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Highly Repressible Expression System for Cloning Genes That Specify Potentially Toxic Proteins

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A highly repressible expression vector system that allows the cloning of potentially deleterious genes has been constructed. Undesired expression of a cloned gene was prevented (i) at the level of initiation of transcription, by the presence of the strong but highly repressible leftward promoter of bacteriophage lambda, λ p_L, and (ii) at the level of transcript elongation or translation, through synthesis of antisense RNA complementary to the mRNA of the cloned gene. The system was tested by measuring the inhibition of expression of *traT*, the gene for the TraT major outer membrane lipoprotein. Direct detection and functional assays indicated that an essentially complete inhibition of *traT* expression was obtained. As a further test of the system, the gene encoding the *Eco*RI restriction endonuclease was cloned in the absence of the gene of the corresponding protective *Eco*RI modification methylase. Transformants harboring this construct were only viable when both repression controls were operational.

A number of the genes encoding biologically interesting proteins have proved difficult or impossible to clone due to the toxicity of their products to host cells. This is particularly true of foreign genes but is also the case for certain native genes when these are overexpressed (e.g., outer membrane protein genes) or when mutant derivatives of them are generated (7, 9, 13, 25).

The TraT protein specified by the *traT* gene of F group conjugative plasmids such as F, R6-5, and R100 is a 26,000-dalton major outer membrane lipoprotein that is exposed on the cell surface of *Escherichia coli* (15). Strains that express the *traT* gene show increased resistance to the bactericidal effects of serum and have a reduced ability to act as recipients when mated with a donor strain carrying a related plasmid, a phenomenon known as surface exclusion. Results with this protein and with several other outer membrane proteins have shown that their overexpression or alteration by mutation is frequently deleterious to bacterial growth (7, 15).

One method by which the expression of a particular gene might be blocked is through the use of antisense RNA, that is, RNA complementary to the mRNA of a specific gene, to inhibit gene expression (12, 19, 24, 26). In this paper, we describe the construction and characterization of a highly repressible expression vector that exploits an antisense RNA system to facilitate the cloning of potentially deleterious genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids that were used are shown in Table 1. When necessary, strains were lysogenized with wild-type bacteriophage lambda (kindly provided by S. Harayama). *E. coli* M72, which specifies a temperature-sensitive lambda repressor, was used as the host strain for plasmids carrying nondeleterious genes.

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Media and chemicals. Strains were cultivated in L broth and antibiotic medium no. 3 (Difco Laboratories) or in minimal A medium (17) supplemented with 2 g of glucose per liter. For maximal induction of the *lac* promoter, strains were cultivated in minimal A medium supplemented with 2 mM isopropyl-β-D-thiogalactoside (IPTG) and 2 g of maltose per liter. Antibiotics were incorporated into medium at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; and tetracycline, 12.5 μg/ml. The self-complementary linker oligonucleotide 5'-GGAATTCC-3' and a 17-base sequencing primer, complementary to coordinates 465 to 481 of the pUC19 sequence (31), were purchased from Pharmacia.

DNA techniques. Standard conditions were used for the preparation of DNA, digestion with restriction endonucleases, fill-in reactions (i.e., repair of cohesive ends with DNA polymerase I Klenow fragment and deoxyribonucleotides), ligations, and transformation (14).

Construction of pDOC19, pDOC22, and pDOC23. Figure 1 shows a schematic representation of plasmid pDOC19 and its derivatives, pDOC22 and pDOC23. The plasmid pDOC6 was used as a source of the *traT* structural gene for the construction of pDOC19, as it has a *lac* promoter situated immediately downstream of and oriented towards the *traT* gene. The position and orientation of the *lac* promoter suggested that it could be used to interfere with expression of the *traT* gene (see Results). pDOC6 contains a 0.82-kilobase (kb) *Nde*I (filled, i.e., repaired, with DNA polymerase I)-*Fnu*DII DNA fragment of pKT107 inserted between the *Hin*dIII (filled) and *Sma*I sites of pUC8 so as to regenerate the *Hin*dIII site of the vector. A 0.86-kb *Hin*dIII-*Fnu*DII DNA fragment from pDOC6, carrying *traT* and the *lac* promoter, was inserted between the *Hin*dIII and *Nde*I (filled) sites of pLV85 to form pDOC19 (Fig. 1). pLV85 is a derivative of the λ p_L expression vector pPLc245 and contains a *Sal*I-*Bam*HI-*Cla*I-*Eco*RV-*Hpa*I-*Hin*dIII polylinker instead of the *Sal*I-*Xba*I-*Pst*I-*Xba*I-*Hin*dIII polylinker of the latter plasmid (C. D. O'Connor and R. W. Pickup, unpublished results). pDOC19 was modified to form pDOC22 by the insertion of a 17-base-pair (bp) *Hin*dIII DNA

TABLE 1. Strains and plasmids used

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
JM83(λ)	F⁻ *ara* Δ(*lac-proAB*) *rpsL thi* φ80d Δ(*lacZM15*) λ⁺	29
LC102	F⁻ *ara argG gal his ile lac leu met mtl proC purE rpsL thi trp xyl tsx*	5
LE392(λ)	*galT22 hsdR514 lacY1 metB1 supE44 supF58 trpR55* λ⁺	20
M72	Δ(*bio-uvrB*) *lacZ*(Am) *rpsL* Δ(*trpEA2*) (λ *N*am7 *N*am53 *c*I857 ΔH1)	3
SH526(λ)	*trp*::Tn*5* derivative of W3110, Kan^r, λ⁺	1
Plasmids		
pBR312-*lac*	17-bp *Hin*dIII fragment carrying *lac* operator cloned in pBR313, Ap^r	2
pDOC6	0.82-kb *Nde*I (filled)-*Fnu*DII DNA fragment carrying the *traT* gene from pKT107 cloned in pUC8, Ap^r	This paper
pDOC19	0.86-kb *Hin*dIII-*Fnu*DII DNA fragment from pDOC6 cloned in λ p_L vector pLV85, Ap^r	This paper
pDOC20	1.2-kb *Hin*dIII fragment carrying the *lacI* gene cloned in pACYC184, Cm^r	This paper
pDOC22	17-bp *Hin*dIII DNA fragment carrying *lacO* cloned into pDOC19, *TraT*⁺, Ap^r	This paper
pDOC23	*Bam*HI-*Hin*dIII deletion derivative of pDOC19, *traT*⁺, Ap^r	This paper
pDOC54	285-bp *Fnu*DII-*Eco*RI DNA fragment from pUC19 substituted for the *Eco*RI fragment in pDOC23, *traT*, Ap^r	This paper
pDOC56	2.2-kb *Nde*I (filled)-*Hae*III DNA fragment from NTP14 cloned in pDOC55, *Eco*RI *hsdR*⁺ *hsdM*⁺, Ap^r	This paper
pKT107	6-kb *Eco*RI fragment from plasmid R6-5 cloned into pACYC184, *traT*⁺, Tc^r	15
pLV56	Derivative of pACYC184 that codes for *Eco*RI modification enzyme, Cm^r	This paper
pLV85	Derivative of pPLc245 carrying λ p_L promoter, Ap^r	This paper
NTP14	Codes for *Eco*RI restriction and modification enzymes, Ap^r	27
pPLc245	Carries strong leftward promoter from phage λ, λ p_L, Ap^r	25
R100-1	Conjugative antibiotic resistance plasmid derepressed for transfer, Cm^r Hg^r Sm^r Su^r Tc^r	5, 11
pUC8 and pUC9	Cloning vectors, Ap^r	29
pUC18 and pUC19	Cloning vectors, Ap^r	21, 31

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Hg^r, mercury resistance; Kan^r, kanamycin resistance; Sm^r, streptomycin resistance; Su^r, sulfonamide resistance; Tc^r, tetracycline resistance.

fragment carrying the *lac* operator between the λ p_L promoter and the *traT* gene. To make several restriction sites in the *traT* gene unique, the polylinker DNA between the *Bam*HI and *Hin*dIII sites of pDOC19 was deleted by removal of the small *Bam*HI-*Hin*dIII fragment and recircularization of the filled-in DNA. The deletion derivative was designated pDOC23.

Construction of pDOC55. A 285-bp *Fnu*DII-*Eco*RI DNA fragment from pUC19, carrying most of the region encoding the *lacZ* alpha peptide, was ligated to *Eco*RI linkers and redigested with *Eco*RI. The resulting *Eco*RI fragment was cloned into *Eco*RI-cut pDOC23 DNA, thereby replacing the *traT* gene-carrying *Eco*RI fragment of this plasmid. Plasmids in which the gene for the *lacZ* alpha peptide was reconstituted were identified by screening transformants of JM83(λ) for a Lac⁺ phenotype on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. To remove the *Eco*RI site nearest to the p_L promoter, DNA of a representative plasmid (designated pDOC54) from a Lac⁺ transformant was linearized by partial digestion with *Eco*RI, treated with DNA polymerase I Klenow fragment to repair the cohesive ends, and recircularized. One of the plasmids that still gave a Lac⁺ phenotype but had lost this *Eco*RI site was designated pDOC55.

Assay for surface exclusion. The frequency of transfer of R100-1, a derepressed conjugative plasmid that specifies resistance to tetracycline, to *E. coli* LE392(λ) carrying pDOC20 (a derivative of pACYC184 that overproduces *lac* repressor) and either pDOC23 (*traT*⁺) or pDOC54 (*traT*) was measured. Bacterial matings were done by the method of Miller (17) with medium supplemented with 2 mM IPTG when necessary. Dilutions of each conjugation mixture were plated on medium containing ampicillin to select for recipients or ampicillin and tetracycline to select for transconjugants.

Temperature induction of protein synthesis. Overnight cultures of *E. coli* M72 carrying derivatives of pDOC23 were diluted 1:20 into fresh prewarmed L broth and grown at 30°C to an A_{600} of 0.5. An equal volume of L broth preheated to 54°C was then added, and the cultures were incubated at 42°C for a further 40 to 120 min. Samples (2 ml) of induced and uninduced cultures were washed with phosphate-buffered saline and suspended in final sample buffer before sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To induce moderate amounts of each mutant protein, cultures were shifted to 37°C rather than 42°C.

Immunodetection of the TraT lipoprotein. Western (immunoblot) and dot blotting of proteins were carried out as described elsewhere (8, 28). The filters were blocked against nonspecific protein binding by incubation in a solution containing 10 mM Tris hydrochloride, pH 7.5, 2 mM disodium EDTA, 150 mM NaCl, 0.5% (wt/vol) bovine serum albumin, and 0.05% (vol/vol) Triton X-100 for 2 h before the addition of monoclonal antibody in the same buffer. Monoclonal antibody binding was detected by the method of Hawkes et al. (8) with 4-chloro-1-naphthol as a chromogenic indicator.

RESULTS

Expression of *traT* from the repressed p_L promoter. The *traT* gene of the antibiotic resistance plasmid R6-5 was placed under the control of the strong but highly repressible λ p_L promoter to form the plasmids pDOC19, pDOC22, and pDOC23 (Fig. 1).

Strain M72(pDOC19), previously grown at 30°C, was incubated at 42°C for 2 h to induce transcription of *traT* from

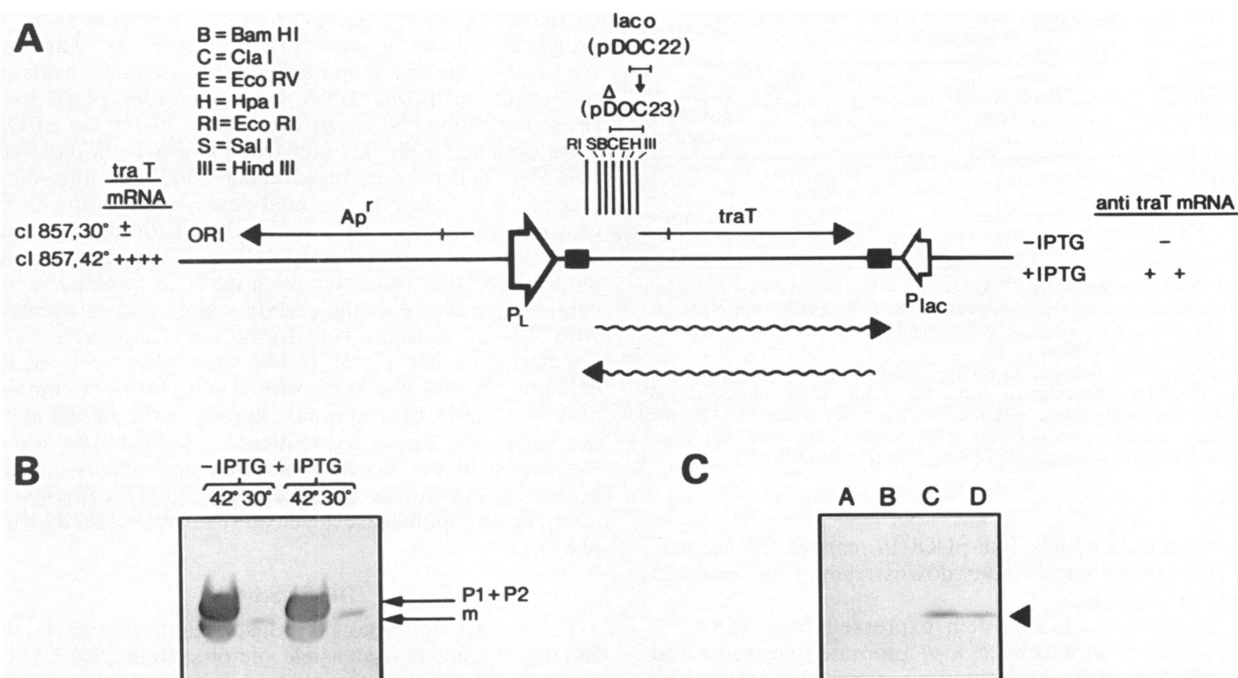

FIG. 1. Expression of the *traT* gene under the control of λ p_L in repressing and nonrepressing conditions and the principle of the expression/repression system of pDOC23. (A) Structure of pDOC19 and its derivatives, pDOC22 and pDOC23. The position of the inserted *lac* operator in pDOC22 (vertical arrow) and the deletion in the polylinker region in pDOC23 (horizontal bar) are shown above the structure of the parent plasmid pDOC19. pDOC19 is 3.7 kb in size. The convergent promoters and operators in the expression/repression system are represented by the large open arrows and filled boxes, respectively, while the long thin horizontal arrows represent the directions of transcription of the ampicillin resistance gene (Ap^r) and the *traT* gene. The wavy lines represent mRNA and anti-mRNA molecules. (B) Immunodetection of TraT protein from strain M72 carrying the *lacO* derivative pDOC22 and pDOC20, a compatible plasmid that overproduces *lac* repressor. Equal amounts of protein from cells grown at 30 or 42°C in L broth in the presence or absence of 2 mM IPTG were transferred by Western blotting to nitrocellulose. TraT protein was detected with monoclonal antibody Mo867 (4; D. Bitter-Suermann, unpublished results). P1 and P2 indicate the position of the two pro-TraT protein species (which comigrate), and m denotes the position of the mature form of the protein (19). (C) Western blot of proteins from SH526(λ) and SH526(λ)(pDOC23). These strains, which specify wild-type lambda repressor, were grown at 37°C in the presence of glucose, maltose, or 2 mM IPTG and maltose. Approximately 10 μg of protein from each whole-cell extract was loaded in each track. Track A, SH526(λ) grown with glucose; track B, SH526(λ)(pDOC23) grown with maltose and IPTG; track C, SH526(λ)(pDOC23) grown with glucose; track D, SH526(λ)(pDOC23) grown with maltose alone. The position of the TraT monomer band is marked.

p_L. In agreement with previous results (18), Western blots with a monoclonal antibody specific for the TraT lipoprotein detected large quantities of both the precursor and the mature forms of the protein when p_L had been induced (Fig. 1B). Densitometer scanning of sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue indicated that the TraT protein constituted ≥15% of the total cell protein under these conditions (data not shown). When λ p_L was repressed, however, very little of the lipoprotein was produced, although it was still possible to detect it by Western blotting and by a surface exclusion assay (data not shown). DNA sequencing of the λ p_L promoter region present in pDOC19 confirmed that no mutation had occurred in this region during genetic manipulations. We wished to know whether it was possible to reduce expression of the *traT* gene to levels below the limits detectable with the assays employed. Deuschle et al. have recently shown that the insertion of a *lac* operator into DNA can block transcribing RNA polymerase molecules when the *lac* repressor is present and hence terminate transcription (6). Therefore, to lower expression of the *traT* gene still further, a 17-bp *lac* operator fragment was cloned between the λ p_L promoter and the structural gene for *traT* to form the plasmid pDOC22 (Fig. 1A). However, for reasons that are not clear, no substantial further repression of the gene resulted when pDOC22 was maintained in strains that overproduced the *lac* repressor, due to the presence of pDOC20 (a compatible plasmid carrying the *lacI* gene) (Fig. 1B).

Design and testing of a highly repressible combined p_L-antisense RNA vector. To reduce gene expression further, it was necessary either to block the low level of initiation of transcription of the *traT* gene or to inhibit the elongation of initiatied transcripts or their translation. One means by which both of these steps in gene expression might be repressed would be to place a convergent promoter immediately downstream of the gene. Figure 1 shows the principle of the repression system. When a gene such as *traT* is cloned between the λ p_L and *lac* promoters so that its transcription is from λ p_L to *lacp*, its expression should be impaired when λ p_L is fully repressed and *lacp* is maximally induced. This can be achieved by growing host cells specifying wild-type lambda repressor with a carbon source such as maltose (to avoid catabolite repression) and in the presence of IPTG. Under these conditions the predominant direction of transcription is through the cloned gene, towards the repressed λ p_L promoter. Apart from producing an antisense RNA complementary to the *traT* mRNA, transcription in this direction could also interfere with transcription from p_L due to collision of RNA polymerase molecules moving in opposite directions (30). To test such a system, we used the

TABLE 2. Surface exclusion of R100-1 in the presence of pDOC23

Plasmid in recipient strain[a]	IPTG (2 mM) present	Transfer of R100-1[b]	
		No IPTG	2 mM IPTG added
pDOC23 (*traT*$^+$)	–	7.6×10^{-4}	5.1×10^{-4}
	+	1.7×10^{-3}	1.5×10^{-2}
pDOC54 (*traT*)	–	2.5×10^{-2}	3.5×10^{-2}
	+	3.0×10^{-2}	1.1×10^{-2}

[a] The recipient strain was LE392(λ) carrying pDOC20, a ColE1-compatible plasmid expressing the *lac* repressor protein, and either pDOC23 or pDOC54, as indicated, and was grown in the absence and presence of 2 mM IPTG. The donor strain was LC102(R100-1).

[b] Per recipient cell. Dilutions of each conjugation mixture were plated on medium containing ampicillin to select for recipients or ampicillin and tetracycline to select for transconjugants. The frequency of transfer of R100-1 to the recipient strains was calculated by dividing the number of transconjugants obtained by the total number of recipients (per milliliter).

plasmid pDOC23 which, like pDOC19, carries the *lac* promoter from pUC8 immediately downstream of and oriented towards the *traT* gene.

The amount of TraT protein expressed from pDOC23, under conditions in which the λ p_L promoter was repressed and in which the *lac* promoter was repressed or induced by the *lac* inducer IPTG, was measured in two ways. First, the frequency of transfer of R100-1 (a conjugative plasmid specifying resistance to tetracycline and derepressed for conjugal transfer) to cells harboring pDOC23 (*traT*$^+$) or pDOC54 (*traT*) was measured in the presence and absence of IPTG. In the presence of IPTG the frequency of transfer of R100-1 to cells carrying pDOC23 was over 20-fold higher than in its absence (Table 2). In fact, the frequency of transfer was indistinguishable from that obtained with recipient cells lacking the *traT* gene.

To measure TraT protein more directly, whole-cell extracts from cells that had been grown in minimal medium supplemented with glucose or maltose and IPTG were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. TraT protein was detectable in the samples from glucose-grown cells but was completely undetectable in samples from cells in which the *lac* promoter had been maximally induced, i.e., grown in the presence of IPTG and with maltose as a carbon source to avoid catabolite repression (Fig. 1C). Taken together with the surface exclusion data, these results indicated that the repression system was highly efficient at inhibiting synthesis of the TraT lipoprotein.

To make the expression/repression system more generally applicable and to facilitate the cloning of DNA fragments, plasmid pDOC55 was constructed (Fig. 2). In addition to the *lac* promoter, pDOC55 contains the region of pUC19 encoding the *lacZ* alpha peptide and hence possesses nine unique restriction sites (*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Sph*I, and *Hin*dIII) downstream of the p_L promoter. Insertion of foreign DNA into any one of these sites and subsequent transformation of JM83(λ) results in a Lac$^-$ phenotype that can readily be detected in medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (29).

pDOC55 was tested by cloning the *Eco*RI restriction and modification genes and subsequently rendering the modification gene nonfunctional. A 2.2-kb *Nde*I (filled)-*Hae*III fragment from the plasmid NTP14 (27), carrying the *Eco*RI restriction and modification genes, was inserted into the *Sma*I site of pDOC55. One of the resulting clones that carried the *Eco*RI genes in the same orientation as the λ p_L promoter was designated pDOC56 (Fig. 2). The *Eco*RI methylase gene was then made nonfunctional by excision of the small *Ava*I-*Sal*I DNA fragment from pDOC56 and recircularization. No transformants (<10 per μg of DNA) were obtained with this construction when the host strain SH526(λ) had been cultured under conditions that did not induce the *lac* promoter located downstream of the endonuclease gene. In contrast, about 10^4 transformants per μg of DNA were obtained with host cells that had been maximally induced for this promoter by growth in minimal medium containing maltose as the carbon source and supplemented with IPTG. Normal transformation frequencies (>10^6 transformants per μg of DNA) were observed when the deletion plasmid was transformed into JM83(λ) containing pLV56, a compatible plasmid expressing the *Eco*RI methylase gene (22). These experiments indicated that although expression of the *Eco*RI restriction endonuclease gene is ordinarily lethal in the absence of the *Eco*RI methylase, the restriction endonuclease gene can readily be cloned by using pDOC55.

DISCUSSION

This report describes the construction, characterization, and use of a highly repressible vector system, pDOC55 (Fig. 2) for the cloning of genes that are potentially deleterious to *E. coli*. Regulated expression of cloned genes is achieved through the presence of the p_L promoter upstream of the multiple cloning sites and heat inactivation of the lambda *c*I857 repressor. Repression of cloned genes lacking native promoters is achieved through growth of host cells at 30°C, at which temperature lambda repressor is active and hence λ p_L is repressed. Additional repression of expression of such genes is achieved through IPTG-induced synthesis of antisense RNA originating from the convergent *lac* promoter located downstream of the cloned genes.

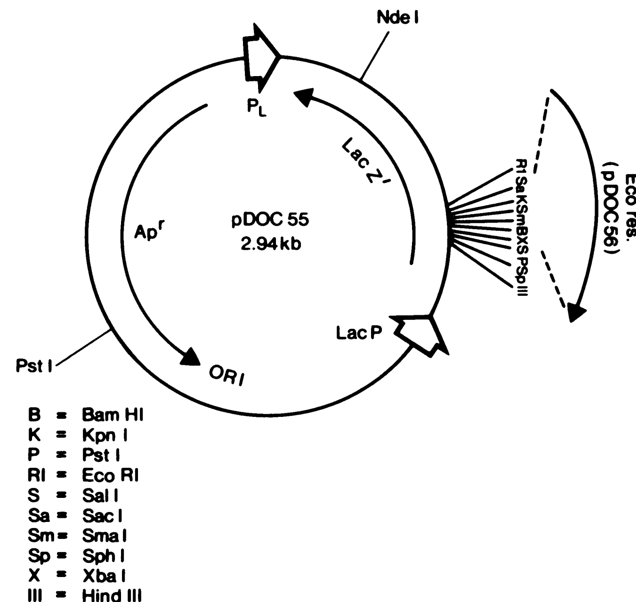

FIG. 2. Schematic representation of the structure of pDOC55. The site of insertion and orientation of the *Eco*RI restriction and modification genes (Eco res) in the multiple cloning site of pDOC55 to form the plasmid pDOC56 are also indicated. See text for further details.

By means of sensitive direct detection (Western blot) and functional (surface exclusion) assays, an essentially complete inhibition of expression of the *traT* gene in pDOC23 was demonstrated when the *lac* promoter was induced. Moreover, the *Eco*RI restriction endonuclease gene was cloned in the absence of the *Eco*RI modification methylase. Although we have not measured the absolute levels of the *Eco*RI restriction endonuclease produced under these conditions, this experiment was probably a particularly severe test of the efficiency of the vector. Thus, it is very probable that the repression system will prove useful for the isolation of other genes that determine equally toxic proteins.

The mechanism by which antisense RNA exerts its regulatory effects is still unclear. Studies on both natural and artificial systems of antisense RNA regulation suggest that in vivo hybridization of the antisense RNA to its complementary mRNA may cause a specific block in the translation of the mRNA (12, 19, 24, 26). However, the possibility of further interference mediated at the transcriptional level, due to the collision of RNA polymerase molecules moving in opposite directions, cannot be excluded (30). In the former situation, the secondary structure and stability of the antisense RNA and its corresponding mRNA, as well as their relative concentrations, is likely to be of crucial importance in determining the degree of inhibition attained. Further work will be needed to assess the relative contributions of each repression mechanism to the overall efficiency of the system. Knowledge of this is not, however, an essential prerequisite to the successful use of the vector. Although we have studied here repression of expression of genes and mutant genes lacking native promoters, this situation is not artificial in the sense that many foreign genes have promoters that function extremely poorly or not at all in *E. coli* (16, 25). However, the system might be ineffective when a cloned DNA fragment contains a strong *E. coli*-like promoter upstream of the gene of interest. In such cases, it should be possible to circumvent the problem either by cloning DNA fragments that contain only the structural portion of the gene or by using a stronger promoter 3′ to the inserted gene.

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