ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1993, p. 2645–2649
0066-4804/93/122645-05$02.00/0

Vol. 37, No. 12

Effect of Mutational Alteration of Asn-128 in the Putative GTP-Binding Domain of Tetracycline Resistance Determinant Tet(O) from *Campylobacter jejuni*

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Received 21 May 1993/Returned for modification 21 July 1993/Accepted 31 August 1993

The deduced amino acid sequence of *Campylobacter jejuni* Tet(O), cloned in *Escherichia coli*, has shown that it contains the five highly conserved sequences of the GTP-binding domain found in other GTPases. Asn-128 belongs to the G4 motif of such a domain and is involved in hydrogen bonding with the guanine ring of the nucleotide. Substitution of Asn-128 by 11 other amino acids resulted in a decrease in tetracycline resistance, indicating that tetracycline resistance conferred by Tet(O) is related to GTP binding. The effect of the mutations on the GTP-binding domain is discussed with the EF-Tu-GDP complex as a model.

Guanine nucleotide-binding domains share local homologies at the protein sequence level and are similar in their three-dimensional structure and interactions with GTP (2, 7, 11, 12). Many GTP-binding proteins have been identified, and the crystal structures of the two GTPases, EF-Tu and $p21^{ras}$, have been determined (8, 12). Certain tetracycline resistance (Tc^r) determinants have been identified, such as Tet(O) and Tet(M), which also possess the highly conserved sequences of the GTP-binding domain (15, 19, 24). Tet(O) and Tet(M) share ~75% sequence homology at both the DNA and protein sequence levels (15). Comparison of the amino acid sequences of these two resistance determinants with other proteins has shown that their N-terminal regions are highly homologous to those of the GTPases participating in protein synthesis, namely EF-Tu and EF-G (5, 15). The GTP-binding domain in these proteins has been found to be located in the N-terminal region and consists of five highly conserved sequence motifs designated G1 to G5 (Table 1). Crystallographic data and computer modelling of the guanine nucleotide-binding domain of EF-Tu and $p21^{ras}$ helped in predicting the structural environment of the bound nucleotide (2, 12). The models show that the motifs G1, G2, and G3 are involved in binding to the phosphate groups of GTP and to the magnesium ion coordinated to these phosphate groups. In contrast, the G4 motif interacts with the guanine moiety of the nucleotide. The G4 motif has two highly conserved residues, aspartate (corresponding to positions 138 and 119 in EF-Tu and $p21^{ras}$, respectively) and asparagine (corresponding to positions 135 and 116 in EF-Tu and $p21^{ras}$, respectively), which hydrogen bonds with the base. In the EF-Tu model, the aspartate is involved in hydrogen bonding with the N-1 and N-2 of the purine ring, whereas the asparagine hydrogen bonds with the O-6 of the base (12). Similar interactions have been shown in the $p21^{ras}$ model, the only difference being that the asparagine residue of the G4 motif does not directly interact with the O-6 but stabilizes other residues of the G4 motif, which in turn are involved in hydrogen bonding with the base (2). The G5 motif of the GTP-binding domain has not yet been clearly identified in most GTPases. In $p21^{ras}$, the residues of this motif interact indirectly with the aspartate and asparagine of the G4 motif. It therefore appears that the overall effect of the G5 motif would be the stabilization of the GTP-G4 interactions (2).

Tetracycline inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome, thereby distorting the A site in such a way that the aminoacyl tRNA can no longer bind to the ribosome (21). Tet(O) is plasmid mediated in *Campylobacter jejuni* and *Campylobacter coli* (27), whereas Tet(M) is frequently chromosomally determined in *Streptococcus* spp. and is often present on conjugative transposons, e.g., Tn*916* (6, 17). The Tet(O) and Tet(M) mechanism of action involves protection of ribosomes such that inhibition by tetracycline does not occur (4, 25). Other mechanisms for Tc^r involve active efflux (16) and the modification of the antibiotic (22). The mechanism of action displayed by Tet(M) and Tet(O) is widely disseminated in the microbial world and is now thought to be the principal mechanism for Tc^r (17).

Burdett (5) has purified Tet(M) and demonstrated it to be a GTPase. Preliminary experiments with Tet(O) protein indicate that it also has GTPase activity (26). Because Tet(O) and Tet(M) possess the highly conserved sequences of the GTP-binding domain, it is likely that the putative GTP-binding motifs of these proteins would exhibit interactions similar to those predicted for EF-Tu and $p21^{ras}$. In this study, we investigated the effect on Tc^r of substituting Asn-128, a highly conserved residue in the G4 motif of the GTP-binding domain of Tet(O), with other amino acids.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial host used was *Escherichia coli* JM83, which was routinely grown in Luria-Bertani (LB) broth or LB agar at 37°C. Phagemid pUOA2E1 and plasmid pUOA2 (29) were the sources of the *tet(O)* gene.

Chemicals. Tetracycline, ampicillin, poly(U), pyruvate kinase, ATP, and GTP were purchased from Sigma Chemical Co. (St. Louis, Mo.); [7-^{3}H(N)]tetracycline (specific activity, 0.8 Ci/mmol) and L-[ring-2,6-^{3}H(N)]phenylalanine (specific activity, 49.7 Ci/mmol) were purchased from DuPont NEN Research Products (Boston, Mass.); phos-

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TABLE 1. Comparison of GTP-binding sequence motifs of EF-G, EF-Tu, and p21^{ras} with homologous regions in Tet(O) and Tet(M)

GTP-binding protein	Sequence of[a]:									
	Motif 1		Motif 2		Motif 3		Motif 4		Motif 5	
Consensus sequence	xooooGxxxxGKSx A T		D-(x)$_n$-T		ojooDxaGjx		ooooNKxD		ooooGSAK(x)$_4$ T L S A C	
EF-G	12	nigisAhidaGKTt	51	Dwmeqeqergi Ti	84	iniiDtpGhv	138	iafvNKmD	219	qnliGSAAease
EF-Tu	13	nvgtiGhvdhGKTt	50	Dnapeekargi Ti	76	yahvDcpGha	131	ivfiNKcD	168	pivrGSALkale
p21^{ras}	5	kivvvGaggvGKSa	30	DgydpTiedsyTi	53	idilDtaGqe	112	vlvgNKcD	140	pfieTSAKtrqr
Tet(O)	5	nlgilAhvdaGKTt	44	Dtmnlerqrgi Ti	70	vniiDtpGhm	124	ifftNKiD	215	pvyhGSAKnnlg
Tet(M)	5	nigblAhvdaGKTt	44	Dntllerqrgi Ti	70	vniiDtpGhm	124	iffiNKiD	214	pvyhGSAKnnig

[a] Symbols: x, any amino acid; j, hydrophilic amino acid; o, hydrophobic amino acid. Highly conserved residues are in uppercase. Other lowercase letters indicate amino acid identities used to align the sequences of various GTPases.

phoenolpyruvate, pyruvate kinase, and *E. coli* MRE600 tRNA were from Boehringer Mannheim (Indianapolis, Ind.). The mutagenic oligonucleotides were prepared by the Molecular Core Facility, Department of Biochemistry, Wayne State University, Detroit, Mich.

Plasmid purification. Plasmids were isolated by the method of Birnboim and Doly (1) and, when required, were further purified by cesium chloride-ethidium bromide density gradient centrifugation (18).

Bacterial transformation. *E. coli* JM83 was transformed by the calcium chloride procedure (18).

Site-directed mutagenesis. The *tet(O)* gene was mutagenized by the method of Kunkel (13) with the Muta-Gene phagemid kit (Bio-Rad Laboratories, Richmond, Calif.). The phagemid pUOA2E1 (ampicillin resistance [Ap^r], Tc^r) containing the wild-type *tet(O)* gene was used to transfect *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1* pCJ105 (Cm^r)] in order to produce uracil-containing single-stranded DNA. The synthetic oligonucleotides were prepared such that all three bases of the codon of interest were replaced. These oligonucleotides were prepared by sequential addition of a particular deoxynucleoside triphosphate (dNTP). The sequence of the oligonucleotides was 5′-GTTAAAAAAAGTAG**XXX** TTTTAACTGGTTC-3′. At the point of the codon of interest (marked **XXX**), all four nucleotides, at equal concentrations, were added to the reaction mixture for each of the three bases, the result being that 64 possible codon combinations could be obtained.

To obtain plasmids carrying the mutated *tet(O)* gene, the oligonucleotides were annealed to the template and the second strand was synthesized by incubation with the four dNTPs, in the presence of T4 DNA polymerase and T4 ligase at 37°C for 90 min. The double-stranded DNA obtained should be completely homologous except for the intended mutation. This double-stranded DNA has one strand containing uracil. Aliquots of the mutagenized plasmids were subsequently used to transform competent *E. coli* JM83 cells such that the uracil-containing strand was inactivated and the non-uracil-containing survivor was further replicated.

Colonies were selected on agar containing ampicillin (25 μg/ml) and then screened for the loss of Tc^r. DNA from Ap^r transformants was sequenced to identify the mutations obtained in the specified region.

DNA sequencing. DNA sequencing was performed according to the method of Sanger et al. (20) with the Sequenase version 2.0 Kit (United States Biochemical Corporation, Cleveland, Ohio).

MIC assay. MICs were determined in microtiter plates with tetracycline (0 to 256 μg/ml) and ampicillin (0 to 1,024 μg/ml) in twofold dilutions. Each antibiotic was assayed with four replicates.

Poly(U)-directed polyphenylalanine synthesis assays. S-100 fractions, capable of synthesizing polypeptides, were prepared from *E. coli* JM83 containing the wild-type plasmid pUOA2E1 and the plasmids mutagenized in the *tet(O)* region. Batches of 500 ml of LB broth containing 25 μg of ampicillin per ml were inoculated with 1 ml of an overnight culture of the desired clone. Cells were grown for 6 h at 37°C to log phase and then were harvested at 16,270 × *g* in a solution of 40 mM Tris-HCl containing 60 mM NH_4Cl, 15 mM $MgCl_2$, and 1.0 mM dithiothreitol (solution I). The pellet was ground to a fine powder under liquid nitrogen and resuspended in solution I. This suspension was centrifuged at 25,500 × *g* for 20 min at 5°C, and then the supernatant was aspirated and recentrifuged for 6 h at 100,000 × *g* to separate ribosomes from the S-100 fraction. This fraction was divided into aliquots and stored at −70°C until further use.

Poly(U)-directed polyphenylalanine synthesis was measured in 100-μl reaction volumes containing 20 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 15 mM $MgCl_2$, 3.0 mM ATP, 0.4 mM GTP, 10 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, 50 μg of tRNA, 100 μg of poly(U), 5 mM dithiothreitol, 100 pmol of *E. coli* JM83 ribosomes, 19 μg of the S-100 fraction, and 3 μl of [^{3}H]phenylalanine (49.7 Ci/mmol). The reaction was incubated at 37°C for 1 h and was stopped with cold 10% trichloroacetic acid. The tubes were placed in ice for 20 min and spun at 14,000 rpm in an Eppendorf 5415L centrifuge for 4 min, and then the resulting precipitates were washed twice with 1-ml aliquots of ice-cold 10% trichloroacetic acid. They were then dissolved in 200 μl of 0.1 N sodium hydroxide by incubation at 37°C for 30 min. Scintillation fluid was added to the reaction mixture, and the radioactivity was determined with a Beckman β-scintillation counter.

Ribosome binding studies. Ribosomes from Tc^r and Tc^s cells were prepared as previously described (28). The binding of [^{3}H]tetracycline to 70S ribosomes was performed according to the procedure of Buck and Cooperman (3). Briefly, 130 pmol of 70S ribosomes was incubated with various concentrations (0.3125 to 80 μM) of cold tetracycline spiked with [^{3}H]tetracycline at a ratio of 5:1 in 50 mM Tris-HCl (pH 7.6) reaction buffer containing 50 mM KCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 0.1 mM EDTA. The reaction was incubated for 15 min at 37°C. Ribosomes were collected on 0.22-μm-pore-size Millipore filters and washed four times with 5 ml of ice-cold reaction buffer. The

TABLE 2. Characterization of mutations at position 128 of Tet(O)

Amino acid substitution in JM83(pUOA2E1)	Codon substitution	Tetracycline MIC (μg/ml)[a]	% Inhibition[b]
E. coli JM83(pUOA2E1)	AAT	64	29
E. coli JM83		2–4	60
Asn→Lys	AAT→AAA	2	ND[c]
Asn→Tyr	AAT→TAC	2	ND
Asn→Ile	AAT→ATA	2	ND
Asn→Leu	AAT→TTA	2	61
Asn→His	AAT→CAC	4	51
Asn→Met	AAT→ATG	4	ND
Asn→Phe	AAT→TTC	4	ND
Asn→Pro	AAT→CCT	8	50
Asn→Thr	AAT→ACT	16	49
Asn→Ala	AAT→GCA	16	ND
Asn→Cys	AAT→TGC	16	ND

[a] All strains of *E. coli* JM83 harboring pUOA2E1 and mutant derivatives were also resistant to ampicillin (MIC, >1,024 μg/ml), whereas *E. coli* JM83, the plasmid-free strain, required a MIC of 2 to 4 μg/ml.

[b] Percentage of inhibition values were calculated as [(polyphenylalanine synthesized in absence of tetracycline − polyphenylalanine synthesized in presence of tetracycline)/polyphenylalanine synthesized in absence of tetracycline] × 100.

[c] ND, not determined.

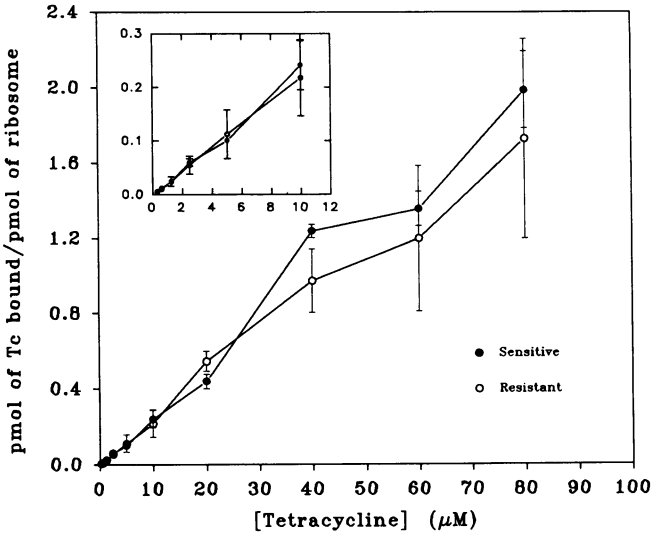

FIG. 1. [^{3}H]tetracycline binding to 70S ribosomes. Binding of [^{3}H]tetracycline to 70S ribosomes isolated from strains JM83 (●) and JM83(pUOA2E1) (○) was determined according to the procedure of Buck and Cooperman (3) as described in Materials and Methods. Each point represents the mean of two independent determinations. The inset shows an enlargement of the time period from 0 to 10 min.

filters were dried, and the radioactivity was measured. The background count of an identical reaction mixture without ribosomes was subtracted to obtain the net radioactivity bound to ribosomes.

RESULTS

Characterization of Tet(O) mutants. Site-directed mutagenesis of the *tet(O)* gene carried out on a pUOA2E1 plasmid resulted in a mixture of clones which were used to transform the host *E. coli* JM83. These transformants were selected on agar plates containing 25 μg of ampicillin per ml. The 95 colonies obtained were individually transferred to microtiter plates containing LB broth and 25 μg of ampicillin per ml, grown overnight, and then plated on 2, 4, and 8 μg of tetracycline per ml.

Determinations of tetracycline MICs were performed with the clones exhibiting little or no growth when screened for susceptibility to the antibiotic. A total of 50 clones requiring MICs lower than that for the wild type (64 μg/ml) were identified. Nearly half of these clones were highly susceptible to tetracycline, with a MIC of 2 μg/ml, indicating a major loss of Tc^r upon mutation of Asn-128 of the Tet(O) protein. The 50 clones were subsequently sequenced for identification of the substitutions at the codon corresponding to Asn-128. The DNA sequencing revealed 11 different amino acid substitutions at the Asn-128 position of Tet(O) (Table 2). As expected, a number of clones were found to have different triplets coding for the same amino acid, and the tetracycline MICs for these clones were identical.

Determination of the MIC for 11 clones (one representative of each amino acid substituted) was then repeated with tetracycline and ampicillin. The results showed that, compared with the wild type, all substitutions either abolished or significantly reduced Tc^r (Table 2). The MIC for the wild type was 4- to 32-fold higher than those for the 11 mutants.

Inability of the mutants to protect poly(U)-directed polyphenylalanine synthesis against the inhibitory action of tetracycline. To further elucidate the effect of mutations corresponding to the Asn-128 residue in Tet(O), the ability of the mutant protein to provide protection against tetracycline in poly(U)-directed polyphenylalanine synthesis was measured (Table 2). A clone representative requiring each MIC (i.e., 2, 4, 8, and 16 μg/ml) was used for the assay. The S-100 fractions from the Tc^s and Tc^r clones were included as controls. An additional control was the S-100 fraction from the clone, Asn-128→Asn, which essentially mimics the action produced by the resistant S-100. Overall, there was a good correlation between tetracycline MIC and the ability of the S-100 to protect poly(U)-directed polyphenylalanine synthesis against the inhibitory action of the antibiotic.

Ribosome binding studies. *E. coli* 70S ribosomes have a single high-affinity site and multiple low-affinity sites for tetracycline binding (9, 23). We have previously demonstrated (14) that ribosomes isolated from Tc^s and Tc^r cells bound almost identical amounts of the antibiotic. The tetracycline concentrations used were in the range of 20 to 100 μM. At such high concentrations of tetracycline, both the low- and high-affinity sites on the 70S ribosomes are occupied by antibiotic and any difference in binding of tetracycline to sensitive or resistant ribosomes at the high-affinity site is masked. In order to see a difference in the amounts of tetracycline bound to ribosomes isolated from Tc^s and Tc^r cells at the high-affinity binding site, lower concentrations of tetracycline in the range of 0.3125 to 80 μM were used. The tetracycline-binding curves (Fig. 1) for ribosomes isolated from Tc^s and Tc^r cells were essentially identical in that the amounts of antibiotic bound to the high- and the low-affinity sites of the ribosomes were the same.

DISCUSSION

The GTP-binding domain of EF-Tu (12) has been widely used as a reference model for elucidating structural and functional details of the respective domains in other GTPases. It

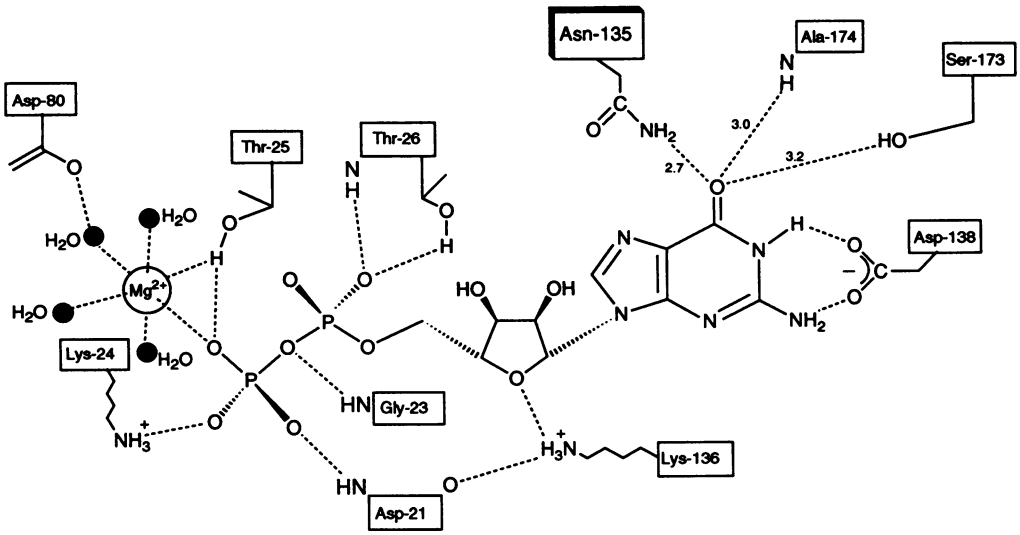

FIG. 2. Model of the EF-Tu-GDP complex illustrating the interactions between the GTP-binding domain of EF-Tu and the nucleotide. Asn-135 (bold box) is equivalent to Asn-128 in Tet(O). The dotted lines represent hydrogen bonds (adapted from reference 12).

has been demonstrated that there are five highly conserved motifs (G1 to G5, Table 1) in the GTP-binding domain which interact with the nucleotide. Similar motifs have also been identified in the Tc^r determinant Tet(O). Crystallographic studies of the EF-Tu-GDP complex indicate that the three motifs G1, G2, and G3 are involved in the binding of the nucleotide phosphate groups and a magnesium ion (Fig. 2). Similar interactions have been suggested for the GTPase p21^{ras}-Gpp(NH)p complex (2). Gpp(NH)p is an analog of GTP. Mutation of the highly conserved residues of these regions drastically affects the GTPase activity of EF-Tu and other GTPases. On the other hand, the G4 motif of the GTP-binding domain in EF-Tu interacts with the guanine moiety via hydrogen bonding (12). The two key residues of this domain are aspartate and asparagine. In both EF-Tu and p21^{ras}, the carboxylate oxygen atoms of aspartate accept two hydrogen bonds from the guanine N-1 and N-2. Moreover, it has been shown that mutation of Asp-138 to asparagine greatly reduces the GTPase activity of EF-Tu (10). The other key residue of the G4 motif is an asparagine which donates an H bond to the exocyclic O-6 of the guanine ring (Fig. 2). It has been suggested that this O-6 can also accept hydrogen bonds donated by other conserved residues, alanine and serine. In the present study, the substitution of Asn-128 belonging to the G4 motif of the putative GTP-binding domain of Tet(O) resulted in a loss of Tc^r conferred by the protein. Our working hypothesis is that there is a GTP-binding domain in Tet(O), and the residues of this domain are involved in interactions similar to those predicted for the EF-Tu-GDP complex. The observed changes in Tc^r of the mutants obtained seem to substantiate the assumption presented above and are discussed below with respect to alterations of the side chain properties of the substituted amino acids.

The mutants in which Asn-128 has been replaced by Lys, Leu, Ile, or Met have side chains with at least one more methylene group than Asn, whereas those with amino acid replacements of His, Tyr, and Phe have side chains with cyclic hydrocarbons. Such modifications of the side-chain lengths or introduction of bulky groups should most likely distort the geometry of the guanine-binding site and disturb the hydrogen-bonding pattern of O-6. The changes observed in the phenotypes of all of these mutants support the hypothesis concerning the significance of the conserved Asn residue in the G4 motif. Finally, the clones with Asn-128 substituted by Thr, Ala, and Cys required MICs only fourfold lower than that required by the wild type. The lack of a ring structure and the presence of shorter side chains most likely render these three amino acids sterically more favorable than the amino acid substitutions seen in mutants requiring a tetracycline MIC of 2 μg/ml. The more modest reduction in Tc^r observed in these three mutants is probably due to the lack of a stable H bond at the O-6 of the guanine moiety.

It should be mentioned that site-directed mutagenesis of the *tet(O)* gene was performed with a mixture of synthetic mutagenic oligonucleotides capable of producing 19 possible substitutions at position 128. By using this method, the mutagenesis procedure resulted in 11 substitutions at position 128 as listed in Table 2. A clone with the Asn-128→Asn substitution was also identified; it exhibited the same phenotype as the wild type when screened on tetracycline. Furthermore, the ampicillin MICs for both the mutants and the wild-type strain were >1,024 μg/ml, which indicates that the mutagenesis procedure did not affect the β-lactamase gene of the plasmid.

The loss in the ability of the mutant protein to protect the ribosomes from tetracycline was roughly paralleled by measurements of the extent of polyphenylalanine synthesized in the presence of the antibiotic. The representative clone Asn-128→Leu requiring a MIC of 2 μg/ml showed the highest degree of inhibition, namely, inhibition similar to that of the sensitive strain. This demonstrates that the mutant Tet(O) completely lost its ability to protect the ribosomes from the antibiotic. For reasons we are unable to explain, the same degree of inhibition, approximately 50%, was obtained for the representatives of the groups with tetracycline MICs between 4 and 16 μg/ml. Assuming that the mutations in Tet(O) affect the GTP-binding ability, the effect of higher concentrations of GTP in stimulating polyphenylalanine synthesis to normal levels was tested. Increasing GTP concentrations had no effect on the amount of

polyphenylalanine synthesized (results not shown). In fact, very high concentrations of GTP inhibited the overall poly(U)-directed polyphenylalanine synthesis even in the absence of tetracycline.

Tetracycline binds to the 30S subunit of ribosomes such that the charged tRNA can no longer bind to the A site (21). Ribosomes have two tetracycline-binding sites, a high-affinity (K_d, <20 μM) and multiple low-affinity (K_d = 20 to 100 μM) binding sites (9, 23). One possible mechanism of action of Tet(O) is that the resistance determinant modifies the tetracycline-binding site on the ribosomes such that the affinity of the ribosomes for tetracycline is decreased. The ribosome-tetracycline-binding curves for ribosomes prepared from Tc^s and Tc^r cells were essentially the same for the range of concentrations tested. Hence, the Tet(O) resistance function does not appear to be a result of the amount of antibiotic bound to the ribosomes.

The exact mechanism of action of the Tc^r determinant remains speculative. Nevertheless, site-directed random mutagenesis of Asn-128 of the putative GTP-binding domain combined with biological screening effectively shows that this conserved residue is essential for the Tc^r function. Hence, the above study further strengthens the prediction that Tet(O) like Tet(M) binds GTP and that this property may be necessary for conferring Tc^r.

ACKNOWLEDGMENTS

We thank Nora Obedeanu and P. Dutton for help in the preparation of the figures and the manuscript.

This research was supported by the Division of Infectious Diseases, Wayne State University, and by a grant from the National Sciences and Engineering Research Council of Canada to D.E.T., who is an Alberta Heritage Foundation for Medical Research Scientist.

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