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In vivo study of the expression of the genes *serA* and *gltD* regulated by Lrp (Leucine-responsive regulatory protein) in *Escherichia coli* K-12

Changfeng Chen

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ABSTRACT

In vivo study of the expression of the genes *serA* and *gltD* regulated by Lrp (Leucine-responsive regulatory protein) in *Escherichia coli* K-12

Changfeng Chen

The plasmid pBAD22 *lrp* Cm^R carrying the *lrp* coding region immediately downstream from the p_{BAD} promoter was used to vary intracellular concentrations of Lrp. The expression of the genes *serA* and *gltD* was determined in the presence of different concentrations of Lrp. The results showed that expression of *serA* and *gltD* was proportional to Lrp concentration, indicating that Lrp activates gene expression. A comparison of the response of the genes *serA* and *gltD* to the variations in Lrp concentrations showed that the promoters of *serA* were more sensitive to Lrp than that of *gltD*. L-leucine's effects on Lrp's function were also investigated. Adding L-leucine to the minimal media decreased Lrp's activation of both *serA* and *gltD*. As for the plasmid-carried *lrp* gene, a decrease in expression was observed as L-serine (500 μg/ml) was added to glycerol minimal media.

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LIST OF ABBREVIATIONS

Amp: ampicillin

CAA: Casamino acids

Cm: chloramphenicol

Crp: cAMP receptor protein

GOGAT: glutamate synthase

Kan: kanamycin

Lrp: Leucine-responsive regulatory protein

ppGpp: guanosine tetraphosphate

Tet: tetracycline

I. INTRODUCTION

The leucine/Lrp regulon in *E.coli* was discovered in 1990 (Lin et al., 1990). Since then, more than 30 genes/operons have been identified as members of this regulon (reviewed in Newman et al., 1996).

serA, which encodes 3-phosphoglycerate dehydrogenase, and *gltD*, which encodes the small subunit of glutamate synthase (GOGAT), are two *E.coli* genes whose promoters require Lrp to function. Lrp's regulation of these two genes, *serA* and *gltD*, has been extensively studied under *in vitro* conditions (Lin, 1992; Ernsting et al., 1992, 1993). However, information about the *in vivo* regulation of gene expression by Lrp still lacks details. This project used these two genes as targets to study the regulation of Lrp on gene expression under *in vivo* conditions, and aimed to answer the following question: how does the expression of these two genes respond to variations in the intracellular Lrp concentration?

This project includes four parts : 1). Construction of a variable *lrp* expression plasmid. Using this plasmid, I was able to vary the intracellular Lrp concentration by varying the inducer's concentration in the media. 2). Construction of host strains required for the regulatory studies. 3). Expression of the genes *serA* and *gltD* in the presence of

different concentrations of Lrp. 4). The effect of overexpression of the *lrp* gene on the growth of *E.coli*.

Before presenting my work, I will review some relevant material.

1. *In vivo* study of the regulation of a regulon

An operon is a group of genes regulated by the same regulator. A regulator of a single operon is defined as a local regulator. A group of operons under the same regulator is defined as a regulon. Such a regulator is termed a global regulator (reviewed in Gottesman, 1984). A regulon is a complex and efficient system allowing the bacteria to respond quickly to environmental changes.

To study a regulon, experiments can be performed under *in vitro* or *in vivo* conditions. *In vitro* experiments can only suggest what potentially could occur inside the cells, while *in vivo* experiments can tell us what is actually occurring. The coupling of *in vitro* and *in vivo* experiments provides strong evidence of how regulons work.

To study the *in vivo* regulation of a global regulator, it is essential to know how levels of the regulator vary inside the cells. Normally there are two methods to detect the concentration of a regulatory protein. Western blot analysis

can be used to quantitate the expression of a regulator directly (Towbin et al., 1979). This method determines the total amount, not the effective concentration of a regulatory protein. Another way to assess the expression of a regulatory protein is to fuse a reporter gene, usually a *lacZ* gene, to a gene encoding the regulatory protein. The β-galactosidase activity produced from *lacZ* reflects the expression level of the regulatory protein.

To determine the *in vivo* regulation of a regulatory protein on its regulated genes, two *in vivo* testing methods are commonly used. They are *in vivo* titration and *in vivo* footprinting. When doing *in vivo* titration, a reporter gene, usually a *lacZ* gene is fused to the target gene, and then the enzyme activities in the presence of different levels of the regulatory protein are measured. *In vivo* footprinting is widely used to study the interaction of a regulatory protein with DNA. A protein bound to the DNA will alter the reactivity of the bases towards chemicals or UV light, depending on whether the bases are located within or outside the stretch of DNA with which the protein makes contact. After living cells are treated with chemical reagents or UV light, DNA is purified and cleaved with enzymes or chemicals. The cleaved fragments can then be detected by sequencing. When analysed on a sequencing gel, differences in the strand-breakage pattern between protein-free DNA and protein-bound DNA will occur at

bases located within regions that interact with proteins. *In vivo* footprinting is extremely informative in studying the mechanism of gene expression (Paul et al., 1995).

The bacterial cell is a very complex system. The expression of a gene may be regulated by many effectors at the same time. In addition to our incomplete knowledge of conditions inside the cells, this complexity can render the interpretation of *in vivo* regulation results very difficult.

2. The Leucine/Lrp regulon

For the Leucine/Lrp regulon, the regulator is Lrp, the leucine-responsive regulatory protein. As a regulator, Lrp activates some genes but represses others. Table 1 lists all the genes/operons so far identified to be regulated by Lrp (reviewed in Newman et al., 1995, 1996).

L-leucine is a coeffector of this regulon. It affects the expression of many, but not all, of its operons (reviewed in Newman et al., 1995, 1996).

Lrp is composed of 163 amino acid residues. Platko et al. (1993), showed that there are three domains in Lrp, which are the domains for DNA-binding, for leucine-binding, and for transcriptional activation. Purified Lrp binds very well to

double-stranded DNA containing an appropriate promoter sequence. Lrp-binding bends DNA. This binding, in some cases, has been shown to help RNA polymerase bind to the DNA, and thus activate gene transcription (Wang et al., 1993). However, in other cases, Lrp's binding to the promoters of some genes, such as *lysU, lrp*, prevents subsequent RNA polymerase binding, and thus represses gene expression (Lin et al., 1992a, Wang et al., 1994).

Lrp is an L-leucine-binding protein. Leucine's binding to Lrp does not prevent the binding of Lrp to its target gene (Ernsting et al., 1993). Leucine is thought to alter the conformation of Lrp, and thus alter the way in which Lrp binds to an Lrp-regulated gene (reviewed in Newman et al., 1995).

Table 1. *E. coli* genes/operons regulated by Lrp

Lrp functions	Operons/ genes
Activate	*daaABCDE, fanABC, fimB, gcvTHP, gltBDF, ilvIH, lacZYA, leuABCD, malEFG, malK, malT, ompF, papBA, pntAB, sdaC, serA, sfaA*
Repress	*fae, glyA, kbl-tdh, livj, livKHMG, lrp, lysU, ompC, oppABCDF, osmY, sdaA*

3. The *serA* gene in *E.coli*

3-1. The *serA* gene and L-serine biosynthesis in *E.coli*

The gene *serA* in *E.coli* encodes 3-phosphoglycerate dehydrogenase, which is the first enzyme in the L-serine biosynthesis pathway. The L-serine biosynthesis pathway of *E.coli* is shown in Figure 1.

The combination of serine, glycine, and C1 biosynthesis forms a major metabolic pathway in *E.coli*. According to Pizer et al. (1964), the carbon assimilated from glucose through the

serine-glycine pathway in *E.coli* acounts for about 15% of the total. Serine and 3-phosphoserine are involved in the biosynthesis of other biomolecules such as cysteine, tryptophan, and pyridoxine (reviewed in Stauffer, 1996).

As shown in Figure 1, the genes *serA, serB,* and *serC* encode the three enzymes required for serine biosynthesis. Unlike most genes involved in amino acid biosynthesis, these three genes are not organized as an operon. On the genetic map, the *serA* gene is located at 65.8 min, *serB* at 99.6 min, and *serC* at 20.6 min.

3-2. Regulation of *serA* in *E.coli*

L-serine, the end-product of the serine biosynthesis pathway, does not affect expression of the *serA* gene. However, it inhibits the enzymatic activity of 3-phosphoglycerate dehydrogenase, the first enzyme in the serine biosynthesis pathway and the translational product of the *serA* gene. Inhibition of 3-phosphoglycerate dehydrogenase by serine is the major form of control of serine biosynthesis in *E.coli*, and occurs by an allosteric process. The inhibition of 3-phosphoglycerate dehydrogenase activity is an effective form of control of the metabolic flow of carbon through the serine-glycine pathway (reviewed in Stauffer, 1996).

Several factors have been found to affect the 3-phosphoglycerate dehydrogenase levels inside the cells (McKitrick et al., 1980; Lin, 1992). Lrp is one of these factors.

Lrp activates expression of *serA*. Primer extension experiments (Lin, 1992) have shown that the gene *serA* has two promoters, P1 and P2. P1 is activated by Lrp, while P2 is repressed. Figure 2 shows the relationship between P1, P2, and the coding region of *serA*. Lin's experiments (1992) also showed that there were at least two Lrp-binding sites in the *serA* promoter region, one related to P1, and one related to P2.

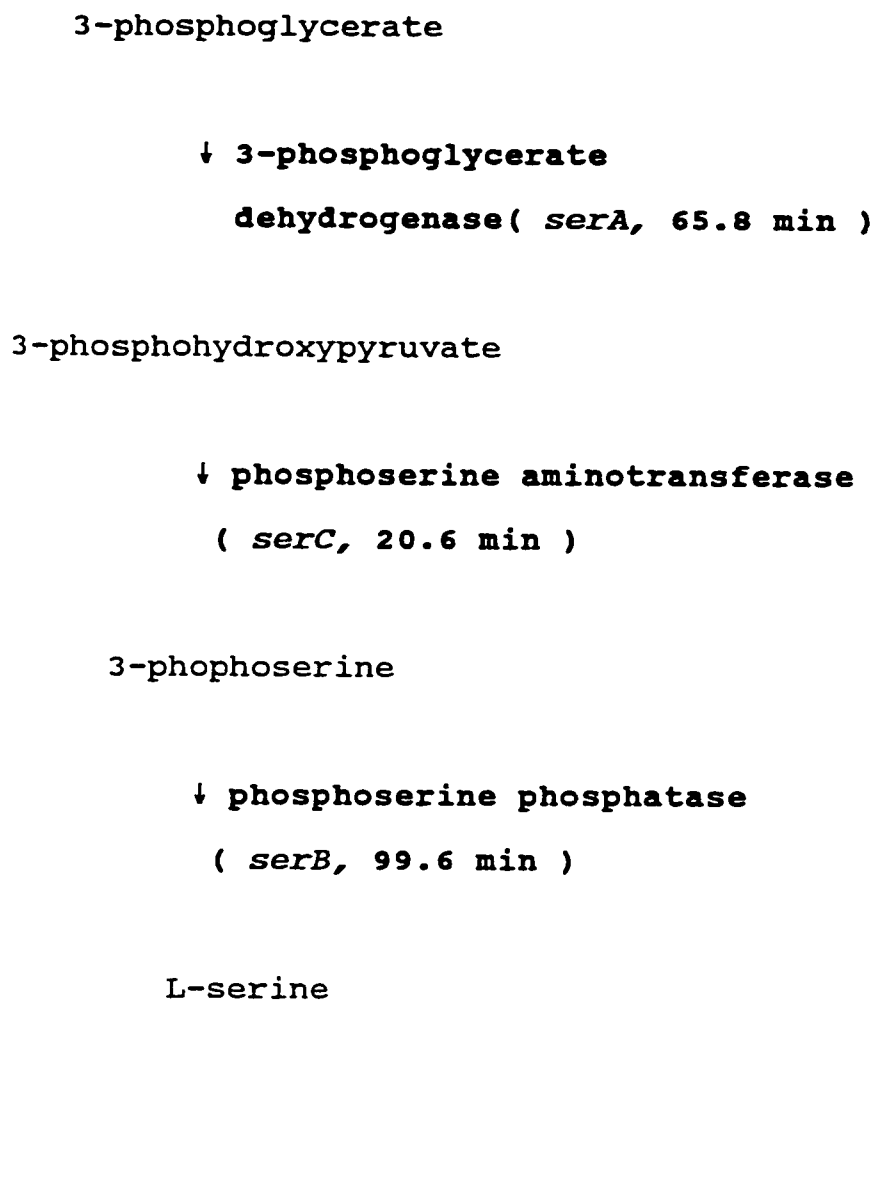


Figure 1. The L-serine biosynthesis pathway in *E.coli*

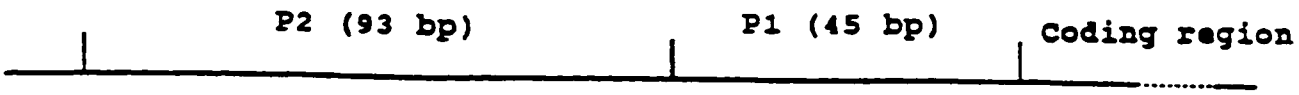


Figure 2. The promoters of *serA* in *E.coli* [graphical data from Lin (1992)]

Lrp activates P1, while represses P2.

4. The *gltD* gene in *E.coli*

4-1. The *gltBDF* operon and ammonia assimilation in *E.coli*

The genes *gltB* and *gltD* encode the large and small subunit of glutamate synthase (GOGAT), respectively. GOGAT catalyzes the reaction shown below:

Glutamine +α-ketoglutarate+ NADPH → 2 Glutamate + $NADP^+$

The *gltBDF* operon is located at 72 min on the *E.coli* chromosome (Berlyn et al., 1996). Besides *gltB* and *gltD,* the *gltF* gene is also a member of this operon. *gltF* encodes a transcriptional regulator. The operon also contains a fourth open reading frame, whose translational product may regulate, to a certain degree, expression of the operon. Two promoters have been found in the operon. The major promoter of the operon precedes the first gene (Oliver et al.,1987), while the minor promoters may exist between *gltB* and *gltD* and after *gltF* (Castano et al., 1992). Figure 3 shows the gene structure of the *gltBDF* operon.

4-2 Regulation of the *gltBDF* operon

Several factors affect the synthesis of GOGAT. These factors include the nitrogen sources, carbon sources, phosphate sources, ppGpp (guanosine tetraphosphate), Crp (cAMP receptor protein), and Lrp (Miller et al., 1972; Prusiner, 1972; Osorio et al., 1993; Ernsting et al., 1993; Helling, 1994).

Lrp has been found to positively regulate the *gltBDF* operon. Ernsting et al. (1993) described the regulation of *gltBDF* expression by Lrp as insensitive to leucine, relative to the regulation of *ilvIH* (encoding acetohydroxy acid synthase III) transcription.

In vivo studies on Lrp's regulation of *gltB* (encoding the large subunit of glutamate synthase) showed that the expression of *gltB::lacZ* rose with increasing the concentration of Lrp up to the level of Lrp found in wild-type strains, at which point the expression was maximum, and further increases of Lrp decreased its expression (Borst et al., 1996).

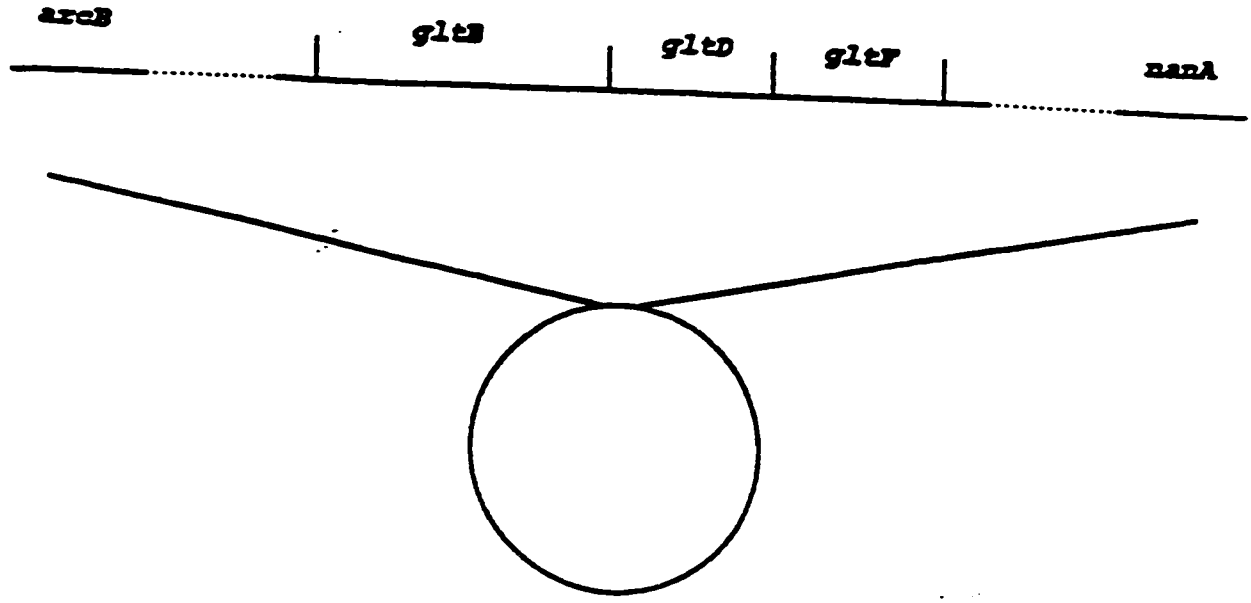


Figure 3. The *gltBDF* operon in *E. coli* [graphical data from Berlyn et al. (1996)]

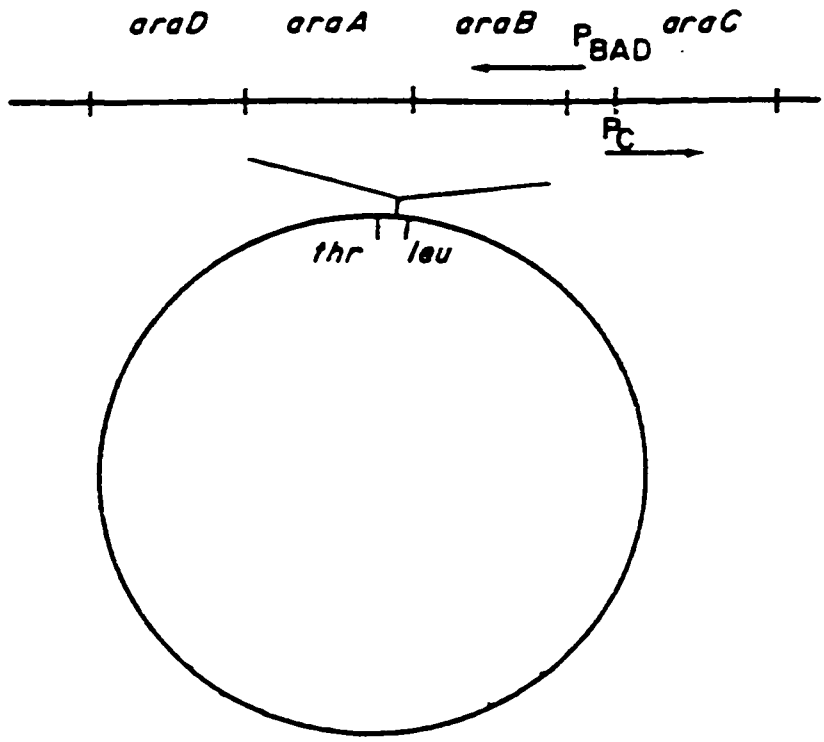
DNA sequences with unknown functions are shown by dotted lines, while DNA sequences with known functions are shown by solid lines.

5. The *araBAD* operon in *E.coli*

The structural genes required for the utilization of L-arabinose by *E.coli*, *araB*, *araC*, and *araD* are clustered at 1 min on the map, and form the *araBAD* operon. *araBAD* is transcribed from a promoter composed of *araO* and *araI*. The *araC* gene, which is located next to *araBAD*, is transcribed from a different promoter (*araCp*) in the opposite direction. The product of the *araC* gene, AraC is required for expression of *araBAD*. However, AraC also acts as a repressor of *araBAD* expression (reviewed in Schleif, 1996). The genes *araBAD* and *araC* are illustrated in Figure 4.

Guzman et al. (1995) have constructed a series of plasmid vectors containing the p_{BAD} promoter of the *araBAD* operon and the *araC* gene. On these vectors, the multiple cloning sites (MCS) are immediately downstream of the p_{BAD} promoter. Thus, the expression of a gene fused in any site within the MCS is under the control of the p_{BAD} promoter. Figure 5 shows the map of the pBAD22 vector. The pBAD22 vector contains an optimized SD sequence (Shine-Dalgarno sequence) between the *NheI* and *EcoRI* sites of the MCS as well as a translation start codon (ATG) at the NcoI site. The pBAD22 vector is suitable for cloning genes which lack sequences for initiation of translation. According to Guzman et al.(1995), expresssion of the gene fused in the MCS on pBAD22 is stable, due to the

optimized SD sequence in the MCS. The pBAD series of vectors are non-leaky plasmids - when no inducer is present, the basal expression is close to zero. In glycerol minimal media, 0.2 μg/ml of arabinose can turn on the p_{BAD} promoter; and 20 μg/ml of arabinose can almost induce the promoter to a maximum. The ratio of maximum expression to basal expression is very high (up to 1,000).



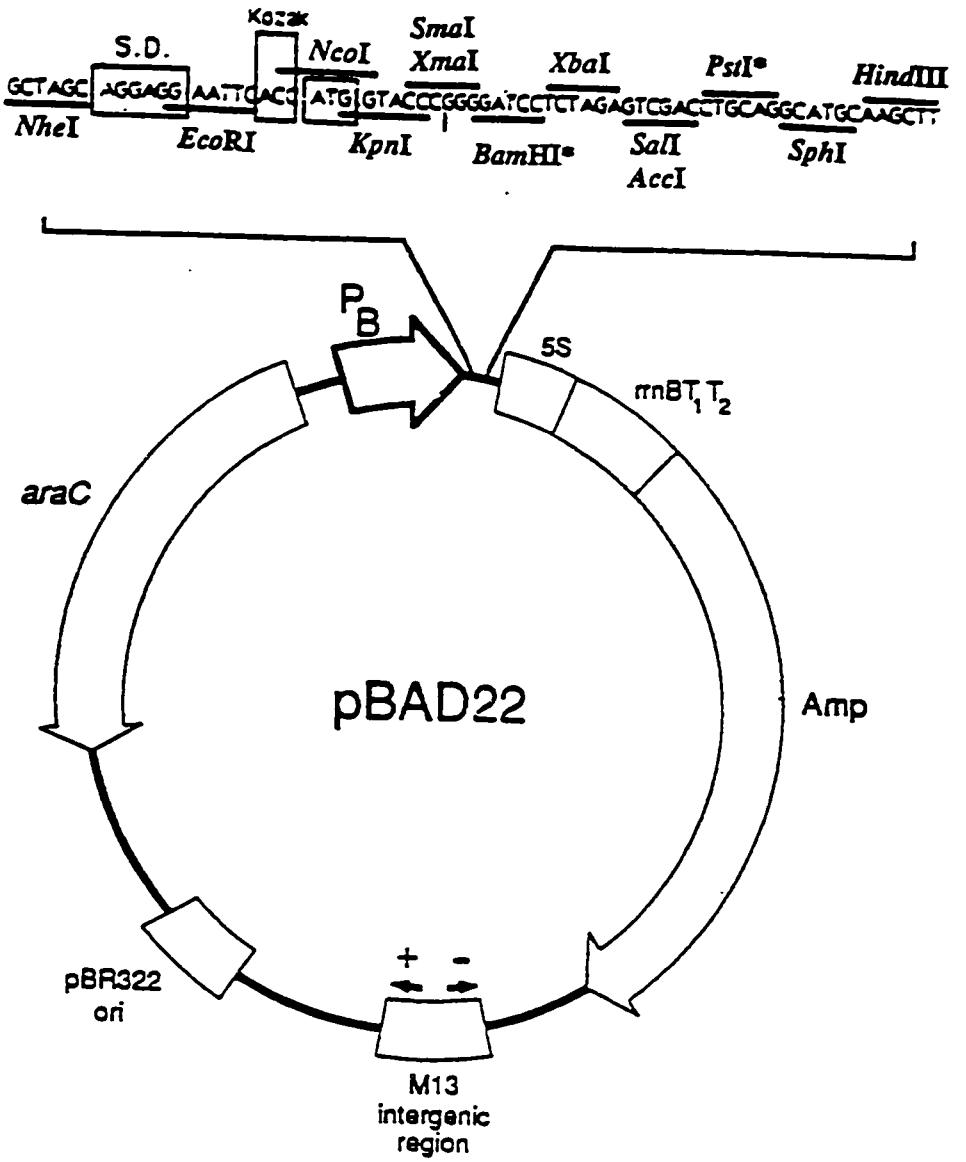
Structural genes and their products:

***araA*: L-arabinose Isomerase**

***araB*: L-ribulo-kinase**

***araC*: L-ribulose-5-phosphate-4-epimerase**

Figure 4. The *araBAD* operon and *araC* gene in *E.coli* [modified from Schleif (1987)]



* = not single sites

Figure 5. The map of the pBAD22 plasmid vector [modified from L. Guzman (unpublished data, personal communication to E.B. Newman)]

II. MATERIALS AND METHODS

1. STRAINS AND PLASMIDS

The strains and plasmids used in this study are described in Tables 2 and 3.

2. MEDIA AND GROWTH CONDITIONS

2-1. Minimal media

Niv-stock solution (pH 7.0): 0.527% KH_2PO_4, 1.500% K_2HPO_4, 0.020% $MgSO_4$ 0.001% $CaCl_2$, 50 μg/ml L-isoleucine, 50 μg/ml L-valine, and 0.2% $(NH_4)_2SO_4$.

Carbon sources were added to the Niv-stock solution in the following concentrations: D-glucose, 0.2%; glycerol, 0.5%; L-arabinose, 0.2%; succinate, 0.2%; and gluconic acid, 0.2%.

For solid media, 0.6% Bacto-agar was added.

2-2. NSIV media

0.2% L-serine was added to Niv-stock solution as a carbon source.

Table 2. Strains

Strains	Genotype	Reference/Source
Cu 1008	*E.coli* K-12 *ilvA*	Williams, L.S.
MEW1	Cu 1008 Δ*lacZ*	Newman et al., 1985
Cp55	MEW1 *leu::*λ*plac*Mu9	Lin et al., 1992
CT4A	MEW1 *ara⁻ lrp⁻*	Shao, Z.Q.[a]
CLE	MEW1 *leu::*λ*plac*Mu9	This work
CA	MEW1 Δ*ara*	This work
CAL	CA *lrp::*Tn*10*	This work
CALS	CAL *serA::lacZ*	This work
CALG	CAL *gltD::lacZ*	This work
Cp67	MEW1 *gcv::*λ*plac*Mu9	Lin et al., 1992
Cup22	MEW1 *sdaA::*λ*plac*Mu9	Su et al., 1989
Cp8	MEW1 *gltD::*λ*plac*Mu9	Lin et al., 1992
Cv975	*ilvIH::lacZ*	Willins, et al., 1992
SP2	MEW1 *lrp::lacZ*	Shao, Z.Q.[a]
CuS2	MEW1 *serA::lacZ*	Shao, Z.Q.[a]
LT10	MEW1 *ara* Δ*714*	Tao, 1995

a: personal communication

Table 3. Plasmids

Plasmids	Genotype/ Characteristics	Reference/ Source
pBAD18	A derivative of pBR322, carrying p_{BAD} promoter	Guzman et al., 1995
pBAD22	A derivative of pBR322, carrying p_{BAD} promoter	Guzman et al., 1995
pMC1871	pBR322 with *lacZ*	Gilbert, W.[a]
pHE1	pBAD18 carrying *lrp* coding region	Shao, Z.Q.[b]
pCm	pBR322 carrying CAT gene	Su, H.S.[b]
pBAD22 *lrp* Amp^R	pBAD22 carrying *lrp* coding region, ampicillin resistance	This work
pBAD22 *lrp* Cm^R	A derivative of pBAD22 *lrp* Amp^R, chloramphenicol resistance	This work
pBAD22 *lrp::lacZ* Cm^R	A derivative of pBAD22 *lrp* Cm^R, carrying *lrp::lacZ* fusion	This work

a: data from GenBank; b: personal communication

2-3. Luria Broth (LB)

1% bactotryptone, 0.5% yeast extract, and 0.5% NaCl.

For solid media, 0.6% Bacto-agar was added.

2-4. Media for the growth of bacteriophage P1

1% Bacto-tryptone, 1% Bacto-yeast extract, 0.8% NaCl.

1.7% Bacto-agar was used for plate, and 0.6% Bacto-agar for top agar. $CaCl_2$ and glucose were added to top agar to a final concentration of 2 mM and 0.1%, respectively.

2-5. SOC media

2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

2-6. Other additions to the media

Antibiotics were added in the following concentrations: Ampicillin (Amp), 100 μg/ml (or as noted); Tetracycline (Tet), 15 μg/ml; Kanamycin (Kan),50 μg/ml; Chloramphenicol (Cm), 25 μg/ml (or as noted).

3. β-GALACTOSIDASE ACTIVITY ASSAY

Cell samples were harvested from exponential-phase cultures. β-galactosidase activity was assayed in whole cells using Miller's Method (1972) and expressed in Miller units.

4. DETERMINATION OF PLASMID MAINTENANCE

An appropriate dilution of cells was first plated on LB plates, and the resulting colonies were replica plated on LB plates containing 25 μg/ml of chloramphenicol (or 100 μg/ml of ampicillin).

5 TRANSFORMATION AND TRANSDUCTION

5-1. Transformation:

Chemical (Ca^{2+}) shock transformation was done according to the method described by Sambrook et al.(1989).

Electro-transformation was done according to the method described in the Pulse Controller Instruction Manual provided by Bio-Rad.

5-2. P1 Transduction:

P1-mediated transduction was done using the method

described by Miller (1972).

6. PLASMID DNA ISOLATION

Plasmid isolation was carried out using the Qiagen miniprep or maxiprep kit.

7. RESTRICTION ENZYME DIGESTION

Restriction enzyme digestion was done according to the instructions accompanying the enzymes.

8. DNA EXTRACTION FROM AGAROSE GEL

DNA was extracted from agarose gels using the Qiagen DNA Extraction Kit.

9. AGAROSE GEL ELECTROPHORESIS

DNA agarose gel electrophoresis was done according to the method described by Sambrook et al. (1989).

10. CONSTRUCTION OF STRAINS

10-1. Construction of the strain CA (MEW1 Δ*ara*)

10-1-1. Construction of the strain CLE (MEW1 *leu::λplac*Mu9)

The strain MEW1 (Δ*lac*) was transduced with P1 phage grown on the strain CP55 (*leu::λplac*Mu9). A strain carrying a defective *leu* gene can only grow on minimal media supplemented with L-Leucine. Thus, the transductants were selected on glucose minimal media agar plates supplemented with L-leucine (50 μg/ml), X-gal(40 μg/ml), and kanamycin (50 μg/ml). In order to verify that this was truly a *leu*$^-$ strain, the colonies grown on such plates were then streaked on glucose minimal media agar plates supplemented with X-gal (40 μg/ml), and kanamycin(50 μg/ml). The leucine auxotroph transductant was named CLE (MEW1 *leu::λplac*Mu9).

10-1-2. Construction of the strain CA (MEW1 Δ*ara*)

The CLE (MEW1 *leu::λplac*Mu9)strain was transduced by P1 phage grown on the strain LT10 (MEW1 *ara*Δ714). Transductants were selected on glucose minimal media agar plates with X-gal(40 μg/ml). All the white colonies from the above plates were streaked on arabinose minimal media agar plates to select *ara*$^-$. The selected *ara*$^-$ strains were further tested on LB plates containing kanamycin(50 μg/ml) to screen for *kan*^{s} to ensure that the resulting strain lost the whole λ*plac*Mu9 insertion. A strain, which was *ara*$^-$, Kan^s, and white on X-gal, was named CA. To check if L-arabinose was toxic to the strain

CA, N. Zografakis measured the growth rate of the CA strain in glucose minimal media with and without 0.2% L-arabinose, and glycerol minimal media with and without 0.2% L-arabinose. His results indicated that adding arabinose to the media was not toxic to the newly constructed CA strain.

10-2. Construction of the strain CAL (CA *lrp::Tn10*)

The strain CA (MEW1 Δ*ara*) *was* transduced with P1 phage grown on the strain CT4A (*lrp::*Tn*10*). The transductants were selected on LB plates containing tetracycline at a concentration of 15 μg/ml. The *lrp*⁻ genotype was confirmed by streaking the transductants expressing tetracycline resistance on NSIV (L-serine minimal media) plates. Only *lrp*⁻ strains can grow on minimal media using L-serine as the sole carbon source.

10-3. Construction of the strain CALS (CAL *serA::lacZ*)

Phage P1 grown on the strain CuS2 (MEW1 *serA::*λ*plac*Mu9) was used to transduce the strain CAL (*ara*⁻ *lrp::*Tn*10*). The transductants were selected on LB plates containing tetracycline (15 μg/ml) and kanamycin (50 μg/ml). The resulting strain was named CALS, which had been confirmed as an L-serine auxotroph by growing the cells in minimal media with and without L-serine.

10-4. Construction of the strain CALG (CAL *gltD::lacZ*)

P1 grown on the strain Cp8 (MEW1 *gltD::*λ*plac*Mu9) was used to transduce the strain CAL (*ara*⁻ *lrp::*Tn*10*). The transductants were selected on LB plates containing tetracycline (15 μg/ml) and kanamycin (50 μg/ml). The *gltD*⁻ genotype was confirmed by growing the cells in minimal media with ammonia at both low and high concentrations. The strain which required ammonia at high concentrations for growth was named CALG.

11. Construction of plasmids

11-1. Construction of the plasmids pBAD22 *lrp* Amp^R and pBAD22 *lrp* Cm^R

The *lrp* coding region was amplified by PCR using primers Lrp-1 (5′-GGGGTACC**ATG**GTAGATAGCAAGA-3′) and Lrp-2 (5′-CTCCA-GGTACAAGCTTTTCC-3′). Here, GGTACC is a new *Kpn*I cutting site, and **ATG** acts as the *lrp* translation start site. Lrp-2 is complementary to the sequences 100 bp downstream of the *lrp* translation stop codon. The plasmid pHE1 containing the coding region of the *lrp* gene, served as a template for PCR. The PCR product obtained was about 600 bp long.

The plasmid pBAD22 was cut with the enzymes *Hind*III and *Kpn*I (the *Hind*III and *Kpn*I cutting sites were located in the

polycloning site of pBAD22, and immediately downstream of the p_{BAD} promoter). The resulting fragments were desalted using the Qiagen desalting kit. During the desalting process, the 40 bp fragments tended to be lost, so that, after desalting, the remaining DNA fragments were the 4.6 kb fragments, which served as vectors for the subsequent cloning.

The PCR products were cut with *Hind*III and *Kpn*I. The resulting fragments were ligated to the 4.6 kb fragment of pBAD22 (*Hind*III-*Kpn*I). The strain CT4A (MEW1 *ara*$^-$ *lrp*$^-$) was transformed with the ligation products, and plated on LB plates containing ampicillin (100 μg/ml).

lrp$^-$ strains can use L-serine as the sole carbon source for growth, while *lrp* wild-type strains cannot grow on L-serine minimal media (NSIV). This characteristic proved advantageous for further screening of the pBAD22-*lrp* fusion constructs. The p_{BAD} promoter on the plasmid pBAD22 is a "non-leaky" promoter, with a very low basal expression in the absence of L-arabinose. Arabinose is required in a concentration of about 20 μg/ml for maximum induction. When transformed into an *ara*$^-$ and *lrp*$^-$ strain, the aforementioned construct should give an Lrp$^-$ phenotype until arabinose is added to the medium. The colonies on LB containing ampicillin (100 μg/ml) plates were picked up and streaked on NSIV plates with arabinose (10 μg/ml) and ampicillin (100 μg/ml), and NSIV

plates with ampicillin (100 μg/ml). Of these, colonies which grew on NSIV plates with ampicillin (100 μg/ml), but failed to grow on NSIV plates with arabinose and ampicillin, were selected. Plasmids were isolated from one of the selected colonies. The agarose-gel electrophoresis showed that the isolated plasmids were of the right size (about 5.2 kb). The new plasmid was given the name pBAD22 *lrp* Amp^R. Figure 6 illustrates construction of the pBAD22 *lrp* Amp^R plasmid.

Since ampicillin is not stable during culture, the ampicillin resistance gene carried by the pBAD22 vector was replaced by the chloramphenicol resistance gene (CAT). In order to do this, the pBAD22 *lrp* Amp^R plasmid was linearized by cutting with the restriction enzyme *Pst*I. The plasmid pCm was cut with the same enzyme, and gave a 1.5 kb fragment which contained the CAT gene. The 1.5 kb fragment was ligated to the linearized pBAD22 *lrp* Amp^R. The ligation products were transformed into the CT4A (*ara*$^-$ *lrp*$^-$) strain. LB plates containing 25 μg/ml of chloramphenicol were used to select chloramphenicol resistant transformants, and plasmids were isolated from one colony with chloramphenicol resistance. Agarose gel electrophoresis showed that the isolated plasmids were of the right size (about 6.7 kb). The recombinant plasmid was named pBAD22 *lrp* Cm^R. Figure 7 illustrates construction of the pBAD22 *lrp* Cm^R plasmid.

Since the expression of this plasmid-carried *lrp* gene was under the control of the *araBAD* promoter, p_{BAD}, I expected that adding different concentrations of L-arabinose to appropriate minimal media could induce different intracellular concentrations of Lrp.

11-2. Construction of the pBAD22 *lrp::lacZ* Cm^R plasmid

In order to measure which levels of arabinose induced, or corresponded to which level of the induced Lrp inside the cells, a *lacZ* reporter gene was fused into the *lrp* coding region of pBAD22 *lrp* Cm^R. The resulting plasmid was named pBAD22 *lrp::lacZ* Cm^R. Figure 8 illustrates construction of the pBAD22 *lrp::lacZ* Cm^R plasmid.

In order to do this, the pBAD22 *lrp* Cm^R was linearized with the restriction enzyme *Bgl*II, dephosphorylated with CIP (calf intestinal alkaline phosphatase), and then isolated with the Qiagen gel purification kit. The plasmid pMC1871 (Figure 9) was cut with *Bam*HI, and a fragment (about 3 kb) containing a promoterless *lacZ* gene was dephosphorylated and subsequently isolated with the Qiagen gel purification kit. The linearized and dephosphorylated pBAD22 *lrp* Cm^R and the 3 kb fragment containing *lacZ* were ligated.

The strain CA (MEW1 *ara*$^-$) was transformed with the ligation

product. Blue colonies on LB plates containing arabinose (0.1%), X-gal (40 μg/ml), and chloramphenicol (25 μg/ml) were isolated, and plasmids were extracted from one of the blue colonies. Agarose-gel electrophoresis showed that the isolated plasmids were of the right size(about 9.7 kb). The plasmids were also digested with *Mlu*I. The *Mlu*I digestion results further confirmed that the construct was inserted in the right orientation.

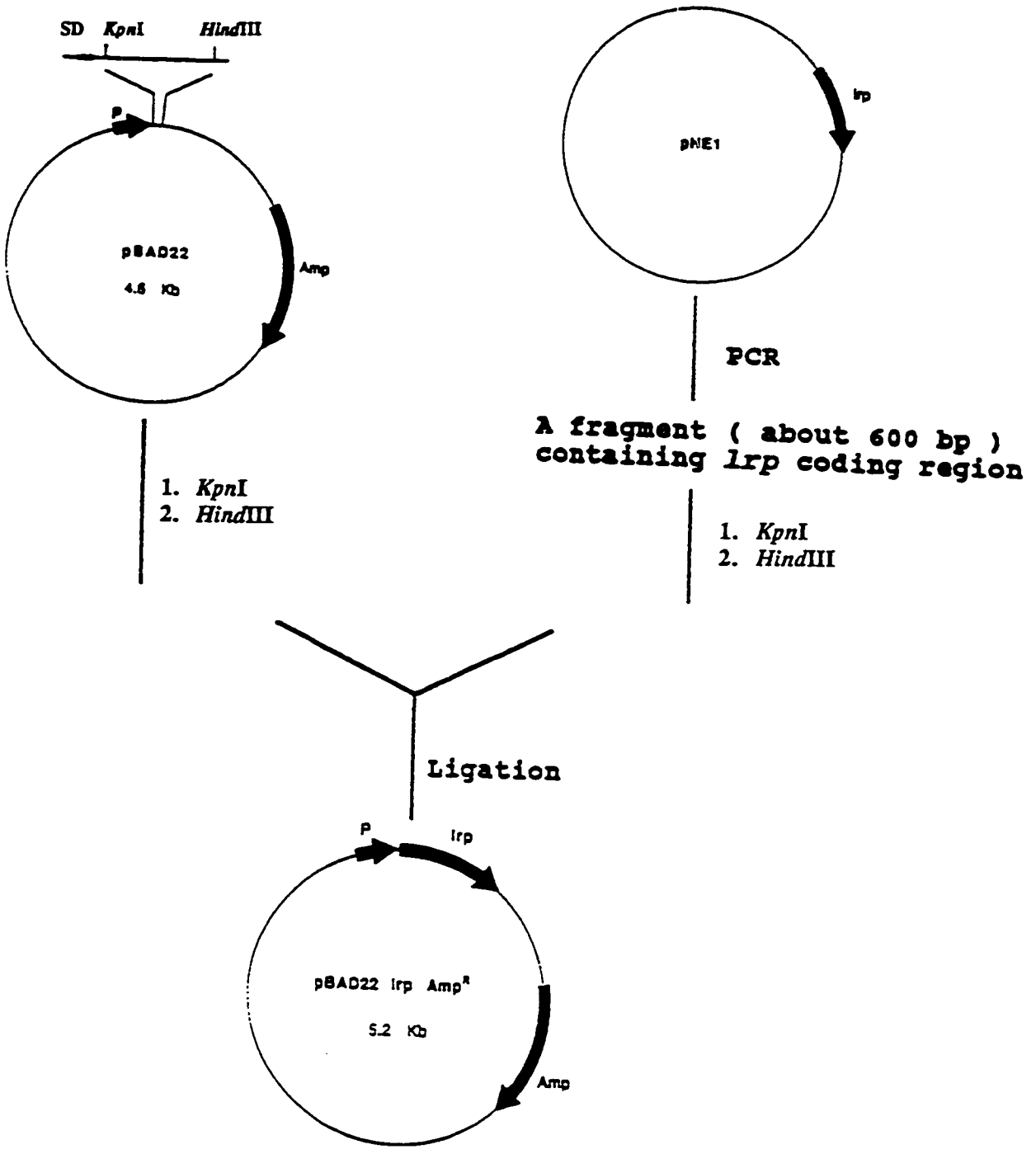


Figure 6. Construction of the pBAD22 *lrp* Amp^R plasmid

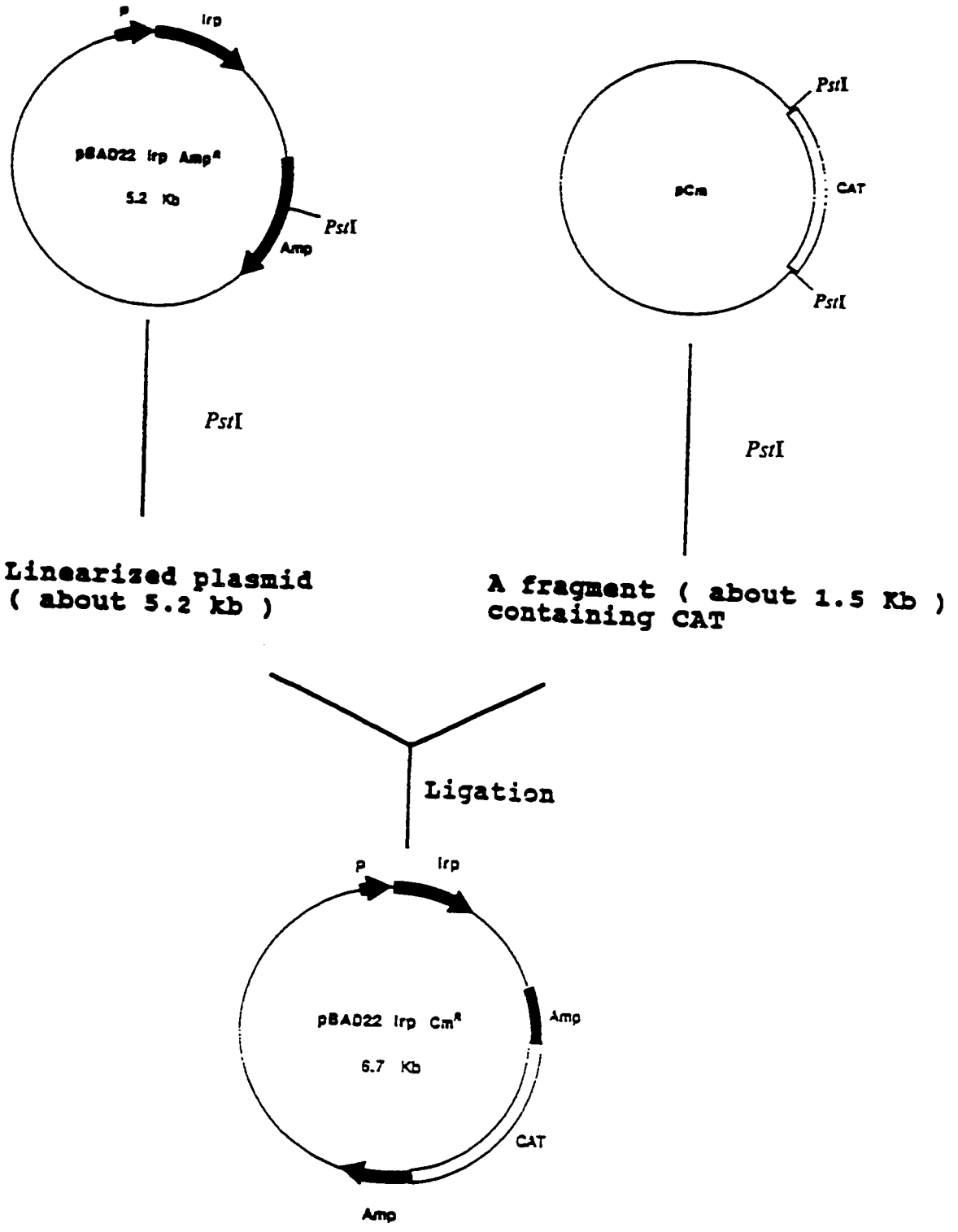


Figure 7. Construction of the pBAD22 *lrp* Cm^R plasmid

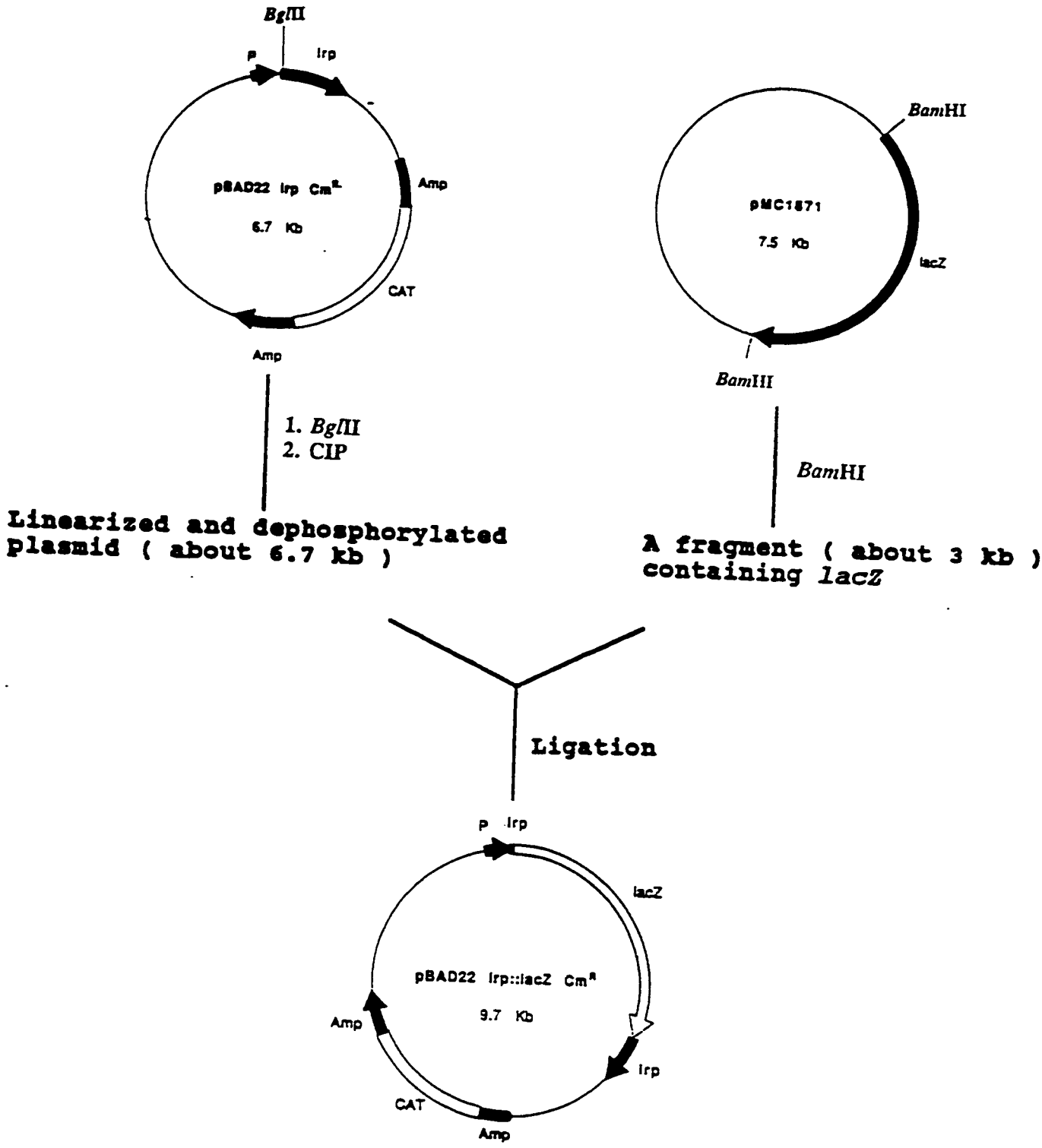


Figure 8. Construction of the pBAD22 *lrp::lacZ* Cm^R plasmid

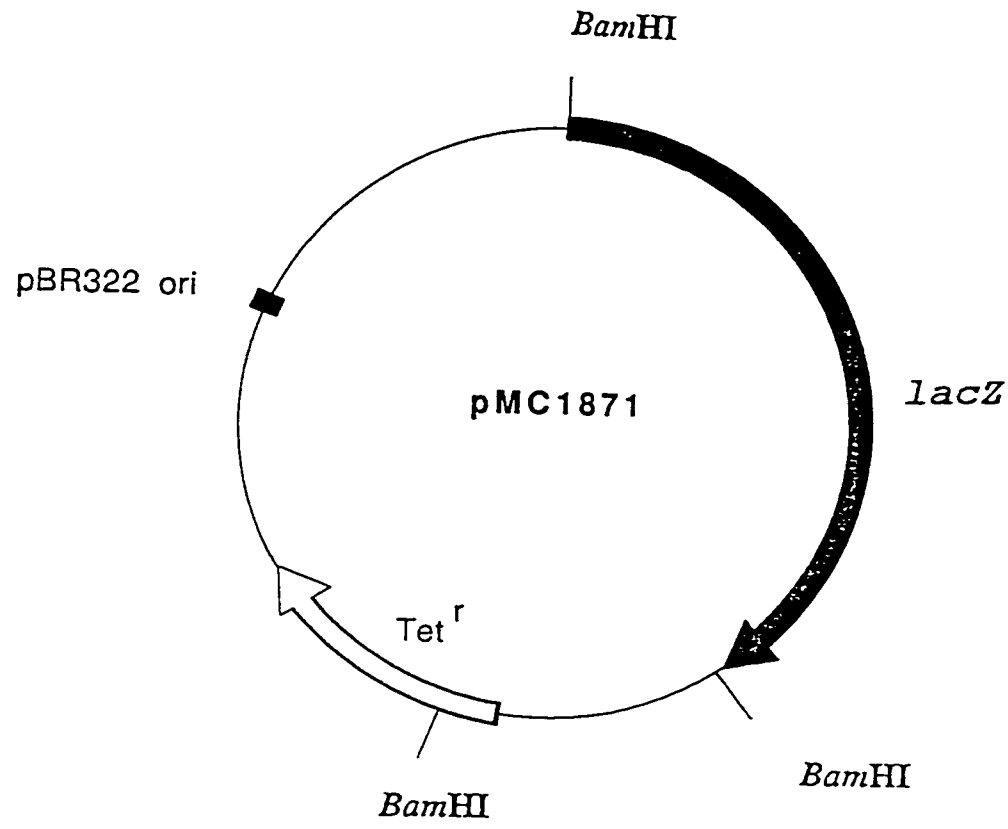


Figure 9. The map of the pMC1871 plasmid (graphical data from GenBank)

12. Determination of the toxicity of Lrp to cell growth

The cells of strain CA (*ara*$^-$ *lrp*$^+$)/pBAD22 *lrp* Cm^R from a culture in glycerol minimal media were subcultured in glycerol minimal media with and without 100 μg/ml of L-arabinose. The starting point of the culture was recorded as 0 hours. Samples were harvested every two hours. Turbidity (OD_{600}) of each sample was measured. To determine the number of cells which can form colonies (colony-forming unit), the cells of each sample were diluted and plated on LB plates. The colony-forming unit (CFU) was calculated and expressed as cells/ml. To maintain plasmids within the cells, 50 μg/ml of chloramphenicol was added to the minimal media.

III. RESULTS

This thesis presents data concerning the *in vivo* regulation of expression from two *E.coli* promoters by Lrp: those from the *serA* and *gltD* genes. The results are divided into four sections.

In section 1, I explain the construction of the strains needed for this study.

In section 2, I present data to show that, with this system, the amount of Lrp produced was indeed regulated by the amount of arabinose provided.

In section 3, I describe how the gene expression of *serA* and *gltD* responds to the variations in the intracellular Lrp concentration.

In section 4, I describe the effect of the overexpression of *lrp* on the growth of *E.coli* cells.

1. CONSTRUCTION OF THE STRAINS AND PLASMIDS NEEDED FOR THIS STUDY

1-1. Variable expression plasmid carrying the *lrp* coding region

The following plasmid was constructed so that Lrp expression would be under the control of the p_{BAD} promoter. With this plasmid, we were expecting to be able to vary the intracellular concentrations of Lrp by adding different concentrations of inducer to the media.

Guzman et al. (1995) constructed a series of cloning plasmids, which carry multiple-cloning sites (MCS) immediately downstream of the p_{BAD} promoter. Any gene fused into the MCS area is under the control of p_{BAD}. Without the inducer, L-arabinose, in the media the basal expression from p_{BAD} is low enough to be neglected. Upon the addition of arabinose, the promoter is turned on very quickly. The expression range from p_{BAD} is very large. According to Guzman et al. (1995), the highest ratio of the maximum expression to the basal expression from p_{BAD} on these vectors is up to 1,000. pBAD22 is one of the plasmids in this vector family. Guzman et al. (1995) concluded that since pBAD22 had an optimized SD sequence (Shine-Dalgarno sequence) in its MCS region, the expression of the gene fused downstream of the SD sequence was

quite stable. Therefore, I selected pBAD22 as a vector, and fused the *lrp* coding region in its MCS. I expected the resulting construct to be able to produce Lrp at intracellular concentrations with a very large range, and the gene expression to be stable.

Within the MCS region of pBAD22, there are two unique restriction sites, *Kpn*I and *Hind*III. The *Kpn*I site is located 10 bp downstream of the SD sequence, while the *Hind*III site is 33 bp downstream of the *Kpn*I site. The *lrp* coding region was amplified from the plasmid pHE1 by PCR. The amplified fragment contained a unique *Kpn*I site, which was located just before the translation start site of *lrp*, and a unique *Hind*III site, which was located 100 bp downstream from the translation stop codon of *lrp*.

The *Hind*III and *Kpn*I sites on the vector pBAD22 were ligated to the same sites of the fragment, which contained the *lrp* coding region, thus putting the *lrp* gene under the control of the p_{BAD} promoter (Figure 6).

The ligation products were transformed into the strain CT4A (*ara*$^-$ *lrp*$^-$). The transformants were selected on LB containing ampicillin (100 μg/ml), and futher screened by streaking them on NSIV (L-serine minimal media) plates, and NSIV plates with 0.1% arabinose. Because only the *lrp*$^-$ strain

can use L-serine as the sole carbon source, I expected that the transformants with the right construct could grow on NSIV but not on NSIV with arabinose. Plasmids were purified from such a transformant. The agarose gel electrophoresis showed that the plasmids were of the right size (about 5.2 kb).

Ampicillin is easily degraded during culture, resulting in the loss of plasmids. Loss of plasmids often resulted in the loss of gene expression from some cells in the population, causing unreproducible expression results for a population of cells.

Chloramphenicol is quite stable. Therefore, I attempted to use it instead of ampicillin to maintain plasmids. The plasmid pBAD22 *lrp* Amp^R contains a unique *Pst*I cut site in the middle of the ampicillin resistance gene. The plasmid pCm carries a chloramphenicol resistance gene (CAT) between two *Pst*I cut sites. To change the pBAD22 *lrp* Amp^R plasmid into a chloramphenicol resistance one, the plasmid was linearized by cutting with *Pst*I, and ligated to a CAT gene fragment with two *Pst*I ends. Thus, by inserting a CAT gene fragment into the plasmid, ampicillin resistance was substituted for chloramphenicol resistance. The new plasmid was named pBAD22 *lrp* Cm^R (Figure 7).

1-2. Fusion of the *lacZ* gene to the *lrp* coding region of

pBAD22 *lrp* Cm^R

In order to assess the relationship between arabinose added to the media and the intracellular induced Lrp levels, a promoterless *lacZ* gene was fused to the *lrp* coding region of the plasmid described in the preceding section, pBAD22 *lrp* Cm^R. The new plasmid was named pBAD22 *lrp::lacZ* Cm^R (Figure 8).

The promoterless *lacZ* gene came from the plasmid pMC1871. The plasmid was cut with the restriction enzyme *Bam*HI, and a fragment (about 3 kb) containing *lacZ* was purified. The *lacZ* fragment obtained this way contains its own translation stop codon, and lacks the translation start codon. On the plasmid pBAD22 *lrp* Cm^R, there is a unique *Bgl*II cut site located within the *lrp* coding region and 10 codons downstream of the translation start codon. The *Bgl*II and *Bam*HI cut sites are compatible. Therefore, the *lacZ* fragments, with two cohesive ends produced by cutting with *Bam*HI, were ligated to the pBAD22 *lrp* Cm^R linearized by cutting with *Bgl*II. The ligation products were transformed into the strain CA (MEW1 *ara*$^-$). The transformants were selected on LB containing chloramphenicol (15 μg/ml), X-gal (40 μg/ml), and arabinose (1,000 μg/ml). Plasmid was purified from one of the blue colonies. Agarose-gel electrophoresis showed that the newborn plasmid was of the right size (about 9.7 kb).

In the *lrp::lacZ* fusion, the bulk of the *lrp* sequence located downstream from the stop codon of *lacZ* is not translated. However, a small fragment (10 codons) of *lrp* located upstream from the *lacZ*, including its translation start codon, ATG, is translated accompanying the translation of *lacZ*.

The expression of *lrp::lacZ* on this construct was used as a measure of expression of *lrp* on the pBAD22 vector.

1-3. Construction of host strains needed for *in vivo* regulatory studies

1-3-1. Construction of the strain CA (MEW1 Δ*ara*) and CAL (MEW1 Δ*ara lrp*⁻)

The intent of this study was to measure gene expression at a variety of arabinose concentrations. It was therefore necessary to keep all the host strains for the regulatory study *ara*⁻.

I used a two-step procedure to create strains with *ara* deletions, first adding an auxotroph due to an insertion in a closely linked gene, and then using a strain with a known *ara* deletion to remove the auxotroph and add the arabinose deletion.

Thus, I first constructed a strain with a defective *leu* gene by transducing the strain MEW1 with P1 phage grown on the strain CP55 (*leu::λplac*Mu9); and then transferred a functional *leu* gene to the resulting *leu*$^-$ strain using P1 phage grown on the strain LT10 (MEW1 *araΔ714*) constructed by L. Tao (1995). Among the *leu*$^+$ transductants obtained, about 10% were *ara*$^-$, and were screened by testing their growth on arabinose minimal media. The strain constructed this way was named CA (MEW1 *ara*$^-$). Since L-arabinose is toxic to certain strains of *E.coli*, the effect of L-arabinose on strain CA was determined by N. Zografakis in our lab. The results showed that L-arabinose was not toxic to CA.

For promoter studies with variable intracellular Lrp levels, I also needed a host strain deficient in Lrp production. To produce this strain, *ara*$^-$ and *lrp*$^-$, I transduced strain CA (MEW1 *ara*$^-$) with P1 grown on strain CT4A (*lrp::*Tn*10*). The transductants were selected on LB containing tetracycline (15 μg/ml). The genotype of *lrp*$^-$ was verified by growing the strain on minimal media using L-serine as the sole carbon source.

1-3-2. Construction of the strain CALS (CAL *serA::lacZ*) and CALG (CAL *gltD::lacZ*)

The purpose of this work was to determine the effect on

transcription of the genes *serA* and *gltD* of the variation of the intracellular Lrp concentration. To realize this aim, I needed strains which were *ara*$^-$ and *lrp*$^-$ and carried a target gene. Since strains carrying chromosomal *serA::lacZ* (CuS2) and *gltD::lacZ* (Cp8) were already available, I decided to concentrate on those genes, both of which are activated by Lrp.

To transfer *serA::lacZ* to the appropriate strain, P1 grown on the strain CuS2 (MEW1 *serA::lacZ*) was used to transduce the fusion into strain CAL (MEW1 *ara*$^-$ *lrp*$^-$), selecting for kanamycin resistance and blue coloured colonies on X-gal. The transductants with such phenotypes were further tested for their ability to grow with and without L-serine. A strain requiring L-serine was selected and named CALS (CAL *serA::lacZ*).

In the same way *gltD::lacZ* was transduced into the strain CAL (MEW1 *ara*$^-$ *lrp*$^-$), selecting for kanamycin resistance and blue coloured colonies on X-gal. The transductants with such phenotypes were further tested for their ability to grow with low and high concentrations of ammonia. A strain requiring a high concentration of ammonia was selected and named CALG (CAL *gltD::lacZ*).

2. REGULATORY STUDIES WITH THE VARIABLE EXPRESSION PLASMID

2-1. Expression of *lrp* under the control of the p_{BAD} promoter

In a cell carrying the plasmid pBAD22 *lrp* Cm^R, production of Lrp should increase with increasing arabinose. I verified this using *lacZ* as a reporter for the p_{BAD} promoter. To study how L-arabinose regulates expression of the plasmid-carried *lrp* gene, the plasmid pBAD22 *lrp::lacZ* Cm^R was transformed into strain CAL (MEW1 *ara*$^-$ *lrp*$^-$). I then measured β-galactosidase activities of the resulting strain CAL/pBAD22 *lrp::lacZ* Cm^R grown with a variety of L-arabinose concentrations in different media. Because expression of the *lrp* gene on the construct pBAD22 *lrp::lacZ* Cm^R is under the control of the p_{BAD} promoter, adding different concentrations of L-arabinose to minimal media was expected to produce different concentrations of LacZ protein, which could be detected by assaying β-galactosidase activity.

2-1-1. Expression of *lrp::lacZ* under the control of the p_{BAD} promoter in glycerol minimal media

To verify that the construct functions as expected, strain CAL (MEW1 *ara*$^-$ *lrp*$^-$)/pBAD22 *lrp::lacZ* Cm^R was grown in glycerol minimal media supplemented with different concentrations of L-arabinose. I also wanted to determine the effect of L-serine and L-leucine on the expression of *lrp::lacZ*, and so did the same experiment in the presence of L-serine (500 μg/ml), L-

leucine (200 μg/ml), and both, measuring β-galactosidase activity in all cases. The results are shown in Table 4 and Figure 10.

Without arabinose in the media, the basal expression of *lrp::lacZ* from the p_{BAD} promoter is quite low (≤40 Miller units) in all the cases. Upon the addition of L-arabinose to the media, the p_{BAD} promoter was turned on. The production of Lrp is proportional to arabinose added to the media before it reaches a maximum.

Without L-serine and L-leucine, the expression curve (Curve 1 in Figure 10) rises abruptly when the arabinose concentration increases from 0 to 15 μg/ml, and changes slowly thereafter. The maximum expression was about 11,200 Miller units (see Table 4).

In the absence of L-serine, the addition of L-leucine to the media does not change the induction pattern of Lrp by arabinose considerably (see Figure 10), when the arabinose concentration is lower than about 7.5 μg/ml. When the arabinose concentration is greater than about 7.5 μg/ml, adding L-leucine (200 μg/ml) to the media lowered the expression, compared with the results obtained in the absence of L-leucine. In this case, about 10 μg/ml of arabinose can induce the expression to the maximum level, which is about

8,500 Miller units. When the arabinose concentration is greater than 10 μg/ml, the expression curve (Curve 2 in Figure 10) levels off.

In the presence of L-serine (500 μg/ml), the sensitivity of the p_{BAD} promoter to L-arabinose is much less than that in the absence of L-serine. In the absence of L-serine (500 μg/ml), 0.1 μg/ml of arabinose can induce about 240 Miller units of expression (about six times greater than the basal expression); while in the presence of L-serine (500 μg/ml), the same amount of arabinose is not enough to induce the p_{BAD} promoter. In the absence of L-serine, about 8-15 μg/ml of arabinose can induce the p_{BAD} promoter to a maximum level no matter whether L-leucine (200 μg/ml) is present or not; while in the presence of L-serine, about 70-100 μg/ml of arabinose is needed.

In the presence of L-serine and absence of L-leucine, the expression curve (Curve 3 in Figure 10) rises with increasing L-arabinose concentration until saturation. The maximum expression under these conditions was about 8,600 Miller units.

In the presence of L-serine, adding L-leucine does not affect the expression pattern when the arabinose concentration is lower than about 30 μg/ml. When the arabinose concentration

is greater than about 30 μg/ml, adding L-leucine (200 μg/ml) decreases the expression. The maximum expression from the p_{BAD} promoter in the presence of L-serine (500 μg/ml) and L-leucine (200 μg/ml) is about 5,000 Miller units.

The results showed that varying arabinose concentration in the media could vary the intracellular Lrp expressed from the plasmid-carried *lrp* gene within a wide range.

Using the curves in Figure 10, I can calculate graphically how much Lrp is induced by a certain concentration of arabinose. For example, using the curve 3, I can estimate that 35 μg/ml of arabinose can induce about 4,000 Miller units of Lrp equivalent in glycerol minimal media with L-serine(500 μg/ml).

2-1-2. Expression of *lrp::lacZ* under the control of the p_{BAD} promoter in gluconic acid minimal media

Entry of arabinose into the cell is inhibited by catabolite repression, so the first experiments were performed with one of the least repressive carbon sources, glycerol. However, some mutant strains did not grow well with glycerol. I therefore grew strain CAL (MEW1 *ara*$^-$ *lrp*$^-$)/pBAD22 *lrp::lacZ* Cm^R in different carbon sources, and assayed β-galactosidase activity as a function of arabinose concentration. Among the

tested carbon sources including maltose, rhamnose, and gluconic acid, gluconic acid proved to be a convenient substitute for glycerol. In gluconic acid minimal media, the cells grew well, and L-arabinose could induce p_{BAD} promoter, though to a lesser degree for a given arabinose concentration as compared with cells grown with glycerol.

The data for expression of *lrp::lacZ* from the p_{BAD} promoter in gluconic acid minimal media is illustrated in Table 5 and Figure 10.

The basal expression of *lrp::lacZ* (10 Miller units) in gluconic acid minimal media is almost the same as that in glycerol minmal media with L-serine (500 μg/ml). The production of Lrp is proportional to arabinose until saturation is seen. Compared with other curves in Figure 10, Curve 5 rises at the lowest rate with increasing arabinose concentrations. The maximum expression of *lrp::lacZ* under this condition was about 4,700 Miller units.

Table 4. β-galactosidase activity expressed from pBAD22 *lrp::lacZ* Cm^R in glycerol minimal media

<arabinose> μg/ml	β-galactosidase activity (Miller units) -ser -leu	-ser +leu	+ser -leu	+ser +leu
0	40	40	15	15
0.1	240	ND	15	ND
0.5	1410	1250	20	25
1	2125	2000	40	40
2	3605	ND	ND	ND
2.5	ND	3395	95	190
4	5940	ND	ND	ND
5	ND	6580	215	190
6	7675	ND	ND	ND
7.5	ND	8235	355	ND
8	8810	ND	ND	ND
10	9945	8415	675	705
12	10715	ND	ND	ND
14	11215	ND	ND	ND
15	ND	8585	1400	ND
16	11185	ND	ND	ND
17.5	ND	8335	ND	ND

(continued)

18	11180	ND	ND	ND
20	12000	8470	1940	2111
25	ND	ND	2540	ND
30	ND	ND	3185	3145
40	ND	ND	5145	ND
50	ND	ND	6270	4255
70	ND	ND	7295	4890
100	ND	ND	8620	4990

β-galactosidase activity produced from *lacZ* fused into the *lrp* coding region on pBAD22 was assayed in the cells of strain CAL/pBAD22 *lrp::lacZ* Cm^R grown in glycerol minimal media with L-arabinose at concentrations noted. When present, L-leucine was added to the media at 200 μg/ml, and L-serine at 500 μg/ml. To keep the plasmid, chloramphenicol was added to all media (25 μg/ml), and the % plasmid retention was tested by plating approximately 500 cells on LB and replicating on LB with chloramphenicol (25 μg/ml). The % plasmid maintenance in this and all similar experiments in this thesis is over 90%. The result is the average of the data from three determinations. ND, not determined.

Table 5. β-galactosidase activity expressed from pBAD22 *lrp::lacZ* Cm^R in gluconic acid minimal media

<arabinose> μg/ml	β-galactosidase activity (Miller units) -ser -leu
0	10
5	135
10	236
15	517
20	757
30	1513
40	2153
50	2698
70	3879
100	4701

Experiments were performed in gluconic acid minimal media as detailed in Table 4.

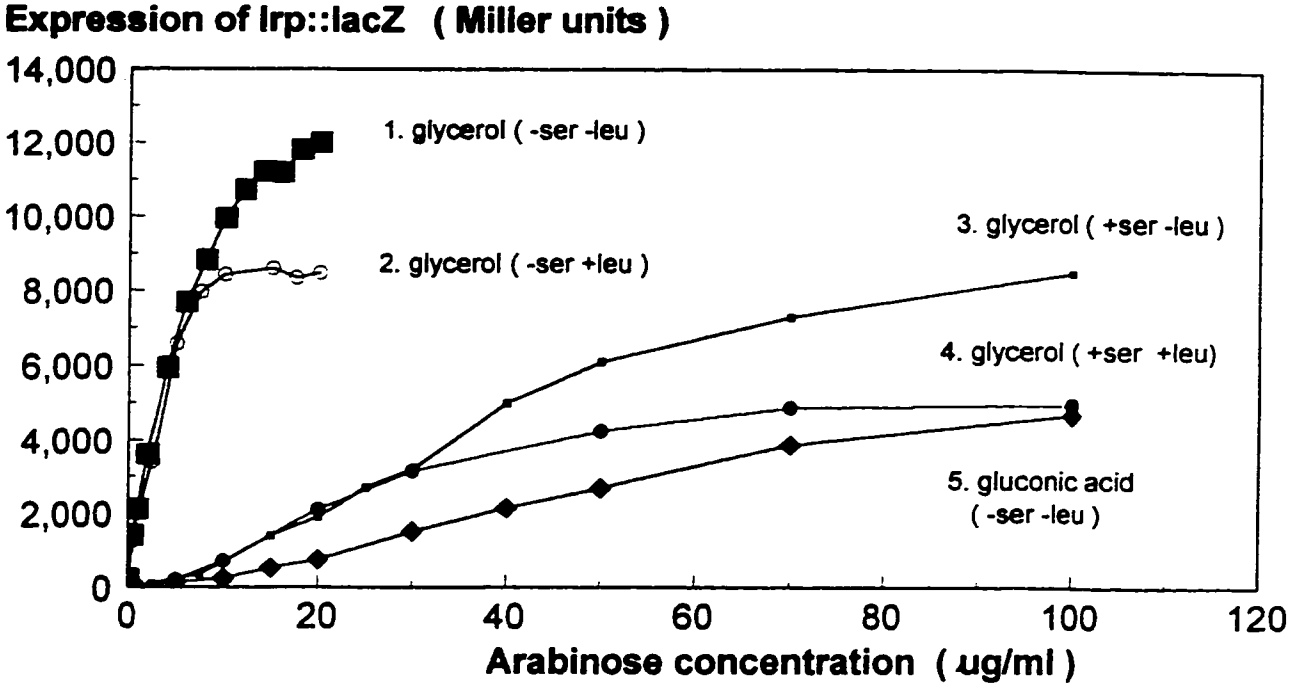


Figure 10. β-galactosidase activity expressed from pBAD22 *lrp::lacZ* Cm^R in minimal media

Graphical representation of data is presented in Tables 4 and 5. The β-galactosidase activities were assayed in the cells of the strain CAL/pBAD22 *lrp::lacZ* Cm^R.

3. *IN VIVO* STUDY OF GENE EXPRESSION REGULATED BY LRP

3-1. The expression of the chromosomal *lrp* gene

In this part, I present data about the expression of the chromosomal *lrp* gene in different media.

The expression of the chromosomal *lrp* gene was determined by measuring the β-galactosidase activity of the strain SP2 (MEW1 *lrp::lacZ*), which carried an *lrp::lacZ* fusion on its chromosome. Table 6 illustrates the expression of the chromosomal *lrp::lacZ* fusion in different culture media.

For mid-exponential phase cells, the expression of the chromosomal *lrp::lacZ* fusion is about 115 Miller units in LB, 100 Miller units in LB supplemented with glucose at a final concentration of 0.2%, 170 Miller units in glucose minimal media supplemented with Casamino acids at a final concentration of 0.1%, 950 to 1,180 Miller units in gluconic acid, arabinose, rhamnose, and glycerol minimal media, and 1,500 Miller units in glucose minimal media. As can be seen, the expression of *lrp::lacZ* in glucose minimal media is higher than that in any of the other conditions tested. This expression was reduced about 14 to 15-fold by growth in LB or LB supplemented with glucose. Adding casamino acid to glucose minimal media decreased the expression about 9-fold,

indicating that amino acids might account, mainly, for the effect of LB, which represses expression of the chromosomal *lrp* gene.

For late-exponential/stationary phase cells, the expression of *lrp::lacZ* was about 300 Miller units in LB, which was about 2.7-fold higher than what is observed for mid-exponential phase cells.

3-2. *In vivo* study of Lrp regulated expression of *serA*

In this study, I varied the intracellular concentration of Lrp by inducing the *lrp* gene on the plasmid pBAD22 *lrp* Cm^R with different concentrations of arabinose, and measured the response of expression of *serA* to these variations.

3-2-1. Expression of *serA::lacZ* in *lrp*$^-$ and *lrp*$^+$ strains

In order to obtain controls for the subsequent experiments on expression of *serA* in the presence of different concentrations of Lrp produced from the plasmid-carried *lrp* gene, I first measured the expression levels of *serA* in strains with intact and defective *lrp* genes.

Two strains, CuS2 (*serA::lacZ*) and CALS (*ara*$^-$ *lrp*$^-$ *serA::lacZ*), were used for this study.

Because of the insertion of *lacZ*, the *serA* gene was defective, rendering CuS2 and CALS L-serine auxotrophs.

Table 7 shows the expression of the chromosomal *serA* gene in glycerol minimal media supplemented with 500 μg/ml of L-serine.

In the *lrp*$^+$ strain, the expression of *serA* was about 860 Miller units. In the *lrp*$^-$ strain, the expression was about 220 Miller units. Without Lrp, the expression decreased about 4-fold, which indicates that Lrp activates *serA::lacZ*.

3-2-2. Expression of *serA::lacZ* in the presence of different concentrations of Lrp

The aim of this study was to investigate how gene expression of *serA* responded to variations in intracellular Lrp concentrations.

The strain CALS (MEW1 *ara*$^-$ *lrp*$^-$ *serA::lacZ*) was tranformed with the plasmid pBAD22 *lrp* Cm^R, which was used to vary the intracellular concentration of Lrp. By adding different concentrations of arabinose to glycerol minimal media, different levels of intracellular Lrp were produced (see Tables 4 and 5). The expression of *serA* was quantified by measuring β-galactosidase activity expressed from the

chromosomal *serA::lacZ* fusion in the presence of different concentrations of Lrp.

To determine the effect of L-leucine on the regulation of *serA::lacZ* by Lrp, 200 μg/ml of L-leucine was added to the minimal media. The β-galactosidase activity of *serA::lacZ* in this case reflects L-leucine's effects on Lrp's regulation.

The results are presented in Table 8, as well as Figures 11 and 12.

The results show that varying the concentration of arabinose/Lrp altered expression of *serA::lacZ*. Without arabinose in the media, the basal expression of *serA* was about 250 Miller units. As the arabinose/Lrp concentration was increased, the *serA::lacZ* expression increased. The maximum expression was about 990 Miller units.

In Figure 11, the expression curve (Curve 1) of *serA::lacZ* in the absence of L-leucine rises abruptly, as the arabinose concentration was varied from about 0 to 20 μg/ml, corresponding to Lrp equivalent at levels from about 0 to 2,000 Miller units. Further increases in arabinose/Lrp concentration did not change the expression of *serA::lacZ* considerably. Upon the addition of about 45 μg/ml of L-arabinose to the media, the expression of *serA::lacZ* reached the maximum level of about 990 Miller units; thereafter, the

expression curve leveled off. The ratio of maximum expression to basal expression is about 3.9.

Adding L-leucine (200 μg/ml) repressed expression of *serA::lacZ* at every arabinose concentration from 0 to 100 μg/ml (see Figure 11). In the presence of L-leucine (200 μg/ml), the basal expression of *serA::lacZ* was about 105 Miller units, about 2.4-fold lower than that in the absence of L-leucine (see Table 8), while the maximum expression was about 385 Miller units, about 2.6-fold lower than that in the absence of L-leucine. This indicates that adding L-leucine represses expression of *serA*. The ratio of maximum expression/basal expression is about 3.6.

3-3. *In vivo* study of Lrp regulated expression of *gltD*

In this part of study, I investigated the response of *gltD* to the variations in the intracellular Lrp concentrations.

Strains Cp8 (MEW1 *gltD::lacZ*), CALG (MEW1 *ara*$^-$ *lrp*$^-$ *gltD::lacZ*), and CALG/pBAD22 *lrp* Cm^R were used for this study.

In order to compare the expression of *gltD::lacZ* with that of *serA::lacZ*, all strains for this part of the study were grown in glycerol minimal media with L-serine (500 μg/ml), same as that for *serA::lacZ* strains.

Similar to the sudies performed on *serA*, I carried out the experiments in two steps: 1). Measuring the expression of *gltD::lacZ* in *lrp*$^-$ and *lrp*$^+$ strains; 2). Measuring the expression of *gltD::lacZ* in the presence of different levels of Lrp.

3-3-1. Expression of *gltD::lacZ* in *lrp*$^-$ and *lrp*$^+$ strains

The expression of the chromosomal *gltD::lacZ* was studied with the strains Cp8 (*lrp*$^+$ *gltD::lacZ*) and CALG (*lrp*$^-$ *gltD::lacZ*).

The results are listed in Table 9. The expression from Cp8 (*lrp*$^+$) is about 85 Miller units, 5.6-fold higher than that

(15 Miller units) from CALG (*lrp*⁻). This indicates that Lrp activates the expression of *gltD*.

3-3-2. Expression of *gltD::lacZ* in the presence of different concentrations of Lrp

Similar to the experiments performed on *serA*, the plasmid pBAD22 *lrp* Cm^R was transformed into strain CALG (MEW1 *ara*⁻ *lrp*⁻ *gltD::lacZ*) to produce different levels of Lrp inside the cells. The expression of *gltD* was detected by measuring β-galactosidase activity expressed from the chromosomal *gltD::lacZ* fusion in the presence of different concentrations of Lrp.

The results are listed in Table 10, and illustrated in Figures 13 and 14.

Similar to the case of *serA::lacZ*, increasing L-arabinose/ Lrp concentration increased expression from *gltD::lacZ*. The basal expression of *gltD::lacZ* (no Lrp was present) was about 15 Miller units. The maximum expression seen was about 175 Miller units. The ratio of maximum expression/basal expression was about 12.

Adding L-leucine to the minimal media repressed Lrp's activation of *gltD::lacZ*. In the presence of 200 μg/ml of L-

leucine, the basal expression of *gltD::lacZ* was about 13 Miller units, while the maximum expression was about 60 Miller units. The ratio of maximum expression/basal expression was about 6.

Table 6. β-galactosidase activity expressed from the chromosomal *lrp::lacZ* in the strain SP2 (MEW1 *lrp::lacZ*)

Media	β-galactosidase activity (Miller units)
LB[a]	115
Lb[b]	300
LB + glucose (0.2%)[a]	100
Minimal media with	
Glucose (0.2%)[a]	1445
Glucose (0.2%)+CAA(1%)[a]	170
Arabinose (0.2%)[a]	1175
Glycerol (0.5%)[a]	1095
Gluconic acid (0.2%)[a]	955
Rhamnose (0.2%)[a]	1080

β-Galactosidase produced by *lacZ* fused to the chromosomal *lrp* gene was assayed in the exponential-phase cells of the strain SP2 grown in the media noted. The result is the average of the data from three determinations. a) β-galactosidase was assayed in the cells from the culture in mid-exponential phase b) β-galactosidase was assayed in the cells from the culture in late-exponential/stationary phase. CAA: Casamino acids.

Table 7. β-galactosidase activity expressed from the chromosomal *serA::lacZ* in *lrp*$^+$ and *lrp*$^-$ strains

Strains	β-galactosidase activity (Miller units)
CuS2	860
CALS	220

β-galactosidase produced by *lacZ* fused to the chromosomal *serA* gene was assayed in exponential phase cells of the strains noted in glycerol minimal media with 500 μg/ml of L-serine. The result is the average of the data from three determinations.

Table 8. *β*-galactosidase activity expressed from the chromosomal *serA::lacZ* in the presence of different concentrations of L-arabinose

<arabinose> μg/ml	*β*-galactosidase activity (Miller units)	
	-leucine	+leucine
0	250	105
0.5	250	110
1	260	110
2.5	285	115
5	340	120
7.5	455	130
10	550	165
15	700	210
20	830	255
30	905	320
40	975	365
50	990	385
70	990	390
100	990	385

Table 8. β-galactosidase activity expressed from the chromosomal *serA::lacZ* in the presence of different concentrations of L-arabinose

β-galactosidase produced by *lacZ* fused to the chromosomal *serA* gene was assayed in exponential phase cells of strain CALS/pBAD22 *lrp* Cm^R grown in glycerol minimal media supplemented with L-arabinose at concentrations noted. The final concentration of L-serine added to the media was 500 μg/ml. When L-leucine was present, its final concentration was 200 μg/ml. To keep the plasmids, 25 μg/ml of chloramphenicol was added to the media. The result is the average of data from three determinations.

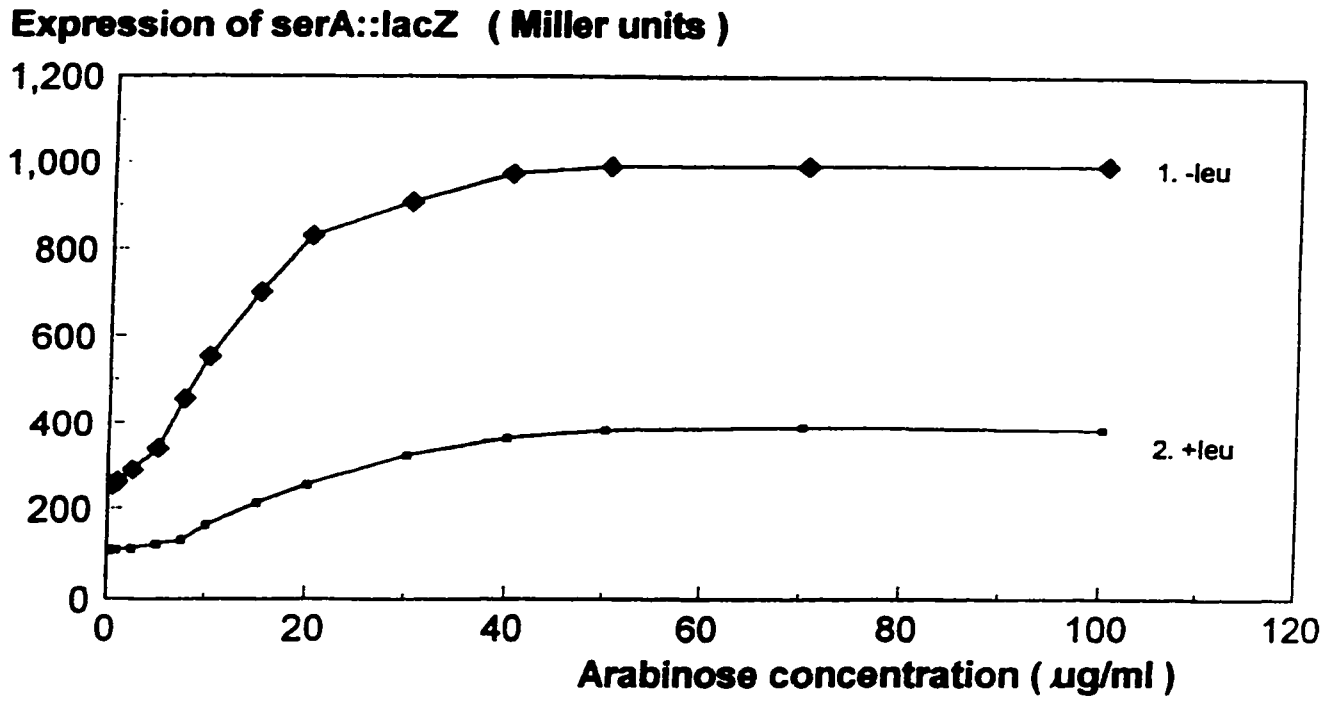


Figure 11. β-galactosidase activity expressed from the chromosomal *serA::lacZ* fusion in the presence of different concentrations of L-arabinose

Graphical representation of the data is presented in Table 8. β-galactosidase activities were assayed in the cells of strain CALS/pBAD22 *lrp* Cm^R.

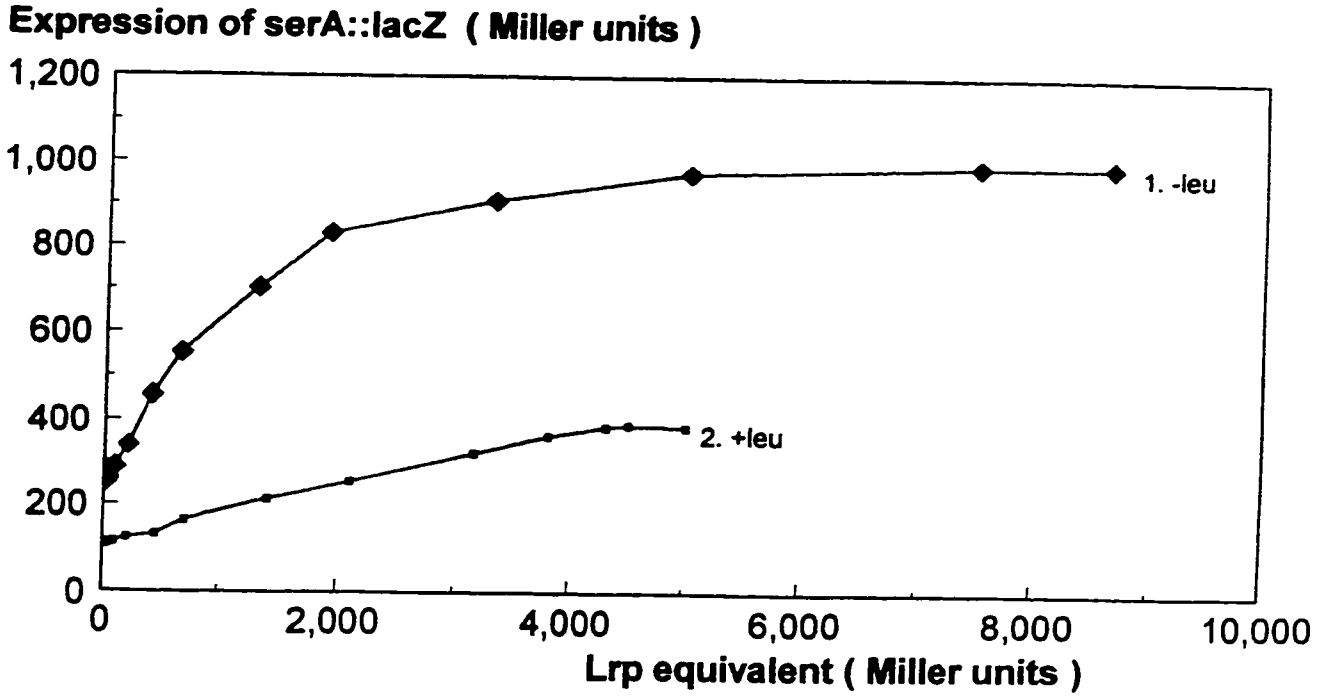


Figure 12. β-galactosidase activity expressed from the chromosomal *serA::lacZ* fusion in the presence of different levels of Lrp

Graphical representation of the data is presented in the Table 8. β-galactosidse activities were assayed in the cells of strain of CAL/pBAD22 *lrp::lacZ* Cm^R.

Table 9. β-galactosidase activity expressed from the chromosomal *gltD::lacZ* in *lrp*$^+$ and *lrp*$^-$ strains in glycerol minimal media

Strains	β-galactosidase activity (Miller units)
Cp8	85
CALG	15

Experiment was performed in glycerol minimal media as detailed in Table 7.

Cp8: MEW1 *gltD::lacZ*; CALG: MEW1 *ara*$^-$ *lrp*$^-$ *gltD::lacZ*.

Table 10. *β*-galactosidase activity expressed from the chromosomal *gltD::lacZ* fusion in the presence of different concentrations of L-arabinose

<arabinose> *μ*g/ml	*β*-galactosidase activity (Miller units)	
	-leucine	+leucine
0	15	13
0.1	16	13
0.5	18	13
1	18	14
2.5	21	15
5	29	19
7.5	32	26
10	39	30
15	47	36
20	58	42
30	81	49
40	111	55
50	128	58
70	160	60
100	175	59

Table 10. β-galactosidase activity expressed from the chromosomal *gltD::lacZ* fusion in the presence of different concentrations of L-arabinose

Experiment was performed in glycerol minimal media supplemented with L-serine (500 μg/ml) as detailed in Table 8.

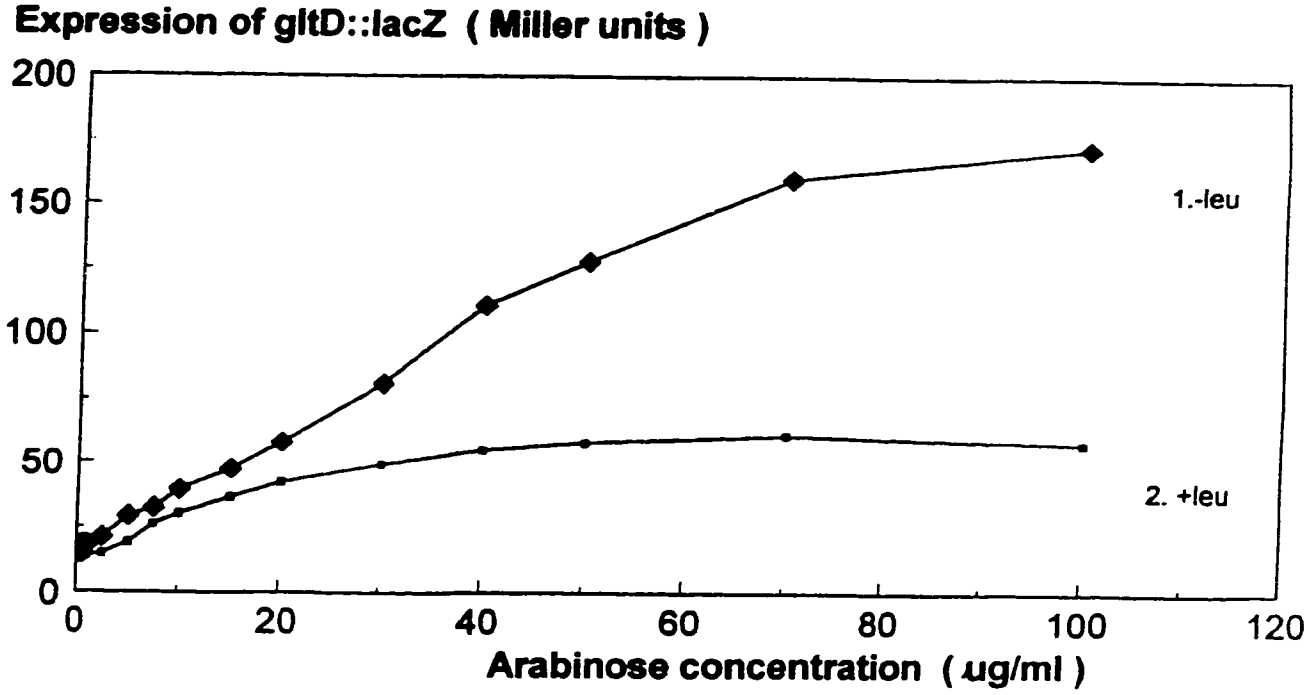


Figure 13. β-galactosidase activity expressed from the chromosomal *gltD::lacZ* fusion in the presence of different concentrations of L-arabinose

Graphical representation of the data is presented in Table 10. β-galactosidase activities were assayed in the cells of strain CALG/pBAD22 *lrp* Cm^R.

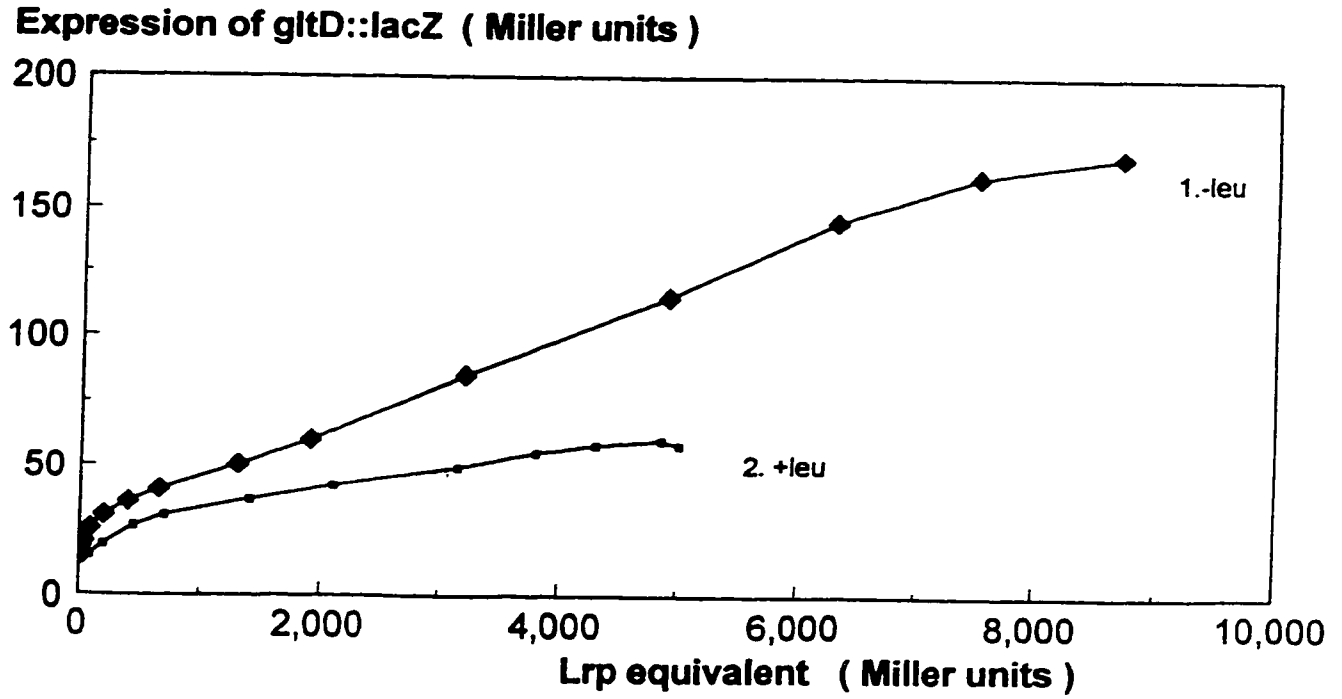


Figure 14. **β-galactosidase activity expressed from the chromosomal *gltD::lacZ* fusion in the presence of different levels of Lrp**

Graphical representation of the data is presented in Table 10. β-galactosidase activities were assayed in the cells of strain CALG/pBAD22 *lrp* Cm^R.

3-4. Expression of some other genes regulated by Lrp

To determine the expression of some Lrp-regulated genes by Lrp, the strain with a wild-type *lrp* gene and a target gene::*lacZ* fusion was first cultured overnight in succinate minimal media, and then subcultured in glucose and succinate minimal media. Cells in the mid-exponential phase were harvested to assay β-galactosidase activity. The results are shown in Table 11.

Lin (1992) showed that Lrp repressed expression of *sdaA*. Thus, I expected the expression of *sdaA::lacZ* to be low when Lrp is expressed. The results indeed coincide with what I expected - in glucose and succinate minimal media, the expression of *sdaA::lacZ* in strain Cup22 (MEW1 *sdaA::lacZ*) was about 50 and 40 Miller units, respectively. Such low gene expression is consistant with the fact the an lrp^+ strain can not grow with L-serine as the sole carbon source.

Lrp activates all other genes tested (reviewed in Newman et al., 1995, 1996). Since the expression level of *lrp* in glucose minimal media is higher than that in succinate (Chen et al., 1997), I expected the expression of these genes to be higher in glucose minimal media than that in succinate minimal media. This is true for *gltD::lacZ* in strain Cp8, *serA::lacZ* in strain CuS2, and *ilvIH::lacZ* in strain Cv975. However, for

gcv::lacZ in strain Cp67, the case is different from what I expected - in glucose minimal media, expression of *gcv::lacZ* was about 570 Miller units, while in succinate minimal media, the expression was about 2.5 times higher, which was about 1,420 Miller units. This strongly suggests that other factors are also involved in the regulation of *gcv*.

Table 11. Expression of some other Lrp-regulated genes in *E.coli*

Strains	β-galactosidase activity (Miller units)		Ratio
	glucose (A)	Succinate (B)	A/B
Cup22	50	40	1.2
Cp8	345	145	2.4
Cp67	570	1420	0.40
Cv975	255	200	1.28

β-galactosidase produced by *lacZ* fused into the target gene as noted was assayed in exponential phase cells of the noted strains, which were grown in minimal media as noted. Cup22: *sdaA::lacZ*; Cp8: *gltD::lacZ* ; Cp67: *gcv::lacZ*; Cv975: *ilvIH::lacZ*. All these strains carry a wild-type *lrp* gene. The result is the average of data from three determinations.

4. TOXICITY OF OVERPRODUCTION OF LRP

In this section, I describe the effect of overexpression of *lrp* on *E.coli* cells.

The strain CA (*ara*$^-$ *lrp*$^+$)/pBAD22 *lrp* Cm^R was employed for this study. I investigated the effect of overexpression of *lrp* on the cells by comparing the growth of this strain in glycerol minimal media with and without 100 μg/ml of L-arabinose. When no L-arabinose was added to the media, the intracellular Lrp came mainly from the chromosomal *lrp* gene, which was carried by the host strain CA. In order to induce the plasmid-carried *lrp* gene, 100 μg/ml of L-arabinose was added to the media - in this case, the intracellular Lrp came mainly from the plasmid-carried *lrp* gene.

Cells from a culture in glycerol minimal media were subcultured in the same media with and without 100 μg/ml of arabinose. The starting point of the culture was recorded as 0 hours. Culture samples were harvested every two hours. Turbidity (OD_{600}) of each sample was measured, which reflected the total amount of cells in the culture. To determine the number of cells which can form colonies (colony-forming unit), the cells from each sample were diluted and plated on LB plates (viable count). In this experiment, 50 μg/ml of chloramphenicol was used to keep plasmids. Results (not shown

in this thesis) showed that most cells kept their plasmids at the end of the experiments.

Without L-arabinose (100 μg/ml) in the media, turbidity (OD_{600}) and the colony-forming unit (CFU) increased with time. After 14 hours of culturing, the OD_{600} of the culture was greater than 1.000, and CFU was about 1.3×10^9 cells/ml (see Table 12, Figures 15(I)-(II)).

With 100 μg/ml of L-arabinose in the media, after 4 hours of culturing, the colony-forming unit (CFU) reached maximum (8.5×10^6 cells/ml). Thereafter, CFU started to decrease. A drastic decrease in CFU occured during the culturing period from 4 to 6 hours. After 6 hours of culturing, CFU is only 25% of that determined after 4 hours of culturing (see Table 12, Figures 16 (II)). The pattern of changes in turbidity is different from that of CFU. The maximum OD_{600} (about 0.060) occured two hours later than the maximum CFU. After 6 hours of culturing, the turbidity of the samples almost remained constant (see Table 12, and Figure 16 (I)).

Table 12. Growth of the strain CA/pBAD22 *lrp* Cm^R in glycerol minimal media with and without L-arabinose (100 μg/ml)

Time (hours)	Glycerol minimal media			
	-arabinose		+arabinose	
	OD600	CFU ($x10^5$)	OD600	CFU ($x10^5$)
0	0.026	14	0.023	19
2	0.028	45	0.029	31
4	0.042	110	0.037	85
6	0.079	140	0.061	21
8	0.136	340	0.059	10
10	0.257	1100	0.056	8
12	0.513	2000	0.051	4
14	1.079	13000	0.052	2

The growth of strain CA/pBAD22 *lrp* Cm^R was determined in glycerol minimal media with and without 100 μg/ml of L-arabinose. The start point of culturing was recorded as 0 hours. Samples were harvested every two hours. Turbidity and the number of cells which can form colonies (CFU) on LB plates of each sample was determined by measuring OD_{600} and viable count. CFU was expressed as cells/ml.

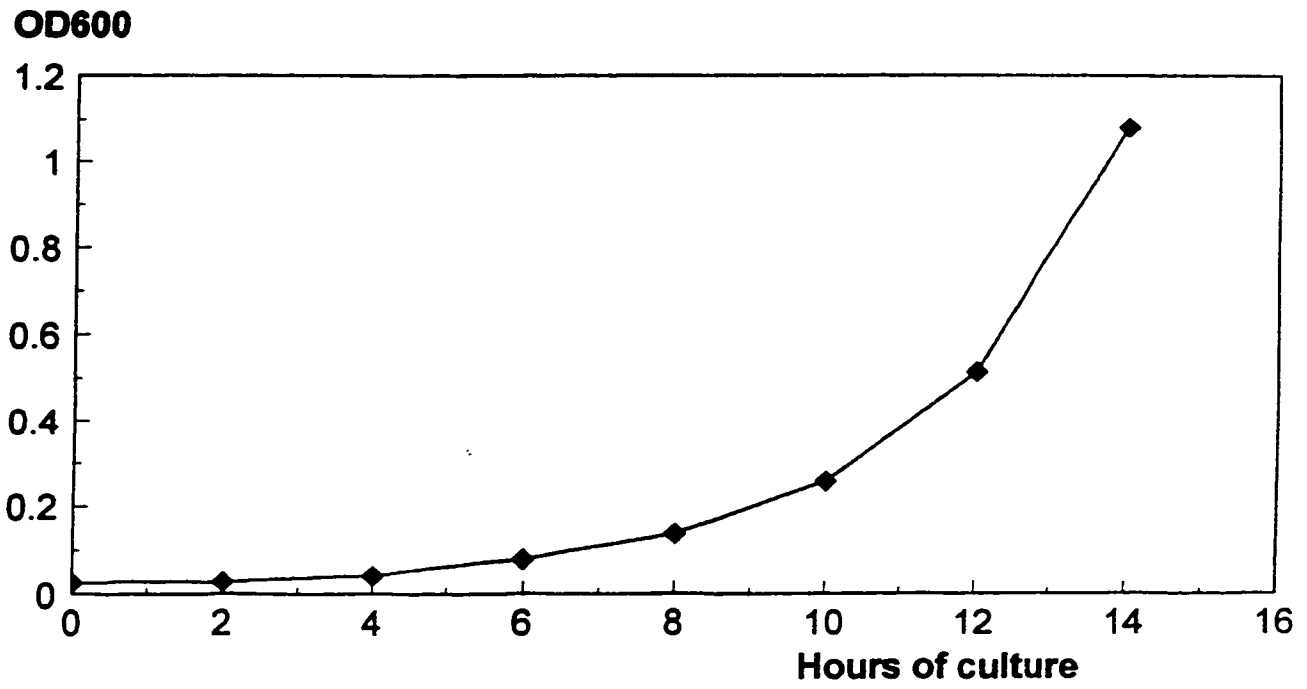


Figure 15 (I). Growth of the strain CA/pBAD22 *lrp* Cm^R in glycerol minimal media without L-arabinose

Graphical representation of the data is presented in Table 12.

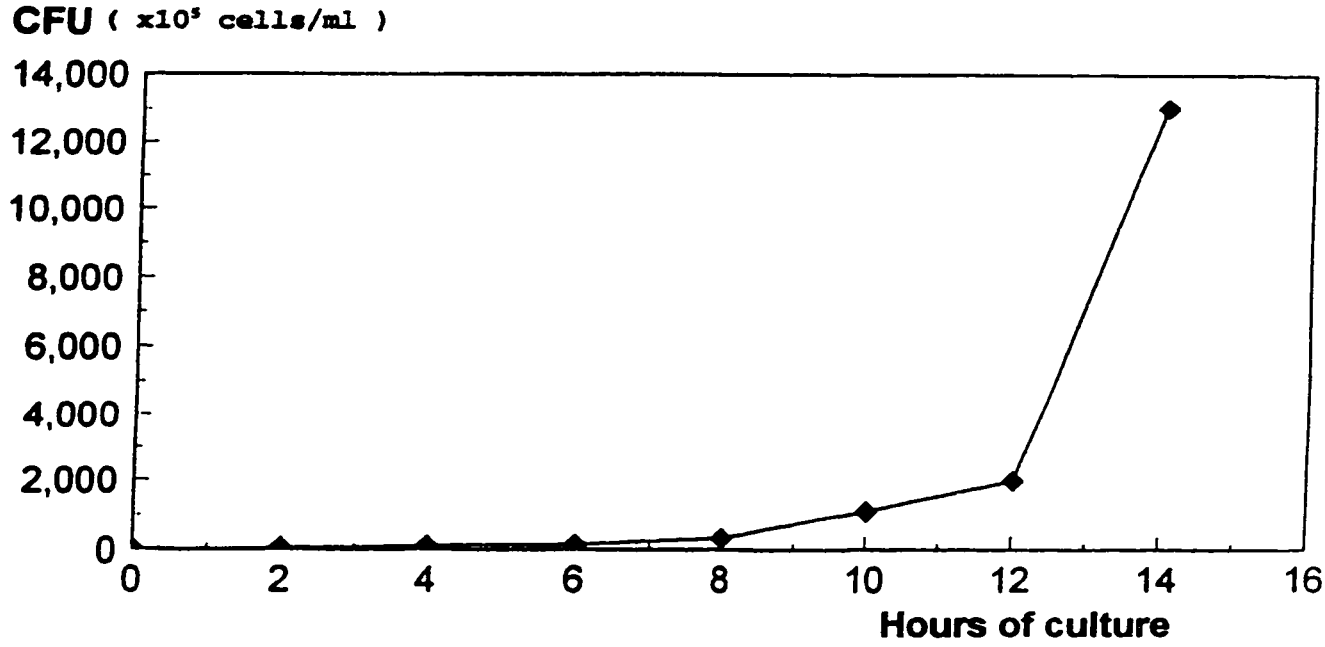


Figure 15 (II). Growth of the strain CA/pBAD22 *lrp* Cm^R in glycerol minimal media without L-arabinose

graphical representation of the data is presented in Table 12.

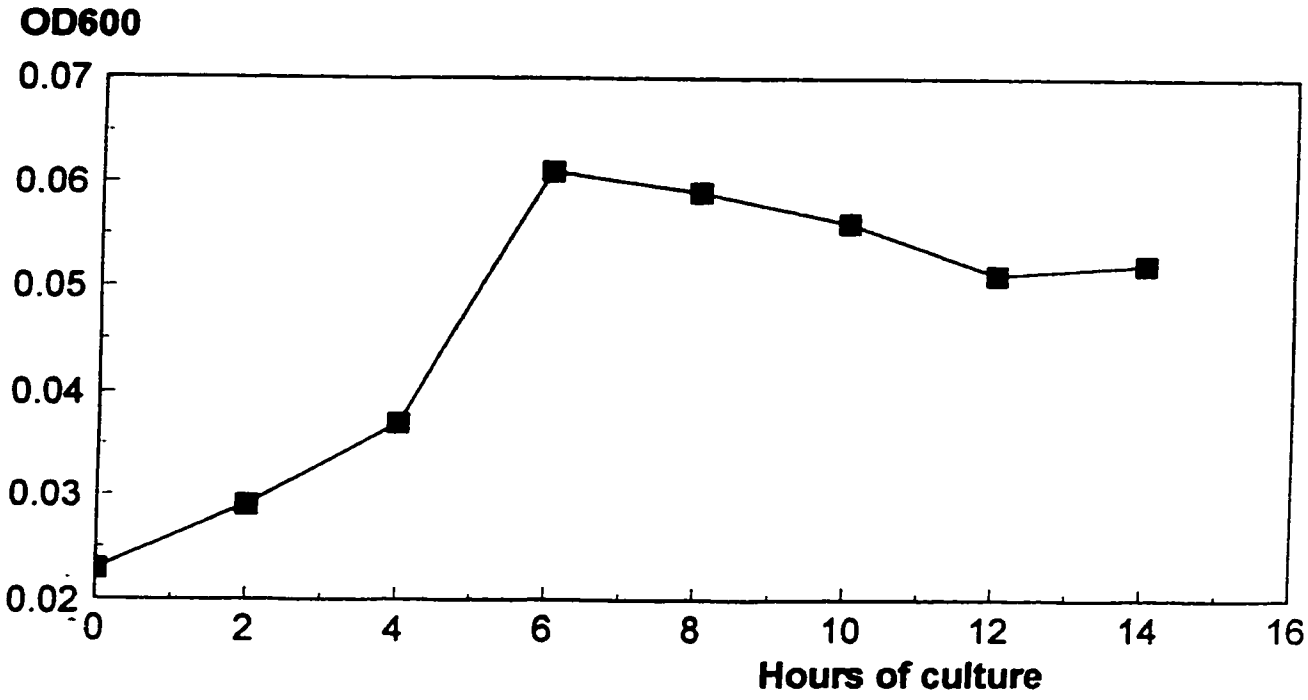


Figure 16 (I). Growth of the strain CA/pBAD22 *lrp* Cm^R in glycerol minimal meida with 100 μg/ml of L-arabinose

Graphical representation of the data is presented in Table 12.

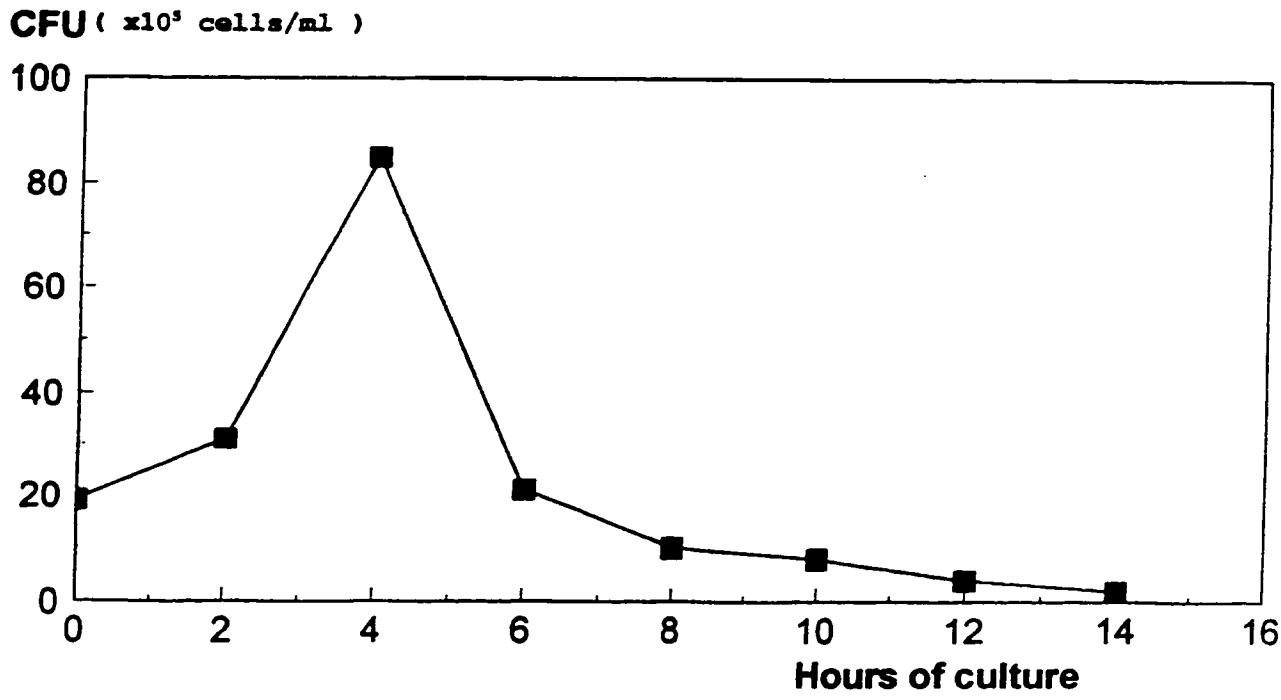


Figure 16 (II). Growth of the strain CA/pBAD22 *lrp* Cm^R in glycerol minimal media with 100 μg/ml of L-arabinose

Graphical representation of the data is presented in Table 12.

IV. DISCUSSION

1. Expression of *lrp* on the pBAD22 vector

1-1 The construct pBAD22 *lrp* Cm^R is suitable for this study

In this work, I used *lacZ* as a reporter to determine the expression level of the plasmid-carried *lrp* gene induced by L-arabinose. The *lacZ* gene was fused into the *lrp* coding region on the plasmid.

In glycerol minimal media without L-arabinose, the basal β-galactosidase activity produced from *lrp::lacZ* on pBAD22 *lrp* Cm^R in the host strain CAL (MEW1 *ara*$^-$ *lrp*$^-$) was about 40 Miller units, when L-serine was absent; and about 15 Miller units, when L-serine (500 μg/ml) was present. This expression is very close to the basal β-galactosidase activity produced in the cells of strain MEW1 (Δ*lacZ*), the parent strain of CAL, which has no *lacZ* gene (Tao, 1995). Thus, when no arabinose is present in the media, the p_{BAD} promoter on the plasmid can be considered to be totally shut off, and almost no Lrp produced.

Upon the addition of L-arabinose, the p_{BAD} promoter was turned on. The expression of the plasmid-carried *lrp* gene increased with increasing arabinose until saturation. Over a wide range, the expression of *lrp::lacZ* from the p_{BAD} promoter

was approximately proportional to the arabinose concentration. The range was about 0-11,000 Miller units in glycerol minimal media, about 0-8,000 Miller units in glycerol minimal media with L-leucine (200 μg/ml), about 0-8,000 Miller units in glycerol minimal media with L-serine (500 μg/ml), and about 0-4,000 Miller units with both L-leucine and L-serine. Therefore, I was able to vary the intracellular Lrp concentration over a wide range by changing arabinose concentrations in the media. This is an advantage for studying the response of a target gene to Lrp at concentrations from very low to very high levels.

Guzman et al.(1995) constructed a series of pBAD vectors. Among them, pBAD22 is capable of stable gene expression, due to an optimized SD sequence (Shine-Dalgarno sequence) in its multiple cloning sites. This is one reason we chose it as a vector. However, this plasmid carries an ampicillin resistance gene. Ampicillin is very easily degraded during cell culture. This instability often leads to loss of plasmids by some cells, leading to unreproducible expression of genes on the plasmid. To overcome this instability, the β-lactamase gene, which confers ampicillin resistance, on the original vector was replaced by the chloramphenicol acetyltransferase gene (CAT), which confers chloramphenicol resistance. This enabled me to use chloramphenicol to ensure maintenance of plasmids in the cells. Chloramphenicol is stable during cell culture, and

when it was used to maintain plasmids in this study, more than 90% of cells retained plasmids at the end of the experiments. The expression of *lrp* on pBAD22 *lrp* Cm^R is quite stable, possibly due to the SD sequence on the vector and the high percentage of plasmid maintenance.

1-2. When the p_{BAD} promoter is used to control gene expression, gluconic acid is a convenient substitute for glycerol as carbon source

L-arabinose is the inducer of the p_{BAD} promoter. Only upon entry into the cell, can L-arabinose function. Entry of L-arabinose into the cell is inhibited by catabolite repression. The presence of glucose can efficiently prevent cells from taking up L-arabinose, and thus glucose cannot be used as carbon source in this case. Glycerol is one of the least repressive carbon sources, and was therefore chosen to grow the cells with the pBAD series of plasmids by Guzman and other researchers. However, glycerol is problematic for the growth of some mutant strains. I therefore decided to look for other carbon sources to replace it. I cultured strain CAL (*ara*$^-$ *lrp*$^-$)/pBAD22 *lrp::lacZ* Cm^R and assayed β-galactosidase activity with different carbon sources including rhamnose, mannose, maltose, and gluconic acid. Among the tested carbon sources, gluconic acid was a suitable substitute for glycerol. In gluconic acid minimal media, the cells grew well (data not

shown in this thesis), and L-arabinose could induce the p_{BAD} promoter, although at a lower range. Table 5 and Figure 10 illustrate that in gluconic acid minimal media, the expression range of the plasmid-carried *lrp* gene is about 0-4,700 Miller units of LacZ equivalent.

2. Nutrients in culture media affect expression of the chromosomal *lrp* gene

Several factors have been shown to affect expression of the chromosomal *lrp* gene. These factors include the concentration of Lrp itself (Lin et al., 1992b, Wang et al., 1994), nutrients in the media (Chen et al., 1997), the growth phase of the population (Chen et al., 1997), and the growth rate of the cells (Landgraf et al., 1996).

In this thesis, I describe the effect of nutrients in the media on expression of the chromosomal *lrp* gene.

Strain SP2 carried a chromosomal *lrp::lacZ* fusion. The β-galactosidase activities produced from *lrp::lacZ* in this strain were measured in different media, and reflected the expression levels of the chromosomal *lrp* gene. Because of the insertion of *lacZ*, the *lrp* gene was defective, and thus Lrp's autogenous regulation did not function in strain SP2.

For cells in mid-exponential phase, the lowest expression of the chromosomal *lrp::lacZ* so far observed was about 100 Miller units, which was determined in LB supplemented with 0.2% glucose, while the highest expression was about 1,500 Miller units, which was determined in glucose minimal media. I calculated expression ratios by dividing the expression in a specific medium by that in glucose minimal medium (see Table 13).

Growth in rich media repressed expression of *lrp::lacZ*. In rich media (LB, and LB with glucose), the expression of *lrp::lacZ* was only 7-8% of that in glucose minimal media, and very close to the expression level in glucose minimal media supplemented with 0.1% Casamino acids, which was about 12% of that in glucose minimal media. This suggested that amino acids might be mainly responsible for the effect of LB, which represses the expression of *lrp*.

In minimal media with L-arabinose, rhamnose, glycerol, and gluconic acid, the expression of *lrp::lacZ* was about 70-80% of that in glucose minimal media. We observed that in acetate and succinate minimal media, the expression was less than the above levels, only 40% of that in glucose minimal media (Chen et al., 1997). However, in minimal media with the carbon sources so far tested, the expression levels of the chromosomal *lrp::lacZ* fusion were much higher than that in

rich media (LB).

Lrp represses genes involved in biodegradation and transport, while activating genes involved in biosynthesis (reviewed in Newman et al., 1995, 1996). Lrp seems likely to act as a lever to balance some biodegradation and biosynthesis processes in the cells in environments with different nutrient contents. In rich media (LB), where nutrients abound, Lrp's concentration is low, possibly mainly due to the amino acid abundance. Low levels of Lrp ensure a relatively low level of expression of some genes for biosynthesis, and a relatively high level of expression of some genes for biodegradation. In minimal media, where nutrients are poor, Lrp's concentration is relatively high. High levels of Lrp ensure a relatively high level of expression of some genes for biosynthesis, and a relatively low level of some genes for biodegradation.

Table 13. Expression ratio of *lrp::lacZ* in mid-exponential phase cells of strain SP2 (MEW1 *lrp::lacZ*)in different media

Media	Ratio
LB + glucose (0.2%)	0.07
LB	0.08
Minimal media with	
Glucose + CAA (0.1%)	0.12
Gluconic acid	0.68
Rhamnose	0.74
Glycerol	0.76
Arabinose	0.81
Glucose	1

All the values used to calculate the ratios in this table are presented in Table 6 . When added to the minimal media, the final concentration of glycerol was 0.5%, and that of any other carbon source listed above was 0.2%. CAA: Casamino acids.

3. Regulation of *serA* and *gltD* by Lrp

3-1. Lrp activates the expression of *serA*

In this part of my study, the strain CALS (*lrp*$^-$ *ara*$^-$ *serA::lacZ*) was transformed with the pBAD22 *lrp* Cm^R plasmid, which was used to produce different concentrations of intracellular Lrp. β-galactosidase activities produced from the chromosomal *serA::lacZ* fusion in glycerol minimal media supplemented with 500 μg/ml of L-serine and different concentrations of L-arabinose were measured, and reflected the expression levels of *serA* in the presence of different concentrations of Lrp.

The expression of *serA::lacZ* is proportional to the amount of Lrp. β-galactosidase activity increases with increasing intracellular Lrp concentration until saturation, indicating that Lrp activates expression of *serA*. The basal expression of *serA::lacZ*, when no Lrp was present, was about 250 Miller units. The maximum expression was about 990 Miller units, which occurred when the arabinose concentration was greater than 45 μg/ml (Lrp equivalent was more than about 6,000 Miller units).

serA has two promoters, P1 and P2. P1 initiates at 45 bp from the translation start site, while P2 at 93 bp further

upstream. Two Lrp-binding sites have been found in *serA* promoter region. One site with a high affinity to Lrp is found upstream of P2. The other one with a low affinity is related to P1 (Lin, 1992).

P1 is activated by Lrp, while P2 is repressed (Lin, 1992). Therefore, when no arabinose was present and therefore almost no Lrp was produced, the basal expression of *serA* must mainly come from P2.

Because the P2 binding site overlaps with the P2 RNA polymerase binding site, Lrp's binding inhibits transcription from P2. However, Lrp's binding to P2, together with Lrp binding to P1, activates transcription from P1 (Lin, 1992). As the concentrations of arabinose added to the media increased, the intracellular Lrp increased. With Lrp's increase, more Lrp bound to the P1 site with a low affinity, resulting in a shift of transcription domination from P2 to P1. When its concentration reached a certain point, Lrp might have totally blocked the expression of *serA* from P2, and all the transcripts came from P1. In fact, Lrp produced from the chromosomal *lrp* gene in glucose minimal media almost totally prevented *serA* from transcribing from P2 (Lin, 1992). Thus, it is reasonable to speculate that the expression at the maximum point on the expression curves in Figures 11 and 12 must mainly, if not entirely, come from P1.

As the Lrp concentration increases, the expression curves in Figure 12 keep rising until saturation is reached, with no loss of *serA* expression. This suggests that transcription from P1 - activated by cooperative binding to the two sites by Lrp - might be sufficient to compensate for the inhibition of transcription from P2. Otherwise, the *serA* expression curves would have shown a decrease in expression, before the smooth increase until saturation was seen.

Since L-serine plays a very important role in *E.coli* metabolism as reviewed earlier, it is very important for the cell to keep the ability to biosynthesize L-serine. When the cell is provided with a nutrient-rich medium (LB), the intracellular Lrp concentration is low. Under these conditions, according to Lin (1992), transcription from P2, which is repressed by Lrp, is mainly responsible for synthesizing 3-phosphoglycerate dehydrogenase, although to a relatively low extent. In fact even in an *lrp* defective strain, *serA* is still expressed. In glycerol minimal media supplemented with 500 μg/ml of L-serine, the expression of *serA::lacZ* in strain CALS (*lrp serA::lacZ*) was about 250 Miller units, which should come from P2. When the cell is grown in a nutrient-poor medium (minimal medium), the intracellular Lrp concentration is high. Transcription from P1 enables the cells to synthesize 3-phosphoglycerate at a relatively higher level.

3-2. Lrp activates the expression of *gltD*

To study the effects of Lrp on the expression of *gltD*, the same experiments were done on the strain CALG (*ara*⁻ *lrp*⁻ *gltD::lacZ*)/pBAD22 *lrp* Cm^R. The host strain CALG carries a chromosomal *gltD::lacZ* fusion.

The expression curves in Figure 14 show that expression of *gltD::lacZ* is proportional to the intracellular amount of Lrp. The β-galactosidase activity produced from *gltD::lacZ* increases with increasing Lrp concentration until saturation, indicating that Lrp activates expression of *gltD*. When no arabinose was present, the basal expression of *gltD::lacZ* was about 15 Miller units, an insignificant amount which could be considered as zero. This is consistent with the fact that Lrp is required for the expression of *gltD* (Ernsting et al., 1993).

gltB and *gltD* are two members of the *gltBDF* operon. gltB is located upstream of *gltD* on the genetic map. A single major promoter, which is located upstream of *gltB*, controls the expression of both genes. The Lrp-binding sites have been found within this promoter. The transcription terminator of these two genes is located downstream of *gltD*. According to these facts, one might expect that the response to the variations in Lrp concentration of these two genes should be

similar. However, a previous study on *gltB* by other researchers illustrated a different regulation pattern by Lrp (Borst et al., 1996).

Those researchers used a chromosomal *lrp* gene, which was fused under the p_{lac} promoter, to create low levels of Lrp, and a plasmid-carried *lrp* gene, which was cloned under the p_{trc} promoter, to produce high levels of Lrp. The β-galactosidase activities produced from *gltB::lacZ* were measured in the presence of different concentrations of Lrp. Their results showed that gene expression rose with increasing Lrp concentrations up to the level of Lrp found in wild-type strains, at which point the expression was maximum, and further increases of Lrp decreased gene expression. This regulation pattern is different from that gained from this study, which revealed that expression of *gltD* keeps increasing with increasing Lrp until saturation was seen.

According to a computer analysis of the DNA sequences, Fraenkel et al. (1995) suggested that there might be a new Lrp-binding site within *gltB*, located at nucleotide +36 relative to the transcriptional start site.

To explain the regulation pattern of Lrp on *gltB*, Borst et al. (1996) suggested that high concentrations of Lrp could bind to the binding site within *gltB*, and thus interfere with

gene transcription, resulting in a decrease in gene expression.

Due to the lack of information about the plasmid toxicity to the host cell and the plasmid maintenance, the regulation pattern of Lrp on *gltB* is uncertain. However, one might ask, if high concentrations of Lrp do bind to the binding site within *gltB*, resulting in decreases in gene expression, why does this binding not decrease the expression of *gltD*? Although there is not enough information so far to support a hypothesis, I can speculate.

Catano et al. (1992) showed that there might be a minor promoter located between *gltB* and *gltD*. If this minor promoter does exist, and Lrp at high concentrations does bind to the site within *gltB,* I speculate that the binding of Lrp at high concentrations to both sites upstream and within *gltB* may facilitate somewhat transcription of *gltD* from the minor promoter, like the case of Lrp's function on *serA* P1 and *serA* P2, and therefore will not cause a decrease in expression of *gltD*.

3-3. A comparison of the response of genes *serA* and *gltD* to the variation in intracellular Lrp concentrations

In this part, I will compare the gene expression of *serA*

and *gltD* in glycerol minimal media supplemented with L-serine (500 μg/ml) in response to different concentrations of L-arabinose.

To facilitate the comparison, all the related values in Table 8 and 10 have had the basal expression level subtracted (resulting data not shown), and the expression ratio were calculated by dividing the expression value at a certain concentration of Lrp by the maximum expression. The resulting ratios are presented in Table 14, and plotted against Lrp concentrations in Figure 17.

In Figure 17, the expression curve of *serA::lacZ* rises sharply, whereas that of *gltD::lacZ* rises relatively slowly.

According to Figure 17, to induce half-maximum expression, about 1,000 Miller units of Lrp equivalent was required for *serA::lacZ*; while about 2,800 Miller units of Lrp equivalent was required for *gltD::lacZ*. The amount of Lrp needed for half-maximum expression of a gene reflects the sensitivity of the promoter of the gene to Lrp. The lower the amount, the more sensitive the promoter is. Therefore, I conclude that the promoters of *serA* are more sensitive to Lrp than that of *gltD* in glycerol minimal media with 500 μg/ml of L-serine.

When no Lrp was present, *serA* was still expressed,

although at a relatively low level (the basal expression of *serA::lacZ* was about 250 Miller units); while *gltD* was almost shut off (the basal expression of *gltD::lacZ* was about 15 Miller units).

Table 14. A comparison of gene expression of *serA::lacZ* and *gltD::lacZ* in the presence of different concentrations of L-arabinose

<arabinose> µg/ml	Ratio (%) *serA::lacZ*	*gltD::lacZ*
0	0	0
1	1.4	1.9
2.5	2.5	3.8
5	6.1	8.8
7.5	9.4	11
10	21	15
15	38	20
20	54	27
30	78	41
40	93	60
50	100	71
70	100	90
100	100	100

All the values used for calculating the expression ratios are presented in Tables 8 and 10, and have had the basal expression value subtracted. The expression ratio listed above was calculated by dividing the gene expression at a certain

concentration of L-arabinose by the maximum expression.

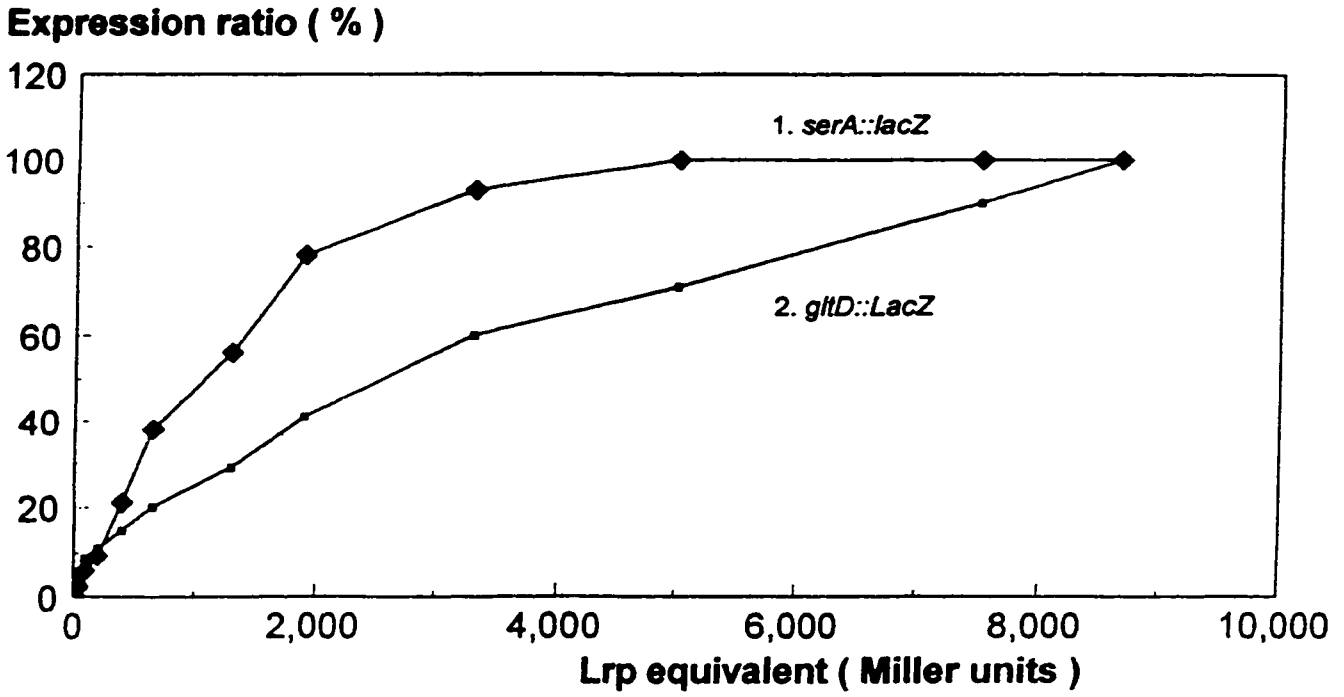


Figure 17. A comparison of gene expression of *serA::lacZ* and *gltD::lacZ* in the presence of different levels of Lrp

Graphical representation of data is presented in Table 14. The arabinose concentrations were converted into Lrp equivalents according to Curve 3 in Figure 10.

4. Exogenous L-leucine represses activation of both *serA* and *gltD* by Lrp

To study the effect of exogenous L-leucine on the function of Lrp, 200 μg/ml of L-leucine was added to glycerol minimal media supplemented with 500 μg/ml of L-serine and different concentrations of L-arabinose, in which strains CALS (*ara⁻ lrp⁻ serA::lacZ*)/pBAD22 *lrp* Cm^R and CALG (*ara⁻ lrp⁻gltD::lacZ*)/ pBAD22 *lrp* Cm^R were cultured. β-galactosidase activities produced from *serA::lacZ* and *gltD::lacZ* were measured under these conditions, and reflected the expression levels of *serA* and *gltD*, respectively, in the presence of L-leucine (200 μg/ml) and different concentrations of Lrp.

Results (see Tables 8 and 10) showed that exogenous L-leucine (200 μg/ml) decreased the activation of both *serA* and *gltD* by Lrp. Adding L-leucine to the media led to a decrease throughout the expression curves of both *serA* and *gltD* (see Figures 11, 12, 13, and 14). For example, when the intracellular Lrp was at an expression level of 4,000 Miller units of LacZ equivalent, in the presence of exogenous L-leucine (200 μg/ml), expression of *serA::lacZ* and *gltD::lacZ* was about 380 and 60 Miller units, respectively. This was 2.4 and 1.5 fold less than those when exogenous L-leucine was absent, which were about 900 and 90 Miller units, for *serA::lacZ* and *gltD::lacZ,* respectively (expression levels

were obtained by the graphical calculation according to Figures 12 and 14).

In solution, as well as when binding to DNA, Lrp appears as a dimer (Willins et al., 1991; reviewed in Calvo et al., 1994). We do not yet know how many molecules of L-leucine are required to bind to an Lrp dimer. I assume that one molecule of L-leucine binds to one Lrp molecule, and thus two molecules of L-leucine are required for each Lrp dimer. Inside the cells, I assume that the following reactions occur.

$$(Lrp)_2 \cdot (leu)_2DNA \underset{+DNA}{\overset{-DNA}{\rightleftharpoons}} (Lrp)_2 \cdot (Leu)_2 \underset{+leu}{\overset{-leu}{\rightleftharpoons}} (Lrp)_2 \underset{-DNA}{\overset{+DNA}{\rightleftharpoons}} (Lrp)_2 \cdot DNA$$

Here, $(Lrp)_2$ refers to the Lrp dimer, and DNA to the Lrp-binding sites in the promoters of *serA* and *gltD*.

Lrp is an L-leucine binding protein. L-leucine's binding to Lrp is thought to change the conformation of the complex of Lrp and promoters. Such changes were unfavourable for RNA polymerase's binding in some cases, leading to a decrease in gene transcription (reviewed in Newman et al., 1995, 1996; Calvo et al., 1994).

Adding L-leucine to the minimal media increases the

concentration of endogenous L-leucine (Quary et al., 1977). The increase in endogenous concentrations of L-leucine caused by adding L-leucine to glycerol minimal media increased the possibility of its binding to Lrp. Therefore, upon the addition of L-leucine to the minimal media, the amount of L-leucine-bound Lrp increased. The relevant β-galactosidase activity values shown in Table 8 and 10 were the total amounts of expression activated by both the L-leucine-bound Lrp and L-leucine-free Lrp (although we do not yet know if L-leucine-bound Lrp activates gene expression, I assume it can activate *serA* and *gltD*). The expression of both *serA* and *gltD* activated by L-leucine-bound Lrp might be less than that activated by L-leucine-free Lrp. The decrease in the ratio of the amount of L-leucine-free Lrp to that of L-leucine-bound Lrp, which was caused by adding L-leucine to the media, resulted in a decrease in the net expression of both *serA* and *gltD*.

5. Overexpression of *lrp* is toxic to cell growth

To study the effect of overexpression of *lrp* on the growth of *E.coli* cells, the growth of the strain CA (*ara*$^-$ *lrp*$^+$)/ pBAD22 *lrp* Cm^R in glycerol minimal media with and without 100 μg/ml of L-arabinose was investigated as mentioned earlier. When 100 μg/ml of arabinose was added to the media, the cell culture grew poorly, and after 4 hours of culturing, CFU (colony-forming units) started to decrease, indicating that this culture condition was lethal to the cell (see Table 12, and Figures 16 (I)-(II)). When no arabinose was added to the media (intracellular Lrp was expressed from the chromosomal *lrp* gene of the host strain CA), strain CA/pBAD22 *lrp* Cm^R grew well, demonstrating that the pBAD22 *lrp* Cm^R plasmid was not toxic to cell growth. Besides these facts, N. Zografakis and S. Lacasse in our laboratory have shown that L-arabinose at a concentration of 2,000 μg/ml is not toxic to the strain used for this study (unpublished results, personal communication). All these results lead to a conclusion that overexpression of *lrp* is toxic to *E.coli*.

Lrp regulates at least 30 genes/operons, which are involved in diverse bio-reactions in *E.coli*. Changes in Lrp concentrations change expression of many genes inside the cells. However, beyond a certain limit the changes in Lrp concentration might cause total disorder in gene regulation,

resulting in toxicity to the cell. In this study, overexpression of *lrp* induced by 100 μg/ml of L-arabinose was lethal to *E.coli*.

V. Summary

The expression of genes *serA* and *gltD* regulated by Lrp was studied *in vivo*. Plasmid pBAD22 *lrp* Cm^R, which carries the coding region of *lrp* immediately downstream of the *araBAD* promoter on the vector, was constructed. By varying the concentrations of L-arabinose in the minimal media, different concentration of Lrp can be induced inside the cells. Adding L-serine (500 μg/ml) decreases expression of *lrp* on the plasmid. This plasmid was transformed into a strain carrying a chromosomal *serA::lacZ* and a strain carrying a chromosomal *gltD::lacZ*. The response in terms of expression of these two genes to the artificial elevation of intracellular Lrp concentrations was determined. Expression of *serA* and *gltD* was proportional to Lrp concentration. Also, exogenous L-leucine's effects on Lrp regulation of these two genes were investigated. L-leucine depressed activation of both *serA* and *gltD* by Lrp. A comparison of gene expression of *serA* and *gltD* in the presence of different concentrations of Lrp leads to the conclusion that the promoters of *serA* are more sensitive to Lrp than that of *gltD*. When 100 μg/ml of L-arabinose was added to glycerol minimal media, the induced Lrp was lethal to the cells, indicating that overexpression of *lrp* was toxic to *E.coli*.

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