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Molecular Studies on the Mechanism of Tetracycline Resistance Mediated by Tet(O)

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The mechanism of resistance to tetracycline in *Escherichia coli* mediated by the *Campylobacter jejuni*-derived resistance determinant Tet(O) was investigated. The cloned Tet(O) protein had no detectable effect on the intracellular accumulation of tetracycline. The presence of Tet(O) markedly diminished the inhibitory effect of tetracycline on protein synthesis both in vivo and in vitro. Ribosomes prepared from tetracycline-resistant and susceptible *E. coli* cells bound almost identical amounts of radiolabeled tetracycline. Thus, a reduction in the binding of the antibiotic to its target site on the ribosome is not the primary mechanism of resistance. Poly(U)-directed polyphenylalanine synthesis revealed that an S-100 fraction prepared from tetracycline-resistant cells made the ribosomes prepared from susceptible cells considerably more resistant to the inhibitory action of tetracycline. The N-terminal portion (1 to 150 residues) of Tet(O) is highly homologous to the GTP-binding domain of elongation factor Tu and to elongation factor G, indicating that the Tet(O) protein has the potential to bind GTP. These data suggest that the Tet(O) protein could function either as a tetracycline-resistant analog of this elongation factor(s) or by modifying the target sites on the ribosomes in a catalytic fashion.

Resistance to tetracycline (Tc^r) in *Campylobacter jejuni* is mediated by a self-transmissible plasmid (pUA466) of 45 kilobases (24, 26). To study the genetic basis of Tc^r in this organism, we previously cloned and expressed the resistance determinant [designated Tet(O)] in *Escherichia coli* (27). Nucleotide sequence analysis of the *tet*(O) gene revealed that it encodes a polypeptide with a calculated molecular mass of 72.3 kilodaltons (kDa) (12). By using an *E. coli* derived-in vitro transcription-translation system, we identified a polypeptide of 68 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12, 27). Amino acid sequence comparisons of Tet(O) with other known Tet proteins revealed that it is unrelated to the Tc^r determinants of the family *Enterobacteriaceae* but closely related (75% homology) to Tet(M) (13), originally isolated from the gram-positive *Streptococcus pneumoniae*. Sougakoff et al. (21) have characterized the Tet(O) from *Campylobacter coli*, and a comparison of the amino acid sequences of *C. jejuni* Tet(O) with those of *C. coli* Tet(O) revealed 98% homology (25). Recently, an almost identical determinant has been characterized from *Streptococcus mutans* (11).

Although the *tet*(O) gene from several organisms has been analyzed, very little is known about the mechanistic basis of Tet(O)-mediated resistance to tetracycline. Three types of Tc^r mechanisms have been reported so far. (i) In members of the family *Enterobacteriaceae*, the net accumulation of tetracycline in resistant cells is markedly reduced by an energy-dependent efflux mechanism mediated by the Tet protein of classes A through E (14). (ii) Unlike the Tet proteins of the family *Enterobacteriaceae*, the streptococcal Tc^r determinant Tet(M) specifies a different Tc^r mechanism by acting at the level of protein synthesis, possibly by protecting the ribosome (2). (iii) In contrast to the efflux and the ribosomal protective mechanisms by which the antibiotic is not destroyed, a recently described Tc^r mechanism specified by the cryptic class F determinant from *Bacteroides fragilis* involves inactivation of the drug (18, 22, 23).

The elucidation of the mechanism of Tet(O)-mediated resistance to tetracycline is dependent upon the correct understanding of the mode of action of tetracycline. Tetracycline binds to a single high-affinity site localized to the 30S subunit within the 70S ribosome ($K_d \sim 2$ μM), as well as to a large number of low-affinity sites. There is evidence that the major inhibitory effect of tetracycline on protein synthesis, the blocking of aminoacyl-tRNA binding to the A site, is a direct consequence of its binding to the high-affinity site. Earlier, we showed that the major protein photoaffinity labeled by [^{3}H]tetracycline in a site-specific manner is S7 (7). More recently, we have used single-protein omission reconstitution studies to demonstrate the strong dependence of tetracycline binding to 30S subunits on proteins S7, S14, and S19 (3). Related studies (30) show that these three proteins are part of a substructure, falling within the head region of the 30S particle, that also includes the 3′ major domain of 16S RNA, comprising bases 920 to 1396. Our results indicate that tetracycline binds within this rather large substructure and has a direct interaction with S7. This is consistent with the results of Moazed and Noller (15), showing evidence for tetracycline interaction with bases A-892, U-1052, and C-1054.

We have investigated the mechanism of Tet(O)-mediated resistance to tetracycline in *E. coli* using the cloned *tet*(O) gene. Here we report that the Tet(O) protein confers resistance to tetracycline by acting at the level of protein synthesis, possibly by the modification of ribosomal function in a catalytic fashion.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* K-12 (strain JM107; 31) and LBE 11001 *recA56* (E. Vijgenboom, University of Leiden, Leiden, The Netherlands; 29) were used. Unless otherwise

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specified, the cells were routinely grown in Luria broth medium. The plasmids pUOA1 and pUOA2 (27) were used as sources of the *tet*(O) gene. The plasmids were amplified in *E. coli* and isolated as previously described (26).

^{35}S labeling of cells. JM107 and JM107(pUOA2) cells were grown to the mid-log phase of growth, washed, and suspended in fresh growth medium at a cell density at an A_{600} of 0.25. Portions (0.5 ml) of the cell suspension were incubated at 37°C for 60 min with [^{35}S]methionine (20 μCi/ml). The radiolabeled cells were washed with ice-cold 10 mM Tris, pH 7.6, and lysed in cracking buffer (6) by boiling for 5 min. The polypeptides were separated by SDS-PAGE (10) and autoradiographed as previously described (1). The extent of [^{35}S]methionine incorporation into polypeptides was taken as a measure of in vivo protein synthesis.

Uptake of tetracycline. Tetracycline-susceptible (JM107) and Tc^r [JM107(pUOA2)] cells were grown in Luria broth medium, harvested by centrifugation, washed, and suspended in fresh medium (A_{600} = 0.25). One-milliliter portions of the cell suspension were incubated with 10 μM [^{3}H]tetracycline at 37°C. At various intervals, duplicate 50-μl portions were removed. The cells were collected on membrane filters (HAWP; Millipore Corp.; 0.45-μm pore size) and washed four times (5 ml each) with ice-cold 10 mM Tris, pH 7.6. The filters were dried for 30 min at 65°C, and the radioactivity associated with them was determined by scintillation counting. Samples without the cells were treated identically, and portions were removed at various intervals to determine the background. To reduce the background count, the filters were prewashed with 1 ml of 1 mM cold tetracycline. This prewashing markedly reduced the nonspecific binding of labeled tetracycline to the filters.

Poly(U)-directed polyphenylalanine synthesis. S-30 fractions capable of synthesizing polypeptides were prepared from JM107 and JM107(pUOA2) cells as previously described (5). Poly(U)-directed polyphenylalanine synthesis was done essentially as described by Ohnuki et al. (17). Typically, 5 μl of an S-30 fraction (5 mg of protein per ml) was incubated in a 50-μl reaction mixture containing 50 mM Tris hydrochloride (pH 7.8), 60 mM ammonium chloride, 7.5 mM magnesium acetate, 1 mM ATP, 5 mM phosphoenolpyruvate (pH 7.0), 0.4 mM spermidine, 25 μM GTP, 20 μM [^{14}C]phenylalanine, 1.5 μg of pyruvate kinase, and 5 μg of poly(U) in the presence of various concentrations of tetracycline at 37°C for 60 min. Acid-insoluble materials were precipitated with 10% trichloroacetic acid, collected on Millipore filters (HAWP; 0.45-μm pore size), and washed with 5% ice-cold trichloroacetic acid (four times, 5 ml each). The radioactivity associated with the materials retained on the filters was determined by scintillation counting. The reaction mixture without poly(U) was treated identically to determine the background. The background value was subtracted to obtain radioactivity associated with polyphenylalanine.

Tetracycline binding studies. Ribosomes from Tc^r and tetracycline-susceptible cells were prepared as previously described (28). The binding of [^{3}H]tetracycline to 70S ribosomes was done as described elsewhere (M. Buck, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1988). Briefly, 130 pmol of 70S ribosomes was incubated in 70 μl of reaction buffer (50 mM Tris hydrochloride, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 0.1 mM EDTA) containing various concentrations (20 to 100 μM) of [^{3}H]tetracycline for 15 min at 37°C. The ribosomes were collected on Millipore filters (HAWP; 0.22-μm pore size) and washed four times (5 ml each) with ice-cold reaction buffer. The washed filters were dried for 30 min at 65°C, and the radioactivity associated with the filters was determined. Reaction mixtures without ribosomes were treated identically, collected on filters, and washed, and the radioactivity associated with the filters was determined for each concentration of [^{3}H]tetracycline used to obtain the background count.

Effect of kirromycin. *E. coli* LBE 11001 and LBE 11001 (pUOA1) were grown at 37°C in Penassay broth (Difco antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.) for 6 h. Cells were then diluted in 0.05 M sodium phosphate and plated on Davis minimal agar containing 1% glucose, 0.5% Casamino Acids (Difco) and 1 mM EDTA (to make cells permeable to kirromycin), and plates were incubated for 18 h at 37°C before being scored for growth.

Amino acid sequence homology analysis. The amino acid sequence homology of Tet(O) to other GTP-binding proteins was analyzed by the Bionet Computer Program as described by Needleman and Wunsch (16). The initial search was done by matching the complete amino acid sequence of Tet(O) with those of the proteins in the data bank. The protein sequences that exhibited 20% or more overall homology were selected and compared with the N-terminal portion (residues 1 to 150) of Tet(O).

RP-HPLC analysis. Total protein derived from the 70S ribosomes (TP70) prepared from Tc^r and tetracycline-susceptible cells were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) according to the procedure of Cooperman et al. (4).

Chemicals and reagents. L-[^{35}S]methionine (1,117 Ci/mmol), L-[U-^{14}C]phenylalanine (512 mCi/mmol), and [7-^{3}H, N]tetracycline (0.6 or 0.5 Ci/mmol) were obtained from Dupont, NEN Research Products of Canada, Montreal, Quebec. Radiolabeled tetracycline was dissolved in 95% ethanol and stored at −70°C covered with aluminium foil to reduce photolysis. Kirromycin was a kind gift from N. Brot (Roche Institute, Nutley, N.J.). Other chemicals used were obtained from either Sigma Chemical Co. (St. Louis, Mo.) or Boehringer Mannheim of Canada, Montreal, Quebec.

RESULTS

Tet(O) acts at the level of protein synthesis. To study the effect of Tet(O) on tetracycline transport, the accumulation of radiolabeled tetracycline in tetracycline-susceptible and Tc^r cells was measured. Both JM107 and JM107(pUOA2) accumulated approximately similar amounts of [^{3}H]tetracycline over a period of 90 min (Fig. 1A). However, identical experiments done in JM107(pBR322) containing the *tet*(C) gene showed a markedly different profile (Fig. 1B).

Both in vivo and in vitro, protein synthesis was resistant to the inhibitory action of tetracycline in the presence of Tet(O). For example, ^{35}S-labeling experiments with JM107 and JM107(pUOA2) cells revealed that 56 μM tetracycline almost completely inhibited the incorporation of [^{35}S]methionine into cellular proteins in JM107 but not in JM107 (pUOA2). A fourfold increase in the concentration of the antibiotic was required for significant reduction of protein synthesis in the Tc^r cells (Fig. 2). Similarly, 56 μM tetracycline inhibited poly(U)-directed polyphenylalanine synthesis in an S-30 fraction prepared from tetracycline-susceptible cells by 75%, whereas 450 μM tetracycline was required to obtain the same level of inhibition in the Tc^r group (Fig. 3). These data indicate that the key to the mechanistic basis of Tet(O)-mediated resistance to tetracycline is its ability to act at the level of protein synthesis.

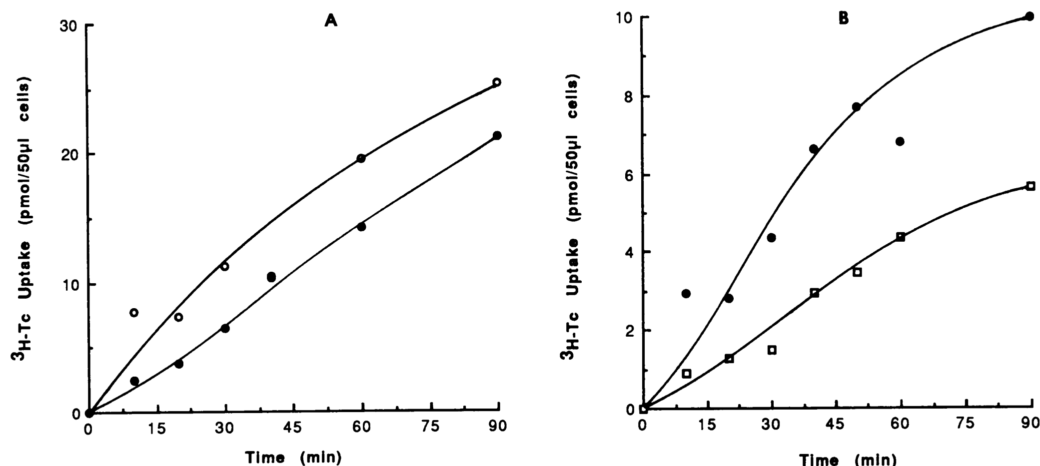

FIG. 1. Comparison of the uptake of [^{3}H]tetracycline in JM107(pUOA2) (●) with that in JM107 (○) (A) and with that in JM107(pBR322) (□) (B). The cultures were grown until they reached mid-log phase, washed, and suspended in fresh growth medium. The accumulation of [^{3}H]tetracycline for various intervals was measured as described in Materials and Methods. The plasmid pBR322 was used as the source of the *tet*(C) gene, whose product, Tet(C) protein, is involved in the active efflux of tetracycline accumulated in the cells. Each point represents the mean value of two independent determinations.

Ribosome binding studies with [^{3}H]tetracycline. Since the binding of tetracycline to the 30S subunit appeared to be a prerequisite for its inhibitory action, we examined the binding of [^{3}H]tetracycline to tetracycline-sensitive and Tc^r ribosomes. Ribosomes isolated from tetracycline-susceptible and Tc^r cells bound an almost identical amount of [^{3}H]tetracycline, indicating that interference of the binding of tetracycline to its target site on the 30S subunit is not the primary mechanism by which Tet(O) mediates resistance to tetracycline (Fig. 4).

Homology of Tet(O) to GTP-binding proteins. The amino acid sequence of Tet(O) was compared with the known sequences of various proteins of the protein synthetic machinery. No ribosomal subunit proteins showed significant homology to Tet(O). The N-terminal portion (residues 1 to 150) of the Tet(O) protein is highly homologous to N-terminal regions of several GTP-binding proteins involved in protein synthesis in particular elongation factors Tu and G (32 and 44% homology, respectively) (Fig. 5). The regions of Ef-Tu that showed similarity to comparable regions of Tet(O) are known to be involved in the binding of GTP/GDP as revealed by X-ray crystallographic studies (9).

Effect of kirromycin. Kirromycin is a specific inhibitor of Ef-Tu (19). We reasoned that if the mode of action of Tet(O) is to replace Ef-Tu, these cells, which are normally susceptible to kirromycin, might become resistant to kirromycin if they contain *tet*(O). Van der Meide et al. (29) demonstrated that *E. coli* LBE 11001 (the parental strain) is inhibited by concentrations of less than 20 µg of kirromycin per ml. We obtained similar results (Table 1) and found that the presence of *tet*(O) carried on pUOA1 did not affect killing of the strain by kirromycin.

Presence of Tet(O) in an S-100 fraction. The hydrophilicity profile analysis of the Tet(O) protein (12) indicated that it is moderately hydrophilic and could be associated with the soluble fraction. To further examine this possibility, we determined the ability of an S-100 fraction prepared from Tc^r cells to make sensitive ribosomes resistant to the inhibitory action of tetracycline on poly(U)-directed polyphenylalanine synthesis. Tetracycline at a concentration of 112 µM inhibited poly(U)-directed polyphenylalanine synthesis by approximately 82% when tetracycline-sensitive ribosomes and a tetracycline-sensitive S-100 fraction were used (Table 2). A similar experiment, in which the components were from Tc^r cells, provided only 23% inhibition. The inhibitory effect of tetracycline was almost completely reversed when a combination of tetracycline-sensitive ribosomes and a Tc^r S-100 fraction was used. However, the Tc^r ribosomes with a tetracycline-sensitive S-100 fraction also provided similar results.

The pretreatment of tetracycline-sensitive ribosomes with S-100 fractions prepared from Tc^r cells failed to protect the ribosomes from the inhibitory action of tetracycline (Table 3), indicating that the continued presence of Tet(O) is required for the protection.

RP-HPLC analysis. RP-HPLC has been successfully used to analyze individual proteins of the 70S ribosome (4). With the use of the appropriate column and solvents, the majority of the ribosomal proteins were well resolved, and the presence of any additional protein(s) associated with the ribosomes can be easily detected. By this technique, we examined the possible association of Tet(O) with Tc^r ribosomes. Our results reveal identical RP-HPLC profiles for Tc^r and tetracycline-sensitive ribosomal proteins (Fig. 6), suggesting that no detectable amount of Tet(O) protein is associated with Tc^r ribosomes.

DISCUSSION

The mechanism of Tet(O)-mediated resistance to tetracycline does not depend on the active efflux of tetracycline from resistant cells. Our results confirm and extend those of Burdett (2), who used the Tet(M) system in *Streptococcus* spp. While, in principle, Tet(O)-mediated resistance to tetracycline could depend on its ability to sequester it, our results suggested that this is not the case. In the absence of purified Tet(O) protein, we used an S-100 fraction prepared from Tc^r cells as the source of Tet(O) for binding studies. S-100 fractions obtained from the Tc^r and tetracycline-susceptible cells failed to bind [^{3}H]tetracycline above the background levels associated with filters, indicating that neither the Tet(O) protein nor any other component of the S-100 fraction is able to bind a detectable amount of [^{3}H]tetracycline. Our experiments, as well as work by Bur-

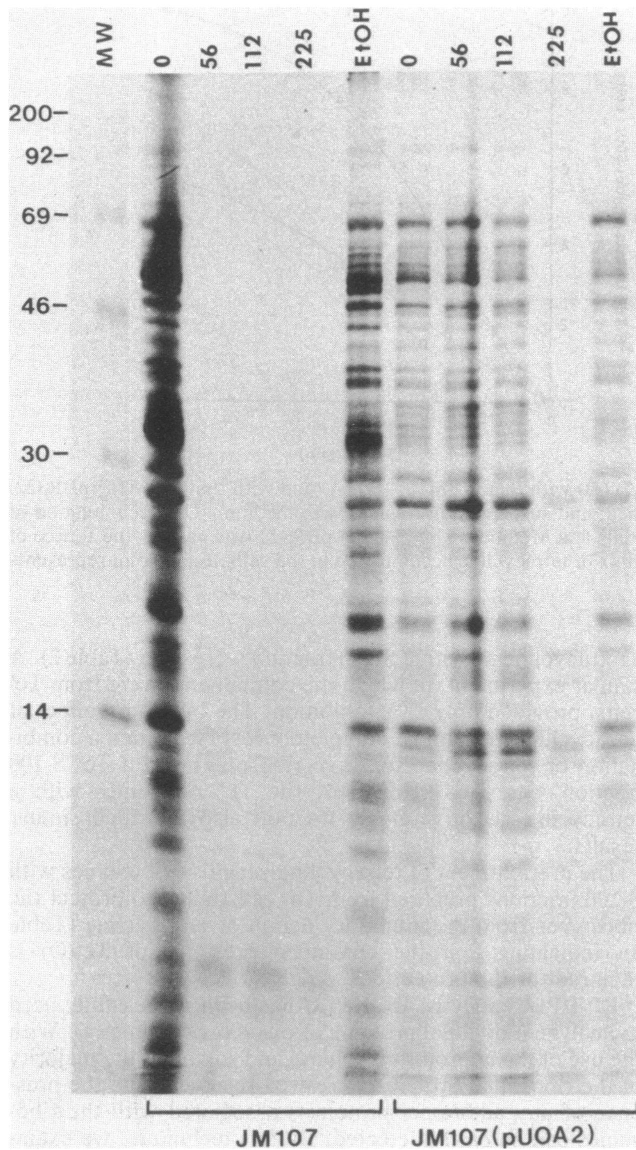

FIG. 2. Effect of Tet(O) on the inhibitory action of tetracycline on in vivo protein synthesis. Tetracycline-susceptible (JM107) and Tc^r JM107(pUOA2) cells were radiolabeled with [^{35}S]methionine in the presence of various concentrations (0 to 225 μM) of tetracycline, washed, and lysed as described in Materials and Methods. The polypeptides were separated by SDS-PAGE and autoradiographed. The extent of [^{35}S]methionine incorporation into the polypeptides was taken as a measure of in vivo protein synthesis. Since ethanol was used as the solvent for [^{3}H]tetracycline, 1% (vol/vol) ethanol (comparable to the highest concentration used in the experiment) was used as the control (EtOH). Lanes marked 0, 56, 112, and 225 represent the different concentrations (micromolar) of tetracycline used. ^{14}C-labeled myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa) (Amersham Canada) were used as molecular size standards (MW).

dett (2) with the closely related Tet(M) protein, have demonstrated that these proteins act primarily at the level of protein synthesis in conferring resistance to tetracycline.

Although the site of action of Tet(O) is at the level of protein synthesis, the actual mechanism by which protein synthesis is made resistant to tetracycline action is not yet understood. Sanchez-Pescador et al. (20) have postulated that either (i) Tet(O) is stoichiometrically associated with the ribosomes affecting the binding of tetracycline to the 30S subunit without interfering with the binding of aminoacyl-tRNA to the A site or (ii) since Tet(O) has a significant amount of regional homology to Ef-Tu and Ef-G, it is possible that Tet(O) could mimic the function of these proteins. In addition, we postulate that Tet(O) may have catalytic function which modifies some key component(s) of the protein synthetic machinery essential for the inhibitory action of tetracycline without affecting protein synthesis.

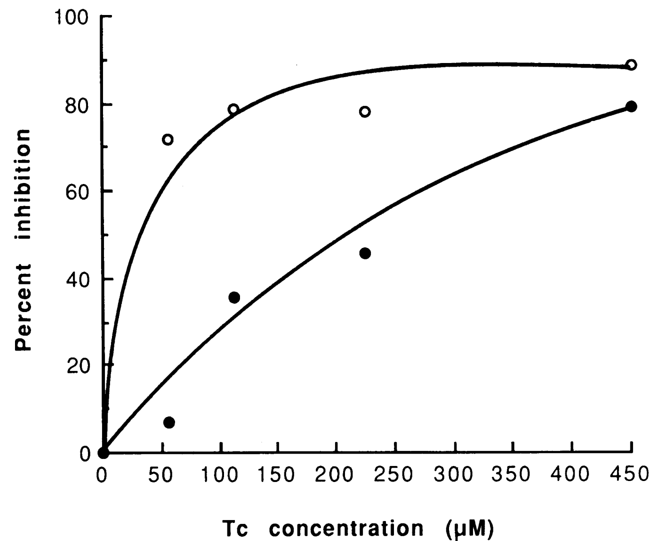

FIG. 3. Effect of Tet(O) on the inhibitory action of tetracycline on polypeptide synthesis. Poly(U)-directed polyphenylalanine synthesis by S-30 fractions prepared from JM107 (○) and JM107(pUOA2) (●) was measured in the presence of various concentrations of tetracycline (0 to 450 μM) as described in Materials and Methods. Each point represents the mean value of two independent determinations.

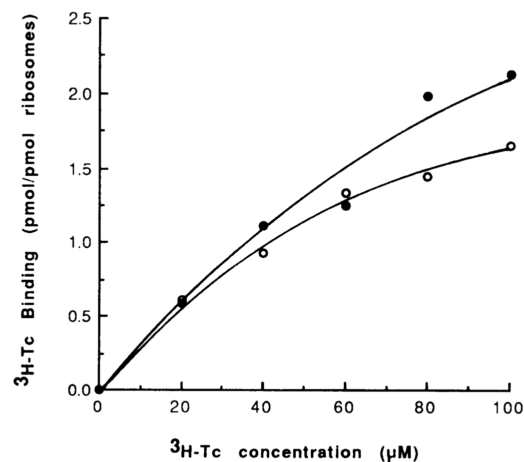

FIG. 4. [^{3}H]tetracycline binding to 70S ribosomes. Binding of [^{3}H]tetracycline to 70S ribosomes isolated from JM107 (○) and JM107(pUOA2) (●) was determined according to the procedure of Buck (Ph.D. thesis) as described in Materials and Methods. Each point represents the mean of two independent determinations. The experiment was repeated twice, and identical results were obtained.

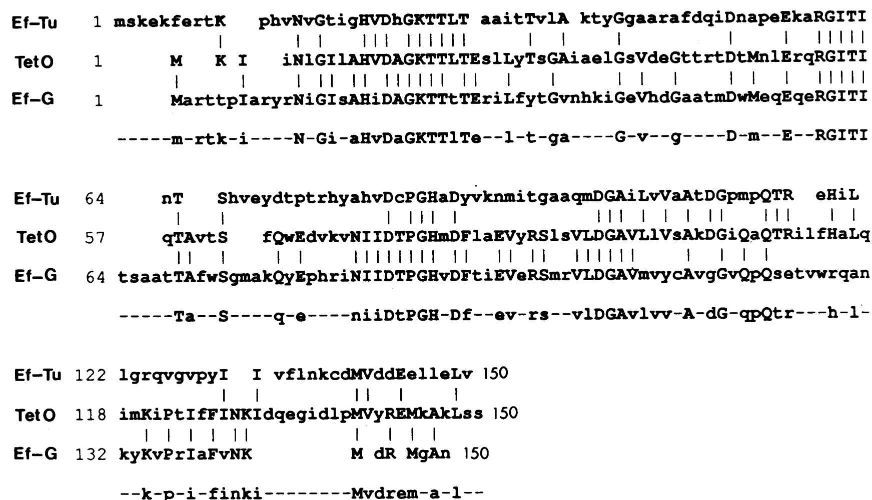

FIG. 5. Amino acid sequence homology of Tet(O) to elongation factors Tu (Ef-Tu) and G (Ef-G). Amino acid residues 1 to 150, as deduced from the nucleotide sequence of the *tet*(O) gene (12), were aligned with those corresponding to the homologous regions of Ef-Tu and Ef-G by using the Genalign program of Bionet as previously described. Uppercase and lowercase letters represent identical and nonidentical residues, respectively. The consensus sequence for the three proteins is shown at the bottom.

Our binding studies demonstrated that [^{3}H]tetracycline binds sensitive and resistant ribosomes to the same extent. Since there is no reduction in the binding of Tc^r cells, compared with that of tetracycline-susceptible cells, it seems unlikely that Tet(O) inhibits tetracycline binding to ribosomes. Analysis of the ribosomal proteins from tetracycline-sensitive and Tc^r ribosomes with RP-HPLC showed identical profiles. Similarly, in an analogous experiment in which the 70S ribosomal proteins were analyzed by SDS-PAGE, no additional protein band representing the 72.3-kDa Tet(O) protein was seen in the Tc^r ribosomes (E. K. Manavathu and D. E. Taylor, unpublished data). Failure to see such a protein would suggest that Tet(O) is not stoichiometrically associated with the ribosomes. However, these experiments cannot rule out the possibility that Tet(O) may be associated with the ribosomes in very small amounts undetectable by the methods we used for the analysis.

The suggestion that Tet(O) could be mimicking Ef-Tu or Ef-G is an attractive one by virtue of the fact that Tet(O) has a high degree of homology to the nucleotide (GTP)-binding regions of these proteins. However, the following points are worth noting: (i) as Ef-Tu is one of the most predominant (5 to 10% of the total) proteins in *E. coli*, it is unlikely that Tet(O) produced in a very small amount could effectively replace Ef-Tu; (ii) Tet(O) was unable to confer resistance to kirromycin, a specific inhibitor of Ef-Tu in vivo; (iii) Ef-G showed the highest degree of resemblance to Tet(O), and the critical Gly residue essential for Ef-Tu function (8) is an Ala in Ef-G and Tet(O); (iv) the molecular masses of Ef-G and Tet(O) are very similar (80 and 72.3 kDa, respectively); and (v) only one molecule of Ef-G per ribosome is found in *E. coli*. Together, these data suggests that if Tet(O) mimics any GTP-binding components of the protein synthetic machinery, it is more likely to be Ef-G.

Whether Tet(O) is acting as a GTP-binding component of the protein synthetic machinery or not, it is likely to be functioning in a catalytic rather than a stoichiometric fashion. Three pieces of evidence are consistent with this notion. (i) S-100 fractions prepared from the Tc^r cells were able to convert tetracycline-sensitive ribosomes to Tc^r during protein biosynthesis as measured by the synthesis of poly(U)-directed polyphenylalanine. (ii) The Tc^r ribosomes were resistant to the inhibitory action of tetracycline even in the presence of a Tc^s S-100 fraction. (iii) Only a small amount of Tet(O) protein is required to render ribosomes Tc^r. RP-HPLC and SDS-PAGE analyses failed to detect the presence of Tet(O) either associated with the ribosomes or in the S-100 fraction, although the same

TABLE 1. Effect of kirromycin on the growth of Tc^r *E. coli* LBE 11001 cells

E. coli strain	Cell concn	Growth in the presence of kirromycin at indicated concn (μg/ml)[a]		
		0	10	20
LBE 11001	2.5×10^6	++	+	−
	2.5×10^4	+	−	−
	2.5×10^2	−	−	−
LBE 11001(pUOA1)	2.5×10^6	++	±	−
	2.5×10^4	+	−	−
	2.5×10^2	−	−	−

[a] Symbols: ++, good growth; +, moderate growth; ±, slight growth; −, no growth.

TABLE 2. Effect of the addition of S-100 fraction containing Tet(O) on the inhibitory action of tetracycline as measured by poly(U)-directed polyphenylalanine synthesis

Ribosome–S-100 fraction combination	Mean % poly(U)-directed polyphenylalanine synthesis ± SEM[a]
Tc^s ribosomes + Tc^s S-100	18.2 ± 6.5
Tc^s ribosomes + Tc^r S-100	94.6 ± 16.6
Tc^r ribosomes + Tc^s S-100	88.9 ± 15.4
Tc^r ribosomes + Tc^r S-100	76.8 ± 3.1

[a] Poly(U)-directed polyphenylalanine synthesis was measured as described in Materials and Methods. Each value represents the mean of two independent determinations. Since each ribosome–S-100 combination could have a different rate of polyphenylalanine synthesis, a corresponding combination without the addition of tetracycline (112 μm) was taken as the control. Percent control values were calculated as follows: (polyphenylalanine synthesized in the presence of tetracycline/polyphenylalanine synthesized in the absence of tetracycline) × 100.

TABLE 3. Effect of pretreatment of Tc^s ribosomes with an S-100 fraction containing Tet(O) on the inhibitory action of tetracycline on poly(U)-directed polyphenylalanine synthesis[a]

Pretreatment	Mean cpm in poly(U)-directed polyphenylalanine synthesis ± SEM[b]
None	2,526 ± 603
Buffer	2,990 ± 359
Tc^s S-100	2,525 ± 883
Tc^r S-100	3,349 ± 284

[a] Tc^s ribosomes (100 U at A_{260}) were preincubated with an S-100 fraction (total volume, 200 μl) for 30 min at 37°C, washed by centrifugation, and suspended. The pretreated ribosomes were combined with a Tc^s S-100 fraction, and poly(U)-directed polyphenylalanine synthesis was measured in the presence of tetracycline (112 μM) as described in Materials and Methods.

[b] Each value represents the mean of two independent determinations.

preparations were resistant to the inhibitory action of tetracycline on poly(U)-directed polyphenylalanine synthesis; also, our attempts to obtain overproduction of the Tet(O) protein by using three different procaryotic expression vectors (*tac*, λP_L, and T7 promoters) into which the Tet(O) determinant had been cloned failed to yield amounts of Tet(O) protein detectable by Coomassie blue staining despite the presence of the Tc^r phenotype. Although it is quite likely that Tet(O) is acting as a catalyst, the exact function remains speculative. It is possible that Tet(O) could modify the ribosomal protein(s) or rRNA such that the inhibitory effect of tetracycline is diminished but protein synthesis is continued. The type of modifications one could envision are phosphorylation or acetylation of protein(s) or methylation of proteins or RNA. Such subtle changes, while possibly crucial for biological function, may not be detected in RP-HPLC or SDS-PAGE. Experiments are under way to examine whether such changes take place in the presence of the Tet(O) protein.

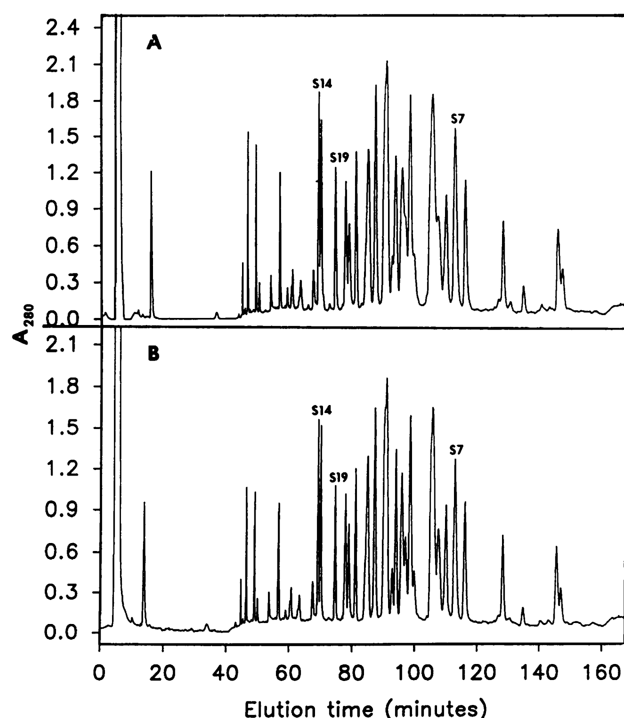

FIG. 6. RP-HPLC separations of total protein (TP70) from Tc^r (A) and Tc^s (B) ribosomes. Samples were injected onto a SynChropak RP-P column. The solvents used were 0.1% (wt/vol) CF_3COOH in water and 0.1% (wt/vol) CF_3COOH in acetonitrile. TP70 (661 μg in panel A; 622 μg in panel B) was eluted with a convex gradient of 15 to 45% of 0.1% (wt/vol) CF_3COOH in acetonitrile in 160 min. Peaks corresponding to the ribosomal proteins (S7, S14, and S19) involved in the binding of tetracycline to the 30S subunit are indicated.

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