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Using chromosomal *lacI*^{Q1} to control expression of genes on high-copy-number plasmids in *Escherichia coli*[1]

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Abstract

Transcription of the *lac* and the hybrid *tac* promoters is repressed by the *lac* repressor and induced by the non-metabolizable substrate IPTG. The degree of repression depends upon the ratio of LacI molecules in a cell to the DNA operator sites. In the absence of an inducer, repression of *Ptac* on a high-copy-number (hcn) plasmid was equivalent in strains containing *lacI*^{Q1} on the chromosome, or *lacI*$^{+}$ on the plasmid, but not from strains with *lacI*$^{+}$ or *lacI*^{Q} only on the chromosome. Induction of *Ptac* on hcn plasmids in strains in which expression was controlled by *lacI*^{Q1} occurred at very low inducer concentrations (3–10 μM IPTG) and reached levels significantly higher than in strains with *lacI*$^{+}$ on the plasmid. Greater than 300-fold induction of a β-LacZ fusion was observed, and >600-fold induction was estimated from recombinant hemoglobin synthesis. Transcription from *PlacI*^{Q1} initiated in the same point as *PlacI*$^{+}$, but was 170-fold stronger, consistent with the *lac* repressor levels required to control LacI-regulated genes on hcn plasmids. The DNA sequence upstream of *lacI* was used to develop a simple PCR test to identify *lacI*^{Q1} by a characteristic 15-bp deletion. This deletion created a consensus −35 hexamer, responsible for the increased *lacI* transcription, and was easily detectable in a variety of strains. Using *lacI*^{Q1} hosts eliminates the requirement to maintain *lacI* on the plasmid to regulate gene expression on hcn expression plasmids.

Keywords: Repressor; Fusion protein; Induction; *tac* promoter; Recombinant DNA

1. Introduction

Tight repression of transcription of heterologous genes in *Escherichia coli* is often desirable or necessary since leaky expression can be detrimental to cell growth or even lethal. Regulated gene expression requires an inducible or repressible system in vivo. The most commonly used systems in *E. coli* are those based on LacI-mediated repression of a promoter, particularly the *lac* promoter (*Plac*; Farabaugh, 1978) and the threefold stronger *tac* hybrid promoter (Russell and Bennett, 1982; De Boer et al., 1983). Expression from these promoters is controlled by the LacI protein, the best-studied member of a large family of bacterial transcriptional regulators (Weickert and Adhya, 1992). Expression may be induced by lactose or non-metabolizable analogues, such as IPTG, that reduce the affinity of the *lac* repressor for its specific DNA-binding site. The *lac* promoter is repressed up to 1000-fold, whereas *Ptac* is repressed only 50-fold in the absence of inducer (Lanzer and Bujard, 1988). Highly inducible expression of genes from T7 polymerase-dependent promoters (Studier and Moffatt, 1986; Dubendorff and Studier, 1991) also relies on the *lac* system to regulate expression of the T7 polymerase gene before induction.

The ratio of functional repressor protein to the repressor-binding site on the DNA, the operator, determines

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Abbreviations: *A*, absorbance; aa, amino acid(s); βGal (or LacZ), β-galactosidase; β-LacZ, protein fusion of β-globin N-terminus with βGal; β-lacZ, gene fusion encoding β-LacZ; bp, base pair(s); hcn, high-copy-number; IPTG, isopropyl-β-D-thiogalactopyranoside; Km, kanamycin; LacI, *lac* repressor; LB, Luria–Bertani broth; oligo, oligodeoxyribonucleotide; *ori*, origin of DNA replication; *P*, promoter; PA, polyacrylamide; PAGE, PA gel electrophoresis; PCR, polymerase chain reaction; ^{R}, resistance; SDS, sodium dodecyl sulfate; TAE, Tris-acetate electrophoresis buffer; Tc, tetracycline; TE, Tris-EDTA buffer; *ter*, terminator of transcription; *tsp*, transcription start point; u, Miller units of βGal; wt, wild type.

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the level of control in the absence of induction. Multiple copies of the *lac* operator in trans can titrate out lesser levels of LacI, thus inducing transcription (Backman et al., 1976; Heyneker et al., 1976; Sadler et al., 1980; Haber and Adhya, 1988). The number of operator-like sequences (Fickert and Müller-Hill, 1992), the position of the operator in a promoter, the stability and kinetics of formation of the repressor–operator complex, and the kinetics of RNA polymerase–promoter complex formation and polymerase clearance also affect the degree of repressibility of a promoter, whereas the promoter strength alone does not correlate with the degree of repressibility (Lanzer and Bujard, 1988).

Repression of *Ptac* on a multi-copy episome is typically maintained by the overproduction of LacI from a $lacI^{Q}$ promoter on the same plasmid, as with pTTQ (Stark, 1987), pTrc (Amann et al., 1988) and pTug (Graham et al., 1995) series plasmids. Without a corresponding *lacI* gene on the expression plasmid, partial expression in the absence of inducer results when the copy number of the plasmid exceeds the copies of LacI in the cell. The $lacI^{Q}$ mutation is a single CG→TA change at −35 of the promoter region of *lacI* (Calos, 1978) which causes a 10-fold increase in LacI expression (Müller-Hill et al., 1968). The wt cell has a concentration of LacI of 10 nM or about 10 molecules, with 99% present as a tetramer (Fickert and Müller-Hill, 1992). Cells containing the $lacI^{Q}$ mutation contain about 100 molecules per cell or 100 nM LacI. Plasmids with a copy number exceeding 100 titrate the available LacI by operator association, and expression from *Ptac* on the excess plasmids is typically de-repressed. pBR322 is present in 39–55 copies per cell, depending on the growth rate of the cells (Lin-Chao and Bremer, 1987). Therefore, *lac* or *tac* promoters on pBR322-based plasmids can be repressed by LacI from a chromosomal $lacI^{Q}$ (Amann et al., 1983). Plasmids based on pUC, having a higher copy number (500–700 copies per cell, at 37°C) are not effectively controlled by $lacI^{Q}$ alone, and expression is leaky.

2. Materials and methods

2.1. Strain construction

The strains used in this study are listed in Table 1. SGE299 (MG1061) was reported to be $lacI^{Q}$ (Hu et al., 1993) but this study demonstrated that it was $lacI^{Q1}$. Plasmids were introduced by transformation into *E. coli* using the TSS method (Chung et al., 1989), or procedure of Hanahan (1985). Transformants were selected on LB supplemented with 15 μg Tc/ml.

The $lacI^{Q1}$ allele was transferred by bacteriophage P1 transduction, essentially as described in Miller (1972, 1992). In general, transductants were either selected for resistance to Tc or Km, or selected for growth on minimal media supplemented with lactose as the sole carbon source. To construct $lacI^{Q1}$ strains, a P1 lysate was prepared on SGE299 (Table 1) and used to transduce recipient strains to Km^{R}, lac^{-}. Candidates were further screened for $lacI^{Q1}$ by resistance to induction by operator titration as described in Section 3.2. Transduction was also used to restore the *lac* operon in $lacI^{Q1}$ strains. SGE765 was made by P1 transduction from a lysate made on MS24 into strain C3000, selecting for lac^{+}, Km^{R} transductants. A P1 lysate prepared on SGE765 (Table 1) was used to transduce $lacI^{Q1}$ strains to lac^{+}. The resulting colonies were screened for sensitivity to Km, and sensitive candidates retained $lacI^{Q1}$ and were lac^{+}.

2.2. Plasmid construction

Two sets of plasmids were used for this study. The first expressed rHb1.1, and contained the di-α and β globin genes behind the *tac* promoter, as found in pSGE705 (Weickert and Curry, 1997). Plasmid variants include higher copy number (pSGE715; Weickert and Curry, 1997), no *lacI* gene (pSGE654), or both (pSGE720; Weickert and Curry, 1997). The second plasmid set was created from each globin plasmid by fusing all but the first 6 aa of the *E. coli lacZ* gene from pMLB1034 (Silhavy et al., 1984) with the N-terminal 46 aa of the β globin gene. The resulting β-LacZ fusion protein was easily assayable by the βGal assay (below; Miller, 1972) and served to establish the repression and induction kinetics. The β-LacZ fusion plasmids pSGE712 and pSGE714 are in Fig. 1. pSGE716 and pSGE721 were made from pSGE715 and pSGE720, respectively. pSGE654 was constructed by deleting the *lacI* gene from plasmid pSGE705 and replacing it with a short oligo containing several new convenient restriction sites as described in Weickert and Curry (1997).

2.3. Plasmid isolation, sequencing, and sequence alignment

Plasmids were isolated from overnight cultures by the reagents and protocol for the Wizard plasmid isolation kit (Promega, Madison, WI). DNA sequencing of plasmid templates was performed with Sequenase® V.2 kit reagents according to the procedures recommended by the manufacturer (USB, Cleveland, OH). Primers used for sequencing were synthesized on an Applied Biosystems 380B DNA synthesizer. Sequence homology was determined by searching the NCBI databases using the BLAST algorithm (Altschul et al., 1990).

2.4. Cell growth and βGal assays

Growth of cells for DNA isolation, or stock cell preparation used LB medium, supplemented with 15 μg

Table 1
Strains used for these studies

Strain	Description or relevant genotype[a]	Source[b]
ATCC47043	MM294; *thi*, *endA*, *supE*44, *hsdR*17 [$r_K^- m_K^+$], *lacZ*ΔM15, *lacI*^{Q}	ATCC
C600	K-12; *thi*-1, *thr*-1, *supE*44, *leuB*6, *tonA*21, *lacY*1	Promega, Inc.
C3000	K-12	ATCC, #15597
D1210	(HB101-based) *ara*14, *supE*44, *xyl*-5, *mtl*-1, *recA*13, *mcrB*, *mrr*, *lacI*^{Q}, *galK*2, *hsdS*20 ($r_B^- m_B^-$), *mcrB*, *rpsL*20 [Str^R]	Stratagene, Inc.
JM109	*endA*1, *recA*1, *gyrA*96, *thi*, Δ(*lac-proAB*), *relA*1, *supE*44, *hsdR*17 [$r_K^- m_K^+$], [F′ *traD*36, *proAB*, *lacI*^{Q}, *lacZ*ΔM15]	Promega, Inc.
MS24	MS24 = CAG18420 = MG1655 *lacI*3098::Tn*10*Km^R, *lacZU*118;	Singer et al. (1989)
RV308	Δ*lac*X74, *thi*, *gal*::IS*2*, *StrA*, *opp308*	C. Hershberger
SGE299	MC1061 (*araD*, Δ*ara-leu*, Δ*lac*X74, *galU*, *galK*, *hsdR*2 [$r_K^- m_K^+$], *mcrB1*, *rpsL* [Str^R]) + (F′128 *lacI*^{Q1}, *lacZ*::Tn*5*Km^R)	Hu et al. (1993)
SGE765	C3000 *lacI*::TnKm^R, lacZ$^+$ P1 transductant	T. Griffin
SGE1442	SGE1661[pSGE712]; medium-copy plasmid with *lacI*	This study
SGE1453	SGE1661[pSGE715]; high-copy plasmid with *lacI*	This study
SGE1459	SGE1661[pSGE716]; high-copy plasmid with *lacI*	This study
SGE1470	SGE1675[pSGE721]; high-copy plasmid without *lacI*	This study
SGE1661	*gyrA*96(Nal^R), *endA*, *hsdR*17, *relA*1, *supE*44, *recJ*, *lacI*$^+$, *lac*$^+$	Weickert and Curry (1997)
SGE1662	SGE1661 transformed to Tc^R with pSGE705	Weickert and Curry (1997)
SGE1669	SGE1661 transduced by phage P1 to *lacI*^{Q1}, *lacZ*::*Tn5*Km^R from SGE299	T. Griffin
SGE1670	SGE1661 transduced by phage P1 to *lacI*^{Q1}, *lacZ*::*Tn5*Km^R from SGE299 (independent sibling of SGE1669)	T. Griffin
SGE1674	SGE1669 (*lacI*^{Q1}) transduced by phage P1 to *lac*$^+$, Km^S	T. Griffin
SGE1675	SGE1670 (*lacI*^{Q1}) transduced by phage P1 to *lac*$^+$, Km^S	Weickert and Curry (1997)

[a]Plasmids are described in Section 2.2. Δ, deletion; IS, insertion sequence; Nal, naladixic acid; Str, streptomycin; Tn, transposon. [ ], denotes plasmid-carrier state.
[b]ATCC, American Type Culture Collection, Rockville, MD.

Tc/ml and 50 μg Km/ml. For βGal assays, the cells were grown in M63 minimal media (Miller, 1972) supplemented with 0.4% glucose (wt/vol) and 0.5% casamino acids (wt/vol) and the appropriate antibiotics as above, at 37°C. The βGal assays were performed essentially as described in Miller (1972), except that activity was maximal when cell permeabilization was by addition of 10–20 μl 0.1% SDS and 10–20 μl chloroform to each tube, followed by 10–15 s of vortex mixing at top speed.

2.5. Cell lysis and SDS–PAGE

One-milliliter cultures were pelleted by centrifugation and lysed, and soluble and insoluble fractions were separated for SDS–PAGE generally as described for rHb in Weickert et al. (1997). Each sample was loaded (10 μl) on to an SDS 9.5% PAGE gel, and proteins were separated by electrophoresis (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue.

2.6. Plasmid copy number measurement

Plasmid DNA was extracted in triplicate from standard samples ($A_{600\ nm} = 2$) of overnight bacterial cultures using the Wizard Miniprep Kit (Promega). The plasmid DNA was linearized with *Eco*RI (NE Biolabs, Beverly, MA) and separated by electrophoresis in a TAE 0.7% (w/v) agarose gel containing ethidium bromide (Sambrook et al., 1989) with seven control samples of *Hin*dIII-digested λ DNA (NE Biolabs) in twofold dilutions from 2.0 mg to 0.03125 mg in adjacent lanes. The mass of plasmid/lane was estimated by comparison of the band intensity of linearized plasmids with that of known quantities of a similar-sized *Hin*dIII-digested λ DNA bands. Assuming 8×10^8 cells per ml of culture at $A_{600\ nm} = 1.0$, the mass of DNA was determined by comparison with the standard, and the molecular weight of the plasmid was used to calculate the copy number. A correction factor of 3, for the loss of DNA during the miniprep procedure, was empirically determined by a series of spike-recovery experiments.

2.7. Chromosomal DNA isolation, PCR, and cloning

Standard procedures were typically used (Sambrook et al., 1989), except where noted. PCR to screen strains for size heterogeneity in the promoter region used cells from 1- to 2-mm-diameter colonies from LB plates, scooped using a sterile micropipette tip into an Eppendorf tube containing 100 μl of water. The suspension was boiled for 10 min, after which 5 μl were removed and used as template in the PCR. Purified genomic DNA was also used as template for PCR. A restriction map-based PCR method similar to that by Sarkar et al. (1993) was used to amplify the region upstream of the *lacI* gene. The *E. coli* genetic map

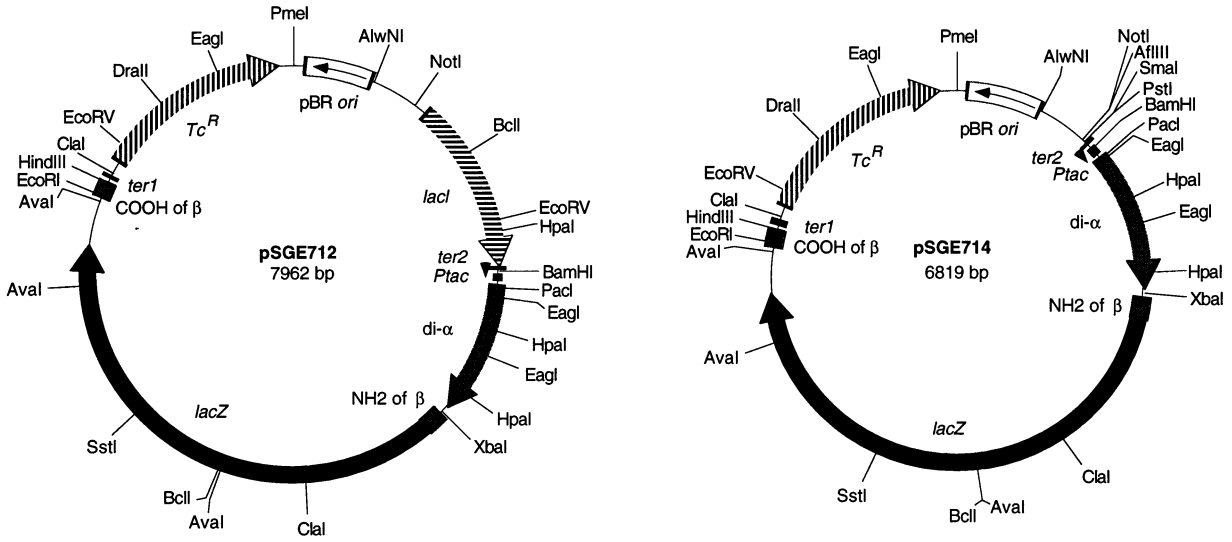

Fig. 1. Medium-copy-number plasmids constructed for expression of a β-LacZ fusion protein. The two plasmids are identical, except that pSGE714 does not contain the *lacI* gene found in pSGE712, only a short, unique linker sequence instead. Plasmid pSGE721 (not shown) is identical to pSGE714 except that it has the pUC *ori* instead of the pBR *ori* found in pSGE714. The recombinant hemoglobin coding regions for β-globin (β) and di-α-globin (di-α) are indicated, as are the two transcription terminators, *ter1* and *ter2*.

(Kohara et al., 1987) predicts a *Pst*I site about 600-bp upstream from the *lacI* promoter. A 12-nt-long oligo (CBG24; 50 pmol) with a degenerate 5′ half and the recognition site for *Pst*I at its 3′ end, and 10 pmol of an oligo complimentary to a region just downstream from the initiatation of translation in the *lacI* gene (TG45 [5′-CTGGCACCCAGTTGATCG]) were used in each 100-μl PCR reaction. Reactions were performed in the Ericomp Twin Block model TCX15A thermocycler using a program whose first cycle consisted of 10 min at 95°C, 5 min of annealing at 50°C, and a 2-min extension at 72°C. The remaining 33 cycles consisted of 1 min at 95°C, 30 s at 50°C, and 1 min at 72°C. The final cycle was 1 min at 95°C, 30 s at 50°C, and 10 min at 72°C. Additional specificity was obtained by using a 2-μl aliquot of this reaction in a second round of PCR amplification with CBG24 and an oligo just upstream of TG45, CBG23 (5′-AGTCAAGCTTAACG-TGGCTGGCCTGGTT), which contained a *Hin*dIII restriction site. Products were examined by agarose gel electrophoresis on 4% NuSieve TAE gels performed as recommended by the manufacturer (FMC Corp., Rockland, ME), or cleaned with the Magic PCR clean-up kit (Promega, Inc., Madison, WI), digested with *Pst*I + *Hin*dIII and ligated into pUC19 plasmid.

2.8. RNA isolation and primer-extension analysis

Cells were grown in shake flasks in M9 minimal media at 37°C to an $A_{600\ nm}$ of about 1.0. RNA was extracted from 25-ml samples of bacterial cells using a small-scale hot-phenol method similar to Lau et al. (1985) and 300–1000 μg were usually obtained. Two primers were used for primer extension; CBG09 (5′-CGGTC-TGATAAGAGACACCGGCATA) was complementary to a region near the 5′ end of the coding region of *lacI* to detect transcription initiating at or near the wt start point, and CBG88 (5′-CACCAGATTCAGACAC-CCAA) was complementary to the region about 200-bp upstream of *lacI* to detect transcripts that initiate farther upstream. Each primer (20 pmol) was radiolabeled with [γ-^{32}P]ATP (Amersham) using polynucleotide kinase (NE Biolabs), according to the manufacturer's instructions. Fifteen micrograms of RNA and labeled primer were mixed with 5× annealing buffer (1.25 M KCl, 50 mM Tris, pH 8.0, 5 mM vanadyl–ribonucleoside complex) in a total volume of 15 μl and heated to 65°C for 10 min, followed by 50°C for 40 min. The mixture was then placed at room temperature and allowed to cool to 37°C. The mixture was placed on ice and incubated for 15 min. Superscript II (Stratagene) was used to synthesize first-strand cDNA at 42°C according to the manufacturer's instructions, except for the following: 1 mM vanadium ribonucleotide complex, 100 mg/ml of actinomycin D, and 20 units of RNasin (Promega) were added. Reactions were precipitated with sodium acetate and ethanol, and centrifuged for 40 min at 4°C. The precipitate was collected and washed with 70% ethanol and then dried. Pellets were resuspended in 3 ml of 0.1 M NaOH, 1 mM EDTA and incubated at room temperature for 10 min. Six milliliters of the sample buffer were then added. Samples were heated at 65°C for 10 min and then loaded on to a 6% PA-7 M urea sequencing gel adjacent to sequencing reactions of a plasmid with wt *lacI* that were performed using the

same primer. Autoradiograms were quantitated using the Discovery Series analysis software from PDI.

3. Results

3.1. Plasmid copy-number measurements

Strains were grown in flasks to stationary phase. The copy number of plasmids with the pUC-type ori of replication (pSGE721) was estimated to be 461 ± 55 ($n=4$) copies per cell, whereas that of plasmids with the pBR322 ori, minus the *rop* gene (pSGE705, pSGE712, and pSGE714), was estimated to be 114 ± 10 ($n=3$) copies per cell. The *rop* gene enhances RNAI–RNAII hybrid formation (Tomizawa and Som, 1984) and its absence increased the pBR322 copy number two- to threefold (Nugent et al., 1986), consistent with the copy number that we measured.

3.2. Identification of a strain which repressed Ptac transcription from a medium-copy number plasmid not containing the lacI gene

The 10-fold overproduction of LacI in *lacI*^{Q} strains should repress LacI-mediated expression from a promoter on a medium-copy number plasmid in the absence of inducer. We attempted to introduce the *lacI*^{Q} allele into SGE1661 (Table 1) by P1 transduction, and six Km^{R} candidates were identified. Plasmids pSGE712 and pSGE714 were introduced into all six candidates and into SGE1661 (wt *lacI*) as a control. Transformants were assayed for β-LacZ after growth in the absence or presence of 100 μM IPTG. Of the six candidates screened for *lacI*^{Q} with pSGE714, only two were able to repress expression in the absence of IPTG (Table 2). Therefore, these two were presumed to contain the *lacI*^{Q} allele, which should produce sufficient LacI to repress β-lacZ transcription in the absence of IPTG. If cells are *lacI*$^{+}$ rather than *lacI*^{Q}, the expression of β-lacZ in the absence of IPTG will be equivalent to the control strain SGE1661. Plasmid pSGE712 was a positive control for repression of *Ptac* because it contained a copy of the *lacI* gene and promoter, thus producing sufficient LacI from the plasmid gene to repress β-lacZ expression in the absence of IPTG in any strain background. Finding only two candidates out of six transductants was an unexpectedly low frequency since the genetic linkage between the *lacZ*::TnKm^{R}, and the *lacI*^{Q} allele should have resulted in at least 90% of the Km^{R} candidates being *lacI*^{Q}.

The lack of further induction of β-LacZ at IPTG concentrations of 100 μM or greater was due to insolubility of β-LacZ at higher concentrations. Examination of the soluble and insoluble protein by SDS–PAGE revealed that at 100 μM IPTG, >80% of the β-LacZ fusion protein was insoluble, and at 1000 μM IPTG, >90% was insoluble (Fig. 2), and therefore not detected in the βGal assay. A similar insolubility has been observed for overproduced βGal (non-fusion) protein in *E. coli* (Cheng, 1983). Di-α globin was also produced from these plasmids in response to inducer, but all was found in the insoluble fraction (Fig. 2), consistent with previous observations that di-α expression in the absence of β resulted only in insoluble protein accumulation (Weickert and Curry, 1997).

3.3. Putative lacI^{Q} transductants could not be titrated with hcn plasmids containing lac operators

The presence of many copies of the *lac* operator on plasmids titrates LacI in *trans* away from the *lac* operon by providing hundreds of alternative binding sites, thus inducing transcription of the *lac* operon. The introduction of pUC19 into strain SGE1661, containing the wt *lac* operon, caused the induction of *lacZ* in the absence of IPTG from 0.3 u without pUC19 to 1815 u with pUC19. However, the introduction of the same plasmid into SGE1675, a transductant containing a putative *lacI*^{Q}, did not result in induction of the chromosomal

Table 2
Plasmid β-LacZ production from *lacI*^{Q} transduction candidates[a]

Strain SGE1661[b]	β-LacZ (u)[c]			
	pSGE712 (*lacI*$^{+}$)		pSGE714 (no *lacI*)	
lacI genotype	−IPTG	+IPTG	−IPTG	+IPTG
lacI^{+} (control SGE1661)	1439	25 473	7726	17 664
Four *lacI*$^{+}$ transduction candidates	1068 ± 119	27 214 ± 866	7713 ± 365	18 464 ± 901
Two *lacI*^{Q} transduction candidates (SGE1669 and SGE1670)	1057 ± 326	26 645 ± 146	902 ± 203	28 329 ± 2298

[a]Each transductant was transformed separately with pSGE712 and pSGE714 (see Fig. 1). IPTG was added to 100 μM, and β-LacZ assayed as described in Section 2.4.
[b]Strains were SGE1661 and derivatives of SGE1661 as described in Table 1. Derivatives were generated by P1 transduction as described in Section 2.1. The four *lacI*$^{+}$ transduction candidates were discarded since they were identical to the parent SGE1661.
[c]±1 SD.

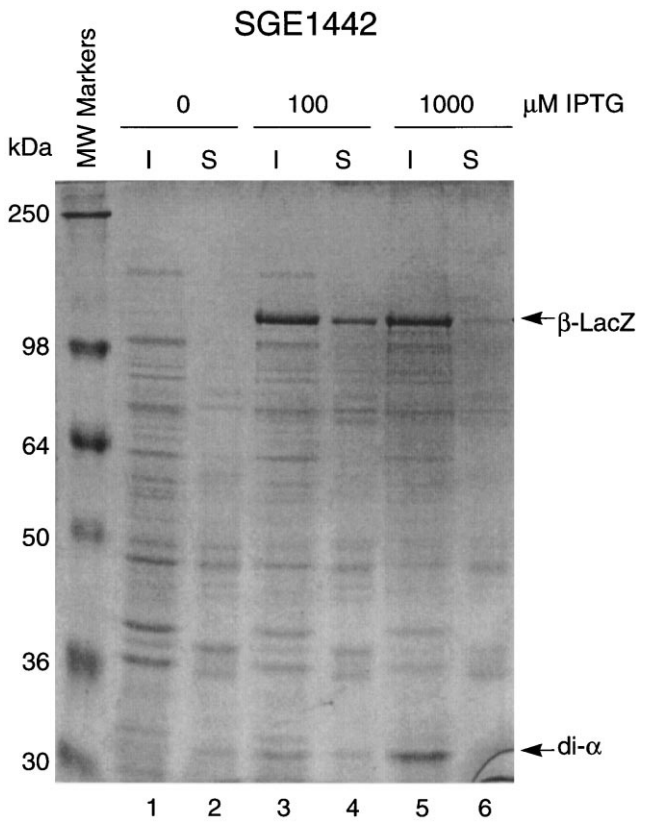

Fig. 2. SDS–PAGE of soluble and insoluble fractions from SGE1442 cell lysate. MW = prestained molecular weight standards (Novex SeeBlue®). Cells were grown in the absence (lanes 1 and 2) or presence of IPTG at 0.1 mM (lanes 3 and 4) or 1 mM (lanes 5 and 6). Soluble samples (S) are in lanes 2, 4, and 6. Insoluble samples (I) are in lanes 1, 3, and 5. Soluble and insoluble cell lysate fractions were prepared as described in Section 2.5.

lac operon. Fusion expression was 19 u in the absence and 20 u in the presence of pUC19. This indicated that the level of LacI in this strain was sufficient to bind to 500–700 copies of pUC19/cell, and repress the *lac* operon on the chromosome, more LacI than expected for an authentic *lacI*^{Q} allele. However, the level of LacZ prior to induction was 60-fold higher in SGE1675 than in SGE1661, the opposite of what would be expected from a strain containing a higher LacI concentration. The identification and characterization of the *lacI*^{Q} promoter reconciled this contradictory result (below).

3.4. *Authentic* lacI^{Q} *did not repress transcription from hcn plasmids*

Authentic *lacI*^{Q} strains were obtained and transformed with medium-copy and hcn plasmids: (1) a medium-copy vector with *lacI* (pSGE712), (2) a medium-copy vector without *lacI* (pSGE714), and (3) a hcn vector without *lacI* (pSGE721). The level of β-LacZ fusion protein activity was measured in the presence or absence of 100 μM IPTG (Table 3). Authentic

Table 3
Inability of *lacI*^{Q} to control hcn plasmid expression

Strain/repressor[a]	Plasmid[b]	β-LacZ (u)[c]	
		−IPTG	+IPTG
D1210/*lacI*^{Q}	pSGE712	3069	52 482
D1210/*lacI*^{Q}	pSGE714	4247	32 783
D1210/*lacI*^{Q}	pSGE721	24 402	25 141
ATCC47043/*lacI*^{Q}	pSGE712	1846	36 511
ATCC47043/*lacI*^{Q}	pSGE714	3957	33 763
ATCC47043/*lacI*^{Q}	pSGE721	9659	39 293
SGE1674/*lacI*^{Q1}	pSGE721	1203	39 770
SGE1675/*lacI*^{Q1}	pSGE721	1778	27 124

[a]see Table 1.
[b]Plasmids as described in Fig. 1 and Section 2.2.
[c]IPTG was added to 100 μM, and β-LacZ assayed as described in Section 2.4.

lacI^{Q} on the chromosome did not control expression of a *β-lacZ* fusion from the hcn plasmid (pSGE721 in D1210 and ATCC47043; Table 3). It controlled expression from a medium-copy-number plasmid (pSGE714) fairly well (Table 3), although uninduced expression was 1.5- to twofold higher than from a plasmid that contained its own *lacI* with the native promoter (pSGE712). Although the cellular level of LacI synthesized as a result of an authentic *lacI*^{Q} promoter mutation was insufficient to repress transcription from *Ptac* on a hcn plasmid, the level of LacI produced from the two putative *lacI*^{Q} transductants was sufficient to repress transcription (Table 3).

3.5. *Identification and characterization of the* lacI^{Q1} *found in SGE1675*

Prior to initiating this study, the published *lacI* promoter region sequence data (Farabaugh, 1978) extended only 50-bp upstream of the *tsp*. Approximately 600 additional bp of the region upstream of *lacI*, including the promoter, were amplified by PCR, subcloned and sequenced from strain C600 (wt *lacI*). The wt sequence upstream of *lacI* has since proved homologous to the recently reported *mhpR* gene, coding for the C-terminal 222 aa (Ferrández et al., 1997). This gene is transcribed in the same direction as *lacI* and terminates just 15 bp upstream of the −35 region of *PlacI*. Between the termination codon and *PlacI*, we located a very short sequence capable of forming an RNA stem–loop with a free energy of −2.6 kcal/mol, followed by a stretch of four uracils. Since expression of *mhpR* is expected to be weak (Ferrández et al., 1997), this may serve as the terminator for the *mhpR* transcript.

From the sequence, oligos were designed and used to amplify the *PlacI* region from SGE1661, SGE1670, and SGE1675. PCR products were analyzed by agarose gel electrophoresis, and two products were observed

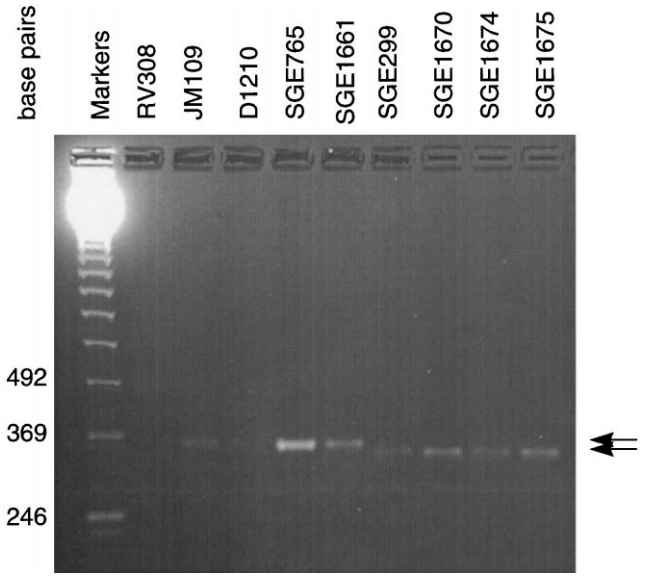

Fig. 3. PCR of *PlacI* region. A 123-bp molecular-weight marker ladder appears in the leftmost lane. A smaller PCR product (lower arrow) was produced from DNA of strains with strong repression of β-LacZ from the hcn plasmid (SGE299, 1670, 1674 and 1675), whereas DNA of strains with the *lacI* or $lacI^{Q}$ alleles (JM109, D1210, SGE765 and SGE1661) produces a slightly larger PCR product (upper arrow). RV308 has a deletion of the *lac* region.

(Fig. 3). The lower band was estimated to be 10–15 bp shorter than the upper. PCR of all strains that repressed expression of β-*lacZ* on a hcn plasmid produced only the lower band (Fig. 3).

The PCR products were digested with *Pst*I + *Hin*dIII, cloned into pBCSK+ between the *Pst*I and *Hin*dIII sites, and sequenced. The *PlacI* sequence in SGE1661 was consistent with wt *lacI*, whereas that found in SGE1670 and SGE1675 was consistent with the $lacI^{Q1}$ allele reported by Calos and Miller (1981). This promoter results from a 15-bp deletion in the *PlacI* region, which fortuitously joins a sequence identical to the −35 consensus σ^{70} hexamer 18 bp upstream of the native −10 region (Fig. 4). This would be expected to create a much stronger promoter, so reverse transcription was used to measure the abundance of *lacI* transcript. This deletion would also abolish the potential terminator of *mhpR* transcription described above.

3.6. *Measurement of* lacI *transcript level*

The *lacI* mRNA *tsp* and relative message abundance were measured by primer-extension analysis of *lacI* transcripts from strains SGE299, SGE1661, SGE1662, SGE1670, SGE1675, and JM109 (Fig. 5). A DNA sequence ladder in the adjacent lanes indicated the sequence at the *tsp*. The transcripts from strains with $lacI^{Q1}$ initiated at the same site as observed in the wt strain SGE1661, the previously mapped native *tsp* (Farabaugh, 1978). No significant upstream initiation was observed (Fig. 5 and additional data with CBG88, not shown). The relative abundance of *lacI* transcript from JM109: SGE1662: SGE299: SGE1670: SGE1675, respectively, was 1: 7: 17: 17: 17.

3.7. *Plasmid induction occurred at lower IPTG concentrations than chromosomal induction, and appeared relatively insensitive to LacI concentration*

The induction of *Ptac* on plasmids was measured by assaying the activity of the β-LacZ fusion expressed from strains with medium (SGE1442, pSGE712) or high (SGE1459, pSGE716; SGE1470, pSGE721) copy number plasmids. Induction of the chromosomal *lacZ* was measured from strains carrying identical plasmids that did not contain the β-lacZ fusion, but instead expressed the rHb1.1 genes (Weickert and Curry, 1997). Therefore, there was no LacZ activity other than that produced from the chromosomal allele, but the amount

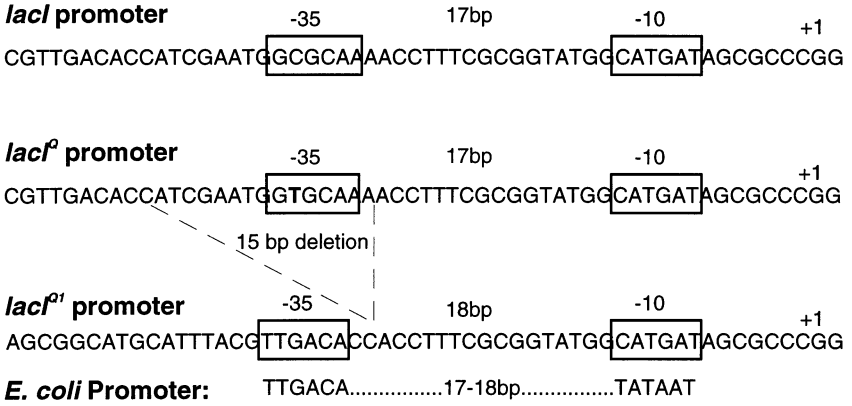

Fig. 4. The sequence of the *lacI*, $lacI^{Q}$, and $lacI^{Q1}$ promoters. The 5′ to 3′ sequence of the top strand is depicted. The *tsp* is indicated by the +1, the −10 and −35 hexamers are boxed, and the distance (bp) between them is indicated. The consensus sequences for *E. coli* σ70 promoter −10 and −35 regions are shown on the bottom line. The $lacI^{Q}$ mutation (C→T in the −35 region) is shown in bold. The 15-bp deletion in the $lacI^{Q1}$ promoter is shown.

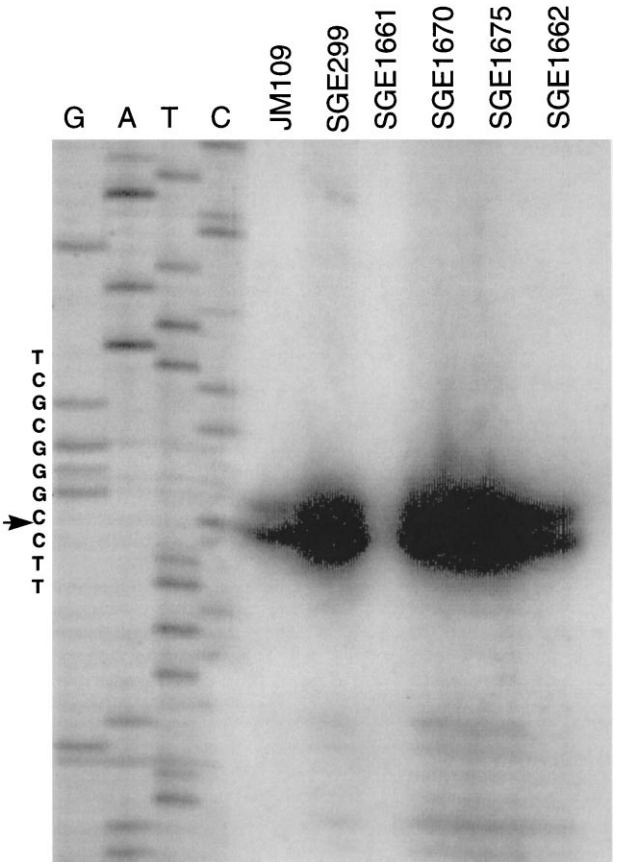

Fig. 5. Primer extension analysis of *lacI* transcripts from various strains. RNA was prepared (as described in Section 2.8) from strains JM109 (F′ *lacI*^{Q}), SGE299 (F′ *lacI*^{Q1}), SGE1661(*lacI*$^{+}$), SGE1670 (*lacI*^{Q1}), SGE1675 (*lacI*^{Q1}), and SGE1662 (*lacI*$^{+}$+medium-copy-number plasmid with *lacI*$^{+}$) and used in primer extension reactions with primer CBG09 (see Section 2.8). The transcripts and DNA sequence were separated by electrophoresis on a PA sequencing gel, as described in Section 2.8. The sequence of the *PlacI* region (complementary to the −7 to +4 sequence in Fig. 4) is shown on the left margin and in the four lanes, G, A, T, and C. The arrow marks the *tsp*.

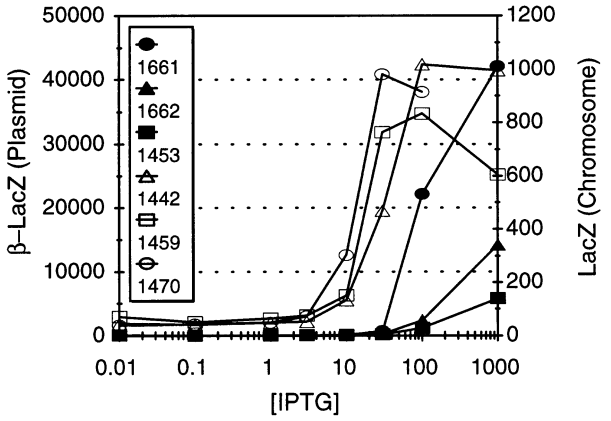

Fig. 6. Synthesis of β-LacZ (plasmid) or LacZ (chromosome) in the absence (no IPTG = 0.01 μM on the scale) or presence of indicated concentrations of IPTG. Cultures were grown in M63 (Section 2.4) + casamino acids and split into eight equal aliquots, which received IPTG to the final concentration (μM) shown for each point. All points are the average of assays of at least three cultures except SGE1661, which was the average of two. The solid symbols denote chromosomal LacZ activity (right axis), and the open symbols denote plasmid β-LacZ activity (left axis). Strains are described in Table 1. Strain SGE1661 is *lacI*$^{+}$; SGE1662 is *lacI*$^{+}$ with a medium-copy-number *lacI*$^{+}$ plasmid (pSGE705); SGE1453 is *lacI*$^{+}$ with a hcn *lacI*$^{+}$ plasmid (pSGE715); SGE1442 is *lacI*$^{+}$ with a medium-copy-number *lacI*$^{+}$, *β-lacZ* plasmid (pSGE712); SGE1459 is *lacI*$^{+}$ with a hcn *lacI*$^{+}$, *β-lacZ* plasmid (pSGE716); and SGE1470 is *lacI*^{Q1} with a hcn *lacI*$^{-}$, *β-lacZ* plasmid (pSGE721).

of LacI contributed from the plasmid-borne *lacI* gene should be consistent.

The amount of LacZ induced from the chromosomal gene was inversely proportional to the plasmid copy number and, therefore, the LacI molar concentration in the cell (Fig. 6). This was in spite of the maintenance of 10:1 stoichiometry between the LacI and *lacO* since each plasmid contained one *lacI* gene with the native promoter, and one *lac* operator in *Ptac*. This indicates that the molar concentration of LacI within the cell is primarily responsible for the magnitude of induction of the chromosomal allele, rather than the stoichiometry of the LacI to *lacO*. At 30 μM IPTG, significant chromosomal LacZ induction was first observed for all three strains, greater than uninduced levels by ~14-fold for SGE1661, ~fivefold for SGE1662, and ~threefold for SGE1453.

Induction of plasmid *β-lacZ* was very different from induction of chromosomal *lacZ* in two important respects: (1) the induction occurred at lower IPTG concentrations, and (2) induction appeared less sensitive to the LacI concentration (Fig. 6). The higher level of specific activity was due to the gene dosage, since the *β-lacZ* fusion gene is on the plasmids. Whereas significant induction from the chromosome occurred at IPTG concentrations between 30 and 100 μM, significant induction from the plasmid occurred at IPTG concentrations between 3 and 10 μM, an order of magnitude lower. As before (Fig. 2), the insolubility of higher amounts of the β-LacZ induced by 100–1000 μM IPTG did not allow us to determine whether significant differences in the magnitude of plasmid expression occurred at these IPTG concentrations (Fig. 6). The induction between 1 and 10 μM IPTG was significantly higher for the strain in which a hcn plasmid was controlled by *lacI*^{Q1}, 6.2-fold for SGE1470 vs. 2.3-fold for SGE1459 and 2.7-fold for SGE1442.

3.8. *Magnitude of induction of hcn* Ptac

The amount of insoluble β-LacZ fusion protein was estimated by densitometry after SDS–PAGE and was used to calculate the total protein and fold induction of protein observed. At 100 μM IPTG, induction of SGE1442 was 135-fold, and at 1000 μM, IPTG was

323-fold. Using data from rHb1.1 expression experiments (Weickert et al., 1997), a >600-fold induction of recombinant human hemoglobin protein was calculated, consistent with these fusion protein results (S. R. Curry and M.J.W., unpublished results). Therefore, $lacI^{Q1}$-controlled expression was >300- to >600-fold inducible from hcn plasmids.

4. Discussion

A desire to simplify plasmids used for gene expression, and reduce the concentration of IPTG necessary to induce expression, led to examination of control strategies that, unlike other approaches (Stark, 1987; Amann et al., 1988; Graham et al., 1995), did not require a *lacI* allele on the plasmid. The allele typically found in *E. coli* cloning strains, $lacI^{Q}$, was not sufficient to control (repress) transcription from the *tac* promoter on hcn expression plasmids since the *lac* operator sites on the plasmid exceed the LacI molecules in the cell by four- to fivefold. The ability to form 'sandwich' associations where one LacI tetramer binds to two operators on separate plasmids (Krämer et al., 1987; Wu and Liu, 1991) may reduce the excess of operators sites to two- to 2.5-fold, but still resulted in significant expression in the absence of inducer. However, this sandwich association is likely to account for the results in Table 3 where partial repression of plasmid promoters was evident in $lacI^{Q}$ strains. Operator titration may affect *Plac* induction from the chromosome less than *Ptac* induction on the plasmid because the native *lac* promoter, with three operator sites, is more effective at capturing LacI than the single operator site on the plasmid (Mossing and Record, 1986), consistent with observations summarized in Fig. 6. Significant repression of transcription of *Ptac* on medium-copy number plasmids (~100 copies per cell) was achieved by $lacI^{Q}$ on the chromosome, consistent with the expected equimolar stoichiometry of LacI to *lac* operator sites on the plasmid (Table 3).

We fortuitously identified a *lacI* chromosomal allele that repressed transcription from *Ptac* on medium and hcn expression plasmids in the absence of inducer. The sequence of this allele was identical to that of $lacI^{Q1}$ previously identified by Calos and Miller (1981), although their text contradicts their Fig. 1b, which shows an additional single bp deletion of a 'G' between the −10 and −35 regions of the promoter, probably in error. However, if this additional deletion is correct, we have a variant of $lacI^{Q1}$ that does not contain it. The $lacI^{Q1}$ 15-bp deletion positions a new −35 sequence identical to the consensus sequence for *E. coli* σ^{A} promoters (TTGACA; Hawley and McClure, 1983), which increased the strength of this promoter 170-fold. A 15-bp smaller product was obtained by PCR from all strains that strongly repressed expression, providing a simple screen for determining whether a strain in question contains the $lacI^{Q1}$ allele.

The $lacI^{Q1}$ allele initiates transcription at the wt location and is 17-fold stronger than that of $lacI^{Q}$, a level that is sufficient to produce the LacI protein levels reported previously (Calos and Miller, 1981; Müller-Hill, 1975) and more than sufficient to account for the ability to repress *Ptac* transcription from a hcn plasmid (~500 copies). It should result in approximately 1700 copies of LacI per cell. In addition, the increased promoter strength accounts for the unexpected phenomenon of much higher than expected uninduced levels of βGal in SGE1675. Since the *lacI* gene is directly upstream of and colinear with the *lacZ* gene, the increased transcription from the $lacI^{Q1}$ promoter is likely to continue into the *lac* operon, resulting in elevated basal levels of *lacZ* expression. The 170-fold increase in $PlacI^{Q1}$ transcription over $PlacI^{+}$ is more than sufficient to account for a 60-fold elevation in downstream uninduced LacZ observed. This elevation of basal LacZ activity may provide a secondary indicator of the presence of $lacI^{Q1}$ in strains: a biochemical confirmation of the PCR test. The $lacI^{Q1}$ deletion also abolishes a potential terminator for *mhpR* transcription upstream of *lacI*. This could result in read-through transcription of $lacI^{Q1}$ from the *mhpR* promoter, further increasing *lacI* transcription. Since expression of *mhpR* is expected to be low (Ferrández et al., 1997), this is not likely to contribute significantly to the effect of the $lacI^{Q1}$ promoter.

Our transcription measurements correlated well with our plasmid copy number measurements. Transcription from the *lacI* promoter on the medium-copy number plasmid pSGE705 in SGE1662 was 70-fold higher than the chromosomal transcription level. Under the growth conditions used for the RNA isolation, we expect about 1.6 copies of the genome per cell (Bremer and Dennis, 1996). Therefore, if plasmid *PlacI* transcription is 70-fold higher than from 1.6 copies of the chromosomal allele, the plasmid copy number of pSGE705 was estimated to be 112 copies per cell, identical to our physical measurement of 114 ± 10 copies per cell.

Strains containing the $lacI^{Q1}$ allele permitted >300-fold to >600-fold inducible expression of heterologous proteins from *Ptac* on hcn plasmids at low IPTG concentrations. The induction of plasmid expression from *Ptac* occurred at an order of magnitude lower IPTG concentrations than *lac* operon induction on the bacterial chromosome in the same host strain. This discrepancy could not be due to LacI concentration or stoichiometry, since it was maintained identically for both the plasmid and chromosomal promoters in the cell. However, the plasmid *Ptac* has only one *lac* operator, whereas the weaker chromosomal *Plac* has three operator sites, two of which simultaneously bind a LacI tetramer, forming a DNA loop sequestering the *lac* promoter (Adhya, 1989; Schleif, 1992). Therefore, the

mechanism of repression is different for the two promoters since the LacI occupancy and topology of the promoter region are different. The presence of only one operator in *lac*, equivalent to the single operator in the *tac* promoter, resulted in only 20-fold repression (Oehler et al., 1990). Differences in supercoiling between plasmid and chromosomal DNA may have also contributed to the difference in inducibility.

The magnitude of induction of the *lac* operon on the bacterial chromosome was proportional to the molar concentration of LacI in the cell estimated from the *lacI* gene dosage (Fig. 6). Consistent with this observation, Law et al. (1993) showed that as the intracellular concentration of LacI increases, the LacZ activity from the chromosomal *lac* operon induced by 1–10 mM IPTG declined by an order of magnitude, even in the presence of plasmids containing *lac* operators.

Tight control of inducible expression of genes on a hcn plasmid was maintained by the chromosomal $lacI^{Q1}$ allele, equivalent to control by plasmid-borne *lacI*. We suspect that some strains reported to be $lacI^{Q}$, may actually be $lacI^{Q1}$, or variants thereof. The $lacI^{Q1}$ allele should improve cloning of potentially detrimental genes by better controlling expression from *Ptac* or other *lac*-based promoters on hcn plasmids such as those with the pUC origin of replication, which typically do not contain a *lacI* gene. The use of $lacI^{Q1}$ induces higher expression of genes under *Ptac* control on hcn plasmids in *E. coli*, at low IPTG concentrations. Perhaps this characterization and demonstration of techniques for manipulating and detecting $lacI^{Q1}$ might encourage its wider use.

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