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Plasmid Vectors Based on Tn*10* DNA: Gene Expression Regulated by Tetracycline[1]

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The regulatory region of the tetracycline resistance determinant from transposon Tn*10* has been used to construct plasmid vectors for gene expression regulated by tetracycline. Plasmids pRS *tet*Bam-8 and pRS *tet*Bam-16 include the *tet* regulatory region, the segment coding for the first four amino acids of the tetracycline resistance protein (*tetA* protein), and a linker region with *Sal*I, *Hpa*II, and *Bam*HI restriction sites for gene fusions. Plasmid pTB-1, a derivative of pRS *tet*Bam-8 and of the β-galactosidase gene-containing plasmid pMC1403, constitutively expresses a *tetA* fragment-β-galactosidase fusion protein. If a multicopy runaway replication plasmid, pMOBglII-16 that includes a 2.7-kb *Bgl*II DNA fragment from Tn*10* that provides *tetR* protein is present along with pTB-1, the expression of β-galactosidase is reduced about eightfold. Tetracycline acts as an inducer of the system and restores the level of β-galactosidase activity measured in transformants containing pTB-1 alone. Plasmid mutants unable to produce active *tetR* protein are ineffective in reducing expression. *Escherichia coli* carrying plasmids that express both *tetA* protein and *tetR* protein show an increase in the tetracycline resistance level after incubation with the drug. The observations are consistent with the previously proposed mechanism of regulation of tetracycline resistance in Tn*10*.

Transposon Tn*10* encodes genes for tetracycline resistance on a 2.7-kb DNA region, flanked by the two unique *Bgl*II restriction sites present in Tn*10* DNA (Jorgensen *et al.*, 1979; Jorgensen and Reznikoff, 1979; Wray *et al.*, 1981; Coleman and Foster, 1981; Beck *et al.*, 1982). Early evidence indicated that tetracycline resistance expression is induced by subinhibitory concentrations of the drug (Franklin, 1967; Franklin and Cook, 1971). At least two proteins are involved in the expression of tetracycline resistance: a 36-KDa resistance protein (*tetA* protein)[2] (Levy and McMurray, 1974; Jorgensen and Reznikoff, 1979) and a 25-KDa repressor protein (*tetR* protein). Deletion mapping, RNA polymerase binding, and nucleotide sequencing studies have shown that two overlapping promotor–operator regions (*tet* regulatory region) control the initiation of synthesis of the two mRNAs which are elongated in opposite direction (Jorgensen and Reznikoff, 1979; Wray *et al.*, 1981; Coleman and Foster, 1981; Hillen and Schollmeier, 1983; Bertrand *et al.*, 1983). According to the model proposed for the expression of *tet* genes, *tetR* protein binds, in the absence of tetracycline, to the *tet* regulatory region and represses both its own synthesis and that of *tetA* protein. In the presence of the drug, the *tetR* repressor activity is inhibited and synthesis of the two proteins takes place (Yang *et al.*, 1976; Jorgensen and Reznikoff, 1979; Wray *et al.*, 1981; Coleman and Foster,

[1] This paper is dedicated to Dr. Ricardo Pastrana who died in Madrid on November 19, 1983.

[2] Abbreviations used: *tetA* protein, tetracycline resistance protein; *tetR* protein, repressor protein; the genes coding for these proteins are referred to as *tetA* and *tetR*, respectively; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Cm, chloramphenicol.

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1981; Beck *et al.*, 1982). The binding of purified *tetR* protein to the *tet* operator region and the inhibition of the interaction by tetracycline have been analyzed by *in vitro* studies (Hillen *et al.*, 1982; Hillen and Unger, 1982a,b).

These observations suggest that the promoter–operator region programming the synthesis of *tetA* protein could potentially provide vectors for gene expression regulated by tetracycline. Also, comparative measurements of gene expression utilizing the promoter cloning vehicle λRS205, indicate that the *tetA* promoter programs gene expression at over twice the level of the *lac* promoter (K. P. Bertrand, K. Postle, J. L. V. Wray, and W. S. Reznikoff, submitted). This is expected since the −35 region includes the consensus sequence of other bacterial promoters, the probable −10 sequence contains several consensus bases (Bertrand *et al.*, 1983; Rosenberg and Court, 1979) and the −35 and −10 regions are located 18 bp apart; only 1 bp more than the consensus distance.

In the present report, the construction of plasmids including the *tet* regulatory DNA segment and a linker region to facilitate gene fusions is described. A β-galactosidase fusion protein, expressed under the control of the *tet* regulatory region, was inducible by tetracycline if a second plasmid that provided *tetR* repressor protein was present. With plasmid mutants unable to synthesise *tetR* protein, β-galactosidase synthesis was constitutive.

MATERIALS AND METHODS

Chemicals and media. Tetracyclin, ampicillin, chloramphenicol, kanamycin, and *O*-nitrophenyl-β-galactopyranoside were obtained from Sigma Chemical Company. *Bam*HI linkers were from Collaborative Research Inc. For plasmid preparation, *Escherichia coli* transformants were grown in LB medium containing the appropriate antibiotic. For the colorimetric assay of β-galactosidase, bacteria were grown in A medium prepared as described by Miller (1972) except that the $MgSO_4$ was substituted by NaCl (6 g/liter). Agar and MacConkey agar were from Difco.

Enzymes and enzyme assays. Restriction enzymes, DNA polymerase I, Klenow fragment, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs and nuclease *Bal*31 from Bethesda Research Laboratories. Enzyme assays were performed according to described procedures (Maniatis *et al.*, 1982). β-Galactosidase activity was measured colorimetrically as described by Miller (1972).

Bacterial strains and plasmids. E. coli MC1061 (*ara* D139, Δ (*ara, leu*)7697, Δ *lacX74, gal* U^-, *gal* K^-, hsr^-, hsm^+, strA) (Casadaban and Cohen, 1980) and *E. coli* C600 (F^-, *thi-1, thr-1, leuB6, lac Y1, tonA21, supE44,* λ^-) were used for transformation. Plasmid pRS*tet* 158-64 (Bertrand, Postle, Wray, and Reznikoff, submitted) is a derivative of pBR322 in which the 650-bp *Eco*RI–*Sal*I segment was substituted by a 158-bp *Taq*I fragment that includes the *tet* regulatory region and the adjacent segments coding for the N-terminal regions of *tetA* and *tetR* proteins (Hillen and Schollmeier, 1983; Bertrand *et al.*, 1983). This was accomplished by a procedure which regenerated *Eco*RI and *Sal*I sites bracketting the *Taq*I fragment (Backman *et al.*, 1976; Wartell and Reznikoff, 1980; Bertrand, Postle, Wray, and Reznikoff, submitted). The runaway replication plasmid pMOB45 (Bittner and Vapnek, 1981) was obtained from Dr. R. Diaz; pMC1403 (Casadaban *et al.*, 1980) was provided by Dr. M. Casadaban; pACYC177 (Chang and Cohen, 1978) was from Dr. F. Cabello. pRT44 has been described (Jorgensen *et al.*, 1979).

Competent, calcium-treated *E. coli* MC1061 (Mandel and Higa, 1970) were transformed with plasmid DNA as described by Cohen *et al.* (1972).

For preliminary characterization, plasmid DNA was prepared by the procedure of Klein *et al.* (1980). Purification by lysis of bacteria and ethidium bromide–CsCl equilibrium density gradients was based on the method of Timmis *et al.* (1978). In bacteria with two plasmids, their relative copy number was estimated from densitometry tracings of electrophoretic separations of DNA prepared ac-

cording to Klein *et al.* (1980), after treatment with RNase A and sodium dodecyl sulfate.

Nucleotide sequences were determined by the method of Maxam and Gilbert (1980).

Tetracycline resistance levels were determined according to Tait *et al.* (1977) and Jorgensen and Reznikoff (1979). Dilutions of bacterial cultures were plated on LB-agar containing 0, 1, 3, 6, 9, 15, 20, 30, and 50 μg/ml of tetracycline for plasmids derived from pMOB45, and 0, 9, 20, 30, 50, 75, 100, 125, 150, 175, and 200 μg/ml of tetracycline for plasmids derived from pACYC177 and for pRT44 and pBR322. Resistance is expressed as the concentration of tetracycline giving 50% plating efficiency (EOP_{50}). The values to be compared were determined in parallel, using the same batch of medium.

RESULTS

Construction of plasmids with the tet regulatory region: pRStetBam and pTB-1. To facilitate gene fusions to the *tet* regulatory region, pRS*tet* 158-64 was modified by linearization with *Sal*I, filling in recessed 3′ ends with DNA polymerase I, Klenow fragment, and ligation to *Bam*HI linkers. Two plasmids, pRS*tet*Bam-8 and pRS*tet*Bam-16, including *Sal*I, *Hpa*II, and *Bam*HI sites in the region coding for the amino terminus of *tetA* protein were obtained (Fig. 1). In pRS*tet*Bam-8 DNA a CG base pair of the *Bam*HI linker was lost, eliminating the *Hpa*II restriction site adjacent to the *Sal*I site (compare sequences in Fig. 1). Substitution of the *Eco*RI–*Bam*HI linker region of pMC1403 β-galactosidase fusion vector (Casadaban *et al.*, 1980) by the *Eco*RI–*Bam*HI *tet* regulatory segment of pRS*tet*Bam-8 resulted in plasmids that yielded Lac$^+$ *E. coli* MC1061 transformants. This result was as predicted from the nucleotide sequence around the *Bam*HI site of pRS*tet*Bam-8 (Fig. 1) and of pMC1403 (Fig. 6 in Casadaban *et al.*, 1980). One of the plasmids, pTB-1, constitutively expressed β-galactosidase, as measured by enzyme activity and by the presence of a protein of about 115Kda in bacterial extracts. pTB-1 was characterized by restriction enzyme mapping (Fig. 1).

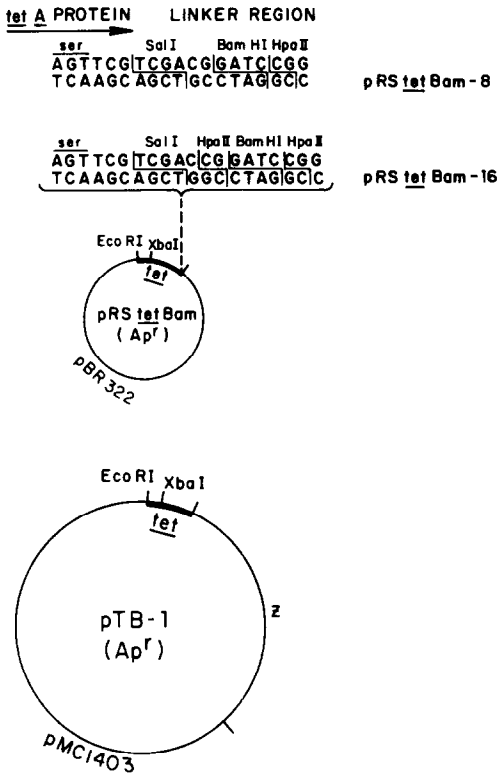

FIG. 1. Plasmids pRS*tet*Bam and pTB-1. pRS*tet* 158-64 DNA (4 μg) was digested with *Sal*I and the reaction mixture was adjusted to 0.1 mM each of dATP, dTTP, dCTP, and dGTP and incubated with DNA polymerase I, Klenow fragment (0.5 u, 30 min at 25°C). The mixture was extracted with phenol and filtered through Sephadex. The DNA was recovered by ethanol precipitation and incubated with a 100-fold molar excess of phosphorylated *Bam*HI linkers and T4 DNA ligase (200 u, 20 h at 6°C). The mixture was then adjusted to 0.1 M NaCl, heated 10 min at 65°C, cooled, and treated with *Bam*HI. The DNA was extracted with phenol, filtered through Sephadex G-50, recovered by ethanol precipitation, and incubated with T4 DNA ligase, as above. The DNA was used to transform *E. coli* C600. Plasmid DNA from 4 of 20 transformants analyzed included a *Bam*HI site. Two of them, pRS*tet*Bam-8 and pRS*tet*Bam-16 were further characterized by restriction mapping and nucleotide sequencing by labeling at the *Bam*HI site. *ser* is the third amino acid of *tetA* protein.

Construction of plasmids with the 2.7-kb BglII DNA fragment from Tn10: pMOBglII-16 and pACBglII2.7–21. Plasmid pMOBglII-16 was constructed by inserting the 2.7-kb *Bgl*II fragment of Tn*10* (Jorgensen *et al.*, 1979) into the *Bam*HI site of a Tc^s-derivative

of plasmid pMOB45 (Fig. 2). Plasmid pACBglII2.7 Ap^r, Km^r, Tc^r was constructed by inserting the 2.7-kb *Bgl*II fragment from Tn*10* at the *Bam*HI site of plasmid pACYC177 (Chang and Cohen, 1978), and contains four unique sites for cloning: *Eco*RI (Tc^s); *Sma*I, *Xho*I (Km^s); *Pst*I (Ap^s). It was made Ap^s by linearization with *Pst*I and endonuclease S1 digestion. The resulting pACBglII2.7–21 plasmid Tc^r, Km^r, Ap^s (6 kb) was characterized by restriction enzyme mapping (results not shown). Since the deletion

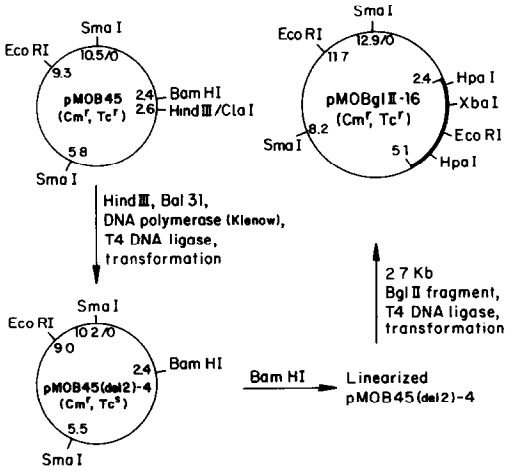

FIG. 2. Construction of pMOBglII-16. pMOB45 DNA (Bittner and Vapnek, 1981) (1 μg) was digested with *Hin*dIII and then with exonuclease *Bal*31 (0.5 u, 2 min, 30°C). The solution was extracted with phenol and the DNA recovered by ethanol precipitation. Then it was incubated with DNA polymerase I (Klenow fragment) (0.5 u, 45 min, 25°C) and 10 μM each of dATP, dGTP, dCTP, and dTTP. The mixture was extracted with phenol, filtered through Sephadex G-50, and the DNA precipitated with ethanol and treated with T4 DNA ligase. DNA from one of the Cm^r, Tc^s transformants, pMOB45(del 2)-4 was purified and characterized by restriction mapping. pRT44 DNA (Jorgensen *et al.*, 1979) (6 μg) was digested with *Bgl*II and the 2.7-kb fragment was separated by agarose gel electrophoresis and recovered by electroelution (Allet *et al.*, 1973). The eluted fragment was mixed with *Bam*HI-treated pMOB45(del 2)-4 DNA and incubated with T4 DNA ligase. Several Tc^r, Cm^r transformants were obtained. DNA from three Tc^r, Cm^r transformants was prepared and characterized by digestion with several restriction enzymes. One of them, pMOBglII16, was purified and further characterized by restriction mapping. Digestion with *Eco*RI + *Xba*I oriented the 2.7-kb *Bgl*II insert, as shown. Numbers refer to kb of DNA measured clockwise from the *Sma*I site.

generated by S1 digestion reached the *Pvu*I site at the Ap^r gene, this plasmid includes a unique *Pvu*I site in the Km^r gene.

Plasmids pMOBglII-16 and pACBglII2.7–21 carry the *tetA* and *tetR* genes from Tn*10* and were used to study the expression of the β-galactosidase gene from pTB-1.

Regulation by tetracycline of the expression of β-galactosidase encoded in pTB-1. Plasmid pTB-1 in *E. coli* MC1061 expresses β-galactosidase activity at a level that is unaffected by the addition of subinhibitory concentrations of tetracycline to the culture medium (Table 1). In transformants which include both pTB-1 and pMOBglII-16 at a relative copy number of 1:10 at 37°C, the activity was about 8-fold lower. If tetracycline was added to the culture medium one hour before the enzyme assay, the β-galactosidase activity was similar to that measured with pTB-1 alone (Table 1). Plasmids pMOB45 or pMOB45 (del 2)-4, lacking the 2.7 Kb-*Bgl*II fragment (Fig. 2) did not cause a reduction of β-galactosidase activity expressed from pTB-1. In the presence of pMOBglII-16, expression of β-galactosidase from pTB-1 was not completely abolished and the activity, measured in the absence of tetracycline, was 50- to 200-fold higher than the background value obtained with *E. coli* MC1061 (Table 1). The repression level appears to correlate with the copy number of the plasmid carrying the *tetR* gene, since plasmid pACBglII2.7–21 caused a 2-fold reduction in β-galactosidase activity (Table 1) and in double transformants its estimated copy number relative to pTB-1 was about 10 times lower than that of pMOBglII-16.

Mutation at the XbaI site of pMOBglII-16 abolishes repression. The hexanucleotide coding for the third and fourth amino acid of *tetR* protein provides an *Xba*I cleavage site (Bertrand *et al.*, 1983) unique in plasmid pMOBglII-16. To generate a selective inactivation of the *tetR* repressor protein, plasmid pMOBglII-16 was modified by cleavage with *Xba*I, filling in the recessed 3'-ends with DNA polymerase, Klenow fragment, and ligation of the resulting DNA. This treatment should generate a 4-bp insertion and a one nucleotide

TABLE 1

β-GALACTOSIDASE ACTIVITY EXPRESSED BY PLASMID pTB-1 IN THE PRESENCE OF Tn*10*-DERIVED PLASMIDS

Plasmid	Tetracycline (μg/ml)	β-galactosidase units[a]
pMC1403	0	35 ± 6
pMC1403	1	27 ± 8
pTB-1	0	1847 ± 174
pTB-1	1	1904 ± 201
pTB-1 + pMOBglII-16	0	227 ± 21
pTB-1 + pMOBglII-16	1	1853 ± 154
pTB-1 + pMOB45(del 2)-4	0	1706 ± 202
pTB-1 + pMOB45(del 2)-4	1	1508 ± 147
pMOB45(del 2)-4	0	3 ± 2
pMOB45(del 2)-4	1	4 ± 1
pTB-1 + pACBglII-2.7	0	717 ± 50
pTB-1 + pACBglII-2.7	1	1731 ± 102
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-1	0	1247 ± 149
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-1	1	1306 ± 201
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-2	0	1401 ± 121
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-2	1	1529 ± 161
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-3	0	1129 ± 104
pTB-1 + pMOBglII-16 (*XbaI*$^-$)-3	1	1079 ± 109
None	0	5 ± 3
None	1	7 ± 4

[a] Plasmids were in *E. coli* MC1061. β-Galactosidase activities were determined in duplicate. The values are the average of at least three experiments. Bacteria were grown in A medium (Miller, 1972). No significant differences were seen in assays with cultures grown in LB medium.

shift in the reading frame of *tetR* m-RNA, near its 5'-terminus. The nucleotide sequence predicts that the protein would terminate at the fifth amino acid from the amino terminus, thus yielding a nonfunctional repressor. Transformants, containing a plasmid of the size of pMOBglII-16 lacking an *Xba*I site were isolated. pMOBglII-16 (*XbaI*$^-$)-1, -2, and -3 showed restriction patterns with *Hpa*I, *Eco*RI, and *Hin*fI identical to those of pMOBglII-16. In particular, the 1.8-kb *Hpa*I fragment yielded undistinguishable *Hin*fI and *Hin*fI + *Eco*RI restriction fragments. Double digestion of pMOBglII-16 DNA with *Hin*fI and *Xba*I yielded fragments of 1044, 600, 180, and 55 bp, in agreement with the restriction map of this DNA region (Jorgensen *et al.*, 1979; Bertrand *et al.*, 1983); DNA from pMOBglII-16 (*XbaI*$^-$)-1 yielded fragments of 1100, 600, and 180 bp, as expected from the loss of the *Xba*I site. *E. coli* MC1061 cotransformed with pTB-1 and either pMOBglII-16 (*XbaI*$^-$)-1, -2, or -3 showed constitutive β-galactosidase expression (Table 1). Thus, mutagenesis at the *Xba*I site eliminated the repressor activity encoded in plasmid pMOBglII-16.

Tetracycline resistance levels of plasmids with the 2.7-kb BglII DNA fragment from Tn 10. Multiple copies of Tn*10* lead to a decrease in the tetracycline resistance level (Taylor *et al.*, 1977; Jorgensen and Reznikoff, 1979; Chopra *et al.*, 1981; Coleman and Foster, 1981; Beck *et al.*, 1982; Moyed *et al.*, 1983; Moyed and Bertrand, 1983). Since different mechanisms have been proposed for this phenomenon, it was of interest to determine the resistance level of *E. coli* harboring either plasmid pACBglII2.7–21 or the runaway multicopy plasmid pMOBglII-16, which is present at 10 times higher copy number. As shown in Table 2, pMOBglII-16 confers about 10 times lower resistance to tetracycline than pACBglII2.7–21. The multicopy effect is also observed with plasmid pMOBglII-16 (*XbaI*$^-$)-

TABLE 2

TETRACYCLINE RESISTANCE (EOP_{50})[a] OF Tn*10*-DERIVED PLASMIDS

Plasmid	EOP_{50} (μg/ml) uninduced	EOP_{50} (μg/ml) induced[b]	EOP_{50} induced/ EOP_{50} uninduced
pRT44	38 ± 10	91 ± 11	2.4
pACBglII2.7-21	77 ± 15	114 ± 16	1.4
pMOBglII-16	3.5 ± 0.5	10.1 ± 1.0	2.8
pMOBglII-16 (*XbaI*$^-$)-1	8.2 ± 0.4	7.5 ± 1.0	0.9
pBR322[c]	107 ± 12	98 ± 10	0.9
None	<1	<1	—

[a] Plasmids were in *E. coli* MC1061. The EOP_{50} was measured in LB medium, as detailed under Materials and Methods.

[b] For induction, tetracycline (1 μg/ml) was added to the cultures 1 h before plating.

[c] Plasmid pBR322 (Bolivar *et al.*, 1977) is included as a representative of a noninducible system.

1 that lacks a functional *tetR*. These results support the view that high-level expression of *tetA* protein is involved in the multicopy effect (Chopra *et al.*, 1981; Coleman and Foster, 1981; Moyed *et al.*, 1983; Moyed and Bertrand, 1983). The results also indicate that a two- to threefold increase in resistance upon preincubation with tetracycline was dependent on a functional *tetR* repressor (compare Table 2).

DISCUSSION

Plasmids pRS*tet*Bam allow the expression of fusion proteins consisting of the first four amino acids (met-asn-ser-ser) of the tetracycline resistance protein *tetA*, plus one to four amino acids derived from the linker region (see Fig. 1). Fusions yielding active human α_1-interferon and VP1 antigenic protein of foot-and-mouth disease virus have been constructed and are under study. In addition, pTB-1 can be used to generate constructions for the expression of additional fusion proteins that can be easily monitored by the β-galactosidase activity (Casadaban *et al.*, 1980). Plasmids that provide *tetR* protein were constructed by cloning the 2.7-kb *Bgl*II DNA fragment of Tn*10* in replicons that are compatible with pRS*tet*Bam-derived plasmids. pMOBglII-16 is derived from replicon RI (Bittner and Vapnek, 1981) and pACBglII2.7-21 includes the P15A replicon of pACYC177 (Chang and Cohen, 1979). The expression of a *tetA* fragment-β-galactosidase fusion protein encoded in pTB-1 suggests that the translation initiation sites proposed for *tetA* (Bertrand *et al.*, 1983) are used in the synthesis of β-galactosidase. The results (Table 1) are consistent with the proposed mechanism of regulation of tetracycline resistance in transposon Tn*10* (Jorgensen and Reznikoff, 1979; Wray *et al.*, 1981; Coleman and Foster, 1981; Beck *et al.*, 1982; Hillen and Schollmeier, 1983, Bertrand *et al.*, 1983): *tetR* protein, which is encoded within the 2.7-kb *Bgl*II fragment, is able to repress expression of β-galactosidase originating at the *tet* regulatory region, and tetracycline acts as an inducer of the system. A similar level of induction has been obtained with concentrations of tetracycline of 0.1–10 μg/ml added to the culture medium 1–10 h before the measurements of β-galactosidase activity; heated 7-Cl tetracycline is as effective an inducer as tetracycline (results not shown).

Vectors such as pRS*tet*Bam, that include a promotor operator system for regulated transcription, are particularly valuable for the expression of proteins that are toxic for the host bacterium. Several such systems are available: *lac, trp,* λ pL, *tac,* etc. They differ in the nature of the inducing signal: addition of a chemical, amino acid depletion, temperature shift, etc. Also, alternative gene fusion

systems will provide different mRNA secondary structures, known to have a marked influence in translation efficiency (Iserentant and Fiers, 1980). For large scale fermentation procedures, the value of a particular regulated expression system would be determined by a variety of considerations such as: (i) the ease with which the inducing signal can be applied; (ii) the cost of the inducer; (iii) the level of the fully induced gene expression, and (iv) the ratio of induced to basal gene expression. The pRS*tet*Bam vector system has significant advantages in regard to the first two considerations. Tetracycline is easy to administer to the culture and tetracycline is very inexpensive (10^{-3} the cost of Isopropyl-D-thiogalactoside for amounts generating an equivalent physiological response). Recent experiments indicate that the *tetR* promoter and the *tetA* promoter, which overlap and are divergent in expression, may be competitive for RNA polymerase binding (L. V. Wray and W. S. Reznikoff, unpublished results). Therefore, mutations which inactivate the *tetR* promoter may enhance *tetA* promoter activity and could be used to increase the maximal level of expression of pRS*tet*Bam. Finally, the basal level of expression of the *tetA* promoter may be decreased (and thus the induction ratio increased) by increasing the amount of repressor protein present, perhaps by programming its synthesis from a constitutive promoter.

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REFERENCES

ALLET, B., JEPPESEN, P. G. N., KATAGARI, K. J., AND DELIUS, H. (1973). Mapping the DNA fragments produced by cleavage of λ DNA with endonuclease RI. *Nature (London)* **241,** 120–122.

BACKMAN, K., PTASHNE, M., AND GILBERT, W. (1976). Construction of plasmids carrying the *cI* gene of bacteriophage λ. *Proc. Natl. Acad. Sci. USA* **73,** 4174–4178.

BECK, C. F., MUTZEL, R., BARBE, J., AND MULLER, W. (1982). A multifunctional gene (*tetR*) controls Tn*10*-encoded tetracycline resistance. *J. Bacteriol.* **150,** 633–642.

BERTRAND, K. P., POSTLE, K., WRAY, J. L. V., AND REZNIKOFF, W. S. (1983). Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. *Gene* **23,** 149–156.

BITTNER, M., AND VAPNEK, D. (1981). Versatile cloning vectors derived from the runaway-replication plasmid pKN402. *Gene* **15,** 319–329.

BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACH, M. C., HEYNEKER, H. L., AND BOYER, H. W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2,** 95–113.

CASADABAN, M. J., CHOU, J., AND COHEN, S. N. (1980). In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of translational initiation signals. *J. Bacteriol.* **143,** 971–980.

CASADABAN, M. J., AND COHEN, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. *J. Mol. Biol.* **138,** 179–207.

CHANG, A., AND COHEN, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15 A cryptic miniplasmid. *J. Bacteriol.* **134,** 1141–1156.

CHOPRA, J., SHALES, S. W., WARD, J. M., AND WALLACE, L. J. (1981). Reduced expression of Tn*10*-mediated tetracycline resistance in Escherichia coli containing more than one copy of the transposon. *J. Gen. Microbiol.* **126,** 45–54.

COHEN, S. N., CHANG, A., AND HSU, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of E. coli by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69,** 2110–2114.

COLEMAN, D. C., AND FOSTER, T. J. (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.

FRANKLIN, T. J. (1967). Resistance of Escherichia coli to tetracyclines: Changes in permeability to tetracyclines in Escherichia coli bearing transferable resistance factors. *Biochem. J.* **105,** 371–377.

FRANKLIN, T. J., AND COOK, J. M. (1971). R factor with a mutation in the tetracycline resistance marker. *Nature (London)* **229,** 273–274.

HILLEN, W., KLOCK, G., KAFFENBERGER, I., WRAY, L. V., AND REZNIKOFF, W. S. (1982). Purification of the TET repressor and TET operator from the tran-

sposon Tn*10* and characterization of their interaction. *J. Biol. Chem.* **257,** 6605–6613.

HILLEN, W., AND SCHOLLMEIER, K. (1983). Nucleotides sequence of the Tn*10* encoded tetracycline resistance gene. *Nucleic Acids Res.* **11,** 525–533.

HILLEN, W., AND UNGER, B. (1982a). Correlation of thermodynamic and genetic properties in the Tn*10* encoded TET gene control region. *Nucleic Acids Res.* **10,** 2685–2700.

HILLEN, W., AND UNGER, B. (1982b). Binding of four repressors to double-stranded tet operator region stabilizes it against thermal denaturation. *Nature (London)* **297,** 700–702.

ISERENTANT, D., AND FIERS, W. (1980). Secondary structure of mRNA and efficiency of translation initiation. *Gene* **9,** 1–12.

JORGENSEN, R. A., BERG, D. E., ALLET, B., AND REZNIKOFF, W. S. (1979). Restriction enzyme celavage map of Tn*10,* a transposon which encodes tetracycline resistance. *J. Bacteriol.* **137,** 681–685.

JORGENSEN, R. A., AND REZNIKOFF, W. S. (1979). Organization of structural and regulatory genes that mediates tetracycline resistance in transposon Tn*10. J. Bacteriol.* **138,** 705–714.

KLEIN, R. D., SALSING, E., AND WELLS, R. D. (1980). A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. *Plasmid* **3,** 88–91.

LEVY, S. B., AND MCMURRAY, L. (1974). Detection of an inducible membrane protein associated with R factor mediated tetracycline resistance. *Biochem. Biophys. Res. Commun.* **56,** 1060–1068.

MANDEL, M., AND HIGA, A. (1970). Calcium dependent bacteriophage DNA infection. *J. Bol. Biol.* **53,** 159–162.

MANIATIS, T., FRITSCH, E. F., AND SAMBROOK, J. (1982). "Molecular Cloning. A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

MAXAM, A. M., AND GILBERT, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. *In* "Methods in Enzymology." (L. Grossman and K. Moldave, eds.), Vol. 65, pp. 499–559. Academic Press, New York/London.

MILLER, J. H. (1972). "Experiments in Molecular Genetics," pp. 352–359. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

MOYED, H. S., AND BERTRAND, K. P. (1983). Mutations in multicopy Tn*10* tet plasmids that confer resistance to inhibitory effects of inducers of tet gene expression. *J. Bacteriol.* **155,** 557–564.

MOYED, H. S., NGUYEN, T. T., AND BERTRAND, K. P. (1983). Multicopy Tn*10 tet* plasmids confer sensitivity to induction to tet gene expression. *J. Bacteriol.* **155,** 549–556.

ROSENBERG, M., AND COURT, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu. Rev. Gen.* **13,** 319–353.

TAIT, R., RODRIGUEZ, R., AND BOYER, H. (1977). Altered tetracycline resistance in pSC101 recombinant plasmids. *Mol. Gen. Genet.* **151,** 327–331.

TAYLOR, D. P., GREENBERG, J., AND ROWND, R. H. (1977). Generation of miniplasmid from copy number mutants of the R plasmids NR1. *J. Bacteriol.* **132,** 986–995.

TIMMIS, K. N., CABELLO, F., AND COHEN, S. M. (1978). Cloning and characterization of Eco RI and Hind III restriction endonuclease-generated fragments of antibiotics resistance plasmids R65 and R6. *Mol. Gen. Genet.* **162,** 121–137.

WARTELL, R. M., AND REZNIKOFF, W. S. (1980). Cloning DNA restriction endonuclease fragments with protruding single-stranded ends. *Gene* **9,** 307–319.

WRAY, L. V., JORGENSEN, R. A., AND REZNIKOFF, W. S. (1981). Identification of the tetracycline resistance promoter and repressor in transposon Tn*10. J. Bacteriol.* **147,** 297–304.

YANG, H. L., ZUBAY, G., AND LEVY, S. B. (1976). Synthesis of an R plasmid protein associated with tetracycline resistance is negatively regulated. *Proc. Natl. Acad. Sci. USA* **73,** 1509–1512.