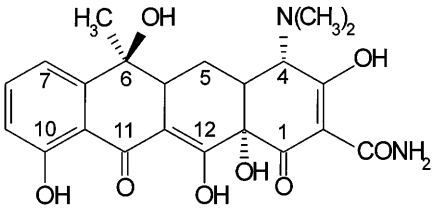


**Fig. 1** Structure of tetracycline

are also used as food additives for growth promotion in animal husbandry. As a result of this extensive application, tetracyclines have been produced on the largest scale of all antibiotics (Johnson and Adams 1992; Levy 1992). The emergence of bacterial resistance against tetracyclines – and the emergence of bacterial resistance against antimicrobial agents in general – has caused severe drawbacks in their use (Service 1995). Therefore, the development of strategies for circumvention of bacterial resistance is one of the most important future goals in the treatment of infectious diseases. An understanding of the antibiotic action and resistance mechanisms would certainly facilitate such developments. After a short description of the antibiotic action and the uptake of tetracycline, this review focuses on the molecular details known about the two most widely distributed modes of tetracycline resistance.

## Antibiotic action of tetracycline

Surprisingly little is known about the mechanism of antibiotic action of tetracycline. Two different groups of tetracyclines are distinguishable by their mode of action: typical tetracyclines such as tetracyclines, chlortetracyclines, doxycycline, or minocycline exhibit bacteriostatic activity, whereas some tetracycline derivatives are bactericidal. The bacteriostatic activity of typical tetracyclines is associated with reversible inhibition of protein synthesis. Several studies have established one strong tetracycline binding site at the ribosome and a large number of

low-affinity tetracycline binding sites accounting for 30–40% of the overall binding (Tritton 1977; Epe and Woolley 1984). The high-affinity site is located on the 30S subunit, while low-affinity binding has been observed for both subunits. Photoaffinity labeling revealed the ribosomal protein S7 as a component of the high-affinity binding site (Goldman et al. 1983). Four other proteins (S3, S8, S14, and S19) are important for tetracycline binding as identified by single protein omission reconstitution (SPORE) experiments: SPORE particles lacking one of these proteins bind tetracycline with less than 30% efficiency as compared to a native 30S subunit (Buck and Cooperman 1990). Proximity of tetracycline to the 16S rRNA has been shown by chemical footprinting (Moazed and Noller 1987): reactivity of A892 toward dimethyl sulfate decreases, while that of U1052 and C1054 is enhanced in the presence of tetracycline. It is not known to what extent the tetracycline-RNA interactions contribute to high- or low-affinity binding. Binding of tetracycline to the ribosome weakens the ribosome-tRNA interaction (Epe et al. 1987). This has been thought to be the cause of inhibition of protein synthesis by tetracycline. The position of S7 on the 30S subunit seems to overlap with the putative aminoacyl-tRNA binding locus. Therefore, tetracycline may interfere with tRNA binding although there is no direct competitive interaction with either the codon-anticodon or the peptidyltransferase sites (Goldman et al. 1983). In our opinion, it has not been proven convincingly that binding to the ribosome reflects interaction with the site of antibiotic activity. The molecular mechanism of tetracycline action will remain unclear until a ribosome-tetracycline interaction can be clearly correlated to inhibition of protein synthesis at concentrations resembling the in vivo conditions.

Atypical tetracyclines like chelocardin, 6-thiatetracycline, or anhydrotetracycline exhibit bactericidal activity and are poor inhibitors of protein synthesis (Chopra 1994). The cytoplasmic membrane, rather than the ribosome, has been suggested to be the relevant target of these agents since they cause morphological alterations of the bacterial cell and trigger release of β-galactosidase from the cytoplasm (Oliva et al. 1992). Accordingly, resistance determinants operating by tetracycline efflux or ribosomal protection do not confer resistance against these tetracycline derivatives (Oliva and Chopra 1992). Despite their ability to kill even those bacteria that contain resistance genes, atypical tetracycline derivatives are not of therapeutic value because their application leads to severe side effects, probably because their action on the membrane is not specific for the prokaryotic cell (Rogalski 1985).

## Uptake of tetracycline by *Escherichia coli*

Bacteria susceptible to tetracycline exhibit a monophasic, rapid uptake of tetracycline (Argast and Beck 1985) that is partially energy-dependent (McMurry et al. 1980). The passive accumulation of tetracycline can easily be explained by binding to cell components such as phospholipids or proteins (Argast and Beck 1984). In contrast, it has been disputed for many years why tetracycline influx is partially energy-dependent. Despite several attempts to prove participation of presumed carrier proteins, no influx pump has been identified (McMurry et al. 1981; Argast and Beck 1985). Instead, a model that explains the energetics of tetracycline accumulation without the contribution of a transport protein has been supported by various results. This model has been discussed in detail by Nikaido and Thanassi (1993) and will, therefore, only be summarized briefly in this review (Fig. 2): tetracycline is thought to pass the outer membrane of gram-negative bacteria through the porins OmpF (Thanassi et al. 1995) and OmpC (Mortimer and Piddock 1993), probably chelating a $M^{2+}$ ion as $[M\text{-}tc]^+$. This assumption has been sustained by the observation that porin-deficient mutants are less susceptible to tetracycline (Pugsley and Schnaitman 1978). The cationic $[M\text{-}tc]^+$ is attracted by the Donnan potential across the outer membrane leading to accumulation in the periplasm, where the $[M\text{-}tc]^+$ complex might dissociate, yielding uncharged tetracycline. This weakly lipophilic compound is able to diffuse through lipid bilayers and does not depend on a protein channel (Argast and Beck 1984). Accordingly, tetracycline is expected to penetrate the cell in its electrically neutral form. In the cytoplasm it may be converted to an ionic compound again since the internal pH and the $M^{2+}$ concentration are higher than in the periplasm (Yamaguchi et al. 1991c; Nikaido and Thanassi 1993; Thanassi et al. 1995). The pH difference is dependent on the proton motive force and explains the energy dependence of tetracycline accumulation. In agreement with this model, the uphill accumulation of tetracycline is only driven by ΔpH and not by the transmembrane electrical potential (Δψ) (Yamaguchi et al. 1991a) and is strongly temperature-dependent (Argast and Beck 1985); in addition, the antibacterial activity is influenced by pH

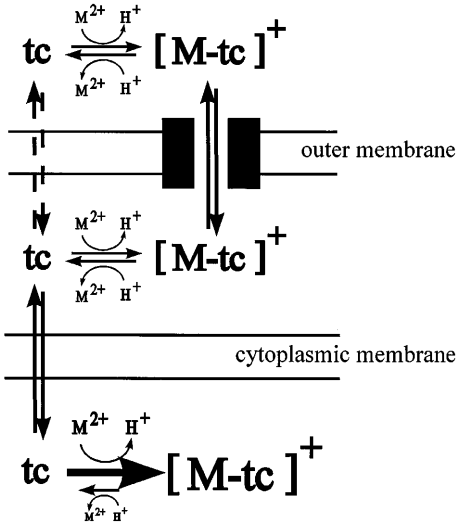


**Fig. 2** Uptake of tetracycline by *Escherichia coli* as described in the text. Tetracycline, protons, metal cations, and the metal-tetracycline complex are depicted as *tc*, $H^+$, $M^{2+}$, and $[M\text{-}tc]^+$ respectively

and by the $Mg^{2+}$ concentration in the extracellular medium (Mitcher 1978). Thus, the uphill concentration of tetracycline into the cytoplasm can – at least for *Escherichia coli* – be explained by the Donnan-potential-facilitated uptake through the outer membrane, by the pH-gradient-driven diffusion across the cytoplasmic membrane, and by the protonation and complexation behavior of tetracycline.

## Mechanisms of resistance to tetracycline

Three different bacterial strategies of resistance have been identified. They interfere with various aspects of the antibiotic activity of tetracycline: (1) protection of the ribosome as the antibiotic target, (2) reduction of the intracellular concentration of tetracycline, and (3) inactivation of the antibiotic by modifying enzymes. Resistance determinants encoding at least one of these mechanisms are widely distributed among all genera of the bacteria. They were first classified based on DNA-DNA hybridization (Mendez et al. 1980). Each *tet* class is designated by a capital letter, and those encoding the same resistance mechanism are grouped together in the alphabet (Levy et al. 1989). Three resistance determinants were found in the oxytetracycline producer *Streptomyces rimosus*: OtrA, OtrB, and OtrC. OtrA and OtrB confer resistance by ribosomal protection and reduced accumulation, respectively, which represent the two non-destructive mechanisms (Ohnuki et al. 1985; Dittrich and Schrempf 1992; Doyle et al. 1991). It is unknown how OtrC mediates tetracycline resistance. The genes *otrA* and *otrB* encode proteins with sequence similarities to the proteins that confer the respective resistance mechanism in non-tetracycline-producing bacteria: the ribosomal protection protein encoded by *otrA* has 40% sequence identity to 300 amino acids of TetO from *Campylobacter jejuni* and TetM from *Streptococcus* (Doyle et al. 1991). OtrB resembles efflux determinants of gram-negative bacteria (Reynes et al. 1988). Furthermore, OtrA and OtrB have also been identified in *Mycobacterium fortuitum* and *Mycobacterium peregrinum*. Both belong to the fast growing, non-tuberculous mycobacteria, a group occupying the same habitat as streptomycetes (Pang et al. 1994). These findings support the hypothesis that resistance genes of opportunistic bacteria may have evolved from ancestral genes in antibiotic-producing streptomycetes (Benveniste and Davies 1973).

Resistance determinants of antibiotic producers are often physically linked to the genes encoding the biosynthetic pathway of the corresponding antibiotic. In contrast, resistance genes of non-tetracycline-producing bacteria are often located on mobile genetic elements such as transposons, conjugative plasmids, or conjugative transposons (for a recent review, see Roberts 1994). Furthermore, tetracycline itself is able to promote the mobility of some elements by stimulating the frequency of conjugation (Stevens et al. 1993). These genetic properties of resistance determinants and the continued use and misuse of tetracycline in medicine, veterinary medicine, and agriculture have probably caused or at least supported their distribution to virtually all groups of bacteria formerly susceptible to tetracycline.

## Protection of the ribosome

Ribosomal protection as a mechanism of tetracycline resistance was discovered in Streptococci (Burdett 1986). To date, six classes of determinants [Tet M, O-Q, S, OtrA] that presumably confer $tc^R$ on the level of protein synthesis have been identified in many gram-positive and gram-negative bacteria (Martin et al. 1986; Taylor 1986; LeBlanc et al. 1988; Doyle et al. 1991; Dittrich and Schrempf 1992; Nikolich et al. 1992; Charpentier et al. 1993; Sloan et al. 1994). Most of the work on the mechanism of ribosomal protection has been done on Tet(M). The ribosomal protection proteins encoded by the other classes have an amino acid sequence similarity of at least 40% to Tet(M) (Sloan et al. 1994). Therefore, the mechanism of action may be similar for all ribosomal protection proteins.

*Streptococcus* spp. bearing Tet(M) accumulates tetracycline to a level similar to that observed in sensitive cells (Burdett 1986). Tetracycline recovered from culture supernatant of resistant strains grown in the presence of tetracycline retained antibiotic activity, and modification of [$^3$H]-tetracycline extracted from the cytoplasm of streptococci could not be detected. Hence, active efflux or chemical drug modification were excluded as the cause for resistance. In vitro protein synthesis was analyzed for tetracycline sensitivity using cell-free extracts prepared from sensitive streptococci and from those expressing *tet*(M). Protein synthesis determined by in vitro incorporation of [$^3$H]-labeled amino acids into polypeptides was inhibited 50% by tetracycline concentrations of about 10 μM in extracts from sensitive cells. In contrast, protein synthesis in extracts from cells bearing *tet*(M) grown in the presence of tetracycline was inhibited 50% by about 300 μM of tetracycline. Fifty-percent inhibition occurred at 30 μM when Tet(M)-bearing cells were grown in the absence of tetracycline, indicating a tetracycline-dependent regulation of resistance expression. Cell extracts were fractionated to determine whether resistance is an intrinsic property of the ribosomes of *tet*(M) cells (Burdett 1991). Ribosomes retained tetracycline resistance if they were extracted under low-salt conditions. But preparations performed under high-salt conditions (1 M ammonium chloride) led to ribosomes which were nearly as tetracycline-sensitive as those prepared from tetracycline-susceptible cells. Therefore, a resistance factor seems to be non-covalently associated with the ribosome.

Tet(M) protein was subsequently purified, and its addition to cell-free extracts led to tetracycline-resistant protein synthesis. Nevertheless, the apparent binding of tetracycline to the resistant ribosome is not altered (Manavathu et al. 1990; Burdett 1993). Cell-free protein synthesis experiments have also indicated that Tet(M) acts cat-

alytically since ribosomes are still in great excess over Tet(M) protein when maximal resistance is achieved. Sequence similarities to elongation factors and other GTP-binding proteins (Sanchez-Pescador 1988; Burdett 1990) have suggested that Tet(M) may have GTPase activity. Purified Tet(M) is indeed able to release $^{32}P_i$ from $\gamma^{32}$P-GTP in the presence of ribosomes. Without ribosomes, the Tet(M)-associated GTPase activity is decreased about 200-fold. Thus, the Tet(M) ribosomal protection protein resembles elongation factors in three properties: (1) it has amino acid sequence similarity to EF-G and EF-Tu, (2) Tet(M) has a ribosome-dependent GTPase activity, and (3) it seems to confer resistance by reversible binding to the ribosome. Tet(O) also shows ribosome-dependent GTPase activity (Taylor et al. 1995). Tet(O) proteins having down-mutations in the motif presumably responsible for GTP binding confer a decreased level of resistance (Grewal et al. 1993). However, despite the similarity to elongation factors, *tet*(M) does not lead to increased survival of a *Bacillus subtilis* strain bearing a temperature-sensitive EF-Tu protein, or of an *E. coli* strain carrying a temperature-sensitive EF-G mutation at non permissive temperature. As pointed out by Burdett (1993), it remains uncertain whether the similarity between elongation factors and ribosomal protection proteins indicates a resemblance in the mechanism of action since EF-G- or EF-Tu-mediated steps of protein synthesis are not inhibited by tetracycline.

Interestingly, a host gene encoding a tRNA modification activity has been shown to be essential for Tet(M) function in *E. coli* (Burdett 1993). Screening for *E. coli* mutants retaining tetracycline sensitivity in the presence of Tet(M) has identified a mutant that shows only 12.5% of the resistance observed in the wild-type. Tetracycline sensitivity of protein synthesis has been assayed in toluenized cells and has shown that protein synthesis of non-Tet(M)-carrying cells, either wild-type or mutant, has the same tetracycline sensitivity (50% inhibition at 10 μM of tetracycline). Tet(M) expression leads to high-level resistance to protein synthesis in wild-type cells (20% inhibition at 250 μM of tetracycline), whereas the resistance of the mutant is of intermediate level (50% at 250 μM of tetracycline). The mutation has been mapped to *miaA*, encoding an isopentenylpyrophosphate (IPP) transferase (Caillet and Droogmans 1988). This enzyme catalyzes the first step of a pathway leading to anticodon loop modification of those tRNA species that recognize codons starting with a U-residue. They typically carry a 2-methylthio-N6-(Δ2-isopentenyl)-adenosine (ms2i6A) at position 37, located at the 3′ side of the anticodon (Björk et al. 1987). Modification of tRNAs is known to be important for translational accuracy. Undermodification of A37 results in pleiotropic effects. For example, *miaA* mutations induce derepression of the *trp* operon (Yanofsky and Soll 1977; Petrullo et al. 1983), increase the spontaneous mutation frequency of *E. coli* (Connolly and Winkler 1989), and decrease the growth rate of *Salmonella typhimurium* (Ericson and Björk 1986). The participation of *miaA* in Tet(M)-mediated tetracycline resistance may indicate that the A37 modification of tRNA is directly involved in the mechanism of Tet(M) action. Tet(M) may act as an accessory translation factor stabilizing ribosome-tRNA interactions (Burdett 1993, Taylor et al. 1995). It may not be able to stabilize (or stabilizes to a lesser extent) the respective interaction with unmodified tRNAs. *miaA* mutations also cause decreased stability of codon-anticodon interactions (Vacher et al. 1984). However, results indicating that Tet(M) increases the affinity of tRNAs to the tetracycline-bound ribosome are lacking. It is also possible that *miaA* supports Tet(M)-mediated resistance indirectly since *miaA* mutations have pleiotropic effects. Elucidation of the mechanism of Tet(M)-mediated resistance requires mapping of the interaction site(s) of Tet(M) on the ribosome. Revelation of the mechanism of Tet(M) function may also provide hints with respect to the mechanism of tetracycline action in protein biosynthesis.

## Reduced intracellular concentration of tetracycline

Since the ribosome is the target, antibiotic activity of tetracycline depends on the presence of the drug in the cytoplasm. Lowering the amount of tetracycline in the cytoplasm results in decreased susceptibility of the cell. A reduced tetracycline concentration in the cytoplasm can be achieved by two means: (1) the permeability of the cell envelope may be lowered, and (2) tetracycline may be pumped out of the cytoplasm in an energy-dependent fashion.

Bacteria differ in their cell-wall composition, causing respective differences in permeability and, hence, insensitivity to antibiotics. The peptidoglycan layer surrounding most gram-positive bacteria has a size exclusion limit of about 100 kD (Scherrer and Gerhardt 1971). It, therefore, does not reduce cytoplasmic accumulation of low-molecular-weight antibiotics such as tetracycline. In contrast, the outer membrane of gram-negative bacteria is an effective permeability barrier for hydrophobic compounds (for a discussion, see Nikaido 1994). Small hydrophilic compounds are able to pass the outer membrane of *E. coli* by diffusion through porin channels. The outer membrane of *Pseudomonas aeruginosa* lacks porins of broad substrate specificity (Yoshimura and Nikaido 1982). This results in a low outer-membrane permeability for many hydrophilic compounds. Accordingly, *P. aeruginosa* shows resistance to a wide variety of antibacterial agents (Nikaido 1994). Mycobacteria are surrounded by one of the most efficient permeability barriers known in bacteria (for a recent review, see Brennan and Nikaido 1995). They also show intrinsic resistance to most antibiotics. Reducing the expression of *ompF* contributes to multiple drug resistance in *E. coli* (Cohen et al. 1988). Porin-deficient *E. coli* mutants are generally somewhat less susceptible to tetracycline. Taken together, reduced permeability of the cell wall contributes to a low antibiotic susceptibility in several instances.

The effects of permeability barriers are usually supported by additional resistance mechanisms to achieve

high-level resistance: porin-deficient mutants of *E. coli* are only moderately resistant to tetracycline, and a low-level, endogenous, active efflux system has also been postulated as contributing to this resistance (Thanassi et al. 1995). Active efflux occurs in conjunction with reduced outer membrane permeability in *P. aeruginosa* (Li et al. 1994a, b) and contributes to multiple antibiotic resistance of *E. coli* (George and Levy 1983). Even in mycobacteria, special mechanisms enhance antibiotic resistance in addition to the low permeability of the cell wall (Jarlier et al. 1991; Nikaido 1994; Jarlier and Nikaido 1994). Therefore, low permeability of the cell wall alone seems to be insufficient to produce high-level resistance phenotypes.

Energy-dependent efflux of tetracycline causes high-level resistance in bacteria by itself. The fact that this even occurs in gram-positive bacteria suggests that this mechanism does not depend on an effective permeability barrier. Two different types of efflux pumps are involved in tetracycline resistance: (1) multidrug resistance pumps, and (2) tetracycline-specific transporters. A multidrug resistance pump belonging to the Acr family is responsible for the reduced tetracycline accumulation in *P. aeruginosa* (Li et al. 1995). It consists of three components: MexB, an inner membrane efflux pump; MexA, a periplasmatic membrane fusion protein (MFP); and OprK, an outer membrane channel. All members of the Acr family possess an inner membrane transporter (typically consisting of 12 transmembrane segments) and an MFP. The MFP connects the efflux pump of the cytoplasmic membrane to a channel protein in the outer membrane. Thus, the multidrug efflux pumps transport their substrate straight out of the cell into the surrounding medium. The outer membrane channel protein interacting with the MFP has so far only been identified for a few members of the Acr family. Acr-type transporters have been reviewed recently and are not discussed here in further detail (Ma et al. 1994). In contrast to the broad substrate range of multiple-drug transporters, many of the efflux pumps identified in gram-positive and gram-negative bacteria specifically transport tetracycline. They are described in detail in the following section.

## Tetracycline-specific efflux pumps

Eleven classes of tetracycline resistance determinants encoding drug-specific efflux proteins are known to date: TetA-E, G, H, K, L, P [*tetA*(P)], and OtrB. TetA-E, G, and H are found in gram-negative bacteria; TetK, L, P [*tetA*(P)], and OtrB occur in gram-positive bacteria. Amino acid identities between tetracycline efflux pumps of gram-negative bacteria range between 45% and 78% (Zhao and Aoki 1992; Allard and Bertrand 1993; Allard et al. 1993; Hansen et al. 1993), but there is only little sequence similarity to the transporters of gram-positive bacteria (Sheridan and Chopra 1991). Efflux determinants from gram-negative bacteria share a common genetic organization, that is different from the one in gram-positive bacteria: *tetR*, encoding a tetracycline-responsive repressor, is located next to *tetA* in a divergent orientation. TetR binds two nearly identical *tet* operators in the short intergenic region in the absence of tetracycline, thereby repressing the expression of *tetR* and *tetA*. The induced TetR-[M-tc]$^+$ complex is formed in the presence of tetracycline and divalent metal ions, and expression of *tetR* and *tetA* is coinduced (for a recent review, see Hillen and Berens 1994).

Resistance mediated by tetracycline transporters of gram-positive bacteria is also inducible (Khan and Novick 1983; Ohnuki et al. 1985; Sloan et al. 1994), but no transcriptional repressor protein that mediates induction has been found. The 5′-ends of *tetK* and *tetL* mRNAs show sequence similarity to mRNAs regulated by translational attenuation, which therefore may be the most probable mechanism for regulating *tetK* and *tetL* expression (Khan and Novick 1983; Speer et al. 1992). *TetA*(P) expression in *Clostridium perfringens* seems to be differently regulated. *TetA*(P) on pCW3, a conjugative 47 kb plasmid that confers inducible tetracycline resistance, exhibits no secondary structures typical for attenuation. Since plasmids that contain only the resistance genes mediate constitutive resistance, an additional gene that perhaps encodes a DNA-binding protein must be involved in regulation (Abraham and Rood 1985). However, sequence analysis revealed no putative regulatory gene in the vicinity of the resistance genes (Sloan et al. 1994). The tetracycline efflux pumps of gram-positive bacteria do not constitute a family of homologous proteins. Only members of Tet(K) and Tet(L) exhibit high levels of sequence similarity sharing of between 64% and 80% identical residues (Ives and Bott 1990), but TetA(P) and *otrB* from *Streptomyces rimosus* show no higher sequence similarity to the TetA-L efflux pumps than to other transmembranal transport proteins (Reynes et al. 1988; Sheridan and Chopra 1991; Sloan et al. 1994).

Most studies of the molecular mechanism underlying tetracycline efflux have been conducted on the transporters of gram-negative bacteria, especially the *Tn10*-encoded TetA protein. An in vitro tetracycline transport assay using inverted membrane vesicles containing the respective tetracycline transport protein was used to biochemically characterize the export reaction. Overproduction of tetracycline transporters is not straightforward. High expression of Tn*10*-encoded TetA protein results in cell death, probably caused by loss of the membrane potential (Eckert and Beck 1989b). Constitutive expression of the Tn*10*- and pSC101-encoded resistance proteins leads to reduced competitive fitness of the respective *E. coli* strain (Lee and Edlin 1985; Nguyen et al. 1989), and cell toxicity has also been attributed to *tetK* expression (Guay and Rothstein 1993). Poor overproduction may, therefore, be caused by toxicity common to all tetracycline-specific efflux pumps.

Several lines of evidence support a two-dimensional topology model originally postulated for TetA(B), but probably commonly valid for the tetracycline transporters (Fig. 3). Hydrophobic pattern analysis suggests that the Tn*10*-encoded efflux pump consists of 12 transmem-

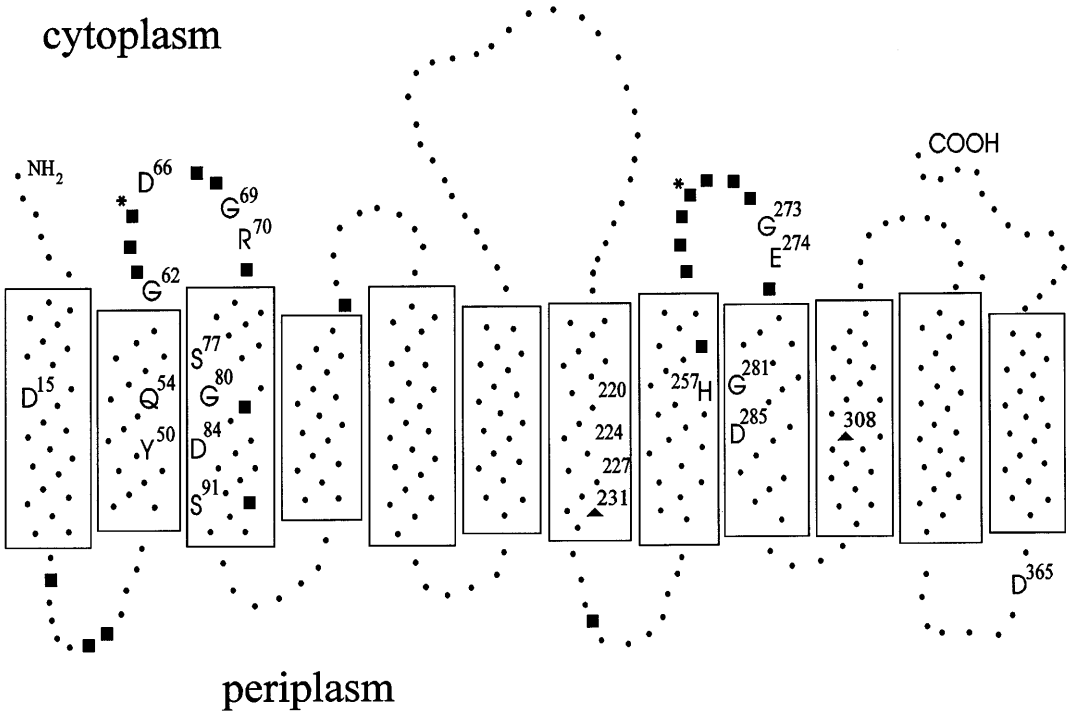


**Fig. 3** Secondary structure model of tetracycline-specific efflux pumps. The model is based on the results presented in Eckert and Beck (1989a) and Yamaguchi et al. (1992a). Putative transmembrane segments are enclosed by *boxes*. Amino acids are given in the one-letter abbreviation for positions at which mutations with a loss of at least 80% activity in TetA(B) have been reported. Positions at which mutations reduce activity by less than 80% are indicated by *filled squares* (■). The *asterisks* (*) indicate positions at which sulfhydryl modifications of mutations to cysteine leads to loss of activity. Positions of intragenic suppression for DN285 are indicated by their respective numbers, and those where mutations changing the substrate specificity of TetA(B) have been reported are indicated by *filled triangles* (▲). All other amino acids are depicted by *filled circles* (●)

branal segments, most probably α-helices, and 11 connecting loops (Eckert and Beck 1989a). Proteolytic digestion susceptibility studies have implied loops protruding into the cytoplasm between segments 2 and 3, 6 and 7, and 10 and 11. Cytoplasmic localization of the N- and C-termini was also demonstrated: the N-terminal Met residue is accessible to modification by cyanate only if the inner membrane is permeabilized by toluene prior to incubation. An antibody against an oligopeptide of the 14 C-terminal amino acids of TetA(B) reacts only with inverted vesicles of *E. coli* cells expressing TetA(B), and not with right-side-out vesicles (Yamaguchi et al. 1990b). Based on localization experiments with TetA-PhoA fusions, an analogous model has been postulated for TetA(C) (Allard and Bertrand 1993), and sequence similarities suggest that all tetracycline transporters of gram-negative bacteria share a similar secondary structure (McNicholas et al. 1992; Varela and Griffith 1993; Allard and Bertrand 1993; Hansen et al. 1993).

Hydropathy analysis revealed that the efflux pumps comprise two structurally symmetrical halves, each containing six transmembranal segments. Based on this similarity, Rubin et al. (1990) have proposed that the two halves of tetracycline efflux pumps have evolved by gene duplication from a single predecessor.

Tetracycline-specific exporters pump their substrate into the periplasm and not across the outer membrane, as found for the multidrug efflux pumps (Thanassi et al. 1995). The energy for active transport is provided by the pH gradient across the cytoplasmic membrane (ΔpH). The transmembrane electrical potential (Δψ) is not necessary for transport (Kaneko et al. 1985), indicating that tetracycline transport is an electrically neutral antiport of protons and a positively charged tetracycline complex. A 1:1 stoichiometry of a monocationic metal-tetracycline/$H^+$ exchange has been confirmed by flow dialysis (Yamaguchi et al. 1991a). Experiments with inverted vesicles have revealed no transport in the absence of divalent cations (Yamaguchi et al. 1990a). The addition of various divalent cations stimulates tetracycline transport to different extents in the order: $Co^{2+} > Mn^{2+} > Mg^{2+}$ $Cd^{2+} > Ca^{2+}$. The respective metal-tetracycline complexes share the same affinity to the efflux protein but show different cation-specific turnover rates.

The specificity of TetA(B) for tetracycline derivatives has been investigated by inhibition of [$H^3$]-tetracycline uptake into inverted vesicles. Effective inhibitors need the four-ring tetracycline structure and a phenolic OH at position 10 of the D-ring conjugated with a 1,3 diketoenolate at position 11-12. Substituents at positions 2, 4, 5, 6, 7, 8, and 9 have seemed to be less important for inhibition (Nelson et al. 1994). Nevertheless, tetracycline derivatives with bulky modifications at position 9, the glycylcyclines (for a recent review, see Tally et al. 1995), have been neither bound nor transported by Tn*10* TetA (Someya et al. 1995a).

Attempts to identify amino acid residues important for tetracycline transport have concentrated primarily on mutagenesis of charged amino acids and conserved sequence motifs. Since tetracycline is transported as a monocationic complex, contributions of especially negatively charged residues to substrate recognition and transport seemed likely. Only mutation of one amino acid of the putative loop regions (see Fig. 3), D66, eliminates tetracycline transport activity of Tn*10* TetA. Mutation of any of the other conserved acidic residues leads to no or only minor effects on tetracycline transport (Yamaguchi et al. 1990c, 1992b). D66 is located in the cytoplasmic loop connecting the transmembranal segments 2 and 3 (loop 2-3). This loop bears a conserved sequence motif, **G**XXXX**R**X-**GRR**, common to tetracycline efflux proteins, glucose uniporters, and sugar/$H^+$ symporters (Yamaguchi et al. 1992c). Mutations of every single residue of the $^{62}$**G**KMSD**R**F**GRR**$^{71}$ motif of Tn*10* TetA have revealed R70 as being the only other essential residue in addition to D66. Replacement of G69 or G62 has led to reduction of activity, with the highest loss of activity obtained by replacement with amino acids which have a low propensity to form a β-turn. Therefore, the β-turn structure of loop 2-3 seems to be crucial to Tn*10* TetA activity. A C-scanning mutagenesis has been conducted in order to investigate if one of the residues of loop 2-3 is part of or in the vicinity of the protein-$[M\text{-}tc]^+$ interaction site (Yamaguchi et al. 1992c). Out of five cysteines at positions 63, 65, 67, 68, and 69, only modification of C65 by N-ethylmaleimide (NEM) leads to inhibition of activity. Binding of $[M\text{-}tc]^+$ accelerates modification of C65, indicating that C65 is exposed to the medium in consequence of the interaction with $[M\text{-}tc]^+$ (Kimura et al. 1995). Thus, loop 2-3 may be part of the entrance gate of $[M\text{-}tc]^+$, and position 65 should be close to $[M\text{-}tc]^+$.

Loop 8-9 of the C-terminal half of Tn*10* TetA corresponds to loop 2-3 of the N-terminal half according to the gene duplication hypothesis (see above). Analyses of TetA proteins mutated in the loop 8-9 residues suggest structural similarities but functional differences to the residues in loop 2-3. None of the amino acids in loop 8-9 is essential for $[M\text{-}tc]^+$ transport (Yamaguchi et al. 1993a, 1994). Based on the structural similarities, Yamaguchi et al. (1993a) proposed that loop 8-9 might also be located at the entrance of the substrate translocation pathway, forming a structurally symmetrical pair with loop 2-3.

Four charged residues, D15, D84, H257, and D285, are located in the putative transmembrane segments according to the topology model of TetA proteins. The effects of H257 mutations depend on the introduced residue. D257 and E257 exhibit 10 and 20% tetracycline transport activity of wild-type TetA(B), respectively. Tetracycline-coupled proton translocation was not detectable for both mutants (Yamaguchi et al. 1991b). In contrast, HY257 has significant proton translocation activity coupled to tetracycline transport. All aspartyl residues in the transmembrane segments are important for activity. Replacement of any of them by neutral amino acids has reduced tetracycline transport activity to less then 10% of wild-type activity (Yamaguchi et al. 1992a; McMurry et al. 1992). D84 and D285 are located at corresponding positions in the N- and C-terminal half of Tn*10* TetA. Conserved residues in the vicinity of D84 (positions 54, 80, and 50) and D285 (positions 261, 281, and 275) further suggest functional similarity (Yamaguchi et al. 1993b): (1) replacement of Q54 and Q261 by alanine results in a decreased substrate affinity; (2) the effects of G80 and G281 mutations seem to depend on the volume of the side chains placed at these positions. GL80 and GL281 showed no transport activity, whereas GA80 and GA281 have 10 and 30% activity of the wild-type respectively; (3) mutations at Y50 and H275 result in a severe reduction of transport activity, although the affinity of these Tn*10* TetA mutants to $[M\text{-}tc]^+$ is only slightly decreased (wild-type $K_m$ = 18 μM, HY257 $K_m$ = 32 μM, YH50 $K_m$ = 20 μM). Double mutations at positions 50 and 257 do not result in an additive loss of activity. In contrast, the activity of the double mutants is similar to or intermediate between the respective single mutants. A model involving pairwise action of Y50 and H257 in the transport function has been proposed; according to this model, helices 2 and 3 and helices 8 and 9 build two sides of one tetracycline transport channel (Yamaguchi et al. 1993b). The importance of helix 3 in $[M\text{-}tc]^+$ transport is further emphasized by two mutations at S77 and S91. SC77 shows no transport activity, and bulky residues at position 91 have hindered substrate translocation (Yamaguchi et al. 1992d). Replacement of the other polar residues in helix 3 have not interfered with Tn*10* TetA function. S77, S91, and D84 are on the same vertical stripe of putative helix 3, which is in accordance with the idea that side chains of this side of helix 3 protrude into the $[M\text{-}tc]^+$ transport channel. Suppressor mutations indicate participation of helix 7 in substrate channel formation. The DN285 mutation is partially suppressed by acidic residues at positions 220, 224, and 227 (Yamaguchi et al. 1993c; Someya et al. 1995b). Suppression is caused by increased affinity of the double mutants for $[M\text{-}tc]^+$. Positions 220, 224, and 227 are on the same side of putative helix 7, with residue 220 in the same depth as residue 285. Thus, putative helices 7 and 8 may contact each other in the TetA(B) structure.

Oligonucleotide-directed mutagenesis of Tn*10* TetA has concentrated on residues possibly important for function because of their charge, their polarity, or their conservation. Random mutagenesis has been used to identify additional residues important for substrate specificity. Tetracycline efflux pumps are not able to confer resistance against glycylcyclines. Guay et al. (1994) has identified TetA(B) variants that confer resistance against glycylcycline 9-(dimethylglycylamido)-minocycline (DMG-Mino). The mutations causing this phenotype have been identified to be WC231 and LS308 (Guay et al. 1994). Both mutations not only increase resistance to DMG-Mino, but also cause diminished tetracycline resistance, and therefore are true altered-specificity mutations. Their location in helices 7 and 10 emphasizes the importance of these helices for transport activity.

In summary, mutational analysis of Tn*10* TetA demonstrates contributions of residues extending over the entire primary structure to tetracycline binding and translocation. Loop 2-3 is the most probable candidate for forming the substrate entrance gate on the cytoplasmic side of the membrane. Amino acids important for tetracycline transport and substrate specificity in helices 1, 2, 3, 7, 8, 9, and 10 suggest participation of these helices in tetracycline transport channel formation.

In contrast to Tn*10* TetA, the pSC101-encoded class C TetA shows potassium-uptake activity. This is valuable for random mutagenesis experiments since screening for complementation of potassium-uptake defects in *E. coli* is possible (Griffith et al. 1988). TetA(C) mutants, lacking tetracycline-resistance activity but retaining the potassium transport activity, have indicated positions specifically important for tetracycline transport (McNicholas et al. 1992). Mutations affecting tetracycline transport mapped to both halves of the pSC101-encoded efflux pump. Suppressor mutations indicated a functional interaction between the N- and C-terminal domains of TetA(C) since mutations in one domain could be suppressed by mutations in the other domain (McNicholas et al. 1995). Some of the residues identified as important for pSC101-encoded TetA are distinct from those characterized in Tn*10* TetA. For example, mutations that abolish tetracycline efflux have been found at four residues of loop 10-11. Further experiments may reveal whether these positions are only important for pSC101 TetA function.

Intragenic complementation suggests that tetracycline efflux pumps function as multimers (Curiale et al. 1984). In addition, Hickman and Levy (1988) have demonstrated that activity of alkaline phosphatase (PhoA) expressed as a fusion protein with Tn*10* TetA is reduced if wild-type Tn*10* TetA is coexpressed. The reduction of PhoA activity might indicate formation of heterodimers containing only one PhoA monomer. PhoA of these heterodimers would be inactive since its activity is dimerization-dependent. Recently, a multimerization domain of Tn*10* TetA has been mapped to the N-terminal half by an in vitro protein-protein interaction assay (McMurry and Levy 1995). Taken together, these results suggest a role for oligomerization in TetA function.

Much less is known about the tetracycline transporters of gram-positive bacteria. Recent studies of *Bacillus subtilis* TetA(L) have revealed differences with respect to the transporters from Enterobacteriaceae. Chromosomally encoded *tetA*(L) confers significant tetracycline resistance only after amplification yielding a multi-copy situation (Ives and Bott 1990). This efflux pump mediates not only tetracycline/$H^+$ antiport, but is also an even more effective $Na^+/H^+$ antiporter. The $Na^+/H^+$ antiport activity is of physiological importance in $Na^+$ resistance and $Na^+$-dependent pH homeostasis (Cheng et al. 1994). These findings may indicate an evolutionary relationship of tetracycline resistance pumps to monovalent cation/$H^+$ antiporters. The antiport catalyzed by TetA(L) has also been shown to be electrogenic, in contrast to the electroneutral antiport of Tn*10* TetA. Similar to the gram-negative efflux pumps, tetracycline uptake by everted membrane vesicles containing TetA(L) requires divalent cations (Guffanti et al. 1995).

*Staphylococcus aureus* TetA(K) resembles TetA(C) in its ability to transport $K^+$ (Guay et al. 1993a). It differs in substrate specificity from Tn*10* TetA and pSC101 TetA since it is less capable of conferring resistance to some tetracycline derivatives (Guay and Rothstein 1993). Hydropathy plotting indicates that Tet(K) contains 14 transmembrane segments (Guay et al. 1993b). Mutagenesis experiments with gram-positive transporters identifying functionally important residues have not been published.

## Conclusion and perspective

Despite the mass application of tetracycline in medicine and feed stock, virtually nothing is known about its mode of action in protein biosynthesis. Structural information on tetracycline interaction with elongating ribosome or efflux pumps is not available. The structural model of $[Mtc]^+$ interaction with Tet repressor (Hinrichs et al. 1994; Müller et al. 1995; Hillen and Berens 1994) can probably not be applied to these complexes due to the lack of apparent sequence similarity. The need for new antibiotics active against resistant pathogenic bacteria is widely acknowledged. Elucidation of the structure of these complexes may support the rational design of tetracycline derivatives with such properties.

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