JOURNAL OF BACTERIOLOGY, May 1982, p. 633–642
0021-9193/82/050633-10$02.00/0

Vol. 150, No. 2

A Multifunctional Gene (*tetR*) Controls Tn*10*-Encoded Tetracycline Resistance

CHRISTOPH F. BECK,†* RUPERT MUTZEL, JORDI BARBÉ,‡ AND WOLFRAM MÜLLER

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, West Germany

Received 3 September 1981/Accepted 20 December 1981

The tetracycline resistance regulatory gene (*tetR*) of transposon Tn*10* was analyzed by a combination of methods involving gene fusion and cloning. This gene is located on a 695-base pair *Hin*cII DNA fragment near the center of Tn*10*. The direction of transcription is opposite to that of neighboring gene *tetA*, which encodes the TetA protein. The gene product of the *tetR* gene (the TetR protein) has a molecular weight of 23,000. *tetR-lacZ* gene fusions encode fusion β-galactosidases that are membrane bound, indicating that the TetR protein itself is membrane associated. Mutants defective in *tetR* result in constitutive tetracycline resistance, but the level of resistance is reduced. Expression of the *tetR* gene is induced by tetracycline; in the absence of tetracycline, the TetR protein turns off its own synthesis.

The widespread use of antibiotics belonging to the tetracycline family has resulted in a rapid spread of resistance against tetracycline in bacteria of clinical interest and concern. In most, if not all, cases resistance in previously susceptible organisms involves the acquisition of new genetic information through plasmids.

To date, at least four different groups of genes responsible for tetracycline resistance in gram-negative bacteria have been analyzed (23). One feature of nearly all of these resistance determinants appears to be their inducibility by the antibiotic (22, 23, 28, 29, 37, 38).

The mechanism of tetracycline resistance has not been elucidated yet in detail (8), although resistance is at least partly due to an active efflux mechanism for tetracycline (4, 22).

The resistance system that has been analyzed in greatest detail is the system encoded by transposable element Tn*10*. Physical mapping with restriction endonucleases has been used to define the structure of the transposon and the location of the tetracycline resistance region (10, 15, 17). Jorgensen and Reznikoff (15) located one gene that is essential for tetracycline resistance on a 1,275-base pair (bp) *Hin*cII fragment of Tn*10* (Fig. 1); we have designated this gene *tetA*. Evidence for the existence of a regulatory gene for tetracycline resistance was provided by mutant analyses, gene fusions, and regulatory studies. From the phenotypes of deletion mutants generated by restriction enzymes, it was concluded that the regulatory region is located on the left side of the *tetA* gene (10, 40), as shown in Fig. 1. It was deduced from studies with *tet-lacZ* fusion mutants that the *tet* regulatory region is involved in the regulation of at least two *tet* genes (6). Zupancic et al. (42) detected two Tn*10*-encoded proteins (molecular weights, 36,000 and 25,000) whose syntheses are induced by tetracycline. Regulatory mutants of Tn*10* that express tetracycline resistance constitutively have been isolated (10–12). Most of the data available are consistent with a model of negative control (6, 40, 41), although the observation that regulatory mutants of Tn*10* often have a dominant constitutive phenotype may indicate that a more complex type of control exists (11). To investigate the regulation of the tetracycline resistance of Tn*10* in detail, we isolated mutants defective in the regulation of resistance. We concluded that partial deletion of regulatory gene *tetR* causes a reduction in tetracycline resistance. This gene is repressed by its own product in the absence of tetracycline but is induced by the antibiotic. The *tetR* gene product also exhibits properties which indicate that it is a membrane protein. Some of these conclusions are supported by the results of Wray et al. (40).

† Present address: Institut für Biologie III, Schänzlestrasse 1, D-7800 Freiburg, West Germany.

‡ Present address: Universidad Autónoma de Barcelona, Facultad de Ciencias, Departamento de Microbiologia, Bellaterra, Barcelona, Spain.

MATERIALS AND METHODS

Chemicals and media. Tetracycline hydrochloride, isopropyl-β-D-thiogalactopyranoside, o-nitrophenyl-β-D-galactopyranoside, and the protein molecular weight standards ovalbumin, α-chymotrypsinogen, and lysozyme were purchased from Sigma Chemical

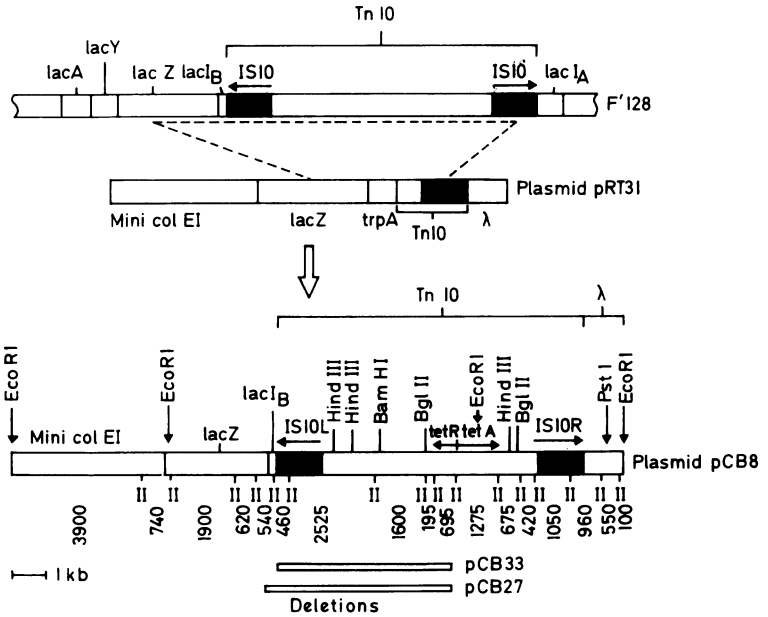

FIG. 1. Construction and restriction map of plasmid pCB8 from F'128 and pRT31 by recombination between regions of homology (indicated by the dashed lines). The following enzymes were used to determine the restriction map for pCB8: *Eco*RI, *Hin*dIII, *Bgl*II, *Bam*HI, and *Hin*cII. The numbers between the *Hin*cII sites (II) are distances (in base pairs). The open bars under pCB8 indicate the extents of DNA deleted in pCB8 derivatives pCB27 and pCB33. kb, Kilobase.

Co. L-[^{35}S]methionine was obtained from Amersham Corp.

AB medium (9) supplemented with a carbon source (concentration, 0.2%) was used as the minimal medium. The complex media used were 0.8% nutrient broth (Difco Laboratories), LB (13), and DYT (24). Solid media contained 0.2% agar (Difco).

Bacterial strains and plasmids. The bacterial strains used are described in Table 1. Plasmid pCB8 (Fig. 1) was constructed by homogenote formation between pRT31 and F'128, which has Tn*10* inserted into the *lacI* gene of a *lacI-Z* fusion (6). pRT31 is a derivative of pRT24 (15) in which the *Hin*dIII-*Kpn*I fragment is replaced by a *Hin*dIII-*Kpn*I fragment carrying the *trp-lacZ* fusion from λ p*trp-lac* deletion derivative W205 (5; R. Jorgensen, personal communication). pRT31 encodes active β-galactosidase but not tetracycline resistance. Recombination between pRT31 and F'128 occurred in strains harboring both plasmids simultaneously, and a pRT31 derivative carrying *lacZ* and Tn*10* was selected by using the plasmid DNA of this strain to transform strain CB40 to tetracycline resistance.

Only plasmids with the structures shown in Fig. 1 for pCB8 were selected in transformation. F'128 is a large plasmid which did not survive the DNA purification procedures. Plasmid pCB13 was derived from pKC7 (27) (see Fig. 3), and pCB38 is a derivative of pKB166 (3) which lacks the *Hin*cII fragment but contains the *tetR* gene of Tn*10* as an *Eco*RI-*Bgl*II fragment. Plasmid pCB57 was derived from pDF41 (16) by cloning the *tet* region of pCB27 as *Eco*RI fragments into the *Eco*RI site. All other plasmids were derived from pCB8 as described below.

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories (*Bgl*II, *Hin*dIII, *Hin*cII) or New England Biolabs (*Eco*RI, *Kpn*I, *Pst*I). T4 DNA ligase was obtained from Boehringer Mannheim.

Preparation of DNA and transformation. Plasmid DNA was prepared from 5-ml cultures grown in DYT by using the procedure of Birnboim and Doly (7). When DNA was prepared from 100-ml cultures, a purification step involving an ethidium bromide-cesium chloride gradient and phenol extraction (32) was added. Transformation was carried out as described by Lederberg and Cohen (20).

Polyacrylamide gel electrophoresis of DNA. The modified E-buffer system used (33) contained 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA. The pH was adjusted to 8.3 with acetic acid. Gels contained 7.5% acrylamide (Serva) and 0.5% bisacrylamide (Serva). DNA fragments were stained for photography under UV light by immersing the gel in a solution containing 10 μg of ethidium bromide per ml for 5 min.

Preparation and labeling of minicells. Derivatives of strain DS410 harboring plasmids were grown with aeration at 37°C in 200 ml of LB medium containing 0.4% glycerol instead of glucose. Minicells were purified at room temperature by the procedure of Larson et al. (19), with the following alterations. The whole cells and minicells were suspended in 4 ml of TNE buffer (50 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, 1 mM EDTA) and were layered over a 25-ml sucrose step gradient (10 ml of 20% sucrose in TNE buffer, 8 ml of 12.5% sucrose in TNE buffer, 7 ml of 5% sucrose in TNE buffer), which was centrifuged at 7,500 rpm (without braking) for 10 min in a Sorvall

TABLE 1. Bacterial strains used[a]

Strain	Genotype	Source
CB40	Δ(*lacZ-lacY*$^+$) *thi rspL recA56*	Derivative of W4680[b]
CB44	Δ(*lacZ-lacY*$^+$) *thi rspE srlA300*::Tn*10*	Derivative of W4680[b]
CB46	Δ(*lacZ-lacY*$^+$) *thi rspE recA56*	Derivative of W4680[b]
CB68	CB40(pCB8)	
CB117	CB46(pCB33)	
CB118	CB46(pCB27)	
CB131	CB46(pCB13)	
CB164	CB46(pCB29)	
CB215	CB46(pCB35)	
CB218	DS410(pCB38)	
CB248	CB46(pCB57)	
DS410	*minA minB thi lacY mtl xyl rspL tonA*	J. Reeve[c]

[a] The nomenclature is that of Bachmann and Low (2).

[b] Strain W4680 carries a nonpolar deletion mutation (*lacZ 4680*) and was isolated and characterized by J. Beckwith.

[c] Strain DS410 is a minicell-producing strain of *E. coli*.

HB-4 rotor in a Sorvall RC-5 centrifuge. The upper 6 to 8 ml of the minicell band was withdrawn from the gradient with a 10-ml pipette and centrifuged at 10,000 rpm for 20 min in a Sorvall SS34 rotor. The minicells were suspended in about 3 ml of AB medium supplemented with 0.4% glycerol at an absorbancy at 578 nm of 1. The minicell suspension (1 ml) was incubated for 10 min at 37°C, and labeling of the proteins was initiated by adding 50 μCi of [^{35}S]methionine (specific activity, 1,400 Ci/mmol). Incubation was continued for 60 min with shaking, and incorporation was stopped by adding 3 ml of LB medium containing 50 μg of L-methionine per ml. The minicells were pelleted, washed with 4 ml of TNE buffer, and suspended in 100 μl of sample solution containing 3% sodium dodecyl sulfate, 100 mM dithiothreitol, and 20% glycerol. The samples were prepared for electrophoresis by heating for 20 min at 60°C. Samples (30 μl) were applied to a sodium dodecyl sulfate-polyacrylamide gel.

Electrophoretic separation of proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Schumacher and Bussmann (31), using the Laemmli buffer system (18). The samples were electrophoresed through a 22% acrylamide slab gel and a 5% stacking gel at a constant current of 18 mA for 16 h. The gels were stained and destained as described previously (31). Ovalbumin (molecular weight, 43,000), maltose binding protein (26) (38,500), α-chymotrypsinogen (25,000), and lysozyme (14,300) were used as molecular weight standards.

Assays. In our β-galactosidase assays we used the assay conditions and unit definition described by Miller (24), except that the pH of Z-buffer was pH 7.5 and the buffer contained 0.1 mM phenylmethylsulfonyl fluoride. NADH oxidase was measured as described by Osborn et al. (25); 1 U of enzyme activity was defined as a change in optical density at 340 nm of 0.001 per milliliter per minute at a light path length of 1 cm.

Preparation of cell membranes and fractionation of inner and outer membranes. Preparation and fractionation of cell membranes by sucrose density gradient centrifugation were done by the method of Osborn et al. (25). At all steps after cell growth, temperatures were kept below 4°C, and the protease inhibitor phenylmethylsulfonyl fluoride was added to all solutions to a concentration of 0.1 mM, since enhanced proteolytic lability has been reported for fusion proteins (14).

Measurement of tetracycline resistance levels. Resistance was measured by determining the tetracycline concentration that caused a 50% reduction in colony-forming units. The cells in cultures growing exponentially in nutrient broth without tetracycline (uninduced) or in nutrient broth supplemented with 1 μg of tetracycline per ml (induced) were harvested at a turbidity equivalent to an optical density at 578 nm of 0.4, centrifuged, and suspended in an equal volume of AB medium. The number of colony-forming units was determined by plating serial dilutions onto nutrient agar plates containing tetracycline at concentrations varying from 0 to 120 μg/ml. These plates were incubated at 37°C for 24 h.

RESULTS

Isolation of *tetR-lacZ* gene fusions. The tetracycline resistance (*tet*) genes of transposon Tn*10* are located in the central part of the transposon. One of these genes was defined and characterized by fusing it with the gene encoding β-galactosidase, *lacZ*. The genetic construction used for the isolation of these mutants is shown in Fig. 1. Plasmid pCB8 carries Tn*10* within the *lacI* gene of a *lacI-Z* fusion (6). Strains harboring this plasmid did not synthesize β-galactosidase because there are no start signals for transcription and translation located between the end of Tn*10* and the start of *lacZ*. The absence of β-galactosidase synthesis also indicated that these signals cannot be provided by the adjacent base sequences of the transposon.

Plasmid pCB8 was transferred into strain CB40, which carries a nonpolar deletion in *lacZ* and a functional lactose permease (*lacY*) gene. Mutants that were able to synthesize β-galactosidase and to grow on lactose as a source of carbon were isolated at a frequency of about 10^{-9} after incubation for 14 days at 37°C. In the majority of these mutants *lacZ* was fused to another gene by deletion of the DNA between genes located on Tn*10* and *lacZ*. The start signals for transcription and translation of *lacZ* were provided by the fused gene, and only genes transcribed in the same direction as *lacZ* could be fused. One class of Lac$^+$ mutants which retained partial tetracycline resistance was investigated in detail. Seven independent mutants of this type were isolated; the two described below (pCB27 and pCB33) (Fig. 1) represent typical examples.

Mapping of deletion endpoints. To locate the gene to which *lacZ* was fused, the extent of the DNA deleted in the fusion mutants was determined by mapping with restriction endonucleases. A restriction map of plasmid pCB8 was constructed from the previously published restriction maps of Tn*10* (15, 17) and our own results (Fig. 1). DNA fragments of the parental and mutant plasmids generated by cleavage with *Hin*cII were separated by electrophoresis on polyacrylamide slab gels (Fig. 2). Of the 16 DNA fragments present in pCB8, 5 were missing in both mutant plasmid pCB27 and mutant plasmid pCB33 (the 2,525-, 1,600-, 695-, 460-, and 195-bp fragments) (Fig. 2). Mutant plasmid pCB27 also lacks the 540-bp fragment but has a new 280-bp fragment, and plasmid pCB33 has a new *Hin*cII fusion fragment about 160 bp long. From the sequence of the fragments on the parental plasmid, we concluded that in both mutants the deletions eliminated the DNA from the beginning of the *lacZ* gene up to and including the 695-bp fragment in the center of Tn*10* (Fig. 1). The 1,275-bp fragment, which is located to the right of the 695-bp fragment, is intact in both mutants. Thus, the *Hin*cII site separating these fragments must also be preserved. From the small size of the new fusion fragments (280 and 160 bp in pCB27 and pCB33, respectively), we concluded that the deletion endpoints in both mutants must be located in the right half of the 695-bp fragment (Fig. 1). The deletion endpoints on the *lacZ* side differ in the two mutant plasmids. In pCB27 some *lacZ* DNA must be deleted because the 540-bp fragment is missing. From the mapping data we concluded that a gene is located on the 695-bp *Hin*cII fragment of Tn*10* and that this gene is transcribed toward IS*10*L. For reasons described below, this Tn*10* gene is referred to as *tetR*.

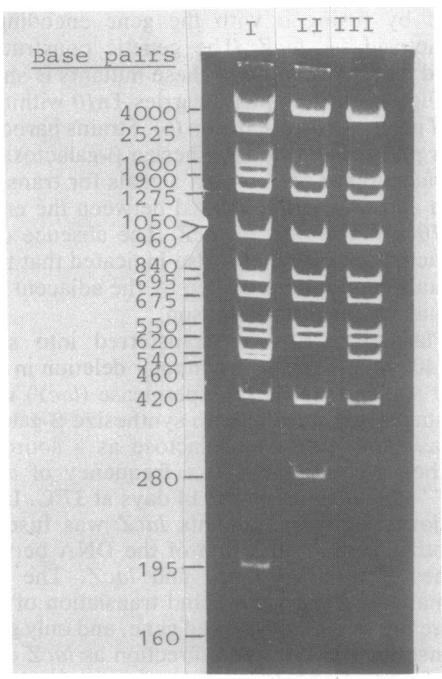

FIG. 2. DNA fragments of pCB plasmids separated on a 7.5% polyacrylamide slab gel after cleavage with *Hin*cII. The sizes of the fragments were determined after electrophoretic separation in a 1.6% agarose gel. Track I, pCB8; track II, pCB27; track III, pCB33.

Phenotype of *tetR-lacZ* fusion mutants. Varying degrees of tetracycline resistance were conferred on the host bacteria by the mutant plasmids, but the levels of resistance were significantly reduced compared with the resistance conferred by the parental plasmid, pCB8 (Table 2). Moreover, tetracycline resistance in strains CB117 and CB118, which harbor mutant plasmids, was no longer inducible by pregrowth in the presence of low tetracycline concentrations (Table 2). The constitutive expression of tetracycline resistance and the lowered level of resistance are probably caused by the partial destruction of the *tetR* gene by fusion to *lacZ*. The reduced level of tetracycline resistance in *tetR*$^-$ strains might be due to the multicopy effect (10) elicited by increased copy numbers of the deletion mutant plasmids. However, enlargement of pCB27 to approximately the size of parental plasmid pCB8 (by insertion of a 4,500-bp DNA fragment into the *Pst*I site of pCB27) did not result in an increase in tetracycline resistance (data not shown). To test whether a defective *tetR* gene affected the level of tetracycline resistance when it was present on a low-copy-number vector, the entire *tet* gene region of pCB27 was cloned on F factor-derived plasmid pDF41 (16), resulting in plasmid pCB57 (data not shown). The levels of resistance caused by pCB57 (CB248) were below those conferred by pCB27 or those conferred by one intact Tn*10* region on the chromosome of an isogenic strain (CB44) (Table 2).

The regulation and rate of expression of *tetR* were estimated by measuring the activity of β-galactosidase synthesized by the fusion mutants. The β-galactosidase activity synthesized by the mutant plasmids varied greatly (Table 2). In the best case (plasmid pCB33), the activity encoded by the multicopy plasmid was similar to the activity in a fully induced strain carrying one copy of the lactose operon per chromosome. The β-galactosidase activities of these mutants (strains CB117 and CB118) could not be induced by adding tetracycline (Table 2), demonstrating that *tetR* was not inducible by the antibiotic in these mutants.

In strains carrying pCB27 (Table 2, strain

TABLE 2. Tetracycline resistance phenotypes and β-galactosidase activities of *tetR-lacZ* fusion mutants

Strain	Plasmid carried[a]	Tetracycline resistance[b]		β-Galactosidase sp act (U)	
		Uninduced	Induced[c]	Uninduced[d]	Induced[e]
CB68	pCB8	42	102	0	ND[f]
CB117	pCB33	14	14	2,264	2,289
CB118	pCB27	28	28	252	237
CB248	pCB57	ND	17	ND	ND
CB44	None	ND	78	ND	ND
CB164	pCB29	18	25	37	163
CB215	pCB35	9	15	14	228
CB131	pCB13	<1	ND	ND	ND

[a] Plasmids pCB33 and pCB27 were *tetR-lacZ* fusions, and plasmids pCB29 and pCB35 were derivatives of pCB27 containing a wild-type *tetR* gene.

[b] Tetracycline resistance is expressed as 50% efficiency of plating (i.e., the tetracycline concentration [in micrograms per milliliter] which resulted in a 50% reduction in colony-forming units).

[c] Induction was carried out by growing the cells before plating in medium containing 1 μg of tetracycline per ml.

[d] Uninduced cells were grown in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside.

[e] Cells were induced by growing them in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside and 1 μg of tetracycline per ml.

[f] ND, Not done.

CB118) β-galactosidase activity occurred only when the chromosomal lactose operon, which contains an internal nonpolar *lacZ* deletion mutant, was also induced. Thus, the activity observed reflected complementation between the fusion β-galactosidase and the β-galactosidase fragment encoded by the mutated chromosomal *lacZ* gene.

Cloning of the *tetR* gene. The next experiment was designed to determine whether the regulation of tetracycline resistance could be restored when the *tetR* gene was inserted into mutant plasmid pCB27 at a different site. It is known that the *tet* genes do not extend to the left of the *BglII* site at the center of Tn*10* (10, 15) (Fig. 1). The DNA inserted into pCB27 contains Tn*10* sequences starting at this *BglII* site and extending to the right. The constructions involved are shown in Fig. 3. In the first step a *BglII-EcoRI* fragment of pCB8, which contains the 695-bp *HincII* fragment (Fig. 1) was cloned in plasmid pKC7. The resulting plasmid, pCB13, was cut with *PstI*, and the fragment containing the Tn*10* DNA was ligated into the *PstI* site of plasmid pCB27. Restriction enzyme analysis showed that plasmid pCB35 carried an intact *PstI* fragment derived from pCB13, but that pCB29 contained a spontaneous deletion mutation that eliminated one *PstI* site and some neighboring sequences.

Effect of the *tetR* gene on the phenotype of *tetR-lacZ* fusion mutants. The tetracycline resistance phenotypes conferred on host strains by the plasmids studied are listed in Table 2. The *tetR* gene by itself (on plasmid pCB13) did not cause resistance. Introduction of *tetR* into the mutant plasmids led to a further reduction in tetracycline resistance, but resistance was clearly and reproducibly inducible in these strains (CB164 and CB215). The reasons for the reduced levels of resistance caused by introduction of an intact *tetR* gene are discussed below.

In the absence of induction with tetracycline, the *tetR-lacZ* fusion plasmids which carry the wild-type cloned *tetR* gene (pCB29 and pCB35) produced levels of β-galactosidase significantly lower than the constitutive levels expressed by fusion plasmids lacking *tetR* (pCB27). The addition of tetracycline led to induction of β-galactosidase synthesis (Table 2). The *tetR* gene located on the newly inserted fragment in plasmids pCB29 and pCB35 repressed expression of the *tetR* gene fused to *lacZ*. We concluded that *tetR* expression is autoregulated. This expression is repressed in the absence of tetracycline and is induced in the presence of this drug. The regulatory signal encoded by *tetR* is acting in *trans*, since its new location is outside Tn*10*, albeit on the same plasmid (Fig. 2).

Protein encoded by *tetR*. The proteins expressed by the plasmids were examined in *Escherichia coli* minicells. Plasmid pCB8 encoded two tetracycline-inducible proteins (Fig. 4). One of these was the previously identified TetA protein (molecular weight, 36,000), and the other inducible protein had a molecular weight of 23,000. The 23,000-dalton protein was not synthesized by minicells containing *tetR-lacZ* fusion plasmid pCB27 (Fig. 4, track 4), but a fusion plasmid carrying the cloned *tetR* gene (pCB38) did encode this protein. As expected, minicells containing pCB35 (with *tetR* cloned in pCB27) also synthesized the 23,000-dalton protein. Since no other proteins encoded by the

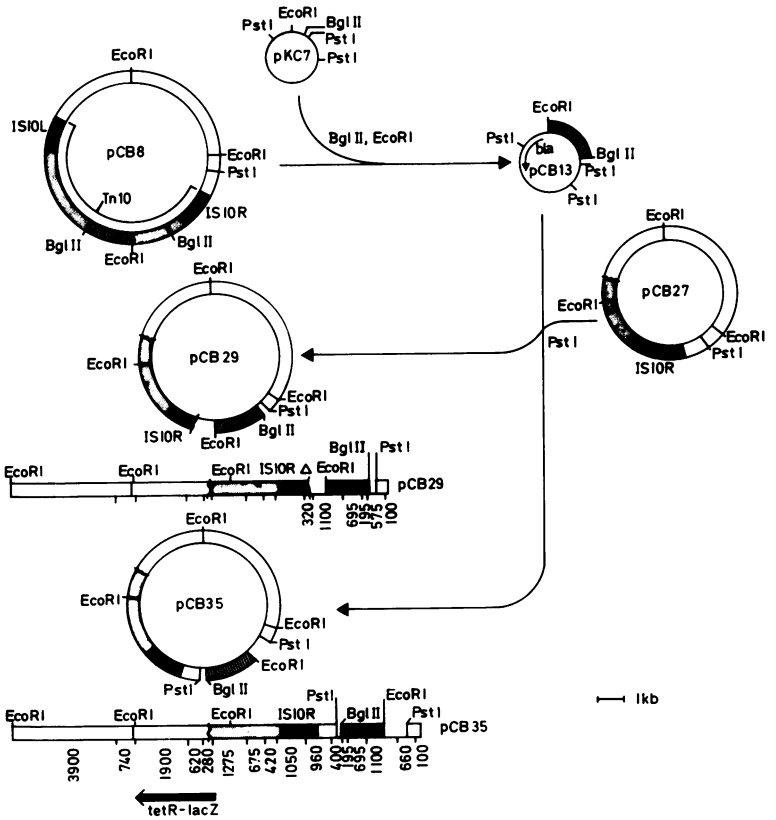

FIG. 3. Construction of *tetR-lacZ* fusion plasmids carrying a second, intact *tetR* gene region. The plasmids are drawn to scale, and the relevant restriction enzyme cleavage sites are indicated. The wavy lines indicate points of fusion. The numbers below the linear maps of pCB29 and pCB35 indicate the locations of *Hin*cII fragments (in base pairs). The extent of the fused *tetR-lacZ* genes is shown below pCB35. kb, Kilobase.

Tn*10* DNA fragment cloned on pCB38 were evident on the gel and since this protein was missing in *tetR*$^-$ mutants, we concluded that the 23,000-dalton protein is the product of the *tetR* gene.

The synthesis of the TetA protein (the product of the *tetA* gene [15, 41]) could be induced in minicells harboring pCB8 (Fig. 4). This protein was synthesized constitutively in minicells harboring *tetR-lacZ* mutant plasmid pCB27, although its level was significantly reduced compared with fully induced pCB8-harboring minicells. The high-molecular-weight proteins encoded by pCB27 and pCB35 presumably represented fusion β-galactosidase.

Cellular location of the β-galactosidase encoded by the *tetR-lacZ* fusion plasmids. In fusion mutants of the protein fusion type, the presence of β-galactosidase molecules in particular cellular fractions can provide information about the cellular location of the protein to which β-galactosidase is covalently attached (34). In six of seven independently isolated *tetR-lacZ* fusion mutants between 30 and 70% of the fusion β-galactosidase activity was associated with the membrane fraction of the cells. The analysis by sucrose gradient centrifugation was performed as described by Osborn et al. (25), and 32% of the total β-galactosidase activity of strain CB118 (carrying pCB27) was recovered after some initial purification steps in the membrane fraction applied to the top of the sucrose gradient. In contrast, strain CB117 (carrying pCB33) yielded 3% of the total activity in the membrane fraction, indicating that strain CB117 encoded a cytoplasmic fusion β-galactosidase. The results of the analysis of strain CB118 by sucrose gradient centrifugation are shown in Fig. 5. Whereas wild-type β-galactosidase only entered the upper part of the gradient, the activity of the fusion β-galactosidase sedimented with fractions containing the inner membrane (identified by the membrane-bound NADH oxidase), as well as heavier fractions of mixed inner and outer membranes. Since we obtained similar results with different *tetR-lacZ* fusions, we concluded that *tetR* en-

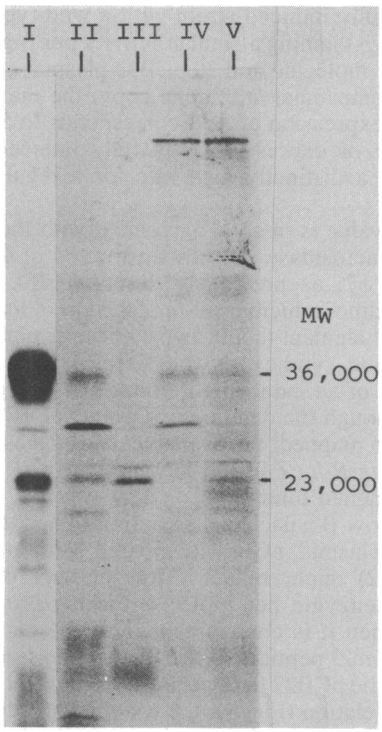

FIG. 4. Autoradiogram of a 22% polyacrylamide gel displaying [^{35}S]methionine-labeled proteins encoded by pCB plasmids in minicells. Track I, pCB8 induced with 1 μg of tetracycline per ml; track II, uninduced pCB8; track III, uninduced pCB38; track IV, pCB27; track V, uninduced pCB35. MW, Molecular weight.

codes a membrane-associated protein. In the fusions, the part of the TetR protein responsible for membrane association is covalently linked to β-galactosidase. Membrane association of the TetR protein could also be demonstrated in minicells (data not shown).

DISCUSSION

Deletions in which Tn*10* DNA sequences encoding tetracycline resistance were partially removed were generated by selecting for mutants in which Tn*10* sequences were fused to *lacZ*. Cells harboring ColE1-derived plasmids carrying such mutations exhibited constitutive tetracycline resistance, although the level of resistance was lower than the level specified by cells harboring plasmids with intact Tn*10* sequences. When tested in a minicell system, these mutant plasmids no longer coded for the synthesis of a 23,000-dalton protein. This protein is presumably identical to the Tn*10*-encoded, tetracycline-inducible 23,000- and 25,000-dalton polypeptides observed previously (40, 42). The TetA protein (the product of *tetA*) was synthesized constitutively by minicells containing these mutant plasmids, although the amounts observed were significantly lower than the amounts produced by induced minicells containing an intact Tn*10*-carrying plasmid (Fig. 4). When the *tetR* gene was reinserted at another site in a *tetR-lacZ* fusion mutant plasmid, the parental phenotype was restored with respect to inducibility of both tetracycline resistance (Table 2) and TetA protein synthesis (data not shown), but the induced and uninduced levels of resistance and TetA protein remained low. No definite reason for this lowered resistance can be given at present. Reduced levels of resistance and TetA protein have been observed previously in cells harboring Tn*10* on a multicopy plasmid (10). The multicopy effect may explain the low expression of tetracycline resistance observed in strains

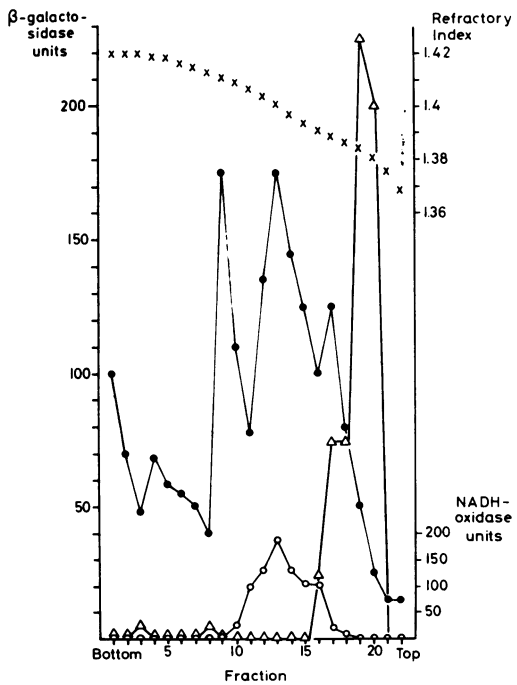

FIG. 5. Fractionation of inner and outer membranes by sucrose density gradient centrifugation of membranes prepared from strain CB118, which carries plasmid pCB27. Membranes from a 100-ml culture grown to an optical density at 578 nm of 0.35 were prepared and separated as described in the text. As a control, commercial β-galactosidase (Boehringer Mannheim) was added to membranes of a *lacZ*$^-$ strain before density gradient centrifugation. Symbols: ● and ○, β-galactosidase and NADH-oxidase activities of strain CB118, respectively; △ activity of commercial β-galactosidase; ×, refractive index.

harboring a wild-type *tetR* gene on *tetR-lacZ* fusion plasmids (pCB29 and pCB35), since these plasmids also encode a TetA protein fragment (from the promoter-proximal part of *tetA* cloned together with *tetR*). In addition, membrane-associated fusion β-galactosidases competing with the wild-type TetR protein for insertion sites in the membrane may cause reduced resistance.

We can say with some certainty that *tetR* constitutes a single gene and that the 23,000-dalton TetR protein is the regulator of the *tet* genes. From the minicell experiments, we know that the cloned *tetR* gene on pCB38 encodes the 23,000-dalton TetR protein as its only major protein product (Fig. 4). Jorgensen and Reznikoff (15) and Wray et al. (40) showed that the *tet* genes do not extend toward IS*10*L beyond the *Hin*cII site separating the 695- and 195-bp fragments in the center of Tn*10* (Fig. 1) On the other side, the *tetA* gene, which is transcribed toward IS*10*R, starts at the *Hin*cII site separating the 695-and 1,275-bp fragments (15). Thus, the 695-bp *Hin*cII fragment contains the whole *tetR* gene and is just large enough to contain this gene, since approximately 630 bp should be required for TetR protein coding (assuming there are no overlapping genes). In a recent report Wray et al. (40) reached similar conclusions.

From these considerations and from the small size of the *Hin*cII fusion fragment in pCB33 (160 bp), we have deduced that the start of the *tetR* gene is close to the *Hin*cII site separating the 695- and 1,275-bp fragments. Thus, the *tetR* and *tetA* genes are neighboring genes that are transcribed in opposite directions. This order of regulatory and structural genes on Tn*10* is similar to the order found on plasmid pSC101, which harbors a different type of tetracycline resistance determinant (23). Recent data have indicated that in pSC101 the regulatory gene and the adjacent structural gene(s) (37) are transcribed in opposite directions from partially overlapping promoters (36).

Insertion elements are active in the formation of deletion mutations, and the termini of such elements have been shown to be preferred deletion endpoints (30, 35). In most of the *tetR-lacZ* fusion mutants, the deletion end-points on the *lacZ* side apparently do not coincide with the termini of the flanking IS*10*L element. This and the low frequency with which the fusion mutants arise (about 10^{-9}) argue against any involvement of the flanking IS*10* elements in the generation of the deletion mutations.

We can obtain a minimal estimate for the rate of *tetR* gene expression by measuring the β-galactosidase activity synthesized by *tetR-lacZ* fusions (6). Cells containing pCB33 exhibit a slightly higher β-galactosidase specific activity than a fully induced strain with a wild-type *lac* operon. Assuming a similar activity per β-galactosidase molecule and 20 pCB33 plasmid copies per chromosomal *lacZ* gene copy, the maximal rate of expression of *tetR* corresponds to 5% of the rate of expression of a fully induced *lac* operon, a distinctly high rate for a regulatory gene.

This value is in good agreement with the rate of β-galactosidase synthesis observed in fusion mutant φ7, as previously described (6). This tetracycline-inducible *tet-lacZ* fusion is located on an F′ element (about two copies per chromosome) and synthesizes β-galactosidase at 10% the rate of an induced chromosomal *lacZ* gene. Even though the deletion end-points of φ7 have not been mapped, the available data suggest that φ7 is a *tetR-lacZ* fusion in which the *tetR* gene has remained functional.

The low β-galactosidase activity encoded by fusion plasmid pCB27 compared with pCB33 (Table 2) might reflect a low activity of the pCB27 enzyme per molecule, which is active only when it is complemented by the chromosomal *lacZ* peptide. Since the amino-terminal part of the pCB27 *lacZ* gene was removed by the fusing deletion (Fig. 1), the enzyme encoded by the fused genes may require complementation by the α-fragment encoded by the chromosomal *lacZ* deletion of strain CB46 (39). Surprisingly, even this complex molecule is associated with the inner membrane of the cells (Fig. 5).

Our understanding of the regulatory properties of the TetR protein is complicated by the observation that this protein appears to be membrane associated. A similar, although unexplained, case is the *traJ* protein, which is located in the outer membrane of the cell envelope but is required for transcription of the *tra* genes (1).

The available data suggest that the TetR protein is itself involved in the resistance mechanism. Independently isolated *tetR-lacZ* fusion mutants exhibit levels of tetracycline resistance ranging from 2.5 to 36 μg of tetracycline per ml (Table 2; unpublished data). These differences in the level of tetracycline resistance argue for a residual TetR protein function which is associated with the fusion β-galactosidases and is required for expression of resistance. In individual mutants, the variation in resistance observed may reflect the extent of *tetR* DNA deleted or different quarternary structures of the fusion proteins or both. Also, the inducibility of *tetR* by tetracycline, the high rate of induced expression of *tetR*, and the membrane association of the *tetR* product may argue for an active role for the TetR protein in the mechanism of resistance. These results differ from recent reports of other workers, in which *tetR*$^-$ mutant strains were shown to exhibit a high level of constitutive

tetracycline resistance (10, 40). The reason for these discrepancies may rest in the differences in the genetic backgrounds of the strains used. Since chromosomal mutations which abolish the low-tetracycline resistance phenotype of *tetR*$^-$ mutants can be isolated easily (unpublished data), many *E. coli* strains may possess mutations that suppress the *tetR*$^-$ phenotype.

Two models can be proposed for the regulation of Tn*10*-encoded tetracycline resistance. The data which these models must explain are as follows: (i) TetR protein synthesis and TetA protein synthesis are induced by tetracycline (Fig. 4); (ii) substantial deletions which extend to various degrees into the DNA coding for the carboxyl end of the TetR protein result in lowered and constitutive levels of TetA protein and reduced resistance (Fig. 4 and Table 2); and (iii) mutants which express tetracycline resistance constitutively at a high level are frequently dominant to the wild type (11). According to one model (model A), tetracycline resistance is regulated through the TetR protein by negative and positive control mechanisms, similar to the mode by which the *araC* protein regulates the expression of the arabinose operon (21). In contrast to the *tetR* gene, which is regulated exclusively by a repressor type of mechanism, *tetA* expression is repressed by the TetR protein in the absence of tetracycline (negative control) and is activated by the TetR protein in the presence of tetracycline (positive control). Thus, *tetR*$^-$ mutants exhibit reduced and uninducible TetA protein synthesis and, consequently, reduced resistance (Fig. 4 and Table 2). According to this model, the dominant constitutive mutants isolated by Foster (11) may possess an altered TetR protein that is able to activate *tetA* expression even in the absence of tetracycline. The evidence for negative regulation of the *tetA* gene comes from the data on the in vitro synthesis of TetA protein of Yang et al. (41), who showed that extracts from Tn*10*-free cells can synthesize TetA protein in the absence of tetracycline, whereas extracts from cells with Tn*10* synthesize TetA protein only when tetracycline is added. In the second model (model B), the TetR protein acts as a repressor for both genes (*tetR* and *tetA*) and, in addition, has a role in the integration, stabilization, or function of the TetA protein in the membrane. In the absence of the TetR protein, *tetA* should be expressed at a high rate, but the TetA protein synthesized is not inserted into the membrane, and it is subsequently degraded. According to model B, the TetR protein is essential for resistance. That tetracycline resistance is observed at all in *tetR* deletion mutants might be due to the location of these helper functions at the amino-terminal ends of the TetR proteins, which are partially preserved in the fusion β-galactosidases. The observation of Coleman and Foster (10) that a small (in vitro-generated) deletion that removes DNA from the region coding for the carboxyl end of the TetR protein results in high-level constitutive tetracycline resistance is in agreement with the hypothesis that the carboxy-terminal end of the TetR protein contains the regulatory domain and the amino-terminal end provides helper functions for the TetA protein. Since mutants selected for high constitutive levels of tetracycline resistance require high rates of *tetA* expression and a functional *tetR* gene product, *cis*-dominant mutants (of the O^c type?) might be expected at a high frequency, and indeed such mutants have been found (11). This model also accounts for the inducibility and high rate of TetR protein synthesis and provides a functional explanation for the association of the TetR protein with the inner membrane of cells.

ACKNOWLEDGMENTS

We thank Marina Kossmann for excellent technical assistance and M. Manson for help with the manuscript. We are very grateful to W. Boos for his kind hospitality and for many helpful discussions. We thank Richard Jorgensen for providing plasmid pRT31. We also thank B. Siebert for typing the manuscript.

This work was supported by a Sonderforschungsbereich 138 grant from the Deutsche Forschungsgemeinschaft to W. Boos.

ADDENDUM IN PROOF

Recent studies by M. S. Curiale and S. B. Levy (J. Bacteriol., July 1982, in press) showed two complementation groups transcribed from the same promoter on the 1,275-base-pair *Hin*cII fragment of Tn*10*. They have named the promoter-proximal locus *tetA* and the promoter-distal locus *tetB*. The latter encodes the 36,000-molecular-weight Tet protein.

LITERATURE CITED

1. **Achtman, M., P. A. Manning, C. Edelbluth, and P. Herrlich.** 1979. Export without processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in *Escherichia coli* minicells. Proc. Natl. Acad. Sci. U.S.A. **76**:4837–4841.
2. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44**:1–56.
3. **Backman, K., D. Hawley, and M. J. Ross.** 1977. Use of phage immunity in molecular cloning experiments. Science **196**:182–183.
4. **Ball, P. R., S. W. Shales, and I. Chopra.** 1980. Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun. **93**:74–81.
5. **Barnes, W. M., R. Siegel, and W. S. Reznikoff.** 1974. The construction of λ transducing phages containing deletions defining regulatory elements of the *lac* and *trp* operons in *E. coli*. Mol. Gen. Genet. **129**:201–215.
6. **Beck, C. F.** 1979. A genetic approach to the analysis of transposons. Proc. Natl. Acad. Sci. U.S.A. **76**:2376–2380.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
8. **Chopra, I., and T. G. B. Howe.** 1978. Bacterial resistance to the tetracyclines. Microbiol. Rev. **42**:707–724.

9. **Clark, D. J., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23**:99–112.
10. **Coleman, D. C., and T. J. Foster.** 1981. Analysis of the reduction in expression of tetracycline resistance determined by transposon Tn*10* in the multicopy state. Mol. Gen. Genet. **182**:171–177.
11. **Foster, T. J.** 1977. Isolation and characterization of mutants of R100-1 which express tetracycline resistance constitutively. FEMS Microbiol. Lett. **2**:271–274.
12. **Franklin, T. J., and J. M. Cook.** 1971. R factor with a mutation in the tetracycline resistance marker. Nature (London) **229**:273–274.
13. **Guerola, N., J. L. Ingraham, and E. Cerda-Olmedo.** 1971. Induction of closely linked multiple mutations by nitrosoguanidine. Nature (London) New Biol. **230**:122–125.
14. **Heidecker, G., and B. Müller-Hill.** 1977. Synthetic multifunctional proteins. Mol. Gen. Genet. **155**:301–307.
15. **Jorgensen, R. A., and W. S. Reznikoff.** 1979. Organization of structural and regulatory genes that mediate tetracycline resistance in transposon Tn*10*. J. Bacteriol. **138**:705–714.
16. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids colE1, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
17. **Kleckner, N., J. A. Swan, and M. Zabeau.** 1978. Restriction enzyme analysis of Tn*10* insertions in the immunity region of bacteriophage lambda. Genetics **90**:450–461.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Larson, T. J., V. A. Lightner, P. R. Green, P. Modrich, and R. M. Bell.** 1980. Membrane phospholipid synthesis in *Escherichia coli*. J. Biol. Chem. **255**:9421–9426.
20. **Lederberg, E., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**:1072–1074.
21. **Lee, N.** 1978. Molecular aspects of *ara* regulation, p. 389–409. *In* J. H. Miller and W. S. Reznikoff (ed.), The operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **McMurray, L., R. E. Petrucci, and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:3974–3977.
23. **Mendez, B., C. Tachibana, and S. B. Levy.** 1980. Heterogeneity of tetracycline resistance determinants. Plasmid **3**:99–108.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3962–3972.
26. **Randall, L. L., S. J. S. Hardy, and L.-G. Josefsson.** 1978. Precursors of three exported proteins in *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:1209–1212.
27. **Rao, R. N., and S. G. Rogers.** 1979. Plasmid pKC7: a vector containing 10 restriction endonuclease sites for cloning DNA segments. Gene **7**:79–82.
28. **Reeve, E. C. R.** 1978. R plasmid R6-5 retains an active *tet* protein repressor gene. Plasmid **1**:581–583.
29. **Reeve, E. C. R., and J. M. Robertson.** 1975. The characteristics of eleven mutants of R-factor R57 constitutive for tetracycline resistance selected and tested in *Escherichia coli* K12. Genet. Res. **25**:297–312.
30. **Ross, D. G., J. Swan, and N. Kleckner.** 1979. Physical structures of Tn*10*-promoted deletions and inversions: role of 1400 bp inverted repetitions. Cell **16**:721–731.
31. **Schumacher, G., and K. Bussmann.** 1978. Cell-free synthesis of proteins related to *sn*-glycerol-3-phosphate transport in *Escherichia coli*. J. Bacteriol. **135**:239–250.
32. **Schumann, W., and E. G. Bade.** 1979. *In vitro* constructed plasmids containing both ends of bacteriophage Mu DNA express phage functions. Mol. Gen. Genet. **169**:97–105.
33. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry **12**:3055–3063.
34. **Silhavy, T. J., P. J. Bassford, and J. R. Beckwith.** 1979. A genetic approach to the study of protein localization in *Escherichia coli*, p. 203–254. *In* M. Inouye (ed.), Bacterial outer membranes. John Wiley & Sons, Inc., New York.
35. **Starlinger, P., and H. Saedler.** 1976. IS-elements in microorganisms. Curr. Top. Microbiol. Immunol. **75**:111–152.
36. **Stüber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. U.S.A. **78**:167–171.
37. **Tait, R. C., and H. W. Boyer.** 1978. On the nature of tetracycline resistance controlled by the plasmid pSC101. Cell **13**:73–81.
38. **Tait, R. C., R. L. Rodriguez, and H. W. Boyer.** 1977. Altered tetracycline resistance in pSC101 recombinant plasmids. Mol. Gen. Genet. **151**:327–331.
39. **Ullmann, A., and D. Perrin.** 1970. Complementation in β-galactosidase, p. 143–172. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Wray, L. V., R. A. Jorgensen, and W. S. Reznikoff.** 1981. Identification of the tetracycline resistance promoter and repressor in transposon Tn*10*. J. Bacteriol. **147**:297–304.
41. **Yang, H.-L., G. Zubay and S. B. Levy.** 1976. Synthesis of an R plasmid protein associated with tetracycline resistance is negatively regulated. Proc. Natl. Acad. Sci. U.S.A. **73**:1509–1512.
42. **Zupancic, T. J., S. R. King, K. L. Pogue-Geile, and S. R. Jaskunas.** 1980. Identification of a second tetracycline-inducible polypeptide encoded by Tn*10*. J. Bacteriol. **144**:341–355.