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Intracistronic Complementation of the Tetracycline Resistance Membrane Protein of Tn*10*

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The structural gene region for tetracycline resistance on Tn*10* consists of two complementation groups, *tetA* and *tetB* (M. S. Curiale and S. B. Levy, J. Bacteriol. **151**:209–215, 1982). Using a series of deletion mutants, we have determined that the *tetA* region is 450 to 600 base pairs long and that the *tetB* region, which is adjacent to *tetA*, is 600 to 750 base pairs long. Point mutations in either *tetA* or *tetB* affected the amount and size of the inducible inner-membrane Tet protein synthesized in *Escherichia coli* maxicells. Moreover, deletions in these regions led to the synthesis of an appropriately smaller Tet protein. A single tetracycline-inducible RNA of about 1,200 bases was detected that was homologous with the tetracycline resistance structural gene region. These results indicate that the *tetA* and *tetB* complementation regions represent two parts of a single gene encoding two domains of the tetracycline resistance protein Tet.

Plasmid-mediated tetracycline resistance similar to that determined by transposon Tn*10* is frequently encountered in nature (20, 30). Five determinant classes (A through E) in *Enterobacteriaceae* have been defined by DNA homology and levels of resistance to tetracycline and tetracycline analogs (25a, 30). Expression of tetracycline resistance from Tn*10* (class B) is negatively regulated by a repressor (38, 39) which also regulates itself (2, 38). Genetic organization of the Tn*10* determinant consists of separate regulatory and structural regions which are transcribed from overlapping promoters in opposite directions (38). Transcriptional analysis of class C determinants (34) and DNA sequence analysis of class A determinants (37) indicate a similar organization.

Using point and deletion mutations which cause tetracycline susceptibility, we have demonstrated two complementation groups, *tetA* and *tetB*, which make up the structural resistance region of Tn*10* (9). Similar findings have been recently reported by others using different mutants (7). Although both complementation groups are essential for the expression of resistance, complementation does not restore full resistance. Instead, resistance levels are typically 2 to 25% of wild-type resistance. However, they are 2- to 80-fold greater than the susceptibility level of the mutants alone. The order of the complementation groups of the resistance region, the *tet* operon, is promoter-*tetA*-*tetB* (9).

Tetracycline-inducible proteins of 25,000 and 36,000 daltons are synthesized by Tn*10* (38, 39). The 25,000-dalton protein is the product of the repressor gene (12, 14, 38). The 36,000-dalton Tet protein is required for resistance (21, 38), and its carboxyl end has been physically mapped to the promoter-distal end of the structural gene region (17, 38). The position of the gene corresponds to the region of the *tet* operon defined by the *tetB* mutations (7, 9). The recently reported nucleotide sequence of the Tn*10* *tet* operon defines a single open reading frame of 1,203 bases specifying a translation product of 43,300 daltons (13).

A single protein was not predicted from our finding that all point mutants fell within one or the other complementation group (9). In this communication, we provide genetic and biochemical data which support the model of a single structural gene product for tetracycline resistance which contains two genetic domains corresponding to the complementation groups described.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* HB101 *leu pro hsdM hsdR recA rpsL lacY* (3) was used throughout this study. Tetracycline susceptibility mutations *tetA1*, *tetA8*, and *tetB2* through *tetB7* were previously described (9) and occur on compatible multicopy plasmids pRT11 (17) and pLR1069 (9). These plasmids contain identical copies of the tetracycline resistance genes on a 2,700 base-pair (bp) *Hpa*I fragment derived from the 9,300-bp transposon Tn*10*. pLR1068 is similar to pLR1069, but its 2,700-bp *Hpa*I fragment is inserted in the opposite orientation. Plasmid R222 (R100) was introduced into strain HB101 by conjugation with D1-7 (19).

Complementation tests and MIC determinations. Cells were grown, induced with autoclaved chlortetracycline (50 μg/ml), and applied to gradient plates containing tetracycline (9). The minimal inhibitory concentration (MIC) was recorded as that level of drug which prevented confluent growth after 48 h at 37°C.

Uptake of [^{3}H]tetracycline. Tetracycline uptake was measured on cells grown in medium A (29) containing 0.5% Casamino Acids, 0.5% glycerol, 100 μg of proline per ml, 40 μg of leucine per ml, 1 μg of thiamine per ml, and 50 μg of autoclaved chlortetracycline per ml. Cells were harvested when they reached an absorbance at 530 nm of 0.8, washed twice with assay buffer (50 mM potassium phosphate, pH 6.1, and 1 mM $MgSO_4$), and suspended to an absorbance at 530 nm of 3.0 in assay buffer supplemented with 20 mM lithium lactate (26, 28). The filtration method of assay (28) was used to measure uptake. The external [^{3}H]tetracycline concentration was 1.0 to 1.6 μM (769 Ci/mol).

Endonuclease- and exonuclease-generated deletions in the *tet* operon. Methods for strain cultivation, DNA purification, and transformation were previously described (9). Enzymes were used according to the recommendations of the manufacturers.

pLR1068 plasmid DNA was purified by CsCl centrifugation with ethidium bromide and was cleaved with either *Bgl*I or *Hpa*I restriction endonucleases. Linear plasmid DNA molecules were partially digested with the exonuclease *Bal* 31 (0.5 U of *Bal* 31 per 5 μg of DNA in 100 μl at 25 or 37°C for 1 to 5 min). The reaction was stopped by agitation with 1 volume of phenol (equilibrated with 0.1 M Tris-hydrochloride [pH 8.0]–0.2% β-mercaptoethanol–0.1% 8-hydroxyquinoline [24]), which extracted the proteins. The treated fragments were circularized with T4 DNA ligase before transformation of competent HB101 cells. Chloramphenicol-resistant (25 μg/ml) transformants were selected and examined for tetracycline susceptibility (5 μg/ml) by replica plating. The extent of each deletion was determined in relation to the restriction enzyme sites for *Xba*I, *Hin*cII, *Hae*II, *Hae*III, *Eco*RI, *Bgl*I, *Hpa*I, and *Hin*dIII by using either 1% agarose gels (9) or 7% polyacrylamide gels (25).

Deletions generated in the tetracycline genes of pLR1068 were cloned into pRT11 by substitution of the *Xba*I-*Hin*dIII fragment of the mutant pLR1068 plasmid for the wild-type fragment in pRT11. Colicin E1-resistant transformants were selected, and tetracycline susceptibility was determined (9). The plasmid size was then analyzed by agarose gel electrophoresis to confirm the presence of the mutant fragment.

The 297-bp *Sau*3A fragment in the *tetA* complementation region was removed by partial digestion of pLR1068 DNA (2 to 5 μg) with *Sau*3A (0.5 U in a reaction volume of 25 μl for 1 min at 25°C). The DNA molecules were circularized with T4 DNA ligase and were used to transform HB101 cells. Chloramphenicol-resistant, tetracycline-susceptible transformants were selected and pooled into six sets of 10 transformants, and the plasmid DNA was purified from each set. The plasmid DNA was used to transform strain HB101 containing the *tetB2* mutant plasmid pLR1053; chloramphenicol-resistant (25 μg/ml), tetracycline-resistant (5 μg/ml) transformants were recovered from one of the six sets. Plasmid DNA was prepared from each of the 10 clones in the positive set to identify the 1 with the mutation (*tetA* deletion) that was complemented and used to transform strain HB101(pLR1053); 1 of the 10 clones produced tetracycline-resistant transformants. The parental clone was named LR1200, and its plasmid was named pLR1200. The absence of the 297-bp fragment from pLR1200 was verified by gel electrophoresis.

Plasmid-directed protein synthesis in maxicells and minicells. Maxicells (33) were prepared from plasmid-containing HB101 cells by irradiation with UV light for 30 s. Induction of the *tet* operon was effected with autoclaved chlortetracycline (50 μg/ml) and commenced with the cycloserine treatment after UV irradiation (16 h before labeling) or with the initiation of sulfur starvation 1 h before labeling. Both induction periods gave identical results. Minicells from strain χ984 were labeled as described (19) without penicillin treatment.

SDS-polyacrylamide gel electrophoresis. Acrylamide gels (120 by 135 by 0.8 mm) were prepared with a stacking gel of 3.9% acrylamide–125 mM Tris-hydrochloride (pH 6.8)–0.1% sodium dodecyl sulfate (SDS) and a separating gel of 12 or 14% acrylamide–380 mM Tris-hydrochloride (pH 8.8)–0.2% SDS. In each gel layer, the acrylamide-to-bisacrylamide ratio was 55:1. The electrode buffer was 50 mM Tris base–380 mM glycine–0.1% SDS. Approximately 10 to 15 μl of sample was used for each gel lane. Electrophoresis was performed for 1 h at 50 V and then for 3 h at 90 V. The gels were stained and destained as previously described (10). Autoradiographic exposures were on Kodak XAR-5 film.

Purification of RNA. RNA was purified from 20-ml HB101 cell cultures grown in L broth with 0.5% glucose to mid-log phase (absorbance at 530 nm, 0.4 to 0.5). Each culture was quickly chilled by adding 5 g of crushed ice, and the cells were harvested by centrifugation at 4°C. At all steps, all solutions and apparatus were at 4°C unless specified otherwise. The cell pellet was washed with 10 ml of Tris-hydrochloride (pH 7.6)–1 mM EDTA and was suspended in 1.8 ml of 50 mM Tris-hydrochloride (pH 8.5)–50 mM EDTA–15% sucrose. Lysozyme was added to a final concentration of 1 mg/ml, and after a 5-min incubation, 2 ml of 2% SDS was added, which resulted in immediate cell lysis. Phenol (2 ml) was added to the lysate, and the layers were mixed by heating to 60°C and passing the mixture through an 18-gauge syringe needle. The emulsion was transferred to room temperature, and 2 ml of chloroform-isoamyl alcohol (24:1) was added and mixed as described above. The phases were separated by centrifugation, and the organic and insoluble layers were discarded. Predigested pronase A was added to a final concentration of 1 mg/ml, and the preparation was incubated at 37°C for 1 h. The proteins were extracted twice with phenol-chloroform-isoamyl alcohol (50:24:1). The nucleic acids were precipitated from the aqueous layer by treatment overnight with 0.3 M sodium acetate, pH 5.0, and 2 volumes of ethanol at −20°C, collected by centrifugation, and suspended in distilled water.

Electrophoretic separation of RNA and transfer to nitrocellulose paper. Denatured RNA was separated by electrophoresis through a 1.1% agarose gel and was blotted onto nitrocellulose paper (36).

Preparation of hybridization probe and hybridization to blots. The 1,300- bp *Xba*I-*Hpa*I fragment of pRT11 containing the *tet* operon was separated, eluted, and concentrated by electrophoresis as previously described for the *Hin*cII fragment (9). The fragment was labeled with [^{32}P]TTP by using a nick translation kit (New England Nuclear Corp.). Hybridization of the heat-denatured probe to the RNA blots was performed as described (36).

RESULTS

Complementation analyses of *Bal* 31-generated mutants with deletions in portions of the *tet* operon genes. To determine the size and location of the two complementation groups within the operon, we used *Bal* 31 exonuclease to produce deletions of different sizes at restriction enzyme cleavage sites in the *tet* operon. The plasmid pLR1068 has unique *Bgl*I and *Hpa*I sites in the *tetB* complementation region at nucleotide positions 784 and 1,161, respectively, relative to the start codon of the *tet* operon (13). Approximately 60 to 900 bp were removed exonucleolytically by *Bal* 31 from plasmid molecules linearized with either restriction enzyme. Nine deletions originating at the *Bgl*I site and three at the *Hpa*I site were retained for further study (Fig. 1). All deletions caused total loss of tetracycline resistance (Table 1).

Complementation analyses of the *tet* deletions showed that they were unable to complement either of the *tetB* point mutations (Table 1). Since the *Bgl*I and *Hpa*I origins of the deletions are in the *tetB* complementation region, this result was anticipated. Six of the *tet* deletions complemented the *tetA1* mutation, indicating that the deletions were in *tetB* only. Since the *Eco*RI site was present in each of these complementing deletion mutants, the 3′ end of *tetA* must occur before or near the *Eco*RI site. Tetracycline susceptibility deletions on plasmids pLR1093, pLR1095, and

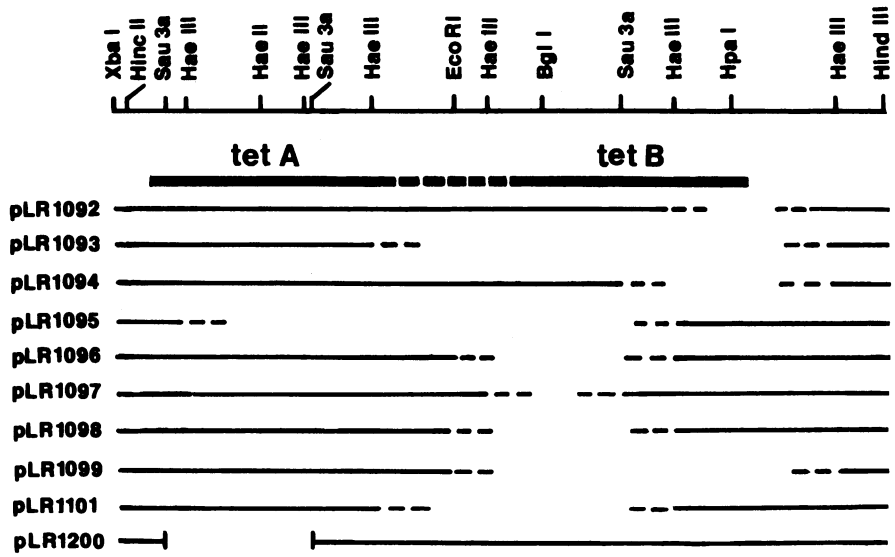

FIG. 1. Physical and genetic map of deletion mutants in the tetracycline structural gene region of the Tn*10* resistance determinant. The deletions were made either with the exonuclease *Bal* 31 or by partial digestion with the restriction endonuclease *Sau*3A. Solid lines indicate sequences known to be present, the blank spaces indicate sequences known to be deleted, and dashed lines indicate regions where the presence of the sequence is not known. The *Xba*I-to-*Hin*dIII fragment is 1,550 bp long. The *tetR* gene lies to the left of *TetA* and is not shown.

pLR1101 complemented neither the *tetA* nor the *tetB* point mutation (Table 1). Two of these plasmids lacked the *Eco*RI site but contained the *Hae*III site to the left (promoter proximal). These data indicate that the terminal end of the *tetA* region was in the 160-bp region lying to the left (promoter proximal) of the *Eco*RI site and to the right of the *Hae*III site (Fig. 1). Consequently, the *tetA* region is estimated to be 450 to 600 bp long. The *tetB* region is then 600 to 750 bp long, provided that it accounts for the remainder of the *tet* operon sequence and that it does not overlap the *tetA* region. Although sequences to the right of the *Bgl*I site (promoter distal) are essential for *tetB*, it cannot yet be concluded that sequences to the left of *Bgl*I are also required.

Complementation of new mutations in the *tetA* region. A spontaneous tetracycline susceptibility mutant of plasmid pLR1068, designated pLR1100, was recovered during the course of this study. The mutant resistance gene reverted to wild-type tetracycline resistance and did not have a detectable deletion or insertion; it is presumably a point mutation. The mutant plasmid was found to complement *tetB2* and *tetB3*, but not *tetA1* (data not shown), indicating that it contained a new *tetA* mutation called *tetA18*. Similar complementation studies were performed on plasmid pLR1200, deleted for a 297-bp (99-codon) *Sau*3A restriction fragment. These tests confirmed that it contained a *tetA* deletion.

To provide additional possibilities for complementation testing with *tetA* mutations carried by plasmids pLR1100 (*tetA18*) and pLR1200 (Δ*tetA20*), three mutant *tet* alleles were transferred from the pLR1068 vector to the compatible vector pRT11 by substitution of the *Xba*I-*Hin*dIII *tet* structural gene fragment for the analogous fragment from the mutants. This construction produced plasmids pLR1127 (Δ*tetB15*), pLR1130 (*tetA18*), and pLR1131 (Δ*tetAB19*). Neither the *tetA18* nor the Δ*tetA20* mutation on the pLR1068 vector complemented the *tetA18* or Δ*tetAB19* mutation on the pRT11 vector; however, both plasmids complemented the *tetB* deletion plasmid pLR1127 (data not shown). The combination of pLR1100 (*tetA18*) and pLR1127 (Δ*tetB15*) increased the level of resistance 10-fold over the level expressed by the plasmids singly, whereas complementation of the two deletion mutants pLR1200 (Δ*tetA20*) and pLR1127 (Δ*tetB15*) showed a threefold increase (data not shown).

Complementation of the tetracycline efflux system. Tetracycline resistance is characterized by a reduction in the cellular accumulation of tetracycline brought about by an energy-dependent tetracycline efflux system (1, 22, 29). In contrast, tetracycline-susceptible cells actively accumulate the antibiotic (26–28). Cells show a net active uptake when greater accumulation occurs in the energized than in the deenergized state; in contrast, active efflux leads to decreased accumulation in the energized state. We assayed our tetracycline-susceptible mutants alone and in pairs for the presence of the tetracycline efflux system. In each instance, whether the

TABLE 1. Complementation analyses of *Bal* 31-generated deletions in the *tet* operon and new *tetA* mutations

Plasmid	Tetracycline MIC (μg/ml)[a]			
	No plasmid	pLR1050 *tetA1*	pLR1053 *tetB2*	pLR1055 *tetB3*
None[b]	0.4	0.3	0.4	0.1
pLR1092 Δ*tetB10*	0.5	**4.2**	0.5	0.5
pLR1093 Δ*tetAB11*	0.5	0.6	0.5	0.5
pLR1094 Δ*tetB12*	0.6	**4.1**	0.5	0.5
pLR1095 Δ*tetAB13*	0.6	0.7	0.6	0.5
pLR1096 Δ*tetB14*	0.4	**5.6**	0.6	0.5
pLR1097 Δ*tetB15*	0.7	**4.6**	0.7	0.7
pLR1098 Δ*tetB16*	0.6	**6.0**	0.6	0.6
pLR1099 Δ*tetB17*	0.5	**6.2**	0.5	0.6
pLR1101 Δ*tetAB19*	0.5	0.5	0.5	0.5
pLR1100 *tetA18*	1.0	1.0	**13.6**	**15.0**
pLR1200 Δ*tetA20*	0.5	0.5	**5.2**	**4.5**

[a] Mean MIC from at least three determinations. The standard deviation of the mean was typically ≤20% of the mean. Values shown in boldface indicate complementation.

[b] Values for no plasmid were previously reported (9).

mutant contained a point or a deletion mutation in *tetA* or *tetB*, active efflux was not detectable; instead, like the susceptible host strain HB101, the mutants actively accumulated tetracycline (Fig. 2). Similarly, the efflux system was not detectable in strains containing noncomplementing pairs of *tet* alleles, such as the two mutations in the *tetB* group (e.g., pLR1053 plus pLR1094; Fig. 2). Typically, most susceptible mutants and noncomplementing pairs showed 12 to 46% lower active accumulaton of tetracycline than HB101; this may represent residual low-level efflux activity. Some, but not all, strains containing plasmids with complementing *tet* alleles showed an active efflux (e.g., pLR1050 plus pLR1092 [Fig. 2]; also pLR1050 plus pLR1096, pLR1050 plus pLR1097, and pLR1050 plus pLR1098 [data not shown]). Others showed active uptake (e.g., pLR1053 plus pLR1200 [Fig. 2]; also pLR1050 plus pLR1099 and pLR1055 plus pLR1200 [data not shown]), and others showed neither a net uptake nor a net efflux (e.g., pLR1055 plus pLR1100 [Fig. 2]). Since the MIC of tetracycline for these complementing pairs was in the range of 4.2 to 15 μg/ml, it is of interest that not all of them demonstrated net efflux. Furthermore, the strength of efflux during complementation was always less than that observed in the wild-type resistant strain HB101(pRT11) (Fig. 2); however, the level of tetracycline uptake was always below that in the susceptible strain HB101. In the absence of induction, all complementing strains showed active uptake of tetracycline (data not shown).

Protein synthesis in maxicells directed by plasmid mutant *tet* genes. In maxicells, plasmids pRT11 and pLR1068, harboring the wild-type tetracycline resistance genes from Tn*10*, directed the synthesis of Tet, the major tetracycline-inducible protein of 36,000 daltons, a 25,000-dalton repressor, and some minor bands between 18,000 and 24,000 daltons (Fig. 3). The minor inducible bands which were observed appeared to represent fragments of Tet and to be peculiar to the maxicell system since Tet protein and repressor were the only reproducible tetracycline-inducible proteins in minicell preparations (data not shown). We found that synthesis of the repressor protein was largely constitutive in the maxicells.

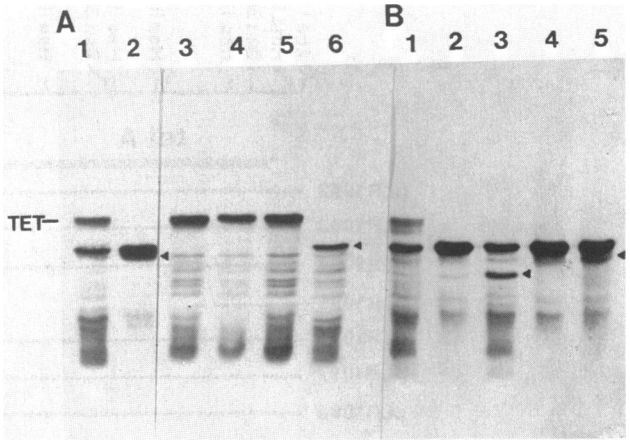

FIG. 3. Synthesis of Tet protein by tetracycline-susceptible mutant plasmids in HB101 maxicells. All cells were induced for Tet synthesis unless specified otherwise. Total protein was separated in a 14% (A) or a 12% (B) polyacrylamide gel. (A) Lane 1, pLR1068; lane 2, pLR1200; lane 3, pRT11; lane 4, pLR1050; lane 5, pLR1053; lane g, pLR1055, (B) Lane 1, pLR1068; lane 2, pLR1097, uninduced; lane 3, pLR1097; lane 4, pLR1200, uninduced; lane 5, pLR1200. Arrowheads indicate truncated inducible Tet proteins.

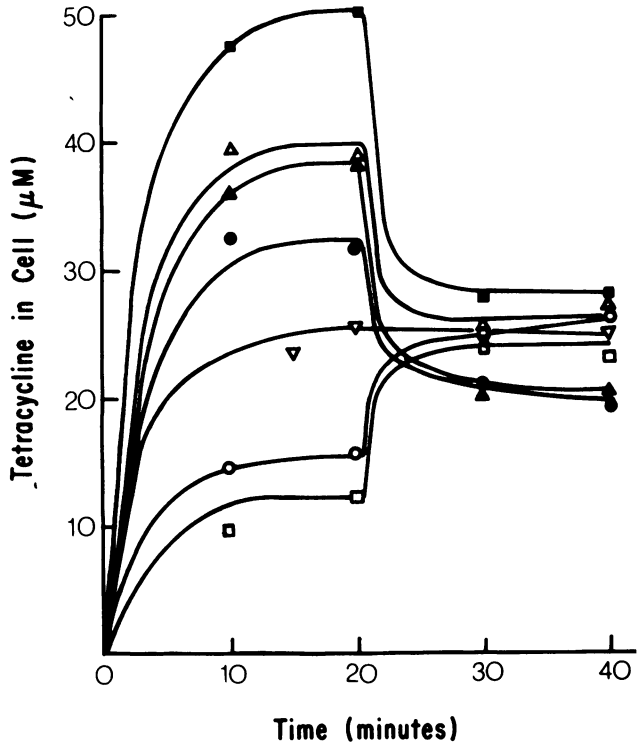

FIG. 2. Accumulation of tetracycline by cells containing mutant tetracycline resistance genes. Membranes were depolarized by the addition of 100 μM CCCP (carbonyl cyanide-*m*-chlorophenyl hydrazone) at 20 min. Symbols: ■, strain HB101 harboring no plasmids; ▲, pLR1200; ●, pLR1053 plus pLR1094; △, pLR1053 plus pLR1200; ○, pLR1050 plus pLR1092; ▽, pLR1055 plus pLR1100; □, pRT11.

We examined the proteins specified in maxicells by the *tetA* point mutation plasmids pLR1050, pLR1076, and pLR1100 (Fig. 3 and Table 2). pLR1050 mediated a normal-sized Tet protein. Neither pLR1076 nor pLR1100 specified Tet protein; however, pLR1100 coded for a tetracycline-inducible protein about 500 daltons smaller than Tet. Of the *tetB* point mutants, only pLR1053 and pLR1075 specified proteins resembling Tet. The point mutant pLR1055 coded for a major inducible protein of 28,000 daltons (Fig. 3). This last finding suggested the presence of a terminator codon in the *tetB* region that led to the synthesis of a truncated Tet protein, and in fact, tetracycline-resistant revertants of pLR1055 made a normal-sized Tet protein (data not shown).

The deletion plasmids were also examined for their ability to specify the synthesis of Tet protein in maxicells (Table 2). None of these mutant plasmids coded for Tet protein; however, five of the mutants specified major tetracycline-inducible proteins which were smaller than Tet (Fig. 3 and Table 2). Of particular note, the in-frame *tetA* deletion in pLR1200 coded for an inducible protein of 25,000 daltons, which is presumably the Tet protein missing 99 amino acids.

Synthesis of tetracycline-inducible mRNA. There is a single promoter for the *tet* operon (38). Hence, a single mRNA of about 1,200 bp, the size of the operon, is expected. We attempted to isolate this RNA from whole cells and to determine its size and also whether it was transcribed in uninduced cells.

TABLE 2. Synthesis of Tet protein in maxicells

Plasmid	Synthesis of:		
	Mutation	Tet protein (36,000 daltons)	New tetracycline-inducible protein[a]
pLR1030	Wild-type	+	−
pLR1050	*tetA1*	+	−
pLR1053	*tetB2*	+	−
pLR1055	*tetB3*	−	+ (28,000)
pLR1068	Wild-type	+	−
pLR1072	*tetB5*	−	−
pLR1074	*tetB6*	−	+ (34,000)
pLR1075	*tetB7*	+	−
pLR1076	*tetA8*	−	−
pLR1092	Δ*tetB10*	−	+ (34,000)
pLR1093	Δ*tetAB11*	−	−
pLR1094	Δ*tetB12*	−	+ (35,000)
pLR1096	Δ*tetB14*	−	−
pLR1097	Δ*tetB15*	−	+ (21,000)
pLR1098	Δ*tetB16*	−	−
pLR1099	Δ*tetB17*	−	−
pLR1100	*tetA18*	−	+ (35,000)
pLR1101	Δ*tetAB19*	−	+ (12,000)
pLR1200	Δ*tetA20*	−	+ (25,000)

[a] Values in parentheses show molecular size in daltons.

RNA was extracted from tetracycline-induced and uninduced resistant cells (containing the entire Tn*10* sequence on plasmid R222), from induced cells that were treated with rifampin (100 μg/ml) for 5 min, and from the plasmid-free host strain HB101. The RNA was denatured, separated on an agarose gel, and blotted onto nitrocellulose paper for hybridization. The DNA hybridization probe was the 1,300-bp *Xba*I-*Hpa*I fragment of pRT11, which contains only the *tetA-tetB* sequences. The probe hybridized to a single RNA species about 1,200 bases long that was present in tetracycline-induced cells (Fig. 4). Sensitivity of this species to rifampin treatment indicated that the sequence was RNA and not DNA (Fig. 4). The RNA extracted from an uninduced strain showed a just-visible hybridization signal at the 1,200-base position after a fourfold longer exposure. No hybridization to nucleic acids of the host strain HB101 occurred (Fig. 4). A similar hybridization profile was observed with pRT11-generated RNA, except that the DNA band migrated farther into the gel, reflecting the lower molecular weight of this plasmid (data not shown). These results show that the *tet* operon makes a single mRNA of the expected size.

DISCUSSION

Genetic evidence has demonstrated that the *tet* operon is divisible into two nonoverlapping complementation groups, *tetA* and *tetB* (7, 9). Point mutations affect one complementation group or the other, but never both groups concurrently (9). Moreover, complementation has now been observed between a plasmid with a *tetA* deletion and others with deletions in *tetB*. Although the level of resistance expressed during complementation did not approach the wild-type level (160 to 180 μg/ml), it was typical that a 10-fold increase in resistance was observed over the noncomplementing level of resistance or the single-mutation plasmid level. These complementation data suggest two separate genes.

The 36,000-dalton Tet protein, the only well-established structural gene product of the *tet* operon (17, 21, 39), is presumed to be the product of the *tetB* region (7, 9). We searched for a second protein, the product of *tetA*, by examining the proteins synthesized by our tetracycline-susceptible mutants in maxicells and minicells. We found that mutations in *tetB* affected the amount or the size of the Tet protein as expected. More important, *tetA* mutations also affected both the synthesis and the size of the Tet protein. This was most clearly demonstrated by the synthesis of a truncated 25,000-dalton Tet protein in maxicells containing the *tetA* deletion plasmid pLR1200. Also, the point mutation *tetA18* caused a slight reduction in the apparent size of the Tet protein. Dramatic alteration of a protein as the result of a single amino acid substitution has been observed before (31). The discovery that mutations in both *tetA* and *tetB* affected the Tet protein suggested that there was a single translation product of the *tet* operon. Moreover, Tet proteins synthesized in maxicells by both *tetA* and *tetB* mutant plasmids were no different from Tet proteins made by the same mutants alone (unpublished data). This result indicates that the gene product of one complementation group is not required for the synthesis of the other. These findings indicate that complementation between *tetA* and *tetB* was intracistronic, with *tetA* and *tetB* defining two domains of the *tet* structural gene.

Recently, nucleotide sequencing of the tetracycline resistance determinant on Tn*10* has shown a single open reading frame encoding a protein of 43,300 daltons (13). A second ribosome-binding sequence and initiation codon was identified within the *tet* operon which would specify a 17,600-dalton protein duplicating the carboxyl protein of Tet. A third reading frame determines a 3,700-dalton protein; however, its ribosome-binding sequence lacks good Shine-Dalgarno homology. Neither of these last two reading frames appears to be a likely candidate for the *tetA* and *tetB*

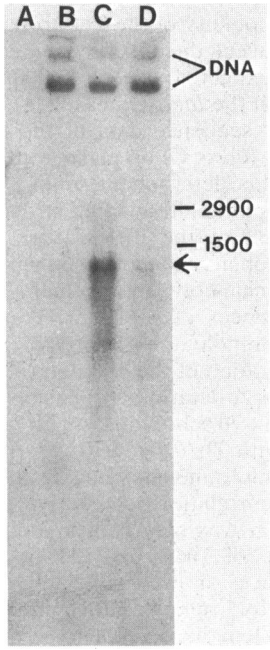

FIG. 4. Identification of *tet* mRNA by hybridization of *tet* DNA to nitrocellulose filter-bound RNA. The 2,900- and 1,500-base ribosomal subunit RNAs (4, 5) were used as molecular weight markers. The RNAs were prepared from HB101 cells (A), HB101(R222) uninduced cells (B), HB101(R222) induced cells (C), and HB101(R222) induced cells treated with rifampin (D). The arrow locates *tet* operon mRNA.

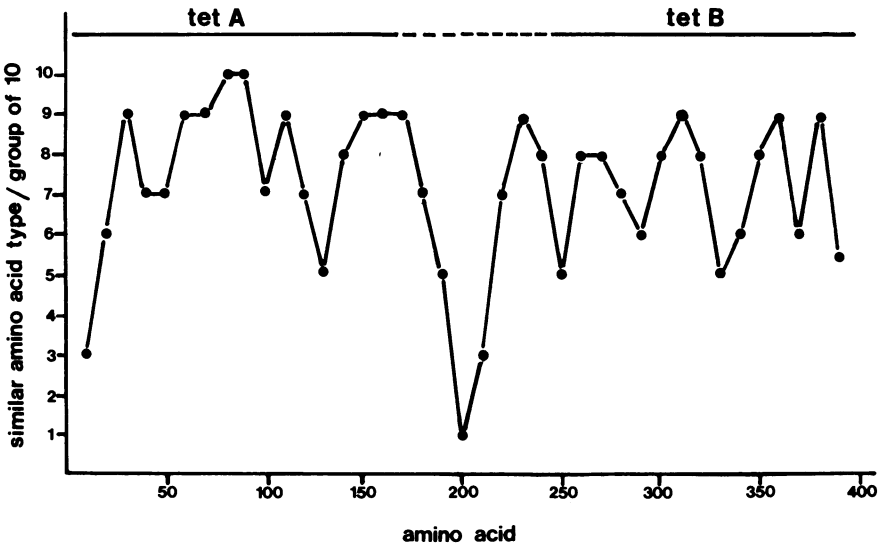

FIG. 5. Conservation of amino acid type among the *tet* determinants of Tn*10*, pBR322, and RP1. The protein sequence of RP1 *tet* was aligned with the Tn*10* and pBR322 *tet* consensus map (13). At each position in the sequence, conservation of side chain was assessed (hydrophobic, nonpolar charged, acidic, or basic) and then grouped into 10 residues from the amino to the carboxyl terminal of the protein for graphing purposes.

complementation groups. Consequently, it appears that the operon determines a single translational product; this is in agreement with our protein data derived from the mutant *tet* alleles. We have detected a single tetracycline-inducible RNA transcript homologous with the resistance structural gene region the size of the *tet* operon, a result which is consistent with the identification of a single promoter for this region. A single transcript, however, may specify one or more polypeptides. Only one protein, Tet, has been identified. The hydrophobicity of the Tet protein may account for the difference between the observed molecular size of 36,000 daltons and the expected size of 43,000 daltons. A similar result is seen with the *lac* permease (11).

The nucleotide sequence data of the tetracycline resistance determinant (class C) on plasmid pBR322 suggests the possibility that this determinant, unlike that on Tn*10*, encodes two overlapping tetracycline structural proteins (35). However, revision of the pBR322 *tet* sequence (23, 32) provides a single open reading frame the size of Tn*10 tet* and consequently a single polypeptide chain. The nucleotide and amino acid sequences of pBR322 *tet* and Tn*10 tet* show about 50 to 45% homology, respectively, suggesting that the functional organization of the two determinants is the same. The RP1 *tet* (class A determinant) has also been sequenced, and it shows about 70% homology with pBR322 *tet* (37) and 50% homology with Tn*10 tet*. RP1 *tet*, like Tn*10 tet*, has a single open reading frame encoding a single *tet* protein (37).

An interesting correlation became evident when the genetic map of Tn*10 tet* was placed over a chart comparing the protein sequences of Tn*10*, pBR322, and RP1 *tet* (Fig. 5). The three sequences showed considerable conservation of amino acid type (hydrophobic, uncharged polar, acidic, and basic) over their lengths, except for a stretch of 20 to 30 amino acid residues around residue 200 (near the *Eco*RI cleavage site) or midway between the two ends of the polypeptide chain. This poorly conserved sequence divides the Tet protein into two portions coinciding with the *tetA* and *tetB* complementation regions. This sequence may divide the protein into the two functional domains as defined by our genetic analysis.

Efflux of tetracycline, a property of tetracycline-resistant cells, was absent from cells bearing plasmids with mutations in either complementation group. Efflux was reestablished by complementation in some, but not all, complementing pairs. In at least four pairs, despite levels of resistance at 4 to 6 μg/ml, a net uptake or no detectable uptake or efflux was noted. These findings support other data (20, 27) which suggest that another mechanism in addition to efflux may be involved in resistance.

The Tet protein is the first example of intracistronic complementation of a membrane protein. This type of complementation has been demonstrated between mutant variants of genes encoding catalytic enzymes or repressor-type proteins (15, 16, 18; for a review, see reference 40). The functional form of these proteins often consists of two or more homologous polypeptide chains. Complementation may then result when mutant polypeptides combine to form an active hybrid protein such that each mutant chain provides or corrects the conformation and site(s) of activity of the protein. It is reasonable to suggest that the native form of the Tet protein in the membrane is a homomultimer.

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