Plasmid **44,** 138–151 (2000)
doi:10.1006/plas.2000.1477, available online at http://www.idealibrary.com on IDEAL®

Construction and Characterization of a Highly Regulable Expression Vector, pLAC11, and Its Multipurpose Derivatives, pLAC22 and pLAC33

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Received November 15, 1999

A number of different expression vectors have been developed to facilitate the regulated overproduction of proteins in *Escherichia coli* and related bacteria. Some of the more popular ones include pKK223-3, pKK233-2, pTrc99A, and the pET family of expression vectors. These vectors were designed to be regulable and can be grown under conditions that repress protein production or under conditions that induce protein production. Unfortunately, however, numerous researchers have found that these vectors produce significant amounts of protein even when grown under repressed conditions. We describe here a new expression vector, pLAC11, which was designed to be more regulable and thus more tightly repressible when grown under repressed conditions. The tight regulation of pLAC11 was achieved by utilizing the O3 auxiliary operator, CAP binding site, promoter, and O1 operator that occur in the wild-type *lac* control region. The pLAC11 vector can be used to conduct physiologically relevant studies in which the cloned gene is expressed at levels comparable to that obtainable from the chromosomal copy of the gene in question. In experiments in which a bacterial cell contained both a null allele in the chromosome and a second copy of the wild-type allele on pLAC11, we observed that cells grown under repressed conditions exhibited the null phenotype while cells grown under induced conditions exhibited the wild-type phenotype. Two multipurpose derivatives of pLAC11, pLAC22, and pLAC33 have also been constructed to fulfill different experimental needs.

Key Words: cloning; inducible; plasmid; repressible.

Most of the routinely employed expression vectors rely on *lac* control in order to overproduce a gene of choice (Brosius, 1988; Balbás and Bolivar, 1990). With the wild-type *lac* promoter/operator, induction ratios of up to 1000× have been observed between repressed versus induced growth conditions (Beckwith and Zipser, 1970). The *lac* promoter/operator functions as it does due to the interplay of three main components (the wild-type *lac* control region is shown in Fig. 1; for general reviews, see Glass, 1982; and Müller-Hill, 1996). First, the wild-type *lac* −10 region (TATGTT) is very weak. c-AMP-activated CAP protein is able to bind to the CAP site just upstream of the −35 region which stimulates binding of RNA polymerase to the promoter. Repression of the *lac* promoter is observed when glucose is the main carbon source because very little c-AMP is present and thus low amounts of c-AMP-activated CAP protein are available. When poor carbon sources such as lactose or glycerol are used, c-AMP levels rise, large amounts of c-AMP-activated CAP protein become available, and thus induction of the *lac* promoter can occur. Second, Lac repressor binds to the *lac* operator which prevents transcription of the *lac* operon. Lac repressor can be overcome by allolactose which is a natural by-product of lactose utilization in the cell or by the gratuitous inducer IPTG.[2] Third, the *lac* operator can form stable loop structures which prevents the initiation of transcription

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[2] Abbreviations used: Δ, deletion; *E., Escherichia;* IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; PCR, polymerase chain reaction; ^{R}, resistance/resistant; SD, Shine–Dalgarno; Tn, transposon; XGal, 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside.

0147-619X/00 $35.00

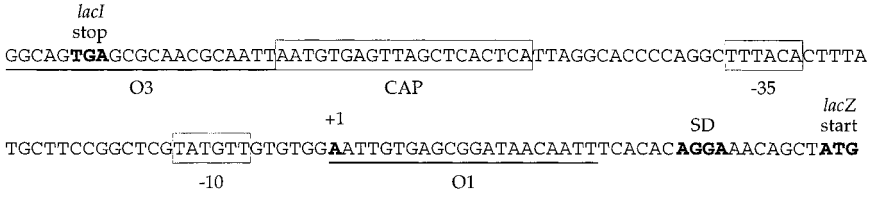

FIG. 1. Control region of the wild-type *lac* operon. The region from the O3 auxiliary operator through the translational start of the *lacZ* gene is shown. DNA binding sites are indicated below the DNA sequence while important RNA sites are shown above the DNA sequence. The Shine–Dalgarno ribosome binding site for *lacZ* is indicated by SD.

due to the interaction of the Lac repressor with the *lac* operator (O1) and one of two auxiliary operators, O2 which is located downstream in the coding region of the *lacZ* gene or O3 which is located just upstream of the CAP binding site.

While binding of Lac repressor to the *lac* operator is the major effector of *lac* regulation, the other two components are not dispensable. Unfortunately, most of the routinely used *lac*-regulable vectors contain either mutations or deletions which alter the effect of the other two components. The pKK223-3 (Brosius and Holy, 1984), pKK233-2 (Amann and Brosius, 1985), pTrc99A (Amann *et al.*, 1988), and pET family of vectors (Studier *et al.*, 1990) contain only the *lac* operator (O1) and lack both the CAP binding site and the O3 auxiliary operator. pKK223-3, pKK233-2, and pTrc99 use a *trp*–*lac* hybrid promoter that contains the *trp* −35 region and the *lac*UV5 −10 region which contains a strong TATAAT site instead of the weak TATGTT site. The pET family of vectors uses the strong T7 promoter. Given this information, perhaps it is not surprising that researchers have found it is not possible to tightly shut off genes that are cloned into these vectors. The promoter/operator control regions utilized by these vectors are shown in Fig. 2.

We wanted to design a vector which would allow researchers to be able to better regulate

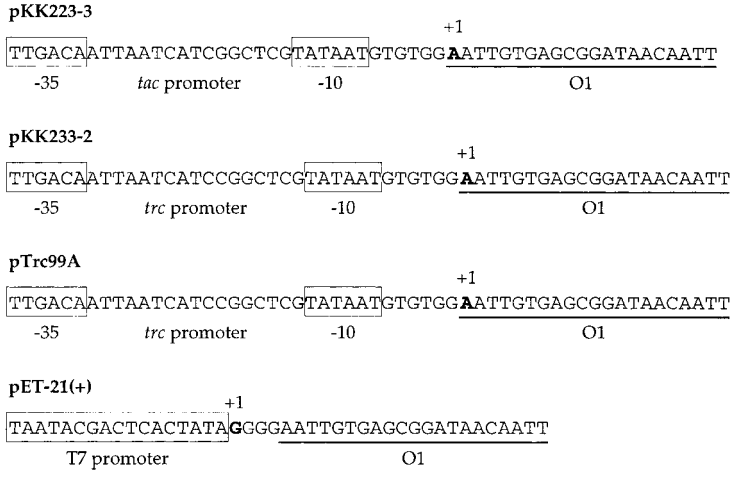

FIG. 2. Control regions of routinely employed expression vectors. The region from the promoter through the *lac* O1 operator for the pKK223-3, pKK233-2, pTrc99A, and pET-21(+) vectors is shown. DNA binding sites are indicated below the DNA sequence while important RNA sites are shown above the DNA sequence. The pET-21(+) control region is typical of the control region that is found in other pET expression vectors. Note that the pKK223-3, pKK233-2, pTrc99A, and pET-21(+) expression vectors do not contain a CAP binding site and lack an auxiliary *lac* operator.

their cloned genes in order to conduct physiological experiments. The expression vectors described here were designed utilizing the wild-type *lac* promoter/operator to accomplish this purpose and include all of the *lac* control region that is contained between the start of the O3 auxiliary operator through the end of the O1 operator (see Fig. 1). As with all *lac*-based vectors, the pLAC11, pLAC22, and pLAC33 expression vectors that we have constructed can be turned on or off by the presence or absence of the gratuitous inducer IPTG.

MATERIALS AND METHODS

Media

Minimal M9 and rich LB media used in this study were prepared as described by Miller (1972). The antibiotics ampicillin, kanamycin, streptomycin, and tetracycline were used in rich media at final concentrations of 100, 40, 200, and 20 μg/ml, respectively. When used in minimal media, tetracycline was added at a final concentration of 10 μg/ml. XGal was added to media at a final concentration of 40 μg/ml, glucose was added to media at a final concentration of 0.2%, and unless otherwise noted IPTG was added to media at a final concentration of 1 mM.

Bacterial Strains and Plasmids

The bacterial strains and plasmids that were used in this study are listed in Table 1. To construct ALS225, ALS224 was mated with ALS216 and streptomycin-resistant, blue recombinants were selected on a rich LB plate that contained streptomycin, XGal, and IPTG. To construct ALS226, ALS224 was mated with ALS217 and streptomycin-resistant, kanamycin-resistant recombinants were selected on a rich LB plate that contained streptomycin and kanamycin. To construct ALS515, ALS514 was mated with ALS216 and streptomycin-resistant, blue recombinants were selected on a rich LB plate that contained streptomycin, XGal, and IPTG. To construct ALS527, ALS524 was mated with ALS224 and streptomycin-resistant, tetracycline-resistant recombinants were selected on a rich LB plate that contained streptomycin and tetracycline. To construct ALS533, a P1 lysate prepared from ALS213 was used to transduce ALS224 and tetracycline-resistant transductants were selected. To construct ALS535, ALS533 was mated with ALS498 and tetracycline-resistant recombinants were selected on a minimal M9 glucose plate that contained tetracycline, leucine, and thiamine (B_1). To construct ALS611, a P1 lysate prepared from ALS420 was used to transduce ALS410 and tetracycline-resistant white transductants were selected on a rich LB plate that contained tetracycline, XGal, and IPTG. To construct ALS749, a P1 lysate prepared from ALS611 was used to transduce ALS221 and tetracycline-resistant white transductants were selected on a rich LB plate that contained tetracycline, XGal, and IPTG.

Construction of the pLAC11, pLAC22, and pLAC33 Expression Vectors

To construct pLAC11, primers 1 and 2 (see Table 2) were used to PCR amplify a 953-bp fragment from the plasmid pBH20 which contains the wild-type *lac* operon. Primer 2 introduced two different base-pair mutations into the seven-base spacer region between the Shine–Dalgarno site and the ATG start site of the *lacZ* which converted it from AACAGCT to AAGATCT, thus placing a *Bgl*II site between the Shine–Dalgarno and the start codon of the *lacZ* gene. The resulting fragment was gel isolated, digested with *Pst*I and *Eco*RI, and then ligated into the 3613-bp fragment from the plasmid pBR322Δ*Ava*I (described below) which had been digested with the same two restriction enzymes. To construct pBR322Δ*Ava*I, pBR322 was digested with *Ava*I, filled in using Klenow, and then religated.

To construct pLAC22, a 1291-bp *Nco*I, *Eco*RI fragment was gel isolated from pLAC21 (described below) and ligated to a 4361-bp *Nco*I, *Eco*RI fragment which was gel isolated from pBR322/*Nco*I (described below). To construct pLAC21, primers 2 and 3 (see Table 2) were used to PCR amplify a 1310-bp fragment from the plasmid pMS421 which contains the

TABLE 1

Bacterial Strains and Plasmids Used in this Work

Laboratory name	Original name	Genotype	Reference or source
		Bacterial strains (*E. coli*)	
ALS213	K5096	*proAB*::Tn10	Miller and Friedman (1980)
ALS216	SE9100	*araD139*Δ(*lac*)*U169 thi f1bB5301 deoC7 ptsF25 rpsE*/F′*lacI*^{Q1} Z^+ Y^+ A^+	Altman *et al.* (1990)
ALS217	SE9100.1	*araD139* Δ(*lac*)*U169 thi f1bB5301 deoC7 ptsF25 rpsE*/F′ *lacI*^{Q1} *Z*::Tn5 *Y*+ *A*+	S. Emr
ALS221	BL21(DE3)	*ompT hsdS*(b) (R−M−) *gal dcm* (DE3)	Studier and Moffatt (1986)
ALS224	MC1061	*araD139* Δ(*araABOIC-leu*)*7679* Δ(*lac*)*X74 galU galK rpsL hsr− hsm*+	Casadaban and Cohen (1980)
ALS225		MC1061/F′*lacI*^{Q1}Z^+ Y^+ A^+	This work
ALS226		MC1061/F′*lacI*^{Q1} *Z*::Tn5 Y^+ A^+	This work
ALS269	CSH27	F^- *trpA33 thi*	Miller (1972)
ALS410	CSH1	F^- *trp lacZ rpsL thi*	Miller (1972)
ALS413	MG1655	*E. coli* wild-type F^- λ^-	Guyer *et al.* (1980)
ALS420	RS1071	*leuB6 fhuA2* zah-281::Tn10 *glnV44*(AS) *gal-6 lambda- trp-31 hisG1*(Fs) *argG6 rpsL104 malT1*(lambda res) *xylA7 mtlA2 metB1*	R. Simons
ALS498	JM101	*supE thi* Δ(*lac-proAB*)/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{Q} Δ(*lacZ*)*M15*	Yanisch-Perron (1985)
ALS514	NM554	MC1061 *recA13*	Raleigh *et al.* (1988)
ALS515		MC1061 *recA13*/F′*lacI*^{Q1}Z^+ Y^+ A^+	This work
ALS524	XL1-Blue	*recA1 endA1 gyrA96 thi−1 hsdR17 supE44 relA1 lac*/F′ *proAB lacI*^{Q} Δ(*lacZ*)*M15* Tn10	Stratagene
ALS527		MC1061/F′ *proAB lacI*^{Q} Δ(*lacZ*)*M15* Tn10	This work
ALS533		MC1061 *proAB*::Tn10	This work
ALS535		MC1061 *proAB*::Tn10/F′*lacI*^{Q} Δ(*lacZ*)*M15 proA*+*B*+	This work
ALS598	CAG18615	zjb-3179::Tn10dKan lambda- *rph*-1	Singer *et al.* (1989)
ALS611		CSH1 zah-281::Tn10	This work
ALS749		BL21(DE3) *lacZ* zah-281::Tn10	This work

Plasmids

Plasmid name	Relevant characteristics	Reference or source
pBH20	Wild-type *lac* promoter/operator, Amp^R, Tet^R, ColE1 replicon	Itakura *et al.* (1977)
pBR322	Amp^R, Tet^R, ColE1 replicon	Bolivar *et al.* (1977)
pET-21(+)	T7 promoter/*lac* operator, *lacI*, Amp^R, ColE1 replicon	Novagen
pGE226	Wild-type *recA* gene, Amp^R	Weisemann and Weinstock (1985)
pKK223-3	*tac* promoter/operator, Amp^R, ColE1 replicon	Brosius and Holy (1984)
pKK233-2	*trc* promoter/operator, Amp^R, ColE1 replicon	Amann and Brosius (1985)
pLysE	T7 lysozyme, Cam^R, P15A replicon	Studier (1991)
pLysS	T7 lysozyme, Cam^R, P15A replicon	Studier (1991)
pMS421	Wild-type *lac* promoter/operator, *lacI*^{Q}, Strep^R, Spec^R, SC101 replicon	Graña *et al.* (1988)
pTer7	Wild-type *lacZ* coding region, Amp^R	R. Young
pTrc99A	*trc* promoter/operator, *lacI*^{Q}, Amp^R, ColE1 replicon	Amann *et al.* (1988)
pUC8	*lac* promoter/operator, Amp^R, ColE1 replicon	Vieira and Messing (1982)
pXE60	Wild-type TOL pWWO *xylE* gene, Amp^R	J. Westpheling

TABLE 2

Primers Employed to PCR Amplify DNA Fragments That Were Used in the Construction of the Various Plasmids Described in This Study

	pLAC11 and pLAC22
1 (for)	GTT GCC ATT GCT GCA GGC AT
2 (rev)	ATT GAA TTC ATA AGA TCT TTC CTG TGT GAA ATT GTT ATC CGC
3 (for)	ATT GAA TTC ACC ATG GAC ACC ATC GAA TGG TGC AAA A
	pBR322/*Nco*I
4 (for)	GTT GTT GCC ATT GCT GCA G
5 (rev)	TGT ATG AAT TCC CGG GTA CCA TGG TTG AAG ACG AAA GGG CCT C
	*Bgl*II–*lacZ*–*Hin*dIII
6 (for)	TAC TAT AGA TCT ATG ACC ATG ATT ACG GAT TCA CTG
7 (rev)	TAC ATA AAG CTT GGC CTG CCC GGT TAT TAT TAT TTT
	*Pst*I–*lacZ*–*Hin*dIII
8 (for)	TAT CAT CTG CAG AGG AAA CAG CTA TGA CCA TGA TTA CGG ATT CAC TG
9 (rev)	TAC ATA CTC GAG CAG GAA AGC TTG GCC TGC CCG GTT ATT ATT ATT TT
	*Bam*HI–*lacZ*–*Hin*dIII (also uses primer #9)
10 (for)	TAT CAT GGA TCC AGG AAA CAG CTA TGA CCA TGA TTA CGG ATT CAC TG
	*Bgl*II–*recA*–*Hin*dIII
11 (for)	TAC TAT AGA TCT ATG GCT ATC GAC GAA AAC AAA CAG
12 (rev)	ATA TAT AAG CTT TTA AAA ATC TTC GTT AGT TTC TGC TAC G
	*Hin*dIII–*recA*–*Hin*dIII
13 (for)	TAC TAT AAG CTT AGG AAA CAG CTA TGG CTA TCG ACG AAA ACA AAC AG
14 (rev)	ATA TAT CCC GGG CAA GCT TTT AAA AAT CCT CGT TAG TTT CTG CTA CG
	*Bam*HI–*xylE*–*Eco*RI
15 (for)	TAC TAT AGA TCT ATG AAC AAA GGT GTA ATG CGA CC
16 (rev)	ATT AGT GAA TTC GCA CAA TCT CTG CAA TAA GTC GT

Note. The regions of the primers that are homologous to the DNA target template are indicated with a double underline, while the relevant restriction sites are indicated with a single underline. All primers are listed in the 5′ to 3′ orientation.

wild-type *lac* operon as well as the $lacI^Q$ repressor. The resulting fragment was gel isolated, digested with *Eco*RI, and then ligated into pBR322 which had also been digested with *Eco*RI. To construct pBR322/*Nco*I, primers 4 and 5 (see Table 2) were used to PCR amplify a 789-bp fragment from the plasmid pBR322. The resulting fragment was gel isolated, digested with *Pst*I and *Eco*RI, and then ligated into the 3609-bp fragment from the plasmid pBR322, which had been digested with the same two restriction enzymes. The pBR322/*Nco*I vector also contains added *Kpn*I and *Sma*I sites in addition to the new *Nco*I site.

To construct pLAC33, a 2778-bp fragment was gel isolated from pLAC12 (described below) which had been digested with *Bsa*BI and *Bsa*I and ligated to a 960-bp fragment from

pUC8 which had been digested with *Afl*III, filled in with Klenow, and then digested with *Bsa*I. To construct pLAC12, a 1311-bp *Pst*I, *Bam*HI fragment was gel isolated from pLAC11 and ligated to a 3232-bp *Pst*I, *Bam*HI fragment which was gel isolated from pBR322.

Compilation of the DNA Sequences for the pLAC11, pLAC22, and pLAC33 Expression Vectors

All of the DNA that is contained in the pLAC11, pLAC22, and, pLAC33 vectors has been sequenced. The sequence for the pLAC11 vector which is 4547-bp can be compiled as follows: bp 1–15 is AGATCTTATGAATTC from primer 2 (Table 2); bp 16–1434 is bp 4–1422 from pBR322 (Accession No. J01749); bp 1435–1442 is TCGGTCGG, caused by filling in the *Ava*I site in pBR322Δ*Ava*I; bp 1443–4375 is bp 1427–4359 from pBR322 (Accession No. J01749); and bp 4376–4547 is bp 1106–1277 from the wild-type *Escherichia coli lac* operon (Accession No. J01636).

The sequence for the pLAC22 vector which is 5652-bp can be compiled as follows: bp 1–15 is AGATCTTATGAATTC from primer 2 (Table 2); bp 16–4370 is bp 4–4358 from pBR322 (Accession No. J01749); bp 4371–4376 is CCATGG which is the *Nco*I site from pBR322/*Nco*I; and bp 4377–5652 is bp 2–1277 from the wild-type *E. coli lac* operon (Accession No. J01636), except that bp 4391 of the pLAC22 sequence or bp 16 from the wild-type *E. coli lac* operon sequence has been changed from a "C" to a "T" to reflect the presence of the $lacI^Q$ mutation (Calos, 1978).

The sequence for the pLAC33 vector which is 3742-bp can be compiled as follows: bp 1–15 is AGATCTTATGAATTC from primer 2 (Table 2); bp 16–1684 is bp 4–1672 from pBR322 (Accession No. J01749); bp 1685–2638 is bp 786–1739 from pUC8 (Accession No. L09132); bp 2639–3570 is bp 3428–4359 from pBR322 (Accession No. J01749); and bp 3571–3742 is bp 1106–1277 from the wild-type *E. coli lac* operon (Accession No. J01636). In the maps for these vectors the *ori* is identified as per Balbás *et al.* (1986), while the *lac*PO is indicated starting with the O3 auxiliary operator and ending with the O1 operator as per Müller-Hill (1996).

Construction of the pLAC11–, pLAC22–, pLAC33–, pKK223-3–, pKK233-2–, pTrc99A–, and pET-21(+)–lacZ Plasmids

To construct pLAC11–*lacZ*, pLAC22–*lacZ*, and pLAC33–*lacZ*, primers 6 and 7 (see Table 2) were used to PCR amplify a 3116-bp fragment from the plasmid pTer7 which contains the wild-type *lacZ* gene. The resulting fragment was gel isolated, digested with *Bgl*II and *Hin*dIII, and then ligated into the pLAC11, pLAC22, or pLAC33 vectors which had been digested with the same two restriction enzymes. To construct pKK223-3–*lacZ* and pKK233-2–*lacZ*, primers 8 and 9 (see Table 2) were used to PCR amplify a 3138-bp fragment from the plasmid pTer7. The resulting fragment was gel isolated, digested with *Pst*I and *Hin*dIII, and then ligated into the pKK223-3 or pKK233-2 vectors which had been digested with the same two restriction enzymes. To construct pTrc99A–*lacZ* and pET-21(+)–*lacZ*, primers 9 and 10 (see Table 2) were used to PCR amplify a 3138-bp fragment from the plasmid pTer7. The resulting fragment was gel isolated, digested with *Bam*HI and *Hin*dIII, and then ligated into the pTrc99A or pET-21(+) vectors which had been digested with the same two restriction enzymes.

Construction of the pLAC11–recA and –xylE Plasmids

To construct pLAC11–*recA*, primers 11 and 12 (see Table 2) were used to PCR amplify a 1086-bp fragment from the plasmid pGE226 which contains the wild-type *recA* gene. The resulting fragment was gel isolated, digested with *Bgl*II and *Hin*dIII, and then ligated into the pLAC11 vector which had been digested with the same two restriction enzymes. To construct pKK223-3–*recA* and pKK233-2–*recA*, primers 13 and 14 (see Table 2) were used to PCR amplify a 1104-bp fragment from the plasmid pGE226. The resulting fragment was gel isolated, digested with *Hin*dIII, and then ligated into either the pKK223-3 or pKK233-2 vector

TABLE 3

β-Galactosidase levels Obtained in Different Expression Vectors Grown under Either Repressed or Induced Conditions

Host strain	Vector	Source of LacI	No. of Miller units observed		Fold induction
			Repressed conditions	Induced conditions	
ALS224	None	None	3	6	—
ALS226	None	F′	4	7	—
ALS226	pLAC11	F′	19	11,209	590×
ALS224	pLAC22	Plasmid	152	13,315	88×
ALS226	pLAC33	F′	322	23,443	73×
ALS226	pKK223-3	F′	92	11,037	120×
ALS226	pKK233-2	F′	85	10,371	122×
ALS224	pTrc99A	Plasmid	261	21,381	82×
ALS749	None	None	3	4	—
ALS749	pET-21(+)	Plasmid	2929	16,803	6×
ALS749	pET-21(+)/pLysE	Plasmid	4085	19,558	5×
ALS749	pET-21(+)/pLysS	Plasmid	1598	20,268	13×

Note. The average values obtained for the four clones that were tested from each vector in two different experiments are listed. Standard deviation is not shown but was less than 5% in each case. Induction ratios are expressed as the ratios of enzymatic activity observed under fully induced conditions versus fully repressed conditions. Because pLysE yielded unexpected results, we restriction mapped both of the pLysE and pLysS plasmids to make sure that they were correct.

which had been digested with *Hin*dIII and dephosphorylated with alkaline phosphatase. To construct pLAC11–*xylE*, primers 15 and 16 (see Table 2) were used to PCR amplify a 980-bp fragment from the plasmid pXE60 which contains the wild-type *Pseudomonas putida xylE* gene isolated from the TOL pWWO plasmid. The resulting fragment was gel isolated, digested with *Bgl*II and *Eco*RI, and then ligated into the pLAC11 vector which had been digested with the same two restriction enzymes.

β-Galactosidase and Catechol 2,3-Dioxygenase Assays

β-Galactosidase assays were performed as described by Miller (1972), while catechol 2,3-dioxygenase assays were performed as described by Zukowski *et al.* (1983).

P1 Transduction Assay

Overnights were prepared from each of the strains to be tested using either rich medium to which glucose was added at a final concentration of 0.2% (repressed conditions) or rich medium to which IPTG was added at a final concentration of 1 mM (induced conditions). The overnights were diluted 1 to 10 into the same medium which contained calcium chloride added to a final concentration of 10 mM and aerated for 2 h to make them competent for transduction with P1 phage. A 0.1-ml volume of cells at an OD_{550} of 1.0 was transduced with either 0.1 ml of P1 lysate which had been adjusted to yield a maximal number of transductants or 0.1 ml of a 10^{-2} dilution of the lysate. After a 25-min incubation at 37°C, 0.2 ml of 0.1 M sodium citrate was added to the cell/phage mixtures and 0.2 ml of the final mixtures was plated onto rich kanamycin plates and incubated overnight at 37°C. The total number of kanamycin-resistant colonies was then counted. As reported in Table 4, ALS225 *recA*$^+$ data points were taken from the transductions which used the 10^{-2} diluted phage, while ALS515 *recA*$^-$ data points were taken from the transductions which used the concentrated phage. The data points for ALS515 *recA*$^-$ strains containing a *recA*$^+$ plasmid were taken from transductions which used the concentrated phage when cells were grown under repressed conditions and trans-

TABLE 4

The Phenotype of a *recA* Null Mutant Strain Can Be Preserved with a pLAC11–*recA* (Wild-Type) Construct under Repressed Conditions

Strain	Repressed conditions: Number of Kan^R transductants	Induced conditions: Number of Kan^R transductants
ALS225 (*recA*$^+$)	178,000	182,000
ALS515 (*recA*$^-$)	5	4
ALS515 (*recA*$^-$ pLAC11-*recA*)	4	174,000
ALS515 (*recA*$^-$ pKK223-3-*recA*)	146	179,000
ALS515 (*recA*$^-$ pKK233-2-*recA*)	143	158,000

Note. The data presented are the numbers of kanamycin-resistant (Kan^R) transductants that were obtained from the different isogenic strains when they were transduced with a P1 lysate prepared from strain ALS598 which harbored a Tn10dKan transposon insertion. Cells were grown in rich medium under either repressed or induced conditions, transduced with equal amounts of the P1 lysate, plated onto rich kanamycin plates, and incubated overnight at 37°C as described under Materials and Methods. The total number of kanamycin-resistant colonies was then counted.

ductions which used the 10^{-2} diluted phage when cells were grown under induced conditions.

Chemicals and Reagents

When amplified DNA was used to construct the plasmids that were generated in this study, the PCR was carried out using native *Pfu* polymerase from Stratagene. XGal and IPTG were purchased from Diagnostic Chemicals Limited.

RESULTS

Construction and Features of pLAC11, pLAC22, and pLAC33

The construction of pLAC11, pLAC22, and pLAC33 is described under Materials and Methods and plasmid maps which indicate the unique restriction sites, drug resistances, origins of replication, and other relevant regions that are contained in these vectors are shown in Fig. 3. Sequences of these three vectors can be compiled as described under Materials and Methods. pLAC11 utilizes the ColE1 origin of replication from pBR322 and Lac repressor is provided in *trans* from either an episome or another compatible plasmid. pLAC22 is very similar to pLAC11; however, it also contains *lacI*^{Q} and thus a source of Lac repressor does not have to be provided in *trans*. pLAC33 is a derivative of pLAC11 which utilizes the mutated ColE1 origin of replication from pUC8 (Lin-Chao *et al.*, 1992) and thus pLAC33's copy number is significantly higher than pLAC11 and is comparable to that of other pUC vectors. Because the cloning regions of these three vectors are identical, cloned genes can be easily shuffled between these three vectors depending on the expression demands of the experiment in question.

To clone into pLAC11, pLAC22, or pLAC33, PCR amplification is performed with primers that are designed to introduce unique restriction sites just upstream and downstream of the gene of interest (see Fig. 4). Usually a *Bgl*II site is introduced immediately in front of the ATG start codon and an *Eco*RI site is introduced immediately following the stop codon. After amplification the dsDNA is restricted with *Bgl*II and *Eco*RI and cloned into the vector which has also been restricted with the same two enzymes. If the gene of interest contains a *Bgl*II site, then *Bam*HI or *Bcl*I can be used instead since they generate overhangs which are compatible with *Bgl*II. If the gene of interest contains an *Eco*RI site, then a site downstream of *Eco*RI in the vector (such as *Hin*dIII) can be

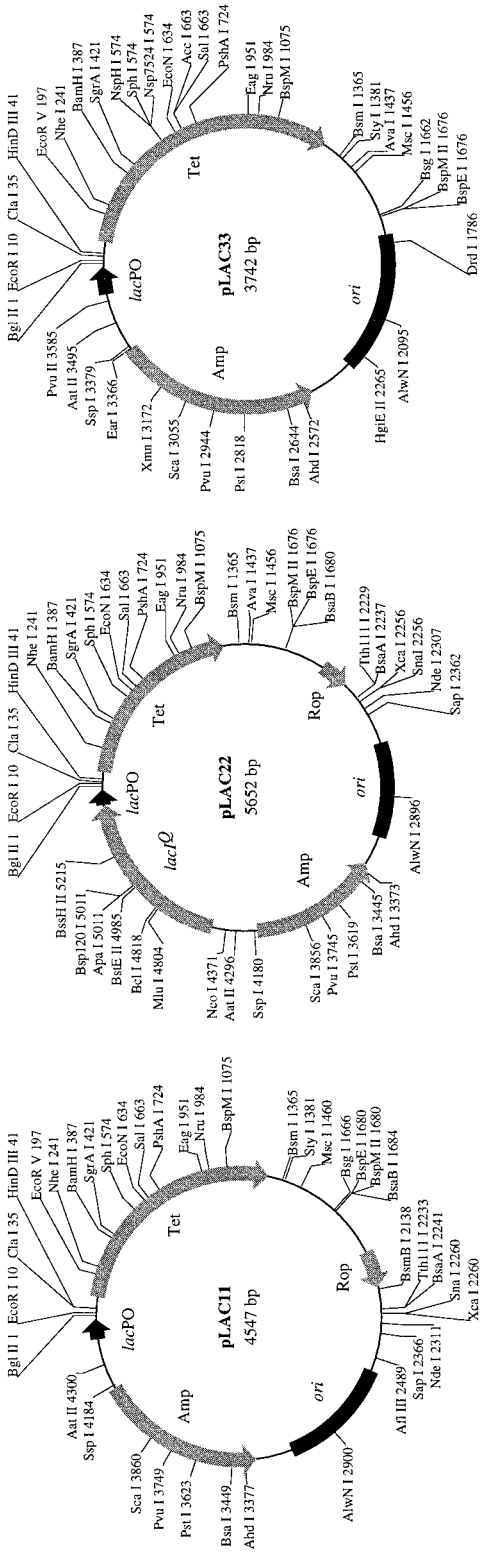

FIG. 3. Map of plasmids pLAC11, pLAC22, and pLAC33. The unique restriction sites and the base pair at which they cut are indicated. Key sites of interest on these plasmids are also shown [pLAC11, Tet (98–1288), Rop (1931–2122), *ori* (2551–3138), Amp (3309–4169), and *lac*PO (4424–4536): pLAC22, Tet (98–1288), Rop (1927–2118), *ori* (2547–3134), Amp (3305–4165), $lacI^{Q}$ (4452–5536), and *lac*PO (5529–5641); and pLAC33, Tet (98–1288), *ori* (1746–2333), Amp (2504–3364), and *lac*PO (3619–3731)].

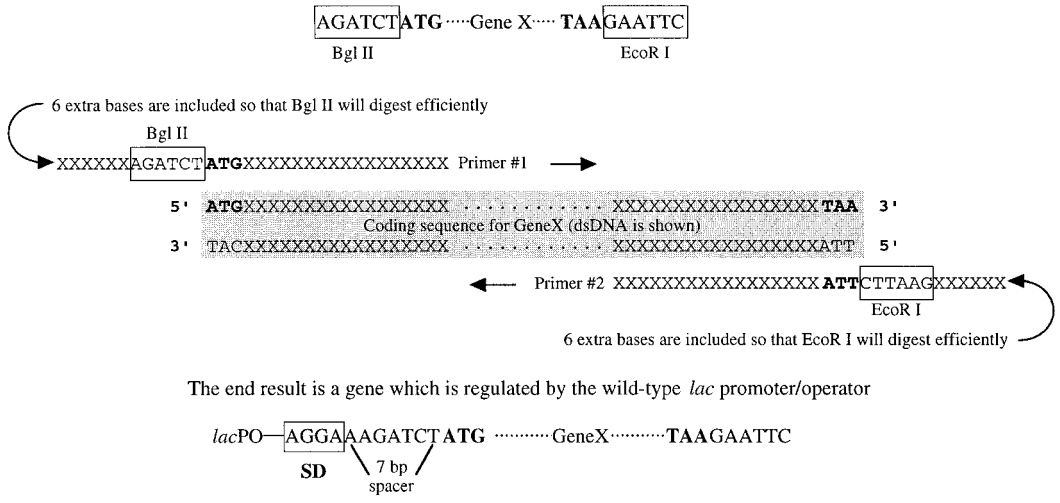

FIG. 4. Cloning into pLAC11, pLAC22, or pLAC33. The coding region of interest can be PCR amplified, cloned into pLAC11, pLAC22, or pLAC33, and placed under *lac* control.

substituted. As shown in Fig. 4, we place an additional six extra bases at both ends of the oligonucleotide in order to ensure that complete digestion occurs.

Comparison of pLAC11, pLAC22, and pLAC33 to Other Expression Vectors

In order to demonstrate how regulable the pLAC11, pLAC22, and pLAC33 expression vectors were, the wild-type *lacZ* gene was cloned into pLAC11, pLAC22, pLAC33, pKK223-3, pKK233-2, pTrc99A, and pET-21(+). Constructs which required an extraneous source of LacI for their repression were transformed into strain ALS226, while constructs which contained a source of LacI on the vector were transformed into strain ALS224. pET-21(+) constructs were transformed into strain ALS749 because they require T7 RNA polymerase for their expression. Four independent *lacZ* clones were chosen from each vector. The plasmids pLysE and pLysS which make T7 lysozyme and thus lower the amount of available T7 polymerase were also transformed into each of the four pET-21(+) clones. Rich ampicillin overnights were diluted 1 to 200 in either rich ampicillin glucose medium (repressed conditions) or rich ampicillin IPTG medium (induced conditions) and grown until they reached midlog ($OD_{550} = 0.5$). Cell extracts were prepared and β-galactosidase assays were performed as per Miller (1972). Table 3 shows the results of these studies and also lists the induction ratio that was determined for each of the expression vectors. As the data clearly indicate, pLAC11 is the most regulable of these expression vectors and its induction ratio is close to that which can be achieved with the wild-type *lac* operon. The vector which yielded the lowest level of expression under repressed conditions was pLAC11, while the vector which yielded the highest level of expression under induced conditions was pLAC33.

Demonstrating That pLAC11 Constructs Can Be Tightly Regulated

To further demonstrate the utility of pLAC11 for physiological experiments, the *rec*A gene was cloned into the pLAC11, pKK223-3, and pKK233-2 vectors and transformed into cells which contained a null *recA* allele in the chromosome. The pKK223-3 and pKK233-2 vectors were chosen as controls because aside from pLAC11, they were the most tightly regulable of all the vectors that were examined in the experiments with *lacZ* shown in Table 2. As the data in Table 4 clearly show, recombination cannot occur in a host strain which contains a nonfunctional RecA protein and thus P1 lysates which provide a Tn10dKan transposon cannot

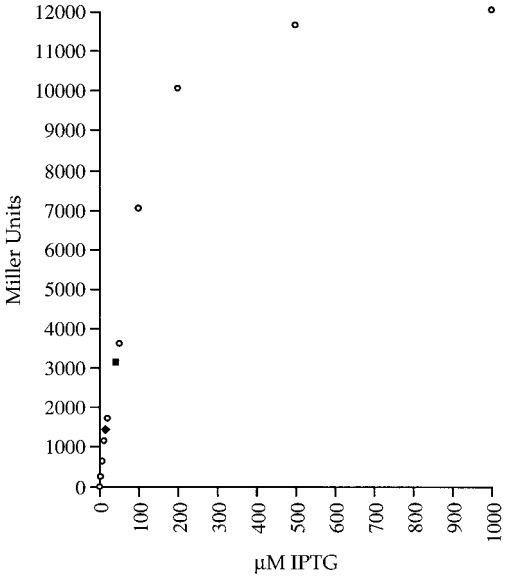

FIG. 5. Response of the pLAC11–*lacZ* construct to varying amounts of IPTG. ALS226 cells containing pLAC11–*lacZ* were grown to midlog in rich medium that contained varying amounts of IPTG and then β-galactosidase activity was assayed. These data points are indicated by open circles. Also indicated in the graph are the average β-galactosidase activities obtained for strains with a single chromosomal copy of the wild-type *lacZ* gene that were grown under different conditions. A filled square indicates the β-galactosidase activity that was obtained when MG1655 or CSH27 cells were grown in rich medium induced with 1 mM IPTG, while a filled diamond indicates the β-galactosidase activity that was obtained when MG1655 or CSH27 cells were grown in M9 minimal lactose medium.

be used to transduce the strain to kanamycin resistance at a high frequency. A *recA*$^-$ strain which also contains the pLAC11–*recA* construct can be transduced to kanamycin resistance at a high frequency when grown under induced conditions but cannot be transduced to kanamycin resistance when grown under repressed conditions. This is not the case for the pKK223-3–*recA* and pKK233-2–*recA* constructs as a significant number of transductants above background can be obtained under repressed conditions.

pLAC11 was designed to provide researchers with an expression vector that could be utilized to conduct physiological experiments in which a cloned gene is studied under completely repressed conditions where it is off or partially induced conditions where it is expressed at physiologically relevant levels. Figure 5 demonstrates how a pLAC11–*lacZ* construct can be utilized to mimic chromosomally expressed *lacZ* that occurs under various physiological conditions by varying the amount of IPTG inducer added.

Testing Various Sources of LacI for Trans Repression of pLAC11

Because pLAC11 was designed to be used with an extraneous source of Lac repressor, different episomal or plasmid sources of LacI which are routinely employed by researchers were tested. Since one of the LacI sources also contained the *lacZ* gene, a reporter construct other than pLAC11–*lacZ* was required and thus a pLAC11–*xylE* construct was engineered. Table 5 shows the results of this study. All of the LacI sources that were tested proved to be adequate to repress expression from pLAC11; however, some were better than others. The basal level of expression that was observed with F′s which provided *lacI*^{Q1} or with the plasmid pMS421 which provided *lacI*^{Q} at approximately six copies per cell was lower than the basal level of expression that was observed with F′s which provided *lacI*^{Q} all three times that the assay was performed. Unfortunately, however, the *xylE* gene could not be induced as high when *lacI*^{Q1} on a F′ or *lacI*^{Q} on a plasmid was used as the source of Lac repressor.

DISCUSSION

Most of the routinely employed expression vectors rely on elements of the *lac* control region for their regulation. While these vectors allow for overexpression of the gene product of interest, they are leaky due to changes that have been introduced into the *lac* control region and gene expression cannot be completely shut off under repressed conditions. Numerous researchers have noticed this problem with the more popular expression vectors pKK223-3 (Posfai *et al.*, 1986; Scrutton *et al.*, 1987), pKK233-2 (Beremand *et al.*, 1987; Ooki *et al.*, 1994), and pTrc99A (Ranie *et al.*, 1993; Ghosh and Singh, 1997), as well as the pET series (Eren and

TABLE 5

Catechol 2,3-Dioxygenase Levels Obtained for a pLAC11–*xylE* Construct When Lac Repressor Is Provided by Various Sources

Host strain	Plasmid present	Source of LacI	Catechol 2,3-dioxygenase activity in milliunits/mg	
			Repressed conditions (rich glucose)	Induced conditions (rich IPTG)
ALS224	No	None	0.2	0.2
ALS224	Yes	None	32.7	432.8
ALS535	Yes	F′*lacI*^{Q} Δ(*lacZ*)*M15 proA*+*B*+ Tn10	0.3	204.4
ALS527	Yes	F′*lacI*^{Q} Δ(*lacZ*)*M15 proA*+*B*+	0.3	243.3
ALS227	Yes	pMS421 *lacI*^{Q}	0.2	90.9
ALS225	Yes	F′*lacI*^{Q1} *Z*$^{+}$ *Y*$^{+}$ *A*$^{+}$	0.2	107.4
ALS226	Yes	F′*lacI*^{Q1} *Z*::Tn5 *Y*$^{+}$ *A*$^{+}$	0.2	85.1

Note. The pLAC11–*xylE* construct was transformed into each of the MC1061 derivative strains listed. ALS224, the parental MC1061 strain, without the pLAC11–*xylE* construct served as a control. Rich overnights were diluted 1 to 200 in either rich glucose or rich IPTG medium and grown until they reached midlog (OD_{550} = 0.5). Cell extracts were then prepared and catechol 2,3-dioxygenase assays were performed as described by Zukowski *et al.* (1983). The average values obtained in three different experiments are listed. Standard deviation is not shown but was less than 10% in each case. It should be noted that some repression is achieved in ALS224 pLAC11–*xylE* under repressed conditions, because the addition of glucose prevents the accumulation of high levels of c-AMP.

Swenson, 1989; Godson, 1991). We describe here a new vector, pLAC11, which relies on the wild-type *lac* control region from the auxiliary *lac* O3 operator through the *lac* O1 operator and thus can be more tightly regulated than the other available expression vectors. In direct comparison studies with pKK223-3, pKK233-2, pTrc99A, and pET-21(+), we found that the lowest level of expression under repressed conditions was achievable with the pLAC11 expression vector. Under fully induced conditions, pLAC11 expressed LacZ protein at levels which were comparable to what could be achieved with the other expression vectors.

Induction ratios of 1000× have been observed with the wild-type *lac* operon. Of all the expression vectors that were tested, only pLAC11 yielded induction ratios which were comparable to what has been observed with the wild-type *lac* operon. It should be noted that the regulation achievable by pLAC11 is actually better than the data indicated in Table 3. Because *lacZ* was used in this test, the auxiliary *lac* O2 operator which resides in the coding region of the *lacZ* gene was provided to the pKK223-3, pKK233-2, pTrc99A, and pET-21(+) vectors which do not normally contain either the O2 or O3 auxiliary operators. Thus the repressed states that were observed in the study in Table 3 are lower than what is normally obtainable with the pKK223-3, pKK233-2, pTrc99A, and pET-21(+) vectors. The studies with RecA in Table 4 demonstrate this as RecA protein expression could only be completely shut off under repressed conditions using pLAC11. Significant expression of the RecA protein occurred in the pKK223-3 and pKK233-2 vectors under repressed conditions.

To meet the expression needs required under different experimental circumstances, we also designed two additional expression vectors which are derivatives of pLAC11. pLAC22 provides *lacI*^{Q} on the vector and thus unlike pLAC11 does not require an extraneous source of LacI for its repression. pLAC33 contains the mutated ColE1 replicon from pUC8 and thus allows proteins to be expressed at much higher levels due to the increase in the copy number of the vector. Of all the expression vectors that we evaluated in direct comparison studies, the highest level of protein expression under fully induced conditions was achieved using the

pLAC33 vector. Because the cloning regions are identical in pLAC11, pLAC22, and pLAC33, genes that are cloned into one of these vectors can be subcloned into one of the other two vectors depending on experimental circumstances. For physiological studies, pLAC11 is the best suited of the three vectors. If, however, the bacterial strain of choice cannot be modified to introduce elevated levels of Lac repressor protein which can be achieved by F′s or compatible plasmids that provide *lacI*^{Q} or *lacI*^{Q1}, the pLAC22 vector can be utilized. If maximal overexpression of a gene product is the goal, then the pLAC33 vector should be utilized.

Numerous experiments call for expression of a cloned gene product at physiological levels, i.e., at expression levels that are equivalent to the expression levels observed for the chromosomal copy of the gene. While this is not easily achievable with any of the commonly utilized expression vectors, these kinds of experiments can be done with the pLAC11 expression vector. By varying the IPTG concentrations, expression from the pLAC11 vector can be adjusted to match the expression levels that occur under different physiological conditions for the chromosomal copy of the gene.

Because the use of Lac repressor is an essential component of any expression vector that utilizes the *lac* operon for its regulation, we also investigated the ability of different sources of LacI to repress the pLAC11 vector. Researchers have historically utilized either *lacI*^{Q} constructs which make 10-fold more Lac repressor than wild-type *lacI* or *lacI*^{Q1} constructs which make 100-fold more Lac repressor than wild-type *lacI* (Müller-Hill, 1975). We found that the greatest level of repression of pLAC11 constructs could be achieved using F′s which provided approximately one copy of the *lacI*^{Q1} gene or a multicopy compatible plasmid which provided approximately six copies of the *lacI*^{Q} gene. However, the induction that was achievable using these LacI sources was significantly lower than the induction that could be achieved when F′s which provided approximately one copy of the *lacI*^{Q} gene were used to repress the pLAC11 construct. Thus if physiological studies are the goal of an investigation, then F′s which provide approximately one copy of the *lacI*^{Q1} gene or a multicopy compatible plasmid which provides approximately six copies of the *lacI*^{Q} gene should be used to regulate the pLAC11 vector. However, if maximal expression is desired, then F′s which provide approximately one copy of the *lacI*^{Q} gene should be utilized. Alternatively, if a bacterial strain can tolerate prolonged overexpression of an expressed gene and overexpression of a gene product is the desired goal, then maximal expression under induced conditions is obtained when a bacteria strain lacks any source of Lac repressor.

ACKNOWLEDGMENT

This work was supported by a Biotechnology Award from the Office of the Vice President for Research, University of Georgia.

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Communicated by D. Chattoraj