JOURNAL OF BACTERIOLOGY, Feb. 1983, p. 921–929
0021-9193/83/020921-09$02.00/0
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Vol. 153, No. 2

Analysis of Tetracycline Resistance Encoded by Transposon Tn*10*: Deletion Mapping of Tetracycline-Sensitive Point Mutations and Identification of Two Structural Genes

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Received 21 September 1982/Accepted 29 November 1982

Deletions in the *tet* genes derived from Tn*10* were formed from different *tet*::Tn*5* insertion mutations by removing DNA sequences located between a *Hin*dIII site in Tn*5* and a *Hin*dIII site adjacent to the *tet* genes. Tetracycline-sensitive point mutations were mapped in recombination tests with the deletions and were thus aligned with the genetic and physical map of the *tet* region. Plasmids carrying point mutations were tested for complementation with derivatives of pDU938, a plasmid carrying cloned *tet* genes derived from Tn*10* which had been inactivated by Tn*5* insertions. Complementation occurred between promoter-proximal *tet* point mutations and distal *tet*::Tn*5* insertions, suggesting the existence of two structural genes, *tetA* and *tetB*. These results, together with the analysis of polypeptides in minicells harboring pDU938*tet*::Tn*5* mutants, suggested that *tetA* and *tetB* are expressed coordinately in an operon. The *tetB* gene encodes the previously characterized 36,000-dalton cytoplasmic membrane TET protein, but the product of *tetA* was not identified. Point mutations in either *tetA* or *tetB* led to the defective expression of the resistance mechanism involving tetracycline efflux. It is suggested that the *tetA* and *tetB* products interact cooperatively in the membrane to express resistance.

Bacterial resistance to the tetracyclines is widespread and is usually plasmid encoded (3, 23). Within gram-negative bacteria, four genetically distinct resistance determinants (TetA to TetD) have been identified (27), of which TetB occurs most frequently (23). The resistance genes of the TetB determinant mediate inducible resistance to both tetracycline and its derivative minocycline (5, 27, 30) and are contained within Tn*10* (16, 17, 27). Recently, much interest has focused upon the identification of the products concerned with the expression of resistance by Tn*10* and the arrangement of the regulatory and resistance genes. The genes controlling resistance are located within a 2,025-base-pair (bp) sequence of Tn*10*, and a model for their organization has been proposed (20, 33) (Fig. 1). The repressor gene (*tetR*), which encodes a 23,500-molecular-weight (23.5K) polypeptide, is located in a 695-bp *Hin*cII fragment (33), and the structural gene for an inducible 36K membrane protein (synonym TET protein) (10, 24), which is required for resistance (20, 34), is located in the adjacent 1,275-bp *Hin*cII fragment.

Since the expression of Tn*10*-mediated tetracycline resistance involves the energy-dependent efflux of the antibiotic (2, 26) and the content of TET protein in the membrane correlates with the ability of cells to exclude tetracycline (I. Chopra, P. R. Ball, and S. W. Shales, volume 18 of the technical series of the Society for Applied Bacteriology, in press), it appears that TET protein is involved in drug efflux. However, Tn*10*-mediated minocycline resistance does not involve drug efflux (5, 30) and is presumably mediated by another Tn*10* product. The gene encoding this product has not been identified and, hence, the number of proteins involved in the expression of resistance to tetracycline and minocycline is unknown.

A combined genetic and biochemical approach is required to define the products that mediate resistance. However, the genetic analysis of *tet* has been hampered by difficulty in performing complementation tests and in obtaining deletion mutations suitable for deletion mapping within the resistance genes (12, 13). The majority of tetracycline-sensitive mutations of Tn*10* were caused by the deletion of the entire resistance region (12, 13). The combination of gene-cloning techniques to obtain the *tet* genes on new vectors in the absence of the Tn*10* inverted repeats and transposon mutagenesis have provided new approaches to the genetic

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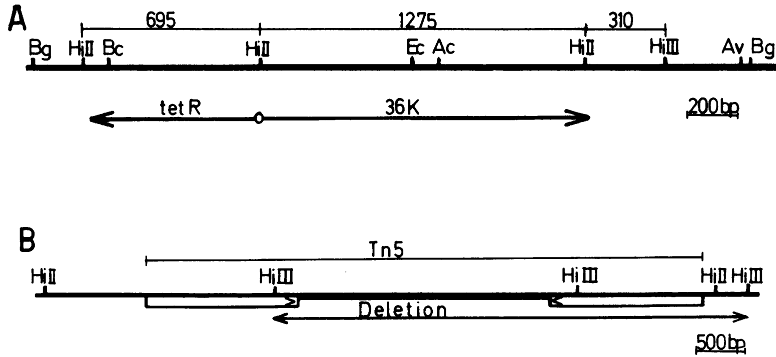

FIG. 1. Restriction maps and isolation of deletion mutations. (A) Restriction map of the 2,800-bp *Bgl*II fragment from within Tn*10* which carries the *tet* genes (20). The symbols for restriction sites are as follows: Bg, *Bgl*II; HiII, *Hin*cII; Ec, *Eco*RI; HiIII, *Hin*dIII; Ac, *Acc*I; and Av, *Ava*I. The 695- and 1,275-bp *Hin*cII fragments which carry the *tet* repressor and the 36K protein structural genes (20, 33) are indicated. The model for the structural organization and expression of the *tet* genes (20, 33) is also shown. (B) Generation of a deletion mutation in *tet* by the removal of DNA distal to a Tn*5* insertion. In this example, Tn*5* is located 1,000 bp from the promoter-proximal left side of the 1,275-bp *Hin*cII fragment. The position of the *Hin*dIII sites and the extent of the deletion are indicated. The scales in (A) and (B) differ as shown by the scale bars. The symbols for restriction sites are as given above.

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analysis of tetracycline resistance (4, 6). This paper describes a method for generating deletion mutations suitable for the fine-structure mapping of tetracycline-sensitive point mutations. These point mutations were tested for complementation with Tn*5* insertion mutations located in the *tet* genes cloned on a compatible plasmid. The data obtained suggest the existence of two *tet* structural genes that are coordinately expressed in an operon.

While the work described here was in progress, Curiale and Levy (7) published a paper which also identifies two structural genes, designated *tetA* and *tetB*, in the tetracycline resistance determinant of Tn*10*. The reported organization of these genes with respect to each other and *tetR* agrees with the findings that we present here.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Tables 1 and 2. The properties of the point mutants of R100 and R100-1 have been described elsewhere (12, 13). The mutants were isolated after mutagenesis with ethyl methane sulfonate. Each reverts spontaneously to Tc^r. Plasmids pDU3, pDU7, pDU9, pDU10, pUB265, and pUB268 are recessive, whereas pDU2, pDU4, pDU6, pDU8, pDU11, pUB261, pUB270, and pUB273 are dominant (13; unpublished data). Wild-type Tc^r cannot be expressed properly in cells also harboring a dominant Tc^s mutant plasmid (13).

Plasmid pNK133 was constructed by cloning a *Bgl*II fragment carrying the *tet* genes from Tn*10* into the *Bgl*II site in pNK75 (6, 16). pNK75 is a derivative of pBR322 carrying the *Salmonella typhimurium hisOGD* genes (16). Plasmid pDU938 was constructed by cloning the *Bgl*II *tet* fragment into the low-copy-number vector pUB5572 (4). pUB5572 was derived from R388 by ligating a *Bgl*II fragment carrying the trimethoprim resistance and replication genes (4, 32).

Chemicals and bacteriological media. The composition of LB broth and agar has been described elsewhere (28). Minicells were harvested from cultures grown in brain heart infusion broth (Oxoid Ltd., London, England). The minicell-labeling medium comprised 1% methionine assay medium (Difco Laboratories, Detroit, Mich.) and 1% glucose in M9 salts (28). An M9 salts medium (28) supplemented with glucose (1%), Casamino acids (Oxoid; 1%), thiamine (0.2 μg/ml), and $MgSO_4$ (10 mM) was used for the selection of trimethoprim resistance.

Tetracycline (Tc), kanamycin (Ka), and chloramphenicol (Cm) were purchased from Sigma Chemical Co., St. Louis, Mo. Ampicillin (Ap) and minocycline were gifts from Beecham Laboratories, Bristol, Tenn., and Lederle Laboratories, Pearl River, N.Y., respectively. Trimethoprim (Tp) was purchased from Wellcome Research Laboratories, Beckenham, England. Antibiotics were incorporated into agar at the following concentrations: tetracycline, 10 μg/ml; kanamycin,

TABLE 1. Bacterial strains

Strain	Genotype	Source/reference
C600	*thr leu thi lac* SuII$^+$	N. Kleckner (Harvard University)
XAcSu$^-$	Δ(*lac-pro*)XIII *ara argE thi rpoB gyrA*	N. Kleckner (Harvard University)
DU5003	Δ(*lac-pro*)XIII *thi rpoB*	Rifampin-resistant mutant of CSH26 (28)
ED2030	Δ*lacX174 thi trp recA56*	N. S. Willetts (Edinburgh University) (18)
DS410	*minA minB rpsL*	G. Dougan (Trinity College, Dublin) (8)

TABLE 2. Plasmids

Plasmid	Phenotypic markers[a]	Source/reference
R100	Cm^r Tc^r Sm^r Su^r Hg^r Tra$^+$ IncFII	
R100-1	Cm^r Tc^r Sm^r Su^r Hg^r Tra$^+$ (*drd*) IncFII	Mutant of R100 derepressed for conjugational transfer (9)
pDU301	Cm^r Tc^r Sm^r Su^r Hg^r Tra$^+$ (*drd*) IncFII	Mutant of R100-1 which expresses tetracycline resistance constitutively (14)
pDU2-pDU11	Cm^r Sm^r Su^r Hg^r IncFII	*tet* point mutants of R100 or R100-1 (Table 3) (12)
pUB261-pUB273	Cm^r Sm^r Su^r Hg^r Tra$^+$ (*drd*) IncFII	*tet* point mutants of pDU301 (Table 3)
pNK133	Tc^r Ap^r	Carries *tet* genes derived from Tn*10* in pNK75 (16)
pDU938	Tc^r Tp^r IncW	Carries *tet* genes from Tn*10* inserted in pUB5572 (4, 6)
PDU1116 pDU1117 pDU1119 pDU1120	Ka^r Tp^r IncW	*tet*::Tn*5* insertions in pDU938 (this paper)
pDU966-pDU987	Ap^r	*tet* deletion mutations derived from pNK133 *tet*::Tn*5* (6) (this paper)
pNK84 pNK83 pNK139	Ap^r	*tet* deletion mutations in pNK80 and pNK133 (6, 16)

[a] Abbreviations: Sm, streptomycin; Su, sulfonamide; Hg, mercuric ion; Tra, conjugational transfer.

chloramphenicol, and trimethoprim, 20 μg/ml; ampicillin, 100 μg/ml. 12a-Deoxytetracycline was a gift from W. V. Shaw (Leicester University, United Kingdom). All other chemicals were obtained from Sigma or were the best grade available from British Drug Houses, Poole, United Kingdom.

Enzymes. Restriction enzymes and T4 ligase were from New England Biolabs, Beverly, Mass. They were used according to the manufacturer's instructions.

Construction of *tet* deletion mutations. The deletion mutations in the *tet* genes were generated in vitro by making use of the *Hin*dIII sites located in the inverted repeats of Tn*5* (19). Transposon Tn*5* insertion mutations located in a number of different sites in the *tet* structural genes on the multicopy chimeric *tet* plasmid pNK133 have been characterized previously (6). This plasmid has a single *Hin*dIII site located 300 bp to the right of the *tet* genes displayed in Fig. 1. The cleavage of pNK133*tet*::Tn*5* plasmids with *Hin*dIII generated three fragments (data not shown). A fragment of 3,540 bp carrying the Ka^r gene was from within the transposon. The other small fragment represented the right junction between Tn*5* and the *tet* plasmid (Fig. 1) and contained the part of the *tet* genes distal to the insertion. The large fragment carried the proximal part of *tet*, the Ap^r gene, and the plasmid replication genes. The *Hin*dIII-cleaved DNA was ligated, and Ap^r Tc^s transformants of C600 were identified. These plasmids were formed by recircularization of the large *Hin*dIII fragment to form the *tet* deletion mutations used for genetic mapping experiments. These elements have lost all Tn*5* sequences apart from the proximal 1,200 bp of the left inverted repeat, as well as *tet* DNA distal to the Tn*5* insertion. The deletion map of the *tet* region is shown in Fig. 2.

Isolation and characterization of pDU938*tet*::Tn*5* mutations. Strain XAcSu$^-$ carrying pDU938 was infected with λ 467 (*b*221 *rex*::Tn*5* 0_{am29} P_{am80} cI857; provided by N. Kleckner, Harvard University, Cambridge, Mass.) at a multiplicity of 1. After incubation at 20°C for 60 min, the phage-infected cells were spread on kanamycin agar. The Ka^r colonies, which should be derived from independent transposition events, were pooled (>10,000 clonies per pool), and plasmid DNA was isolated. Samples of DNA were transformed into C600, and Ka^r colonies were selected. These carried

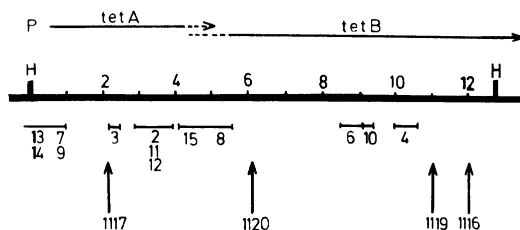

FIG. 2. Map of mutations in *tet*. The thick horizontal line depicts the 1,275-bp *Hin*cII fragment that carries the *tet* structural genes. The *Hin*cII sites are indicated by H. The numbers above this line are physical coordinates in hundreds of base pairs. The lower thin horizontal lines show deletion intervals in which *tet* point mutations have been mapped. The interval number and the physical coordinates are listed in Table 3. The vertical lines show the positions of the *tet*::Tn*5* insertions in pDU938 used for minicell analysis and complementation tests. Insertions carried by pDU1117, pDU1120, pDU1119, and pDU1116 are located at 220, 610 or 660, 1,095 and 1,205 bp, respectively, from the left *Hin*cII site. At the top is the approximate position of the *tetA* and *tetB* genes. The overlapping region is not meant to imply that the *tetA* and *tetB* genes overlap but that there is some doubt about their precise coordinates. P shows the location of the *tet* promoter (20).

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pDU938*tet*::Tn*5* derivatives. Tc^s mutants were identified by replica plating. Independent pDU938*tet*::Tn*5* mutants were mapped by restriction enzyme analysis. First, the Tn*5* elements were positioned with respect to the *Eco*RI site located in the center of the 1,275-bp *Hin*cII *tet* fragment (Fig. 1). Next, the plasmids were cleaved with *Hin*cII. Tn*5* has *Hin*cII sites located 190 bp from its outer ends (19). In each mutant, the 1,275-bp *Hin*cII fragment was missing and was replaced by junction fragments containing the outer 190 bp of the Tn*5* inverted repeat fused to *tet* DNA to the left or right of the insertion. The smaller junction fragment was measured, and the distance of the Tn*5* insertion from the nearest *Hin*cII site was calculated.

Recombination experiments. Recombination tests were performed by selecting for Tc^r derivatives of DU5003 strains carrying both an R100 *tet* point mutant plasmid and a *tet* deletion derivative of pNK133. The heterozygotes were constructed by a plate-mating technique (with the exception of pDU10, which is transfer deficient). Agar plates containing chloramphenicol and ampicillin were flooded with 0.1 ml of DU5003 carrying the deletion mutant plasmid. When the surface of the agar was dry, 0.01 ml of exponential cultures of the conjugative R100 plasmid derivatives were applied to the plate. After overnight incubation, confluent growth occurred in the area of the donor inoculum. Plasmid pDU10 is a transfer-deficient mutant of R100 which has an amber-suppressible mutation in *tet* (12). It was introduced into DU5003 (Su$^-$) carrying the deletion mutant plasmids by transduction with bacteriophage P1 *vir*. Transductants were selected on agar containing chloramphenicol and ampicillin, and single colonies were purified twice to remove phage particles before testing for recombination.

To select for recombinants, the heterozygous cells were inoculated into broth and grown to saturation. Samples (0.1 ml) were applied to plates containing 20 or 50 μg of tetracycline per ml. When recombination occurred, at least 100-fold more Tc^r colonies grew compared with the controls carrying the R100 point mutants alone. Endpoints were verified by performing five replica experiments and controls. Here the number of Tc^r colonies was 10- to 50-fold greater than for the controls.

Recombination between Tc^s mutations located on R100, R100-1, and pDU301 was tested by a phenocopy mating technique to construct transient heterozygotes with incompatible plasmids (14). Stationary-phase cultures were used as recipients because they exhibit reduced surface exclusion (1). Exponential cultures of the derepressed R100-1 and pDU301 derivatives were used as donors. Equal volumes of donors and recipients were incubated at 37°C for 2 h to allow heterozygotes to form and recombination to occur before 0.1 ml was plated on agar containing 20 or 50 μg of tetracycline per ml.

Complementation tests. Complementation was tested by measuring the tetracycline resistance levels of ED2030 *recA* carrying pairs of Tc^s derivatives of the compatible R100 and pDU938 plasmids. The *recA* host was used to prevent Tc^r colonies being formed by homologous recombination. Both parental Tc^r plasmids have a low copy number, and both express high levels of tetracycline resistance in C600 (6). These were chosen because multicopy plasmids carrying Tn*10* often fail to express high levels of tetracycline resistance due to the multicopy effect (6). Many Tc^s mutations on multicopy plasmids still express the multicopy phenotype in *trans* (6), and this interferes with complementation experiments (unpublished data). The R100 Tc^s plasmids were transferred by conjugation or P1 transduction into ED2030 carrying the pDU938*tet*::Tn*5* elements as described above. The heterozygous strains were selected on chloramphenicol plus trimethoprim agar. Complementation was tested by measuring the tetracycline resistance levels as described below.

Resistance level determinations. The tetracycline resistance levels of ED2030 (*recA*) carrying R100 *tet* and pDU938*tet*::Tn*5* mutant plasmids either together or singly were measured as follows. Overnight broth cultures were diluted 1:50 in fresh broth and incubated for 4 h. One hour before plating, 1 μg of the gratuitous inducer 12a-deoxytetracycline per ml was added to each culture. The cultures were diluted to 10^{-3}, and 0.01-ml volumes were inoculated on the surface of LB plates containing 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 20, 30, 40, 50, 60, 70, or 80 μg of tetracycline per ml. Up to 25 samples were applied to a single plate with a multipoint inoculator. The plates were incubated at 37°C for 36 h to allow the slow-growing *recA* strain to form normal colonies. The resistance level was defined as the highest concentration of tetracycline supporting the growth of normal-sized colonies without any reduction in numbers.

Analysis of polypeptides in minicells. Minicell-producing strain DS410 carrying wild-type Tc^r plasmids or the *tet*::Tn*5* insertion mutants of pDU938 were grown with shaking in brain heart infusion broth (200 ml) for 18 h at 37°C. The minicells were purified by sedimentation through three successive 5 to 20% sucrose gradients and were finally suspended in 2 ml of labeling medium. After incubation at 37°C for 30 min, a 1-ml sample was withdrawn and induced by the addition of 1 μg of 12a-deoxytetracycline per ml. Both uninduced and induced cultures were incubated at 37°C for 30 min before the addition of 50 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.). The incubation was continued for 60 min. The minicells were pelleted and suspended in 50 μl of 62.5 mM Tris-hydrochloride–10% glycerol–2% sodium dodecyl sulfate–5% 2-mercaptoethanol (pH 6.8) buffer and immediately boiled for 3 min. Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide) at 20 mA (22). After being stained with Coomassie blue, the gels were dried, and autoradiography was performed for 20 to 30 days with Kodak X-Omat-H paper.

Determination of tetracycline accumulation. Tetracycline accumulation was determined by a spectrofluorimetric technique (2, 4, 5, 30).

RESULTS

Deletion mapping *tet* point mutations. A set of overlapping deletion mutations covering the *tet* genes was constructed in vitro (Fig. 3). The Tc^s point mutants of R100, R100-1, and pDU301 were transferred into strains carrying deletion mutant plasmids. The resulting heterozygotes were tested for the formation of Tc^r recombinants. The deletion interval in which the *tet*

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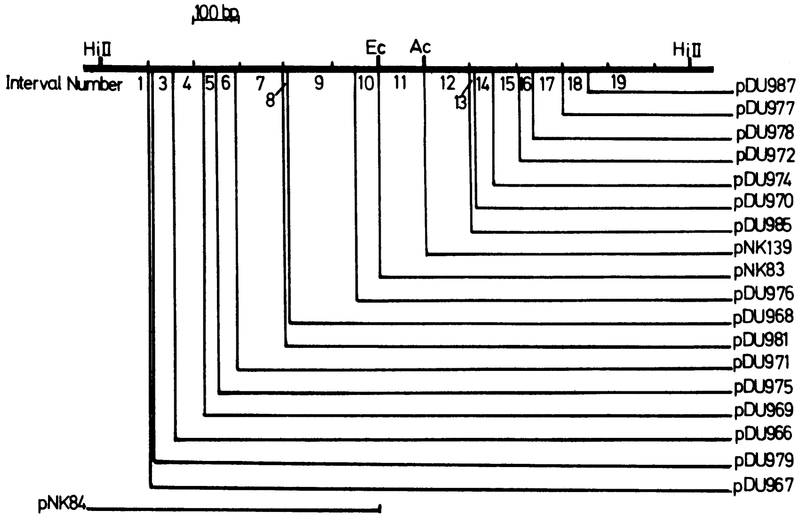

FIG. 3. Deletion map of the *tet* region. The extents of the deletions in the *tet* region are indicated by the series of horizontal lines. Each deletion ends within the 1,275-bp *Hin*cII *tet* fragment shown in Fig. 1A. The deletion interval numbers referred to in the text and Table 3 are shown. Plasmids pNK83, pNK84, and pNK139 carrying deletions in *tet* have been described before (6, 16). The plasmids with a pDU prefix were derived from *tet*::Tn5 insertions. The symbols for restriction sites are as described in the legend to Fig. 1A.

mutations were mapped is summarized in Table 3 and Fig. 2. The mutational sites span the 1,275-bp *Hin*cII fragment in which the *tet* structural genes are known to be located (6, 20, 34).

Four mutations (*tet-7*, *tet-9*, *tet-13*, and *tet-14*) mapped in deletion interval 1 spanning the first 100 bp of the *Hin*cII fragment. The fidelity of the mapping technique was confirmed by the fact

TABLE 3. Properties of Tc^s mutants and complementation tests

Plasmid	*tet* mutation[a]	Parental plasmid	Map position deletion interval	Coordinates[b]	Tetracycline uptake[c] (R^+:R^-)	Tetracycline resistance levels (μg/ml)[d] in complementation tests with: R100	pDU1117	pDU1120	pDU1116
pUB265	*tet-13*	pDU301	1	1–100	1.03	9.0	9.5	9.0	9.0
pUB268	*tet-14*	pDU301	1	1–100	0.67	15.0	15.0	14.5	14.5
pDU7	*tet-7*	R100	1	1–100	0.61	6.0	7.0	11.0	13.5
pDU9	*tet-9*	R100-1	1	1–100	0.98	6.0	6.5	10.0	11.5
pDU3	*tet-3*	R100-1	5	220–255	0.95	2.0	2.0	4.5	6.0
pDU11	*tet-11*	R100	7	290–395	0.71	7.0	7.0	12.0	14.5
pUB261	*tet-12*	pDU301	7	290–395	1.13	8.0	9.0	12.5	14.5
pDU2	*tet-2*	R100-1	7	290–395	0.81	6.0	7.5	11.5	15.0
pDU8	*tet-8*	R100-1	9	405–550	0.72	1.0	1.0	3.5	6.0
pUB270	*tet-15*	pDU301	9	405–550	0.12	10.0	12.0	11.5	10.5
pDU6	*tet-6*	R100	15	850–905	0.29	2.0	2.0	2.0	2.0
pDU10	*tet-10*	R100	16	905–935	NT	0.5	0.75	0.75	0.5
pDU4	*tet-4*	R100-1	18	1,000–1,055	0.73	1.0	1.0	1.0	1.0
pUB273	*tet-16*	pDU301	19	1,055–1,275	0.93	NT	NT	NT	NT
pDU1116	*tet*::Tn5	pDU938			NT	0.5			
	tet$^+$	R100			0.16	70.0			
		R100-1			0.28	70.0			
		pDU301			0.20	70.0			
		pDU938			NT	70.0			
	Plasmid-free control				1.0	0.5			

[a] *tet-10* is amber suppressible (12). The other mutants are presumed to be point because they revert to the wild type, and many retain partial resistance to tetracycline and are thus probably missense. *tet-2*, *-3*, *-4*, *-6*, *-7*, *-8*, and *-9* are not suppressible by amber or ochre suppressors (12, 13).

[b] The map position coordinates are the lengths of the deletion intervals in base pairs (Fig. 3).

[c] Values are based on the mean of replicate determinations.

[d] Strains were grown in 1 μg of 12a-deoxytetracycline per ml for 60 min before plating. NT, Not tested.

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that mutations *tet-7* and *tet-9* did not form recombinants. Similarly, *tet-13* and *tet-14* did not recombine. Both pairs of mutations must be located at identical or closely linked sites, possibly in the promoter or in the amino terminus of a structural gene. Three other mutations (*tet-2*, *tet-11*, and *tet-12*) mapped in the same deletion interval (number 7; 290 to 395 bp) and failed to undergo recombination with each other.

Complementation tests. If more than one structural gene is involved in the expression of tetracycline resistance, it should be possible to detect complementation between mutations in these genes when they are located on different replicons in heterozygous cells. The *tet* point mutations have been mapped to sites within the 1,275-bp *Hin*cII fragment as described above. They were tested for complementation with derivatives of pDU938, which have Tn*5* insertion mutations in *tet*. The physical locations of the insertions were known from restriction enzyme analysis (Fig. 2).

The point mutations fell into two classes on the basis of the complementation tests. Seven mutations (*tet-2*, *tet-3*, *tet-7*, *tet-8*, *tet-9*, *tet-11*, and *tet-12*) which mapped in the proximal 550 bp of the *tet* region expressed a two- to threefold-higher level of tetracycline resistance in the presence of pDU1120 and pDU1116 (pDU938 *tet* mutants with Tn*5* inserted at promoter-distal sites in the 1,275-bp *Hin*cII *tet* fragment). It was inferred that complementation was occurring and that the R100 point mutations and the pDU938 insertion mutations were located in different structural genes. These six point mutations did not complement pDU1117, which has Tn*5* located at a site closer to the promoter. Here the point mutations and the insertions are thought to be located in the same gene.

No increase in tetracycline resistance was observed when the heterozygotes were constructed with the remaining point mutations. Four of these (*tet-4*, *tet-6*, *tet-10*, and *tet-15*) mapped within or distal to deletion interval 9. The failure of complementation with the proximal Tn*5* insertion could be explained by this mutant exerting polarity on a distal gene. No complementation occurred with pDU1120 and pDU1116 because the point and insertion mutations are in the same gene. The promoter-proximal complementation group corresponds to *tetA*, and the distal group corresponds to *tetB*, as designated by Curiale and Levy (7).

Analysis of polypeptides in minicells. Minicells carrying R100 specified a 36K tetracycline-inducible polypeptide (Fig. 4), which corresponds to the 36K TET protein identified by others (10, 20, 24, 25, 34). This protein was also specified by pDU938 (data not shown). Polypeptides coded by mutants of pDU938 with Tn*5* inserted at known positions in the 1,275-bp *Hin*cII fragment

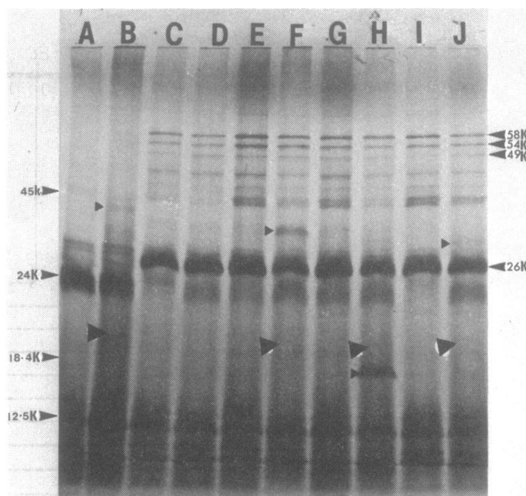

FIG. 4. Autoradiogram of ^{35}S-labeled polypeptides synthesized in minicells by *tet* plasmids. Each pair of tracks from left to right has material derived from uninduced and induced cultures of the same strain. Induction was achieved by exposing minicells to 1 μg of 12a-deoxytetracycline per ml for 30 min before labeling. Tracks A and B show R100-specified polypeptides. A 36K tetracycline-inducible polypeptide is indicated by the small arrow. Plasmid pDU938 also expressed this polypeptide (data not shown). The track locations of samples from the pDU938*tet*::Tn*5* mutants are as follows: pDU1117 (C and D), pDU1119 (E and F), pDU1120 (G and H), and pDU1116 (I and J). The positions of the 36K protein and its truncated variants are indicated by small arrows. Large arrows show the position of molecular weight standards. Bands of 58, 54, 49, and 26K were internal standards specified by Tn*5* (tracks C to J). The other standards were ovalbumin (45K), chymotrypsinogen (24K), β-lactoglobulin (18.4K), and cytochrome *c* (12.5K).

which contains *tetA* and *tetB* were also examined. Mutants pDU1120, pDU1119, and pDU1116 with Tn*5* located in the *tetB* region (Fig. 2) failed to direct the synthesis of the 36K TET protein (Fig. 4, tracks E to J). In each case, smaller tetracycline-inducible polypeptides, which are presumed to be truncated derivatives of the 36K protein, were detected (Fig. 4). The size of the shortened TET protein increased with the distance of the Tn*5* insertion from the promoter. Thus, pDU1120, pDU1119, and pDU1116 specified polypeptides of 18, 29, and 32K, respectively. Plasmid pDU1117 contained Tn*5* located in the *tetA* gene (Fig. 2). This plasmid also failed to synthesize the 36K TET protein (Fig. 4, tracks C and D), but there was no detectable loss of other pDU938-coded proteins.

Effect of mutations in *tetA* and *tetB* on tetracycline uptake and minocycline resistance. The expression of Tn*10*-mediated resistance to tetracycline involves drug exclusion (2–5, 23–26, 30).

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This was confirmed by showing that plasmids R100, R100-1, and pDU301 accumulated only 16, 28, and 20%, respectively, of the plasmid-free control strain (Table 3). Transport studies were performed on only two point mutants in the *tetB* region (*tet-4* and *tet-6*), but both mutations affected tetracycline exclusion (Table 3). With the exception of *tet-15*, all mutations in the *tetA* region abolished or impaired the ability of bacteria to exclude tetracycline (Table 3). The anomalous behavior of *tet-15* in the tetracycline transport assay is considered below.

Point mutations in either *tetA* or *tetB* lowered minocycline resistance (data not shown). In some cases, resistance was totally eliminated as in mutants *tet-3*, *tet-9* (mapping in *tetA*) and *tet-4* (mapping in *tetB*).

DISCUSSION

The genetic complementation tests suggest that there are two structural genes involved in the expression of Tn*10*-encoded tetracycline resistance. Both structural genes (*tetA* and *tetB*) are contained within the 1,275-bp *Hin*cII fragment in the *tet* region. The genes are located at proximal (*tetA*) and distal (*tetB*) positions from the *tet* promoter (Fig. 2). These findings are in agreement with those recently published by Curiale and Levy (7).

We presume that the *tetB* gene codes for the 36K TET protein because it maps in the region known to encode TET protein (20, 33) and Tn*5* insertions in *tetB* abolish the synthesis of the 36K protein (Fig. 4). These insertions lead to shortened variants of TET protein which probably arise by premature termination of translation at nonsense codons within the first 30 bp of the outer ends of the Tn*5* inverted repeats (H. Schaller, personal communication). Point mutations within *tetB* (*tet-4* and *tet-6*) affect tetracycline exclusion (Table 3), which implies that TET protein has a role in energy-dependent tetracycline efflux. This conclusion is also consistent with the location of this protein in the cytoplasmic membrane (10, 23), which is the site of energy-dependent transport processes.

The approximate location of the beginning of the *tetB* gene can be deduced from the sizes of the truncated polypeptides and from the position of Tn*5* insertions in pDU938. The estimations that follow were calculated, assuming that the average molecular weight of an amino acid is 120. Plasmids pDU1119 and pDU1116 specified peptides of 29 and 32K, respectively, which would be encoded by 725 and 800 bp of DNA. The Tn*5* insertions in these plasmids were 1,095 and 1,205 bp from the promoter-proximal *Hin*cII site, suggesting that the beginning of *tetB* is 370 to 405 bp from this site. This conclusion is strengthened by the report that a deletion which removes DNA to the right of the 1,275-bp *Hin*cII fragment shortens the 36K protein by 2,000 daltons (20). Thus, 50 bp of the *tetB* coding sequence spans the right *Hin*cII site. In this case, the estimate for the beginning of *tetB* is 425 bp from the *Hin*cII site in the promoter region.

The data obtained with pDU1120 seem to contradict the above conclusions. Here the Tn*5* element is located either 610 or 665 bp from the promoter-proximal *Hin*cII site and causes the formation of an 18K truncated protein. This would place the beginning of *tetB* 270 to 315 bp from the right *Hin*cII site. However, there is some doubt about the precise location of this insertion, and it is possible that up to 1,000 daltons could be added to the carboxy terminus of the *tetB* fragment as a result of the translation of adjacent Tn*5* sequences.

The arrangement of *tetA* and *tetB* in relation to the *tet* promoter (Fig. 2) implies that they are expressed sequentially in an operon. The complementation that occurred between some of the *tet* mutations provides further evidence for the *tet* operon model. In each case, complementation tests were performed between *tet* point mutant R100 derivatives and Tn*5* insertion mutants of pDU938. All of the point mutations which mapped in the promoter-proximal 550 bp of the 1,275-bp *Hin*cII fragment (with the exception of *tet-13*, *tet-14*, and *tet-5*) were complemented by distal *tetB* insertion mutations carried by pDU1116 and pDU1120. Thus, pDU2, pDU3, pDU7, pDU8, pDU9, pDU11, and pUB261 each have point mutations in *tetA*.

Two of the mutations which mapped in deletion interval 1 (*tet-13* and *tet-14*) did not complement the *tet*::Tn*5* mutants. It is possible that they have lesions in the *tet* promoter rather than in the structural gene. Two of the complementing point mutations mentioned above (*tet-7* and *tet-9*) also mapped in deletion interval 1 and are thus presumed to have lesions within the coding sequence of *tetA*. DNA sequence analysis (K. Bertrand, personal communication) has indicated that an open reading frame for a potential *tet* structural gene (possibly *tetA*) starts 60 bp from the *Hin*cII site in the promoter and would thus fall within deletion interval 1.

The complementation behavior of the two mutations that map in deletion interval 9 suggests that *tetA* ends and *tetB* begins in this region. Thus, *tet-8* complemented the distal Tn*5* insertions and could affect the carboxy terminus of the *tetA* gene product, whereas *tet-15* did not complement the distal *tet*::Tn*5* mutants and could alter the amino terminus of the *tetB* protein.

The simplest interpretation of these data is that *tetA* and *tetB* are two sequentially transcribed and translated genes and that the distal part of *tetA* occurs in deletion interval 9, 410 to 550 bp from the promoter-proximal *Hin*cII site.

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If the coding sequence for *tetA* begins 60 bp from the *Hin*cII site, then at least 350 bp of DNA is available up to the beginning of deletion interval 9. This could encode a polypeptide of about 17K. Alternatively, the distal part of *tetA* and the proximal region of *tetB* might overlap, a situation that may also exist in the *tet* region of pBR322 (31).

The Tn*5* insertion in *tetA* that produced plasmid pDU1117 led to the loss of the 36K protein (Fig. 4). Here, Tn*5* is thought to be inserted in *tetA* and prevents the expression of *tetB* because of the strong polar effects characteristic of transposon insertion mutations (21). We hoped that the use of pDU1117 in minicells would identify the *tetA* product, but unfortunately pDU1117 and the parent plasmid pDU938 apparently differed only in their ability to synthesize TET protein. This could imply that the *tetA* protein is produced only in small amounts.

Although we failed to detect the *tetA* product, it, like the TET protein, is required for the expression of tetracycline exclusion. Thus, with the exception of *tet-15*, all other point mutations in the *tetA* region affected exclusion (Table 3). Since a fluorimetric assay for tetracycline transport was adopted, the anomalous behavior of *tet-15* could result from the synthesis of a mutant TET protein that decreases the fluorescence quantum yield, thus apparently indicating normal tetracycline exclusion. Although minocycline resistance does not involve drug exclusion (5, 30), the expression of this phenotype also requires the *tetA* and *tetB* products. These results suggest a complex interaction between the gene products, probably at the level of the cytoplasmic membrane, whereby they cooperate functionally to express resistance both to tetracycline and minocycline. Curiale and Levy (7) also conclude that there is a cooperative relationship between the *tetA* and *tetB* products.

Intergenic complementation is expected to generate a wild-type phenotype, which in this case would produce tetracycline resistance levels in excess of 50 μg/ml. At best, the increase in resistance was six-fold higher than in the controls with a single mutant plasmid and was usually only two- to threefold higher. It is conceivable that the small increases in resistance represent intragenic complementation, such as that which sometimes occurs between different point mutations in a gene encoding a subunit of a homomultimeric protein (11). However, intragenic complementation usually occurs between a small proportion of missense point mutations and would rarely involve deletion or translation-terminating mutations (11). The failure to obtain wild-type phenotypes is explicable if the two *tet* gene products interact cooperatively in the cytoplasmic membrane to generate tetracycline resistance. It is possible that this can be achieved only if the two polypeptides are translated from the same mRNA molecule. Another factor that could have contributed to the low level of resistance is the different copy numbers of the complementing plasmids. R100 exists at one to two copies per chromosome (29), whereas pDU938 (a derivative of pUB5572) has six to eight copies per chromosome (4). The dominance exhibited by some Tc^s mutations (13) might possibly interfere with the level of resistance generated by complementation, but both dominant and recessive point mutants complemented to similar levels.

One further comment that can be made about the Tc^s point mutants in the light of the mapping data is the apparent correlation between the dominant phenotype (13) and map position. With the exception of translation-terminating mutations, such as nonsense (pDU10) and the Tn*5* insertions, all dominant mutants map distal to deletion interval 5. Thus, recessive missense point mutations map in the *tetA* gene. This indicates that dominance is a manifestation of wild-type *tetA* expression in conjunction with a mutant *tetB* protein.

ACKNOWLEDGMENTS

This work was partially supported by project grants from the United Kingdom Medical Research Council to I.C.

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