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Identification of Additional Genes on Transposon Tn*10*: *tetC* and *tetD*

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Two genes (*tetC* and *tetD*) were identified and located on transposon Tn*10* between gene *tetA* and insertion sequence IS*10*$_R$. Genes *tetC* and *tetD* encode proteins of apparent subunit molecular weights of 23,000 and 18,000, respectively. The TetD protein was found to be membrane associated. Tetracycline resistance levels promoted by transposon Tn*10* were found to be unaffected in *Escherichia coli* K-12 when mutants lacking *tetC* or *tetC* and *tetD* were tested. The nucleotide sequence of genes *tetC* and *tetD* is reported in the accompanying article (K. Schollmeier and W. Hillen, J. Bacteriol. 160:499–503, 1984).

Transposon Tn*10*, with a total length of 9,300 base pairs (bp), consists of a core region of unique sequence flanked by the 1,300-bp insertion sequences IS*10*$_L$ and IS*10*$_R$ in inverted orientation (11). Within the core region, comprising 6,700 bp, two genes, *tetA* and *tetR*, have been identified (5, 12, 15, 31). These genes are transcribed in a divergent manner from a common control region and code for proteins whose synthesis is inducible by tetracycline (5, 15, 31). The gene product of *tetA* (apparent molecular weight, 36,000) has been shown to be essential for tetracycline resistance; *tetR* encodes a repressor protein (apparent molecular weight, 23,000) (5, 15, 31). Together, these two genes, located on the IS*10*$_R$ side of the core region, comprise a length of 1,900 bp (Fig. 1). The expression of two additional proteins of unknown functions has been attributed to Tn*10*, although no genes for these proteins have so far been identified. The synthesis of one of these proteins (molecular weight, 15,000) was found to be inducible by tetracycline (32), whereas the synthesis of the second polypeptide (molecular weight, 13,000) was not affected by the antibiotic (33).

Jorgenson and Reznikoff (15) presented data that indicated that a region of Tn*10*, now known to comprise genes *tetA* and *tetR*, is sufficient for the expression of inducible tetracycline resistance. Higher levels of induced and uninduced resistance were, however, observed when mutants lacking DNA sequences distal to *tetA* were tested, implying that genes affecting the expression of tetracycline resistance are located on DNA sequences between *tetA* and IS*10*$_R$ (15).

Here we report the identification of two genes, *tetC* and *tetD*, located on Tn*10* between *tetA* and IS*10*$_R$. Deletion of these genes had no effect on the level of tetracycline resistance encoded by the transposon in *Escherichia coli* K-12.

MATERIALS AND METHODS

Chemicals and media. Tetracycline hydrochloride and lysozyme were from Sigma Chemical Co. The protein molecular weight standards bovine serum albumin, ovalbumin, chymotrypsin, myoglobulin, cytochrome *c*, and trypsin inhibitor were purchased from Boehringer Mannheim. L-[^{35}S]methionine and D-[1-^{14}C]galactose were obtained from Amersham Corp. 2-Acetyl-2-decarboxyamide tetracycline was a gift of Pfizer Inc. The media used have been described previously (5).

Bacterial strains and plasmids. The bacterial strains used are derivatives of *E. coli* K-12. All enzyme measurements were done with plasmid carrying derivatives of CB454 Δ(*lacZ-lacY*$^+$) *thi rpsL recA56 galK*, a *galK* mutant of CB40 (5). Resistance determinations were carried out with strain W4680 (5). The minicell-producing strain 312a is a *recA* derivative of strain P678-54 (1) described by Rak and von Reutern (25). The plasmids used are listed in Table 1.

Enzymes. The following restriction endonucleases were purchased from Bethesda Research Laboratories: *Hin*dIII, *Bgl*II, *Pst*I, *Hin*cII, and *Eco*RI. T4 DNA ligase was obtained from Boehringer Mannheim.

Preparation of DNA and transformation. Plasmid DNA was prepared from 1- or 800-ml cultures grown in LB medium (5) by the procedures of Birnboim and Doly (7). DNA from the 800-ml cultures was purified by an ethidium bromide-cesium chloride gradient (29). Transformation was carried out as described by Lederberg and Cohen (20).

Gel electrophoresis of DNA. The buffer system and staining of the gels were as described previously (5). Horizontal agarose gel electrophoresis employed agarose concentrations ranging from 0.7 to 1.6%. DNA fragments were isolated after electroelution essentially as described by Wienand et al. (30).

Plasmid constructions. For the construction of plasmid pCB144, plasmid pCB8 was completely digested with *Pst*I and partially digested with *Hin*dIII. A 15-kilobase DNA fragment, comprising all of pCB8 except the DNA between the right *Hin*dIII restriction site within the Tn*10* core region and the *Pst*I site, was isolated and ligated in the presence of a 38-bp *Hin*dIII-*Pst*I linker fragment isolated from the polylinker region of pFD51.

Plasmid pCB149 was constructed in two steps. First, in a subclone of pCB8 that lacked all Tn*10* sequences between the outside border of IS*10*$_L$ and *tetA* (pCB27 [5]), the 320-bp *Hin*dIII-*Bgl*II fragment neighboring IS*10*$_R$ was replaced by a 640-bp λ DNA fragment isolated from pKB166 (3) after digestion with *Hin*dIII and *Bgl*II. From a clone containing the insert, a 3.1-kb *Hin*dIII-*Pst*I DNA fragment which comprised IS*10*$_R$ was isolated and ligated into the 15-kb pCB8 DNA fragment generated by digestion with *Pst*I and *Hin*dIII (partial) as described above.

Galactokinase assay. We used the galactokinase assay conditions described by Rak and von Reutern (25).

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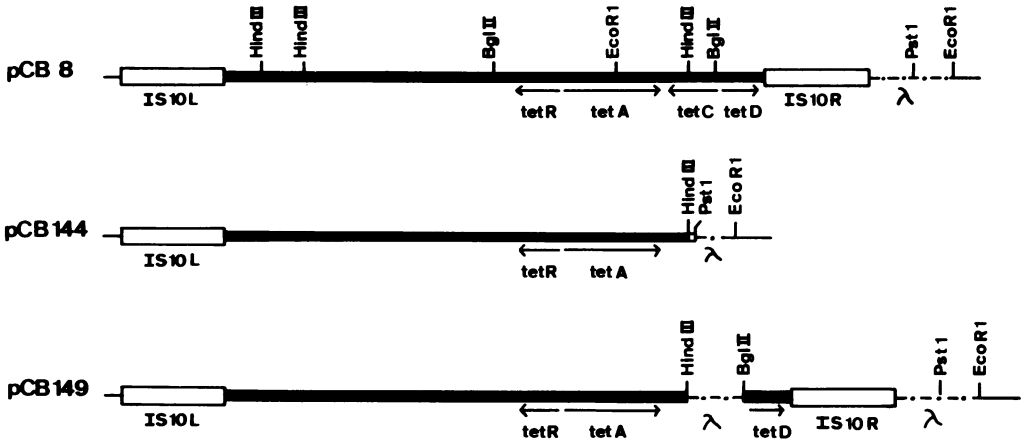

FIG. 1. Map of transposon Tn*10* on plasmid pCB8 and of mutant derivatives. The thick solid line represents the Tn*10* core region. The genes identified and the relevant restriction sites are shown. The construction of plasmids pCB144 and pCB149 is described in the text.

Preparation and labeling of minicells. Derivatives of minicell-producing strain 312A harboring plasmids were grown with aeration in 300 ml of LB at 37°C until the stationary phase. Minicells were isolated in two steps. First, whole cells were separated from minicells by centrifugation in a Sorvall RC-2 centrifuge (rotor GS3, 4,000 rpm for 15 min). Minicells were pelleted (Sorvall RC-2 centrifuge, rotor GS3, 8,000 rpm for 35 min) and suspended in buffer (10 mM Tris-hydrochloride [pH 7.4], 150 mM NaCl). Further purification of minicells was carried out by centrifugation through continuous sucrose gradients as described by Reeve (26). Minicells were suspended in AB medium supplemented with 0.4% glycerol and 20 μg of cycloserine per ml to an absorbancy at 578 nm of 1. To the minicell suspension (0.5 ml) a mixture of amino acids (containing all except methionine) was added to a final concentration of 0.22 mM and, this suspension was incubated at 37°C for 30 min. Labeling of proteins was initiated by adding 100 μCi of [^{35}S]methionine (specific activity, 1,400 Ci/mmol) and continued for 45 min at 37°C. Induction was performed by the addition of the non-inhibitory tetracycline analog 2-acetyl-2-decarboxyamide tetracycline at the time of addition of the [^{35}S]methionine. Minicells were pelleted and suspended in 25 μl of sample solution containing 62 mM Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, and 5% mercaptoethanol. The samples were prepared for electrophoresis by heating for 20 min at 65°C.

TABLE 1. Plasmids used

Plasmid	Relevant plasmid markers[a]	Source or reference
pFD51	Ap^r GalK$^-$	Derivative of pKO1 from B. Rak (21, 25)
pKB166	IcE1 λ^I	(3)
pRT31	IcE1 LacZ$^+$	(5)
pCB8	IcE1 Tc^r	(5)
pCB88	Ice1	Deletion mutant of pCB8 lacking all Tn*10* sequences but IS*10*R
pCB110	Ap^r GalK$^+$	This article
pCB111	Ap^r GalK$^+$	This article
pCB120	Ap^r GalK$^-$	This article
pCB143	Ap^r	pCB111 derivative with a 600-bp *Hin*dIII fragment from pHL222 (19) inserted into the *Hin*dIII site
pCB144	Ice1 Tc^r	This article
pCB149	Ice1 Tc^r	This article

[a] The nomenclature follows that proposed by Bachmann and Low (2) and Novick et al. (24).

Fractionation of minicell proteins. The method used for fractionation of minicell proteins was modified from that described by Neu and Heppel (23). [^{35}S]methionine-labeled minicells were pelleted and suspended in 200 μl of a solution containing 50 mM Tris-hydrochloride (pH 7.8), 50 mM EDTA, 20% sucrose, and 0.2 mg of lysozyme per ml. After incubation for 1 h at 4°C, spheroplasts were pelleted by centrifugation in an Eppendorf centrifuge. Proteins were precipitated from the supernatant by the addition of 1/10 volume of 80% trichloroacetic acid, washed with acetone, dried, and suspended in 20 μl of sample solution (periplasmic protein fraction). Sedimented spheroplasts were lysed in 100 μl of cold water and twice frozen at −20°C and thawed at room temperature. The membrane fraction was separated by pelleting (Sorvall RC-2 centrifuge, rotor SM24, 20,000 rpm for 2 h at 4°C) and suspended in 20 μl of sample solution (membrane protein fraction). Proteins were precipitated from the supernatant by the addition of 1/10 volume of 80% trichloroacetic acid, washed with acetone, dried, and suspended in 20 μl of sample solution (cytoplasmic protein fraction). Equal volumes of all fractions were applied to the sodium dodecyl sulfate-polyacrylamide gels after heating for 20 min at 65°C.

Electrophoretic separation of proteins and autoradiography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by a modified Laemmli system (18). The samples—containing about 40,000 cpm each—were electrophoresed through a 10 to 20% acrylamide gradient slab gel with a 5% stacking gel at a constant voltage of 300 V for 12 h. The gels were stained and destained as described by Schumacher and Bussmann (28). Bovine serum albumin (molecular weight, 66,000), ovalbumin (45,000), chymotrypsin (25,000), myoglobin (17,800), cytochrome *c* (12,900), and trypsin inhibitor (6,500) were used as molecular weight standards. Fluorography was carried out as described by Chamberlain (8) with Du Pont Cronex film.

Measurement of tetracycline resistance levels. Tetracycline resistance levels were measured as described previously (5).

RESULTS

Detection and mapping of *tetC* by gene fusions. We previously described a genetic system designed for the isolation of gene fusions of the protein fusion type. With this system genes located on transposon Tn*10* were fused with *lacZ* (4, 5). In an extension of these studies we isolated several fusion mutants in which the deletions extended from the outside border of IS*10*$_L$ to various sites within the Tn*10* DNA segment delimited by the *Bgl*II and *Hin*dIII restriction sites neighboring IS*10*$_R$ (Fig. 1; data not shown). From these data we drew the tentative conclusion that a gene (or part of it) must be located between these two restriction sites. Since *lacZ* was located next to IS*10*$_L$, the direction of transcription of this gene pointed in the direction of IS*10*$_L$. These initial observations were verified and extended by the ligation of distinct DNA fragments of Tn*10* in front of the galactokinase structural gene (*galK*) on the pKO1-derived promoter detection plasmid pFD51 (25). The structures of these plasmids are shown in Fig. 2. The DNA fragment comprising the right end of Tn*10* (including IS*10*$_R$) was isolated after digestion of pCB8 with restriction endonucleases *Eco*RI and *Hin*dIII and ligated into pFD51 digested with *Eco*RI and *Hin*dIII, resulting in plasmid pCB111. Transformation of pCB111 into CB454 (*galK recA*) resulted in clones that produced galactokinase (Table 2). Since the *Hin*dIII site of the Tn*10* core region was located next to the galactokinase structural gene, we concluded that the DNA segment at this *Hin*dIII site is transcribed in vivo with the orientation from IS*10*$_R$ toward the *tetA* gene. The following constructions were carried out to define the promoter more precisely. We isolated the 320-bp *Bgl*II-*Hin*dIII Tn*10* DNA fragment of pCB8 located between *tetA* and IS*10*$_R$ (Fig. 1) and ligated it into pFD51 deleted for the *Bgl*II-*Hin*dIII fragment. Plasmid pCB120 contained the *Bgl*II-*Hin*dIII fragment with the *Hin*dIII site located next to *galK* (Fig. 2). Transformation of pCB454 with pCB120 did not result in the production of galactokinase (Table 2), and we concluded that this *Bgl*II-*Hin*dIII DNA fragment does not harbor a promoter transcribing in the direction from IS*10*$_R$ toward the *tetA* gene. The tentative conclusion that the origin of the transcription activity observed with pCB110 was located between IS*10*$_R$ and its neighboring *Bgl*II site was tested by ligating the isolated *Eco*RI-*Bgl*II fragment of pCB8 (including IS*10*$_R$; Fig. 1) into plasmid pFD51 deleted for the *Eco*RI-*Bgl*II fragment. In the resulting plasmid, pCB110, the *Eco*RI-*Bgl*II fragment was inserted with the *Bgl*II site neighboring *galK* (Fig. 2). CB454 harboring plasmid pCB110 produced galactokinase. The transcriptional activities observed at the IS*10*$_R$ neighboring *Bgl*II and *Hin*dII sites thus originated from the same promoter that we presume to be located between IS*10*$_R$ and the *Bgl*II site. From these data as well as from the results obtained with protein fusion mutants we concluded that a gene, which we named *tetC*, is located between *tetA* and IS*10*$_R$ on Tn*10* and that the direction of transcription of this gene is toward IS*10*$_L$ (Fig. 1).

TABLE 2. Expression of *tetC* fused to *galK*

Plasmid[a] carried by strain CB454	Galactokinase sp act (U)[b]
pFD51	<1
pCB111	17
pCB110	26
pCB120	<1

[a] The structures of the plasmids are given in Fig. 2.
[b] Galactokinase units are expressed as nmol of [^{14}C]galactose phosphorylated per minute and per unit of absorbancy at 578 nm of bacteria.

The *tetC* gene codes for a protein of 23,000 daltons. Analysis of the DNA sequence in the region where we had mapped the *tetC* gene revealed an open reading frame, ORFL (27),

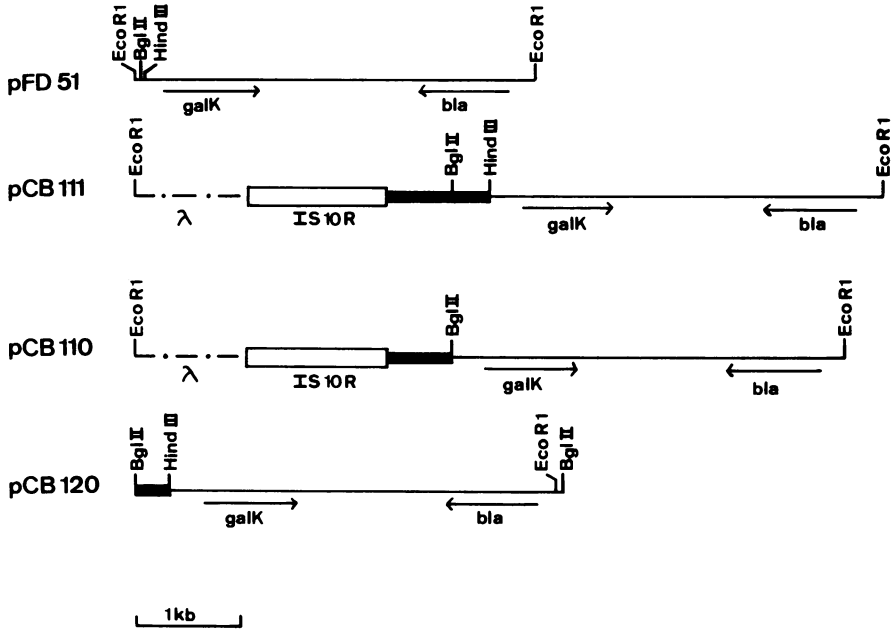

FIG. 2. Relevant structures and restriction sites of plasmids used for the detection of promoter activities. Plasmid pFD51 (25) is a derivative of pKO1 (21). It contains the galactokinase structural gene (*galK*) including its translation start signals, but lacks a promoter in front of the gene. The solid thick line represents DNA from the Tn*10* core region. The construction of plasmids pCB110, pCB111, and pCB120 is described in the text.

which could represent the structural *tetC* gene. This reading frame could encode a protein of 22,733 daltons. *E. coli* minicells were used for the detection and determination of size of the *tetC* gene product. In track d of Fig. 3, the proteins encoded by minicells harboring pCB8, containing a complete Tn*10*, incubated in the presence of 2-acetyl-2-decarboxyamide tetracycline (a tetracycline analog that does not exhibit bacteriostatic effects, but can induce the resistance genes [13]), are shown. A weak band can be seen at a molecular weight of about 23,000. We then used a plasmid (pCB111), in which the C-terminal portion of the potential *tetC* gene had been replaced by a DNA fragment of known sequence, to direct the synthesis of protein in minicells. The open reading frame of pCB111 should encode a shorter protein of 21,810 daltons instead of the 22,733-dalton protein. The fusion protein ($TetC_1$) encoded by pCB111 with an apparent molecular weight of 22,000 is evident in track c of Fig. 3. Its synthesis was again not significantly affected by the presence of 2-acetyl-2-decarboxyamide tetracycline (Fig. 3, compare tracks b and c). Since the fusion protein exhibited the predicted, shift in molecular weight, we concluded that the open reading frame ORFL (27) represents the structural *tetC* gene.

Evidence for gene *tetD*. The synthesis of a protein with a molecular weight of 18,000 became apparent when the tetracycline analog that induced expression of *tetA* and *tetR* (data not shown) was added to minicells that harbored plasmid pCB111. This protein was not synthesized by minicells that contained the parental vector pFD51 (compare tracks b and c with track a, Fig. 3). Synthesis of this protein must therefore be directed by a Tn*10* gene, which we named *tetD*. The TetD protein was encoded by pCB8, which harbors an intact Tn*10*, but not by pCB88, a derivative of pCB8 in which all of Tn*10* except for $IS10_R$ and about 100 bp of the Tn*10* core region had been deleted (Fig. 3, compare tracks d and f). Plasmid pCB110, which carried DNA from the very right end of the Tn*10* core region (Fig. 2), encoded TetD protein, but not TetC protein (data not shown). These data are consistent with a map location of *tetD* between the inner border of $IS10_R$ and *tetC*.

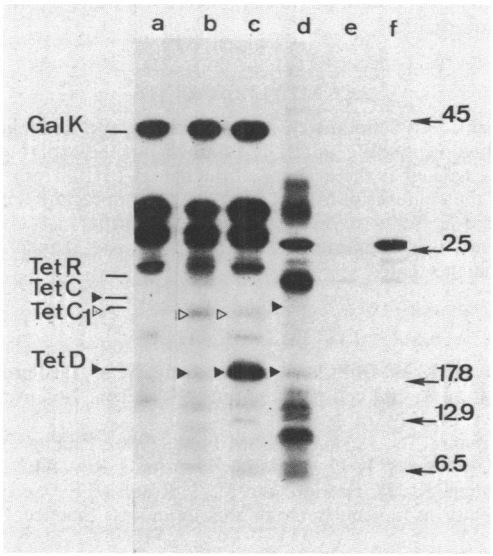

FIG. 3. Autoradiogram of a 10 to 20% polyacrylamide gradient gel displaying [^{35}S]methionine-labeled proteins encoded by various plasmids in minicells. Tracks: a, pFD51; b, pCB111; c, pCB111 induced with 5 μg of 2-acetyl-2-decarboxyamide tetracycline per ml; d, pCB8 induced with 5 μg of 2-acetyl-2-decarboxyamide tetracycline per ml; e, pRT31; f, pCB88. $TetC_1$ denotes the TetC fusion protein. The arrows and numbers on the right side of the autoradiogram indicate the position of the proteins of corresponding molecular weight × 10^{-3}.

Cellular location of the *tetD* gene product. Fractionation of minicells synthesizing the TetD protein encoded by plasmid pCB143 into cytoplasmic, periplasmic, and membrane fractions gave the results presented in Fig. 4. The results clearly demonstrate that the TetD protein was associated with the membrane fraction. Other proteins encoded by pCB143 served as internal controls for the fractionation: galactokinase, β-lactamase precursor, and mature β-lactamase were found in the cytoplasm, membrane, and periplasm, respectively.

Phenotypes of mutants defective in *tetC* and *tetD*. To test the effect of genes *tetC* and *tetD* on the expression of Tn*10*-encoded tetracycline resistance, deletion mutants lacking *tetC* or *tetC* and *tetD* were generated by in vitro techniques. Using pCB8 (with complete Tn*10*) as starting material, we constructed two types of mutants. (i) $tetC^-$ deletion mutants were obtained by replacement of the 320-bp *Bgl*II-*Hin*dIII DNA fragment on the right side of Tn*10* (Fig. 1) by a *Bgl*II-*Hin*dIII DNA fragment containing part of the lambda *c*I gene isolated from plasmid pKB166 (3). This construction yielded plasmid pCB149 (Fig. 1). (ii) *tetC tetD* deletion mutants were generated by replacement of the *Hin*dIII-*Pst*I DNA fragment of pCB8 by a 38-bp *Hin*dIII-*Pst*I DNA fragment obtained from the linker fragment of pFD51, resulting in plasmid pCB144 (Fig. 1).

The levels of tetracycline resistance generated in isogenic strains by pCB8 (with intact Tn*10*), pCB149 ($tetC^-$), and pCB144 ($tetC^-$ $tetD^-$) are given in Table 3. Tn*10* on mini-ColE1-derived plasmid pCB8 caused levels of resistance approximately 150% higher than those conferred by a single Tn*10* on the chromosome of an isogenic strain (data not shown) and thus, with the strain employed, did not exhibit the multicopy effect (10, 22), i.e., a reduction in resistance due to the presence of Tn*10* on a multicopy vector. Deletion of *tetC* or *tetC* and *tetD* did not affect levels of resistance of cells grown with (induced) or without (uninduced) tetracycline (Table 3). From these results we concluded that in *E. coli* K-12 *tetC* and *tetD* have no evident function in Tn*10*-encoded tetracycline resistance. Using plasmids containing only *tetC* and *tetD*, we tested whether these two genes together were able to confer resistance on the host strain. No increase in tetracycline resistance was observed.

DISCUSSION

We identified two genes, *tetC* and *tetD*, on transposon Tn*10* and located them on a DNA segment between gene *tetA* and $IS10_R$ (Fig. 1). The orientation of *tetC* was found to be opposite to that of *tetA*, pointing toward $IS10_L$. Gene *tetD* is located between *tetC* and $IS10_R$. Evidence obtained by cloning *tetC* gene fragments in front of the *galK* structural gene suggested that the promoter for *tetC* is located between the right *Bgl*II restriction site of the Tn*10* core region and $IS10_R$. A promoter of correct orientation was located in this region by in vitro methods (27). Analysis of the DNA sequence of this region of Tn*10* (27) revealed an open reading frame that may code for a protein of 22,733 daltons. The data presented suggest that this reading frame, called ORFL, is identical with that of gene *tetC*. An open reading frame of opposite polarity to that of *tetC*, located between *tetC* and $IS10_R$ (ORFR), was deduced from DNA sequence data (27).

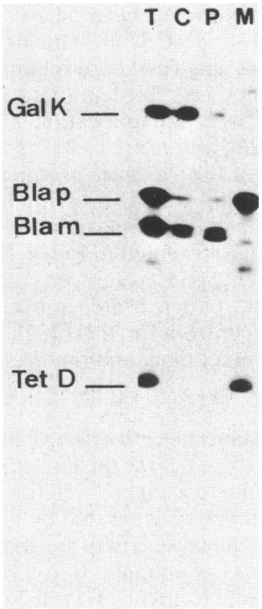

FIG. 4. Autoradiogram of a 10 to 20% polyacrylamide gradient gel displaying [^{35}S]methionine-labeled proteins encoded by plasmid pCB143 in minicells. Minicells were fractionated into cytoplasmic (C), periplasmic (P), and membrane (M) fractions. Proteins of total minicells (T) are also shown. GalK, Bla p, Bla m, and TetD indicate galactokinase, β-lactamase precursor, mature β-lactamase, and TetD protein, respectively.

The data available indicate that this reading frame, which may code for a protein of 16,769 daltons, may represent the structural *tetD* gene.

The expression of *tetC* is rather weak, as can be seen by inspection of the band corresponding to TetC protein on the autoradiogram (Fig. 3) and from the data obtained with *tetC-galK* fusions (Table 2). The level of galactokinase activity encoded by multicopy plasmid pCB111 corresponds to about 60% of the enzyme activity, which could be determined from the chromosomal copy of *galK* when the *gal* operon was induced (data not shown). Higher levels of *tetC* expression were consistently observed with strains harbouring pCB110, in which *galK* was fused to the beginning of *tetC*. Whether the difference in enzyme activity observed between pCB110 and pCB111 reflects some intrinsic properties of the *tetC* gene or is due to differences in DNA sequence between the fusion points of *tetC* and the translational start signal of *galK* is unknown.

Judging from the intensity of the band corresponding to the TetD protein on the autoradiogram, minicells harboring an intact Tn*10* synthesize only small amounts of this protein even in the presence of the non-inhibitory inducer of genes *tetA* and *tetR*, 2-acetyl-2-decarboxyamide tetracycline (Fig. 3, track d). Expression of *tetD* in minicells was substantially increased by the addition of 2-acetyl-2-decarboxyamide tetracycline when plasmid pCB111—which contains a *tetC* gene (altered in the region coding for the carboxy end of the protein), but lacks other sequences of the Tn*10* core region—was tested (compare tracks b and c of Fig. 3). These data are consistent with the assumption that the synthesis of the TetD protein is regulated. The details of the regulatory system controlling the expression of *tetD* remain to be elucidated.

Levels of tetracycline resistance encoded in *E. coli* K-12 by Tn*10* were found to be unaffected by the presence or absence of these genes. Since they are maintained on Tn*10* in a functional state, the question arises as to their function. In considering this question, it must be kept in mind that the core region on the IS*10*$_L$ side of Tn*10* contains 2,700 bp of unknown genetic structure and function. The possibility that genes *tetC* and *tetD* and the 2,700 bp next to IS*10*$_L$ have no function in the tetracycline resistance mechanism cnanot at present be excluded. The inducibility of gene *tetD* by a tetracycline derivative suggests a role of these genes in tetracycline resistance. Possibly, the additional genetic information present on Tn*10* is of importance for the expression of resistance in various groups of gram-negative bacteria other than *E. coli* K-12. A core region considerably larger than required for genes *tetA* and *tetR* (4,500 to 6,500 bp) has also been determined on various tetracycline resistance transposons isolated from gram-negative bacteria (6, 14, 16, 17). Although functions have so far not been identified on these extra DNA sequences, it is tempting to speculate that they may contain genetic information that is of selective advantage. Work is in progress to investigate the function of these sequences.

TABLE 3. Levels of tetracycline resistance caused by plasmids carrying Tn*10* and Tn*10* mutant derivatives[a]

Plasmid	Relevant Tn*10* genotype	Tetracycline resistance[b]	
		Uninduced	Induced[c]
pCB8	*tetA*$^+$*R*$^+$*C*$^+$*D*$^+$	130	170
pCB149	*tetA*$^+$*R*$^+$*C*$^-$*D*$^+$	130	170
pCB144	*tetA*$^+$*R*$^+$*C*$^-$*D*$^-$	130	170

[a] The host strain for the plasmids used was W4680 (5), and the structures of the plasmids are presented in Fig. 1.

[b] Tetracycline resistance is expressed as 50% efficiency of plating (i.e., the tetracycline concentration [in micrograms per milliliter] that resulted in a 50% reduction in CFU. Resistance levels were determined as described previously (5).

[c] Cells were induced by pregrowth in the presence of 2 μg of tetracycline per ml.

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