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MINIREVIEW

Tetracycline Resistance Mediated by Ribosomal Protection

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Resistance to tetracycline may be mediated by one of three different mechanisms: (i) an energy-dependent efflux of tetracycline brought about by an integral membrane protein (20); (ii) ribosomal protection by a soluble protein (5, 23), or (iii) enzymatic inactivation of tetracycline (33), which occurs rarely. This minireview concentrates exclusively on the second mechanism of tetracycline resistance.

OCCURRENCE OF RIBOSOMAL PROTECTION DETERMINANTS

A recent review (28) has documented the epidemiology of the various tetracycline resistance determinants, and only pertinent points will be summarized here. Ribosomal protection mechanisms identified so far fall into six classes: TetM, TetO, TetP, TetQ, TetS, and OtrA. For details of the nomenclature for tetracycline resistance determinants, refer to reference 21. The Tet M determinant was first identified in *Streptococcus* spp. (3, 25), where it is often present on conjugative transposons such as Tn*916* and Tn*1545*. It has subsequently been identified in a wide variety of gram-positive and gram-negative species as well as mycoplasmas and ureaplasmas (28). Tet O appears most often to be plasmid-mediated in *Campylobacter jejuni* (36) and *Campylobacter coli* (32), but it has also been identified in several gram-positive organisms, where it is probably chromosomal (28). Tet Q is from *Bacteroides* spp. (26), Tet S is from a *Listeria monocytogenes* plasmid (7) but was subsequently found in *Enterococcus faecalis*, where it probably resides on the chromosome (8), and Tet P is from a *Clostridium perfringens* plasmid (31). The Otr A determinant is found in the chromosome of *Streptomyces rimosus*, which produces oxytetracycline (12). Recent work has also demonstrated *otr*(A) in mycobacteria and in clinical isolates of *Streptomyces* spp. (27). Thus, the ribosomal protection determinants are widely distributed within the microbial world, and movement of specific resistance determinants from antibiotic producers to clinical microorganisms has been suggested, even though it has not yet been demonstrated unequivocally (27, 28).

RIBOSOMAL PROTECTION PROTEINS

The ribosomal protection proteins (RPPs) are all polypeptides of approximately 72.5 kDa, ranging from Tet(O) with 639 amino acids to Otr(A) with 661 amino acids (Fig. 1). Tet(M) and Tet(O) have 75% sequence similarity (35), and Tet(S) is also closely related to these Tet determinants [with 70% sequence identity to Tet(M)] (7). Otr(A) and Tet(P) are closely related to one another (57% similarity, 37% identity), whereas Tet(Q) is more distantly related to the other RPPs, with only 44% identity (26). Both Tet(M) and Tet(O) proteins have apparent molecular masses of 68 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5, 24).

Regulation of expression by transcription attenuation has been proposed for the Tet M determinant of Tn*916* (34). Although the Tet O determinant is not regulated in a similar fashion, an upstream DNA sequence is required for the full expression of tetracycline resistance (40). The function of the Tet(O) upstream sequence is not understood, and regulation of the other RPP determinants has not been documented.

STRUCTURAL COMPARISONS OF ELONGATION FACTORS AND RPPs

Comparison of the amino acid sequences of Tet(M) and Tet(O) has shown that their N-terminal regions are highly homologous to those of GTPases participating in protein synthesis, namely EF-Tu and EF-G (5, 24, 29, 30). The GTP-binding domain in these proteins has been found to be conserved in the N-terminal region and to consist of five highly conserved sequence motifs designated G1 to G5 (15). The G4 motif interacts with the guanine moiety of the nucleotide (19) and contains the highly conserved residues aspartate and asparagine. Within Tet(O), substitution of the corresponding Asn residue at position 128 (Asn-128) by several other amino acids resulted in a decrease in tetracycline resistance, indicating that tetracycline resistance is dependent on GTP binding (15).

Recently the three-dimensional structure of EF-G from *Thermus thermophilus* has been determined (1, 10). EF-G is a translocase which catalyzes the translocation step during protein synthesis. During this process the ribosome undergoes a conformational transition from the pre- to the posttranslocational state, with movement, relative to the ribosome, of mRNA and associated tRNAs. This conformational change positions the next mRNA codon ready for translation during the subsequent elongation cycle. EF-G catalyzes translocation by binding of the EF-G–GTP complex to the ribosome, rapidly inducing the GTPase activity of EF-G. After GTP hydrolysis, EF-G dissociates from the ribosome, GDP is released, and EF-G binds to GTP again to complete the cycle (22).

Determination of the crystal structure of EF-G from *T. thermophilus* to 0.285 nm demonstrated that it contains five domains (1, 10), which are shown in Fig. 2. The N-terminal domain (G domain), comprising nucleotides 1 to 280, is concerned with GTP-GDP binding. Although the crystal structure of an RPP has not yet been determined, on the basis of amino acid analysis, major homologies can be predicted among EF-G, EF-Tu, and RPP in the G domain (Fig. 2). Within the G

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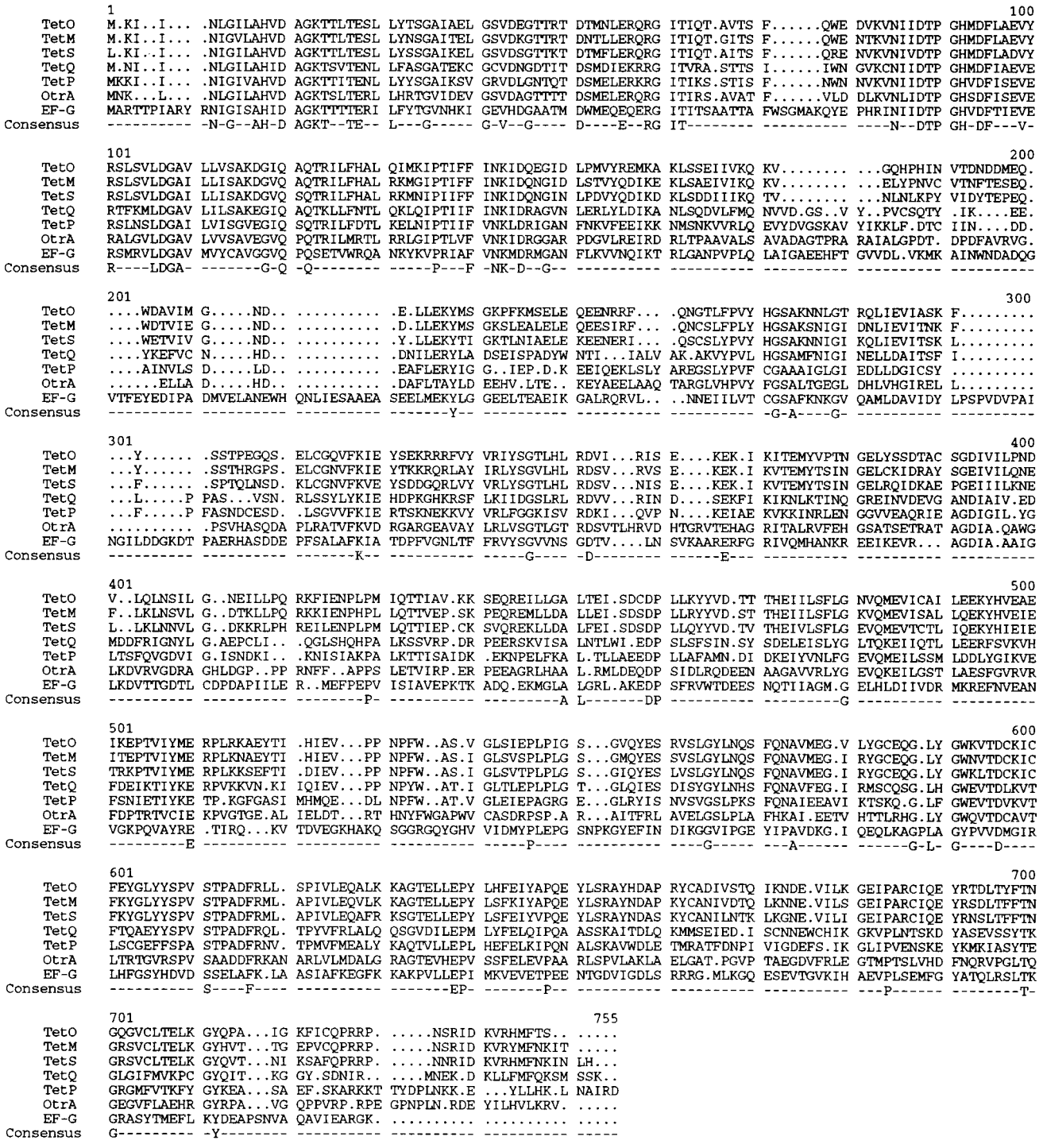

FIG. 1. Amino acid sequence alignment of Tet proteins and EF-G. Each dot represents a gap inserted because of dissimilarities in the alignment. The sequence of Tet(O) is from *C. jejuni* (24), that of Tet(M) is from *S. faecalis* (4), that of Tet(S) is from *L. monocytogenes* (7), that of Tet(Q) is from *Bacillus thetaiotamicron* (26), that of Tet(P) is from *C. perfringens* (31), that of Otr(A) is from *S. rimosus* (12), and that of EF-G is from *E. coli* (17). Alignment was performed with the Pileup program from the GCG analysis software by using previously published programs (11).

domain of EF-G from *T. thermophilus* is a region of approximately 90 amino acids (residues 158 to 253) which has been termed the G′ subdomain (1). Structural analysis suggests that G′ functions as an intrinsic exchange factor modulating the binding of guanine nucleotides and facilitating their exchange. EF-Tu does not have this domain because it requires EF-Ts as a specific guanine nucleotide exchange factor. The RPPs contain substantial portions of the G′ subdomain, but some of the G′ subdomain is also missing (Fig. 2).

Domain II of *T. thermophilus* EF-G (residues 288 to 400) is

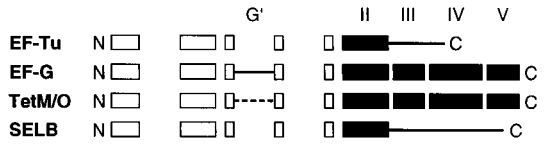

FIG. 2. Schematic structural alignment of Tet(M) and Tet(O) and translation and elongation factors, respectively. Conserved structure is shown as boxes, and only major inserts (≈20 residues or more) are indicated as bold lines between boxes. The white boxes correspond to the G domain, and the shaded boxes correspond to domains II to V in EF-G. The figure is adapted from Ævarsson et al. (1), with permission of Oxford University Press. References for sequence information are as follows: EF-Tu (19), EF-G (1), Tet(M) and Tet(O) (4, 24), and SELB (13).

also conserved in EF-Tu and the RPP. The level of conservation in both the G and the II domains suggested that they form a common structural unit which interacts with the ribosome (1). Substitution of the amino acid Val in place of Gly at position 280 in *Salmonella typhimurium* domain II of EF-Tu disrupts the ternary complex (EF-Tu–GTP–amino acyl-tRNA) binding to the ribosome (39). A conserved Gly residue is found at the corresponding position in all of the RPPs (amino acid 279 in Fig. 1) as well as in SELB, a translation factor (Fig. 2) responsible for the incorporation of selenocysteine into proteins (13).

Domain III (residues 405 to 482) and domain IV (residues 606 to 673) of *T. thermophilus* EF-G resemble ribosomal proteins in having a characteristic fold (1) and may be involved in the rRNA interaction. Similar domains are present in the RPPs (Fig. 2). Domain IV (residues 483 to 605 and 674 to 691) of EF-G is also present in the RPPs, with the residues being well-conserved.

EF-G contains a stretch of amino acids in domain III which are believed to have a direct role in conformational transitions (Gly-X-Gly-Glu-Leu-His, residues 453 to 458 in *T. thermophilus* and corresponding to amino acids 476 to 482 in Fig. 1). Examination of the RPPs shows the amino acid sequence X-X-Gly-X-X-X, or that only one Gly is maintained. Although some replacements are conservative, e.g., Leu replaced by Val in all RPPs except Tet(Q), which contains Thr, some amino acids show great variability in this region both between EF-G and the RPPs as well as among the RPPs themselves (Fig. 1). Similar results were found within a second domain believed to be essential for efficient translocase function (residues 485 to 494 of *Escherichia coli* EF-G, which corresponds to amino acids 504 to 513 in Fig. 1), where the amino acids 485-$PX_1{}^{V}X^{Y}RE^{T}$X-494 have been observed (16). Pro is conserved in Tet(M), Tet(O), and Tet(S), but it is replaced by Thr (related) in Otr(A) and isoleucine (unrelated) in Tet(P) and Tet(Q). Only Glu is invariant in this stretch of amino acids. These results could imply that the RPPs do not undergo a conformational change as significant as that of EF-G.

EF-G has been found to contain several regions associated with resistance to fusidic acid (Fus) (18), with clusters of mutations at residues 66 to 161 (*S. typhimurium* EF-G) in the G domain, at residues 413 to 471 within domain III, and at residues 628 to 681 within domain V. Fus inhibits EF-G by binding to it, forming a ribosome–EF-G–GDP–Fus complex (18, 22). Examination of *S. typhimurium* EF-G Fus^r mutants identified a number of critical amino acids in EF-G which had undergone substitutions (18). Of 10 amino acids in the G domain, four are identical in RPPs (at positions 88, 108, 120, and 122 in Fig. 1), four of seven are identical or conservative substitutions in domain III (amino acids 429, 450, 454, and 460 in Fig. 1), and three of four are identical or conservative substitutions in domain V (at positions 653, 664, and 684 in Fig. 1). We noted the presence of a large number of amino acids in domains G, III, and V of the RPPs which are implicated in resistance to Fus in both *S. typhimurium* EF-G (17) and *E. coli* EF-G (Fig. 1). This finding suggests that tetracycline resistance mediated by the RPPs may be influenced by Fus. However, it should be noted that many single amino acid substitutions in *S. typhimurium* EF-G, e.g., substitution of Val-125 by Leu (17), resulted in Fus resistance. A valine residue is found at this position (amino acid 126 in Fig. 1) within the G domain of *E. coli* EF-G; however, all of the other RPPs contain Leu at this position.

INFLUENCE OF tRNA MODIFICATION ON TETRACYCLINE RESISTANCE

Recently, mutations in the *miaA* gene of *E. coli* were shown to interfere with the ability of Tet(M) to confer tetracycline resistance (6). The MICs of tetracycline for strains carrying the *tet*(M) determinant from Tn*916* on a low-copy-number plasmid were reduced from 25 μg/ml for a wild-type *E. coli* strain to 3.12 μg/ml for a *miaA* mutant (6). A reduced effect was found for the *tet*(O) gene from pUOA466 on a high-copy-number (pUC) vector (36), with the MIC of tetracycline reduced from 64 μg/ml for a wild-type *E. coli* strain to 32 μg/ml for a *miaA* mutant (38). These reductions in MICs may tell us something about the mechanism of resistance.

The *miaA* gene product is Δ^2-isopentenylpyrophosphate transferase (9), an enzyme required for the first step in the modification of the adenosine at position 37 (A37) in tRNA to 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine. In the *miaA* mutant, the A37 immediately 3′ of the anticodon of tRNAs which read codons beginning with U, including those for cysteine, leucine, phenylalanine, serine, tryptophan, and tyrosine, remains unmodified. The *miaA* mutation has a number of pleiotropic effects, including a mutator phenotype (9) and altered translation properties including derepression of the *trp* operon (41). tRNA lacking modification of A37 has been shown to bind to the ribosome with a lower affinity (14, 41). The last property may explain the reduced level of tetracycline resistance in *E. coli miaA* mutants. If the ribosomal A site for aminoacyl-tRNA is distorted by the RPP, the tRNA molecule without modification at A37 might be less able to bind. This might account for the reduced tetracycline MICs for these mutants. Further studies are needed to identify other host mutations which interfere with tetracycline resistance and to clarify the precise role of tRNA modification in ribosomal protection.

MECHANISM OF RPP RESISTANCE

Tetracycline inhibits protein synthesis by binding to a single high-affinity site on the 30S ribosomal subunit, likely to proteins S7, S14, and S19 (2). With tetracycline in this position, the binding of aminoacyl-tRNA to the A site is blocked. Thus, the step in the protein synthesis cycle inhibited by tetracycline is that which is catalyzed by EF-Tu. Yet, the RPPs most closely resemble EF-G (1, 5) (Fig. 2), which catalyzes the translocation reaction, the step preceding the binding of tetracycline in the protein synthesis cycle.

Biochemical studies with isolated Tet(M) or Tet(O) proteins have shown that RPPs bind to both GDP and GTP (5, 37). They have GTPase activities which are stimulated by ribosomes (5, 37). These properties are consistent with the structural features of the proteins (1, 10) discussed above, as well as

with mutagenesis studies of the GTP-binding domain of Tet(O) (15). The purified Tet(M) protein, in fact, protects translation from tetracycline inhibition by using components from susceptible cells (5).

Binding studies with isolated ribosomes from resistant and susceptible Tet(O) strains have demonstrated that [^{3}H]tetracycline binds equally well to both resistant and susceptible ribosomes over a wide range of concentrations (15, 24). Yet, the most logical explanation for resistance is that RPPs inhibit the binding of tetracycline to functioning ribosomes engaged in protein synthesis. Other explanations that have been proposed include the catalytic modification of ribosomal proteins or rRNA (24) or that RPPs substitute for EF-G or EF-Tu (24, 29). This latter explanation seems less likely because Tet(M) is unable to complement *E. coli* EF-G or *Bacillus subtilis* EF-Tu mutants (5). The ability of RPPs to allow the entry of amino acyl-tRNA to the A site when tetracycline is in place must also be considered a likely explanation.

The structural analysis of EF-G and the related RPPs elucidates their ability to undergo conformational changes. Such changes may be related to their ability to associate with the ribosome and perhaps dislodge tetracycline at the critical stage of protein synthesis. Alternatively, the RPP may distort the ribosome in such a way that amino acyl-tRNA may bind even in presence of tetracycline. If this latter hypothesis is correct, then *miaA* mutants might be more susceptible to the distortion and the unmodified amino acyl-tRNA may be less able to bind, resulting in a lower tetracycline MIC. Alternatively, the reduced tetracycline MICs observed when tRNA is unmodified may simply be a nonspecific effect. Knowledge of the structural features of RPP in comparison with those of EF-G raises additional questions, such as the role of the modified G′ subdomain in RPP and the influence of Fus on tetracycline resistance mediated by ribosomal RPPs. Work on these questions is under way in our laboratory.

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