# Modulated Expression of the *lrp* Gene on an Expression Vector in *E. coli* K-12

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## ABSTRACT

**Modulated expression of the *lrp* gene on an expression vector in *E. coli* K-12**

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This thesis describes the expression of the *lrp* gene under the control of the promoter of the arabinose gene, and the effects of varying amounts of Lrp on expression of Lrp-regulated genes. When cloned into the multiple cloning site next to the arabinose promoter, *lrp* expression increased in cells grown in glycerol minimal medium with arabinose concentrations from 0-20 ug/ml. This increase in Lrp production coincided with increased transcription of two target genes, *gcv* and *gltD*, both activated in vivo by Lrp. Increasing Lrp brought about a decrease in the third gene, *sdaA*, one usually repressed by Lrp. Sensitivity of the three genes to Lrp varied considerably.

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# I. INTRODUCTION

Bacteria assure their survival with a variety of mechanisms of adaptation. These responses are well programmed and very economical. Some of them operate by changing the rate of transcription of certain genes, usually in response to an external signal. While some controls involve only a few genes, organized into operons, others regulate whole areas of metabolism, through groups of genes known as regulons. The leucine/Lrp regulon in *E. coli* is a recently defined regulon (Lin et al, 1992), in which the genes of at least 30 operons are directly or indirectly regulated by a leucine-responsive regulatory protein, Lrp.

Since the discovery of the leucine/Lrp regulon, more and more Lrp regulated operons/genes have been identified. Many advances have been made both in physiological and molecular aspects, such as the physiological functions, the interaction of Lrp with DNA, the leucine effects and the mechanism of regulation of the operons in the regulon (Calvo et al, 1994, Newman et al, 1995). The work reported here was focused on understanding how the different promoters of Lrp regulated genes interact with Lrp to different extent, and how these genes react to Lrp at different concentrations. For this purpose, I constructed an Lrp modulation system with an arabinose promoter $p_{BAD}$, in which the Lrp could be expressed at different levels in growing cells. To do this, I used the

plasmid pBAD1rp on which the *lrp* gene was under the control of the *araBAD* promoter $p_{BAD}$ of a convenient vector pBAD18 (Guzman et al ,1995). The *araBAD* promoter is not expressed in the absence of arabinose. In the presence of arabinose, its expression varies over a range of at least 2 orders of magnitude. Using this system, I measured the synthesis of Lrp from the plasmid at different arabinose concentrations, and also the expression of various target genes, in all cases using *lacZ* as a reporter gene. Results on the expression of the Lrp regulated genes *gcv*, *gltD*, and *sdaA* at different Lrp concentrations will be presented in this thesis. This data is used to compare the regulatory patterns of these genes as a function of Lrp concentration, in order to clarify the role of the Lrp protein in the regulation of *E. coli* metabolism. The effect of leucine is also determined as a function of Lrp concentration. This system also provides a convenient way to search for as yet unidentified genes which may be regulated by Lrp.

Before I describe my research work in this thesis, I will survey the general area of regulation of gene expression, and review some recent studies of the interaction of regulators of gene expression with the promoters they regulate. I will compare the effects of small molecule regulators on different promoters, and then describe in detail the mechanism of regulation of *ara* promoter $p_{BAD}$ by the AraC protein and

arabinose.

## 1. Definition of operons and regulons

### 1-1. Operon

The discovery of *lac* operon in *E. coli* by Monod and Jacob began the elucidation of how the gene expression is regulated, for which Monod and Jacob won the Nobel Prize in 1966. The word 'operon' is used to refer to several genes coding for products with a related metabolic function, all co-regulated by a single regulator.

In the particular case studied by Monod and Jacob, the *lac* operon, the three genes, *lacZ*, *lacY* and *lacA* are adjacent on the chromosome and under the control of a single promoter. The definition of an operon required that a single regulator be involved, e.g. ArgR, but the genes may be scattered on the chromosome as long as the promoters respond to the same regulator.

In the *lac* operon, *lacZYA* are structural genes. *lacZ* encodes the enzyme ß-galactsidase (ß-gal), which cuts lactose into glucose and galactose. *lacI* is the structural gene for the *lac* repressor. Its active form can bind to the operator region of the *lac* operon, and block the transcription of *lac*

mRNA. If the repressor is associated with the inducer, lactose or its derivatives such as IPTG, it will be removed from the DNA in the promoter region. Then RNA polymerase can bind to the promoter to start the transcription of the *lac* operon.

This allows for accurate regulation of expression of lactose-degrading capacity in the cell, and allows the genes to be expressed only when they are useful- i.e. when lactose is present in the medium. Much of the genome is regulated in this way, although many genes are expressed constitutively and thus do not depend on the presence of an external regulator. Even regulated genes may be expressed at lower levels in the absence of their inducers, i.e. regulation is not 100% efficient. A given inducer or repressor may also be more or less efficient, so that the induced level seen with one inducer may be less than that seen with another.

**1-2. Regulon**

Operons thus represent a method for the coregulation of genes of directly related function. Cells are also able to regulate groups of operons, using for this purpose a single global regulator. Such a group of coregulated operons is called regulon (Maas, 1964; Gottesman, 1984). The criteria that define a regulon are that 1) it should have more than one operon, 2) the products of the genes in these operons must be

involved in more than one metabolic pathway, and 3) the expression of the operons is regulated by a single regulator, rather than a common stimulus.

Several such regulons have been described, and others are gradually being added. The best studied of all is the cAMP/Crp regulon (Kolb et al, 1993). The LexA regulon has also been studied in considerable detail (Knegtel et al, 1995; Dri et al, 1994). Among the more recent arrivals on the regulon scene is the leucine/Lrp regulon, the subject of this thesis.

## 2. Interaction of regulators with promoters

In all these cases, the regulation of transcription is mediated by the interaction of regulator(s) with the region of the DNA upstream of the start site of the coding region of the regulated gene. The regulators, DNA-binding proteins of varying specificity, bind to this region of the DNA, the promoter region, and affect gene expression in various ways.

### 2-1. Promoters

The first step in the synthesis of mRNA is the binding of RNA polymerase to the promoter regions of the gene to be transcribed. In most cases,binding of RNA polymerase depends on the nature of the DNA sequences centred 35 and 10 base

pairs upstream of the transcription start site. These sequences, known as the -35 and -10 sequences, have a consensus in E. coli of 5'-TTGACA-3' at -10, and 5'-TATAAT-3' at -35, and are recognized by the $\sigma$ subunit of RNA polymerase (Gross et al,1992). The strength of a given promoter is mainly dependent on the closeness of the agreement of its -35 and -10 elements with the consensus sequences.

Recently, a third important sequence element, called the UP element, has been discovered (Newland et al, 1992; Busby et al, 1994). It is an AT-rich sequence of ~20 bp located immediately upstream of the -35 region, which results in an unusually high affinity of the promoter for RNA polymerase. This UP element is seen in particularly highly transcribed genes like the seven *E. coli rrn* genes encoding ribosomal RNA. The UP element is recognized by the C-terminal 85 base pairs of the $\alpha$ subunit of RNA polymerase, a region known as the C-terminal domain. The location of the UP element with respect to the transcription start varies from one gene to another.

It seems then that promoter strength is a function of at least three promoter elements. The very strong promoters have near-consensus -35, and -10 sequences, and in addition a third upstream sequence known as UP. Weak promoters deviate from consensus at any or all of these areas. (Busby, 1994).

The point at which transcription by RNA polymerase starts depends on where it binds. Some genes have only one polymerase binding site, which is called the promoter, and the base pair at which transcription begins is known as +1. However other genes have two or more promoters, with a different -35, -10, and +1 for each- though in all cases the translation start site may be the same. An example of this is the Lrp-regulated *ilvIH* and *serA* promoters. P1 of *serA* is 45 bp from the transcription start site, P2 93 bp further upstream (Lin et al, 1992). These two promoters are used *in vivo* in different environments, the choice depending on concentrations of Lrp, leucine and other factors.

**2-2. Regulatory DNA binding proteins**

Regulatory DNA binding proteins directly bind to DNA, functioning as repressers or activators- often depending on the architecture of the promoter and on interaction with other proteins. A prime example of this is Crp, the cAMP receptor protein, which represses transcription of some genes and activates others (Kolb et al, 1993). AraC and Lrp also have multiple functions. DNA binding proteins such as these must have a DNA-binding domain to recognize a given DNA sequence, and also domains to interact with other binding proteins.

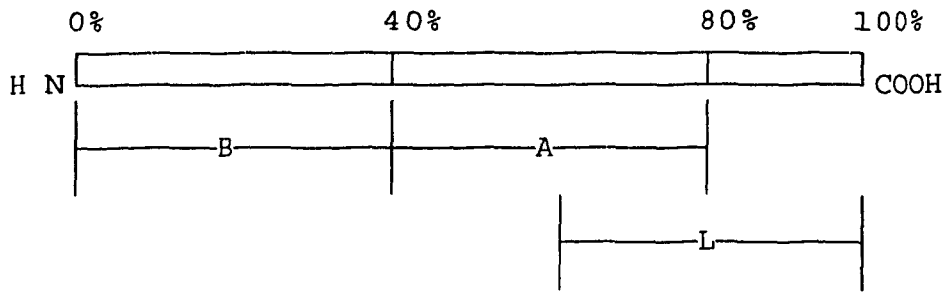
Like several other DNA binding proteins, Lrp is a small

basic DNA binding protein (dimer, 19 kDa). Its function has not been studied yet in very great detail. However three functional domains have been suggested as is schematically shown in Fig. I-1.

**2-2-1. DNA binding sites**

Regulatory DNA binding proteins usually bind to specific DNA binding sites in order to affect transcription. Without association of cAMP, Crp binds to nonspecific sites on DNA. When cAMP is bound to it, Crp shifts to its specific binding sites (de Crombrugghe, 1984). The target DNA binding sites of Crp has been deduced by consensus among the many Crp-regulated genes. It is a 22 bp sequence containing an inverted repeat recognition sequence with the core motif 5'-TGTGA-3' (Kolb et al, 1993). In different promoters, the location of the Crp binding sites for activation are different, -41.5 in *gal* promoter, -61.5 in *lac*, -70.5 in *malT* (Raibaude et al, 1990). The Crp binding sites varies from -40 to -200 over different promoters (Collado-Vides et al, 1991). Usually, Crp recognizes only one site a particular promoter, but two Crp binding sites have been identified in several promoters (Kolb et al, 1993).

Lrp does not recognize a single short sequence as Crp does. Indeed there is some doubt as to whether it recognizes




B: DNA-binding

A: transcriptional activation

C: leucine-binding

**Fig. I-1 Functional domains of Lrp**

a consensus sequence at all. Three groups have proposed the consensus sequence- a very AT-rich sequence with a core of TTTATtCT as reviewed by Newman in 1995. As judged by foot-printing studies, Lrp recognizes multiple binding sites on the promoters of Lrp-regulated genes. This is particularly evident in *ilvIH* where Lrp binds to 6 binding sites at -250, -219, -137, -103, -74, and -54 (Wang et al, 1993).

Similarly there are two long Lrp binding sites at the *serA* promoter, one in -155 to -80 region of P1 with high affinity to Lrp, another in the second promoter, P2. The promoter of *sdaA* has at least 2 Lrp binding sites, one high affinity site, and one low (Lin et al, 1992). Lrp bound cooperatively to one or more sites upstream of the *gltBDF* promoter (Ernsting et al, 1993).

**2-2-2. Affinities of the DNA binding proteins for their promoters**

Some genes are very sensitive to a given regulatory protein, and others less so- i.e. even among the genes regulated by a given regulatory protein, promoter reaction to the particular protein may vary. It is clear that Lrp directly interacts with the promoter of *gcv*, and *gcv* is highly sensitive to the presence or absence of Lrp (Lin et al, 1992). In this thesis, I try to assess this interaction

quantitatively.

## 3. Transcriptional regulation

### 3-1. RNA polymerase and transcription

The RNA polymerase holoenzyme of *E. coli* is composed of core enzyme $\alpha_2$ßß' and one of the many possible $\sigma$ subunits. Each of the subunits plays a different functional role. The catalytic site of RNA polymerase is on the ß subunit. The ß' subunit binds to DNA nonspecifically. The $\alpha$ subunit, in particular its N-terminal domain, is involved in the assembly of the holoenzyme- the alpha subunits assembling first, and this allowing the addition of ß and ß', forming the core enzyme complex.

Core enzyme assembles without $\sigma$ subunit, and the purified DNA polymerase often lacks $\sigma$ factor. There are several alternative $\sigma$ subunits, each recognizing a different set of promoters. $\sigma$ factors then are major determinants of transcription specificity. For example, $\sigma^{70}$ recognizes the most commonly transcribed genes of E. coli, those used for instance in glucose minimal medium. $\sigma^{32}$ recognizes the heat shock genes, $\sigma^{24}$ those involved in extreme heat shock. $\sigma^{54}$ the genes of nitrogen metabolism $\sigma^{28}$ the Flagella-chemotaxis genes, and $\sigma^{38}$ the oxidative stress response genes and

stationary phase-specific genes (Ishihama, 1993).

Some promoters are recognized by holoenzyme $\alpha_2\beta\beta'\sigma$( core enzyme + some $\sigma$), but most promoters require in addition, some transcription activator. These may directly contact $\alpha$ or $\sigma$, or both for the activation (Ishihama, 1993; Busby, 1994).

**3-2. Association of transcriptional factors with other factor(s)**

The association of transcriptional factors with other factor(s) may modify their effect, either in whole or in part. Secondary factors may be necessary for activation or repression by a given binding protein (e.g. cAMP with CRP). They may intensify the effect of the protein, or alleviate it (e.g. leucine with Lrp). These other factors can be proteins, amino acids or others, which act as mediators of signals from the intracellular and extracellular environment. Thus cAMP binds to Crp, and activates it. The level of cAMP is an indicator of the internal catabolite pool. When the availability of catabolites decreases, cAMP is synthesized, and with Crp, activates transcription of a variety of operons which specify degrading enzymes, e.g. the *lac*, *ara* and *mal* operons.

Leucine binds to Lrp (Lin et al, 1992), intensifying its

action at some promoters, and decreasing it at others. Thus leucine may increase or decrease activation at genes for which Lrp is an activator, and may increase or decrease repression where Lrp acts as a repressor. At other operons, even though Lrp is essential to their function, leucine has no or little effect: e.g. on transcription of the *gcv* operon (Lin et al, 1993), and the *lrp* operon (Wang et al, 1992). The effect of leucine on Lrp is complex, but it is generally believed that binding leucine changes the conformation of Lrp, and modifies the affinity of Lrp for DNA. In this thesis, effects of leucine are assessed at promoters which have different affinity for Lrp.

### 3-3. Mechanisms of transcriptional regulation

The regulation of transcription has been the focus of intense research in recent years in many laboratories. One can consider transcription as involving two steps- first the binding of polymerase at the promoter site (with its -10 and -35 sites, and possibly also an UP element) followed by changes in the form of the DNA, and then, the synthesis of the first bases of mRNA. Transcription has been shown to be regulated at each stage of this process, different promoters being affected at different steps.

### 3-3-1. Mechanism of transcriptional repression

A simple mechanism of repression involves the blocking of RNA polymerase binding. This can occur in a variety of ways, including the following:

1) At some promoters, the repressor binding site (operator) overlaps with the RNA polymerase binding site. This is true for Crp-regulated genes where the Crp binding site is situated between -60 and +20, and Crp, in the absence of cAMP, will directly interfere with polymerase binding. Moving the Crp site slightly, by deleting one or two base pairs, may bring about this overlap and convert Crp from an activator to a repressor (Lavigne et al, 1992; Busby et al, 1982).

2) The binding of repressor may change the architecture of the DNA and thus inhibit transcription. The repressor may, for instance, allow the looping of DNA and decrease the affinity of the promoter for RNA polymerase, e.g. the looped *araBAD* promoter (see below). On the other hand, the repressor may interfere at a later stage, and impede function of the bound RNA polymerase (Collado-Vides, 1991).

### 3-3-2. Mechanism of transcriptional activation

In principle, the mechanism of positive transcriptional

regulation is similar to that of repression- the activators facilitate or enhance the binding of RNA polymerase with DNA, or they enhance its function, once bound. Analogous to repression, activators may bind and make contact with RNA polymerase, helping it bind (cooperative binding) or they may change the architecture of the DNA, as by DNA bending, and increase its affinity for RNA polymerase.

Contact between transcription activators and RNA polymerase usually involves the $\alpha$ and $\sigma^{70}$ subunits of RNA polymerase. The C-terminal domain of $\alpha$ ($\alpha$CTD) carries targets for such interaction (Ishihama, 1993). For example, Crp activation at the *lac* promoter requires direct protein-protein contact with target amino acids in the $\alpha$CTD (Busby, 1994). It not only increases the affinity of RNA polymerases for the promoter, but also makes a potentially stipulatory interaction with $\sigma^{70}$. Other activators interact with $\sigma^{70}$ instead of $\alpha$CTD. For instance, activator bacteriophage $\lambda$CI protein binds to a site centred at position -42 and overlapping the -35 element to directly contact a target in $\sigma^{70}$ region 4 (Ishihama, 1993). Moreover, RNA polymerase may be contacted simultaneously by two or more activators (Joung et al, 1994). This may be part of the explanation for the long and multiple binding sites of some regulatory proteins, such as Lrp and Crp.

In the second case, regulatory proteins convert DNA to a form with higher affinity for RNA polymeraes (Kolb et al, 1993). Crp binding bends DNA, resulting in a different angle of bending depending on the number and position of the Crp binding sites (Kim et al, 1989). Lrp binding at a single site caused a bend of about 52°, which increased to at least 135° when Lrp bound to two adjacent sites (Wang et al, 1993).

### 3-2-3. Possible mechanisms of the regulation by Lrp

The global transcriptional regulator Lrp can act as an activator or as a repressor for a variety of operons known collectively as the leucine/Lrp regulon. It has been suggested the mechanism of regulation by Lrp may be similar to that of Crp (Lin, 1992). Lrp binds to the operator position in some Lrp repressed promoters, such as *lysU, lrp* promoters, to block the RNA polymerasae binding (Lin and Ernsting et al, 1993, Wang, 1994). The footprint studies suggested that the bindings of Lrp in Lrp activated promoters cause the bending of the DNA or interact with RNA polymerase, facilitating the binding of RNA polymerase to its binding site.

On the basis of the studied Lrp binding sites in those promoters, at least two questions are very interesting: 1) how do the different Lrp binding sites affect the reaction of the regulated genes to Lrp? 2) Is there some relationship between

the binding sites in the promoters and the regulatory patterns?

**4. Transcriptional systems adaptable for in vivo studies**

In the preceding sections of this introduction, I reviewed the interaction of regulators with promoters and summarized the mechanisms of transcriptional regulation, mostly based on in vitro studies. Very little work has been done on the mechanism of regulation in vivo. To study this, it would be useful to have a system with variable expression of Lrp so that cells could be grown with Lrp at different concentrations. One of the prime requirements for such a system is that it should be shut off as tightly as possible when synthesis of Lrp is not requires, and turned on to a variable extent over as wide a range of concentrations as possible.

This could be carried out with a variety of plasmid vectors carrying a variety of multiple cloning sites. Many of these have been constructed, and are even commercially available- under the name expression vectors. These are designed primarily for high expression in the permissive conditions. Since we also require as low as possible expression in nonpermissive conditions, we chose one such vector, pBAD18, from the laboratory of J. Beckwith. Other

possible choices include $p_{LAC}$, $p_{TAC}$, $p_L$, $p_R$ and $p_{T7}$.

## 4-1. A survey of well-regulated promoters for possible use in *in vivo* studies

In the $p_{LAC}$ expression system, the expression vectors carry the most famous and intensely studied promoter, $p_{LAC}$, and a multiple cloning site such that genes cloned into the vector will be under the control of this promoter. The vectors also carry *lacI* gene, which encodes the lac repressor. The repressed promoter can be induced by inducer isopropyl ß-D-thiogalactoside (IPTG) (Yanisch-Perron et al, 1985; Chen et al, 1991), so that variable expression in vivo can be brought about by varying the IPTG concentration.

$p_{TAC}$ is a hybrid promoter derived from the sequences of the *trp* and *lac* $UV_5$ promoters (Boer, 1983). Briefly, the -35 element of *lac* $UV_5$ promoter was replaced with that of the stronger *trp* promoter. Boer et al (1983) constructed two hybrid promoters *tac*I and *tac*II. The *tac*I promoter directs transcription approximately 11 times more efficiently than the derepressed parental *tac* $UV_5$ promoter and approximately 3 times more efficiently than the *trp* promoter in the absence of the *trp* repressor. The hybrid promoter can be repressed by the *lac* repressor and derepressed with IPTG. This high expression makes the hybrid promoter particularly useful for high and

controlled expression from genes cloned into the vector. Systems like this are widely used in the biotechnology industry.

$p_L$ and $p_R$ are the strong bacteriophage λ promoter. They are repressed by λ repressor encoded by λ*cI*857 gene. Transcription from the strong promoter is repressed in cell growing at 30°C, but is induced at 42°C because the thermolabile repressor is inactivated (Elvin et al, 1990). This works efficiently but has the defect of requiring abrupt temperature changes which may themselves influence our experimental system.

In bacteriophage T7 RNA polymerase/promoter system, the gene to be expressed is fused after promoter $p_{T7}$ and the gene encoded for T7 RNA polymerase is under the control of promoter $p_L$, which is repressed by the product of *cI*857 at 30°C and induced at 42°C. This means that the cloned gene will be expressed only when the T7 RNA polymerase is made. Since that polymerase is expressed only at high temperature, the system should be essentially turned off at low temperature. If rifampicin is added when the temperature is shifted to 42°C, no E. coli gene can be transcribed by host RNA polymerase. Then the only product that is made comes from the T7 RNA polymerase which recognises only T7 promoters, including the one used in this vector. Expression is thus turned off at 30°C

and on at 42°C. However the system is not optimized for variable regulation. (Tabor et al, 1985).

### 4-2. Arabinose promoter p*BAD*

Arabinose promoter $p_{BAD}$ of *ara* operon has been well studied (Schleif, 1995). The intensive studies of this promoter not only made a great contribution to understanding the mechanism of transcriptional regulation in *E. coli*, but also made it possible to utilize the well-understood properties of pBAD for construction of a modulatable expression system.

#### 4-2-1. *ara* operon

*E. coli* can transport and catabolize the sugar L-arabinose. AraBAD are three arabinose-inducible enzymes converting L-arabinose to D-Xylulose-5-Phosphate. The system also involves AraC, the arabinose-responsive transcription activator protein. Transport of arabinose is assured by the *araEFGH* and/or the *araJ* gene products. The *araBAD* operon is positively regulated by AraC.

#### 4-2-2. Regulation mechanism of the $p_{BAD}$ promoter

A two-state model was suggested to interpret the

regulation of $p_{BAD}$ promoter (Fig. I-2). In the absence of arabinose, most copies of the araBAD genes in cells are in a looped state. One subunit of dimer AraC contacts the $araI_1$ half-site and one attaches the $araO_2$ half-site. The looped state of the DNA largely blocks the activity of the promoter $p_C$. The promoter $p_{BAD}$ is not active because AraC is not bound at $araI_2$ half-site and therefore the activation domain of AraC is not properly positioned to activate the promoter.

However, the DNA loop opens on addition of arabinose and with the assistance of CRP protein. AraC loses its ability to contact both $araO_2$ and araI, and shifts to a state of binding to two adjacent half sites energetically preferred. Then, $p_C$ is accessible to RNA polymerase until AraC binds to the $araO_1$ site. Because of the relocation of the DNA binding domain from $araO_2$ to $araI_2$, an activation domain is properly positioned to assist transcription initiation of $p_{BAD}$, which is necessary and sufficient for the activation.

Upon arabinose addition to growing *E. coli*, transcription initiation at $p_{BAD}$ begins within five seconds (Hirsh, et al, 1973). The presence of arabinose increases the affinity of AraC for DNA by about 50 fold (Hendrickson et al, 1984).

One major role of the Crp protein at $p_{BAD}$ promoter is to assist opening the DNA loop between $araI_1$ and $araO_2$, after

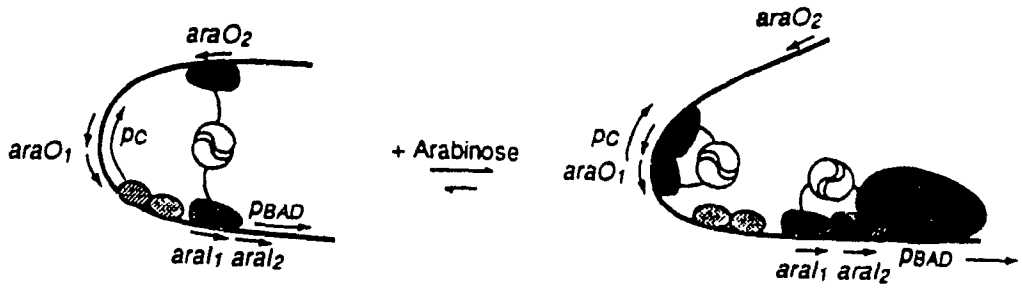



**Fig. I-2 Mechanism of the regulation of $p_{BAD}$ promoter.** A representation of the regulation mechanism which functions in the *araCBAD* regulatory region. Arabinose derives the equilibrium towards the right. The drawing on the right indicates the situation early after the addition of arabinose, before an appreciable fraction of DNA copies in the population have had AraC to *araO1* to repress it (Scheif, 1995)

which the DNA binding domain of AraC can bind to $araI_2$ to activate transcription. However, CRP probably also contacts RNA polymerase directly in the bent or coiled DNA regulatory region.

### 4-3. *araC*-$p_{BAD}$ system

Guzman et al constructed a series of expression vectors which carry the activator gene *araC*, the arabinose promoter $p_{BAD}$ followed by a polylinker, a gene for antibiotic resistance to permit the selection of the plasmid-containing cells and two origins of replication, e.g. M13 and pBR322 in pBAD18 (Fig. II-2). With these pBAD expression vectors, cloning is easy, and the expression of the gene can be easily controlled by the inducer arabinose. In the absence of arabinose, the expression is shut off, and in the presence of arabinose, the expression is turned on.

This system has several advantages.

1) High level expression is possible. Using the *phoA* gene as a reporter, Guzman et al (1995) reported a ratio of repression/induction of about 1000, compared with 50-fold variation for $p_{TAC}$ based vectors.

2) Regulation is rapid and efficient.

3) Expression from this vector can be modulated over a wide range, and is almost totally arabinose-dependent.

4) Experimental manipulations are simple. Arabinose can be added easily and no temperature shift is required.

However the system has one major defect- namely that expression is not possible in the presence of glucose, or in rich media like Luria broth. This is a decided disadvantage though it did not hinder this work greatly. A gratuitous inducer, like IPTG for the lac system, would be very useful but has not yet been described.

## II. MATERIALS AND METHODS

### 1. Strains, bacteriophages and plasmids

The strains, bacteriophages and plasmids used in this work are listed on Table II-1.

### 2. Media

#### 2-1. Minimal medium

The minimal medium used contained 0.527% $KH_2PO_4$, 1.500% $K_2HPO_4$, 0.2% $(NH_4)_2SO_4$, 0.020% $MgSO_4$ and 0.001% $CaCl_2$. 50 $\mu$g/ml L-isoleucine and L-valine were added to compensate for the deletion in *livA* carried by MEW1 and all its derivatives. Carbon sources were added as noted at the following concentrations: D-glucose, 0.2%; L-serine, 0.2%; glycerol, 0.5%. For solid media, 0.8% gelrite was added.

#### 2-2. Minimal medium with alternative nitrogen or carbon sources

When other nitrogen or carbon sources were used, ammonium sulphate or glucose was omitted, and the desired nitrogen or carbon source was added as indicated. e.g. 0.2% glycine; 0.2% serine.

**Table II-1 Strains, bacteriophages and plasmids**

| Strain, phage and plasmid | Description | Reference or source |
|---|---|---|
| Strain | | |
| CU1008 | *E. coli* K-12 *ilvA* | Williams, L. S. |
| MEW1 | Δ*lacZ* derivative of CU1008 | Newman et al, 1985 |
| JP131 | MG4100 *ara*Δ714 | Beckwith, J. |
| Cp55 | MEW1 *leu::λplacZ*Mu9 | Lin et al, 1992 |
| LT10 | MEW1 *ara*Δ714 | this work |
| MEW26 | MEW1 *lrp::Tn10* | Lin et al, 1992 |
| LT20 | LT10 *lrp::Tn10* | this work |
| LT21 | LT20 *gcv:lacZ* | this work |
| LT30 | LT20 pBAD*lrp* | this work |
| LT31 | LT20 pBAD*lrp::lacZ* | this work |
| Cp67 | MEW1 *gcv::λplac*Mu9 | Lin et al, 1992 |
| Ca67 | Cp67 *lrp::Tn10* | Lin et al, 1992 |
| LT32 | LT30 *gcv::λplac*Mu9 | this work |
| Cp8 | MEW1 *gltD::λplac*Mu9 | Lin et al, 1992 |
| Ca8 | Cp8 *lrp::Tn10* | Lin et al, 1992 |
| LT33 | LT30 *gltD::λplac*Mu9 | this work |
| Cup22 | MEW1 *sdaA::λplac*Mu9 | Su et al, 1989 |
| Cap22 | Cup22 *lrp::Tn10* | Lin et al, 1992 |
| LT34 | LT30 *sdaA::λplacMu9* | this work |

**Cont.**

| Phage | | |
|---|---|---|
| P1 | | |
| λ*plac*Mu9 | λ*lac*Mu $Km^r$ | Bremer et al, 1985 |
| λ*plac*Mu507 | λ*c1ts*875 *sam* 7 MuA+B+ helper phage | Bremer et al, 1985 |
| λ*Tn10* | λ*cts samS*53 | Wood, 1981 |

| Plasmid | | |
|---|---|---|
| pBAD18 | carries *araC*-$p_{BAD}$ promoter | Guzman et al, 1995 |
| pBAD*lrp* | pBAD18 carrying *lrp* | Shao, Z. Q. |
| pMC1871 | carries *lacZ* gene | Gilbert, W. |
| pBAD*lrp::lacZ* | pBAD*lrp* fused *lacZ* in *lrp* | this work |

## 2-3. Rich media

For routine culture, Luria broth of the following formulation was used: 1.0% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl. This was solidified when desired with 1.8% Bacto-agar.

## 2-4. Medium for the growth of P1 phage

For production of phage, a rich medium was made as follows: 1.0% Bacto-tryptone, 0.1% Bacto-yeast extract and 0.8% NaCl, with 2mM $CaCl_2$ and 0.1% glucose before use. This was solidified with 1.7% Bacto-agar for use in plates, and 0.6% Bacto-agar for top agar.

## 2-5. TB medium for growth of λp*lac*Mu9 and λp*lac*Mu507

For production of these phage, a rich medium consisting of 1.0% Bacto-tryptone, 0.5% NaCl, with 0.01M $MgSO_4$ and 0.2% maltose was prepared, and solidified with 1.1% Bacto-agar for plates and 0.4% Bacto-agar for top agar.

## 2-6. Other additions to the media

Antibiotics were added to the media at the following

concentrations: 15 μg/ml tetracyclines, 50 μg/ml kanamycin and 200 μg/ml ampicillin. 40 μl 20 mg/ml 5-Bromo-4-Chloro-3-indolyl-ß-D-galactoside (X-gal) was sprayed on media.

### 2-7. Determination of growth rates at different Lrp levels

For determination of growth rates, cells were grown overnight in glycerol minimal medium at 37°C, and subcultured in sidearm flasks in the same medium supplemented with arabinose at concentrations noted in the text. Culture turbidity was measured with a Klett colorimeter using a #42 filter.

## 3. Enzyme assays

### 3-1. L-serine deaminase assay

L-SD was assayed as previously described in toluene-treated whole cells (Newman et al, 1985).

### 3-2. ß-galactosidase assay

ß-galactosidase activity was assayed in whole cells according to the method described by Miller and expressed in his units (1972).

### 3-3. Assessment of plasmid maintenance

To estimate the percentage of cells carrying the plasmid, a dilution containing about 500 cells was plated on LB plates and incubated at 37°C. The resulting colonies were replicated on LB with and without ampicillin and the plasmid maintenance estimated as the per cent of antibiotic resistant cells.

## 4. Strain constructions

### 4-1. Construction of *ara* deletion in MEW1

To construct a strain carrying a deletion of the *ara* degradative genes on the strain background used in this lab, I took advantage of the relatively close linkage between *ara* and *leu*. Strain CP55 carries an insertion in the *leu* operon. It was transduced to leucine prototrophy with P1 phage grown on strain JP131 (*ara*Δ714), obtained from J. Beckwith. The resulting transductants were screened for their ability to grow with glucose only. Those which were able to grow on glucose minimal medium but not with arabinose were used under the name LT10 (*ara*Δ714).

### 4-2. Construction of the strains with *lrp::Tn10(tet$^r$)*

As in earlier work, *lrp::Tn10(tet$^r$)* strains were

constructed by transduction selecting for tetracycline resistance and verified by growing on 0.2% serine minimal medium (Lin et al, 1990).

### 4-3. Construction of the strains carrying plasmid pBAD*lrp*

Plasmid pBADlrp and its derivative were transformed by selecting ampicillin resistance carried by the plasmid.

### 4-4. Construction of the strains with Lrp-regulated genes carrying λp*lac*Mu9 inserts

Since λp*lac*Mu9 carries both *lacZ* and a kanamycin-resistance gene, all genes with λp*lac*Mu9 were transduced with P1 phage, selecting antibiotic resistance and screening simultaneously for transfer of the *lacZ* gene on glycerol minimal medium containing kanamycin and X-gal. Where arabinose was necessary to activate the gene, it was also added (50 μg/ml).

### 4-5. Screening for Lrp-regulated genes using the pBAD*lrp* vector

λp*lac*Mu9(kan$^r$) inserts into strain LT30 (*ara*Δ *lrp*$^-$ pBAD*lrp*) were made according to previously published methods

(Bremer et al, 1985, Lin et al, 1992). Kanamycin-resistant blue colonies were selected. Colonies showing different degrees of colour with and without arabinose were assayed for ß-galactosidase.

## 5. Construction of plasmid pBAD*lrp::lacZ*

### 5-1. Principle

To insert *lacZ* into the *lrp* coding region so that ß-galactosidase activity could be used as a reporter of *lrp* transcription, I used the BglII site in *lrp* 30 bp after the start codon ATG, and ligated it with the *lacZ* gene which had been cut with BamHI compatible with BglII.

### 5-2. Details of the procedure

The part of the sequence of the *lrp* gene from trhe first ATG site through a BglII site early in the gene is showed on Fig. II-1. The *lacZ* gene is cut from pMC1871 with flanking BamHI sites (Fig. II-1).

To isolate the *lacZ* gene, plasmid pMC1871 was cut with BamHI, and the mixture analyzed on a 1% agarose preparatory gel. A *lacZ* fragment of the expected size, about 3 kb, was cut from the gel and treated with QIAET kit (QIAGEN Inc.). The

```
        |lrp->
5'-AGGGATTAatgGTAGATAGCAAGAAGCGCCCTGGCAAAGATCTCGACCGTATCG-3'
   SmaI-SspI                                BglII
          |start->
```

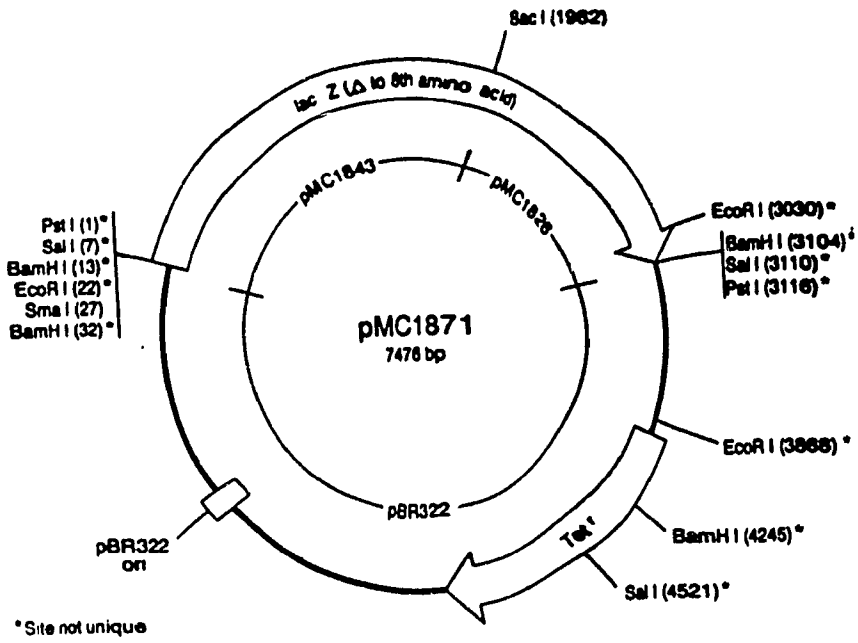



**Fig. II-1 Part of the sequence of *lrp* gene and the map of a *lacZ*-carrying plasmid.** This figure shows the map of the plasmid from which the *lacZ* was isolated in order to construct pBAD*lrp::lacZ*.

size of the putative *lacZ* DNA thus isolated was verified on 1% agarose gel again.

To insert the *lacZ* gene into *lrp*, I isolated the pBAD*lrp* plasmid, cut it with BglII, added the 3kb BamHI cut *lacZ* fragment and ligated with DNA T4 ligase. The ligated plasmid was transformed into strain LT20 (*ara*Δ *lrp::Tn10*), selecting ampicillin resistance on glycerol minimal medium containing X-gal, tetracycline, ampicillin and arabinose 50 μg/ml. The blue colonies were purified, and the constructed plasmid was isolated, and its size verified on a 1% agarose gel.

**6. DNA sequencing**

The DNA sequence of the region from the promoter $p_{BAD}$ to the first codons of *lrp* on pBADlrp was determined by dideoxy-chain-termination method described Sanger et al (1977) with the kind help of Dr. F. Lang in whose lab it was done. The plasmid pBAD*lrp* was transformed into XL1 to prepare single stranded plasmid DNA using the M13 intergenic origin on the plasmid (Fig. II-2). The methods for isolation of single strand DNA, and the sequencing reaction, and the sequencing gel system used in this work were as developed by Dr. Lang et al (1990). The primer used began 57 bases downstream of the start codon of *lrp*, and was directed toward the 5'end of the gene (Fig. II-2).

```
<-polylinker|lrp->
3'......TGGGATTAatgGTAGATAGCAAGAAGCGCCCTGGCAAAGATCTCGA
         SmaI-SspI
                |start->

    CCGTATCGATCGTAACATTCTTAATGAGTTGCAAAAGGATG...5'
                        complementary to primer
```

**Fig. II-2** ***lrp*** **sequence complementary to the primer for the sequencing**

## 7. Other genetic methods

### 7-1. Plasmid isolation

The plasmid were isolated following the protocol described by Sambrook et al (1989).

### 7-2. Transduction

P1 mediated transduction was conducted according to the method described by Miller (1972).

### 7-3. Transformation

Transformation was performed following the protocol described by Sambrook et al (1989).

## III. RESULTS

The purpose of this work was to grow cells with varying amounts of Lrp and determine the effects of this variation on expression of some of the genes affected by Lrp. This would allow a comparison of the relative reaction of the various promoters to Lrp.

To do this, it was necessary to choose a promoter which could be completely turned off in some conditions, and regulatable over a wide range of concentrations. This would allow me to determine the effect of complete deficiency of Lrp, and then measure target gene function as a detailed function of Lrp concentration.

I chose for this purpose a plasmid prepared by Guzman in Dr. Beckwith's lab, which facilitates cloning any gene under the promoter of *araBAD*. Section 1 of the result section explains how the test system was made. In section 1-1 I describe the construction of an appropriate host strain. In 1-2, I describe the subcloning of the *lrp* gene onto the Guzman plasmid and the construction of the Lrp modulation system with promoter $p_{BAD}$. In section 1-3, I verify that the characteristics of the plasmid carrying *lrp* are as would be expected.

In section 2, I assess the function of the Lrp-producing plasmid in various conditions. To do this, I cloned the *lacZ* gene into the coding region of the plasmid-carried *lrp* gene (section 2-1-1). This allowed me to use assays of ß-galactosidase as an indicator of *lrp* expression (section 2-1-2). I then determined whether the plasmid would also permit Lrp production in rich medium (section 2-1-3). I observed the growth of the cells carrying plasmid pBAD*lrp* (section 2-2).

Section 3 deals with the effects of Lrp on target genes. It begins with the details of the construction of the appropriate strains (section 3-1), and goes on to describe the effects of Lrp on 3 target genes (section 3-2-1 to 3-2-3) and compare the sensitivity of the three genes to Lrp(3-3).

In section 4 I show how the arabinose-controlled gene *lrp* can be used to search for as yet unidentified genes regulated by Lrp. The strategy for the screening and preliminary results are described.

## 1. Construction of a plasmid carrying Lrp under the control of $P_{BAD}$, a promoter with variable expression

To investigate the response of target genes to Lrp, as discussed in the Introduction, I needed to control *lrp* expression such that Lrp production could be turned off

totally or maintained at different levels as desired. For this purpose, I used the arabinose $p_{BAD}$ promoter on plasmid pBAD18, putting the *lrp* gene under the control of that promoter. It was then necessary to be sure that this was the only source of Lrp in the cell, so the host needed to be deficient in chromosomal *lrp* gene expression. Further, the cell had to be deficient in arabinose catabolism- otherwise the arabinose concentration in the medium would vary during growth. The ability to take up arabinose from the medium was provided by host chromosomal genes which are located far from *araBAD*.

## 1-1. Construction of strain deficient in *ara* and *lrp* genes

The Lrp-regulated operons had been studied in the host background of *E. coli* K-12 strain MEW1, the reference strain in this lab. Therefore, this work started with the construction of a derivative of strain MEW1, deficient in arabinose catabolism (*ara*$^-$) and in Lrp production (*lrp*$^-$).

### 1-1-1. Choice of the appropriate strain deficient in arabinose catabolism

*AraBAD* are the structural genes encoding the enzymes for utilization of arabinose (see Fig. III-1) Therefore, mutants deficient in any of those genes would be unable to utilize

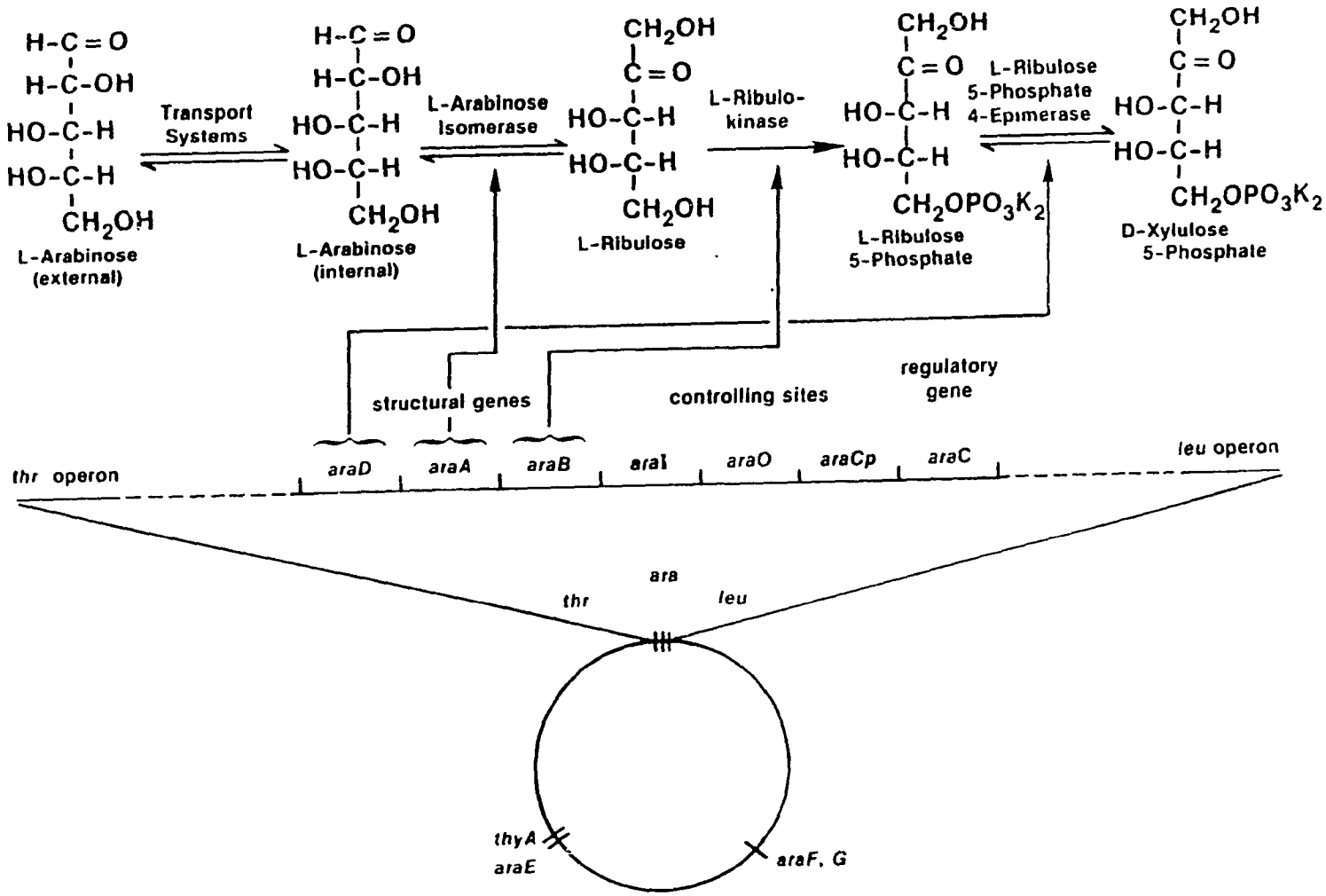



Fig. III-1 Genes and the gene products involved in arabinose degradation. This figure is taken from Silhavy et al (1984).

arabinose. However, a strain carrying an *araD* mutation alone produces a toxic product, D-xylulose phosphate. It is therefore easier to make a complete deletion of the arabinose degradation genes and avoid toxicity problems. Strain JP313 (strain MG4100 *ara*Δ714), which carries a well-known deletion, *ara*Δ714 was obtained from J. Beckwith. I verified that this strain JP313 could not grow with arabinose as sole carbon source. Because arabinose turned out to be toxic in many backgrounds, I also verified that JP313 grew on glycerol minimal medium, and produced uniform size colonies whether or not arabinose was also added to the medium. This indicated that the arabinose deletion in this strain led to a deficiency in arabinose catabolism without causing arabinose toxicity, and thus could be used in this work.

### 1-1-2. Construction of the strain deficient in *ara* gene

The next step involved transferring *ara*Δ714 to the lab host strain, MEW1. This was actually done by transferring the *ara* deletion to a previously constructed derivative of CU1008 deficient in the leucine biosynthetic operon. This strategy takes advantage of the fact that the leucine biosynthetic operon is closely adjacent to the *araBAD* operon (Fig. III-1). Strain CP55, constructed in our lab by R.T. Lin, is a

*leu::kan*$^r$ mutant in the MEW1 background, which can grow in

glucose-minimal medium only when leucine is provided. P1 phage grown on strain JP313 were used to transduce strain CP55 to leucine-independence. Some of the transductants would be expected to be unable to use arabinose because of concomitant transfer of the *ara*Δ714 deletion.

I verified that some of the transductants were able to grow without leucine and had lost their resistance to kanamycin. I then selected for further study those which had lost the ability to make colonies on arabinose-minimal medium plates, and verified that these were able to grow on glycerol even in the presence of arabinose (i.e. were not arabinose-sensitive).

One such strain was used in further studies under the name LT10 (*ara*Δ*714*).

### 1-1-3. Construction of the strain deficient in *ara* and *lrp* genes

To make the preceding strain deficient also in the *lrp* gene, the *lrp::Tn10(tet$^r$)* was transduced onto the chromosome of LT10 (*ara*Δ) by P1 transduction, as has been previously described (Lin et al, 1990). The transductants, selected on LB tetracyclines plates, were shown to be unable to use arabinose as carbon source (*ara*Δ), able to use L-serine as carbon source

(Lrp deficient), and tetracycline-resistant (maintaining the *Tn*10 insert in *lrp*).

The *lrp*$^-$ mutation derepresses the expression of *sdaA*, which encodes L-serine deaminase I(L-SD I). Therefore an *lrp* mutant can use serine as the only carbon source, while the parent strain MEW1 cannot. Thus it is clear that the transductants isolated were deficient in *lrp* and one of the above transductants was selected for further use under the name LT20 (*ara*Δ*lrp*$^-$).

**1-2. Construction and selection of the plasmid pBAD*lrp* in which *lrp* expression is dependent on arabinose**

**1-2-1. Description of plasmid pBAD18 and a summary of its advantages for this work**

Plasmid pBAD18 is a 4612 bp plasmid derived from pBR322 (Guzman et al 1995). It carries the pBAD promoter of *araBAD* and the *araC* gene encoding arabinose-responsive activator protein, which activates *araBAD* transcription. A polylinker is located immediately downstream of the $p_{BAD}$. The plasmid also carries the *amp*$^r$ gene, the pBR322 origin and the M13 intergenic region (Fig. III-2). 1% agarose gel electrophoresis showed that the linear plasmid was 4.6 kb.

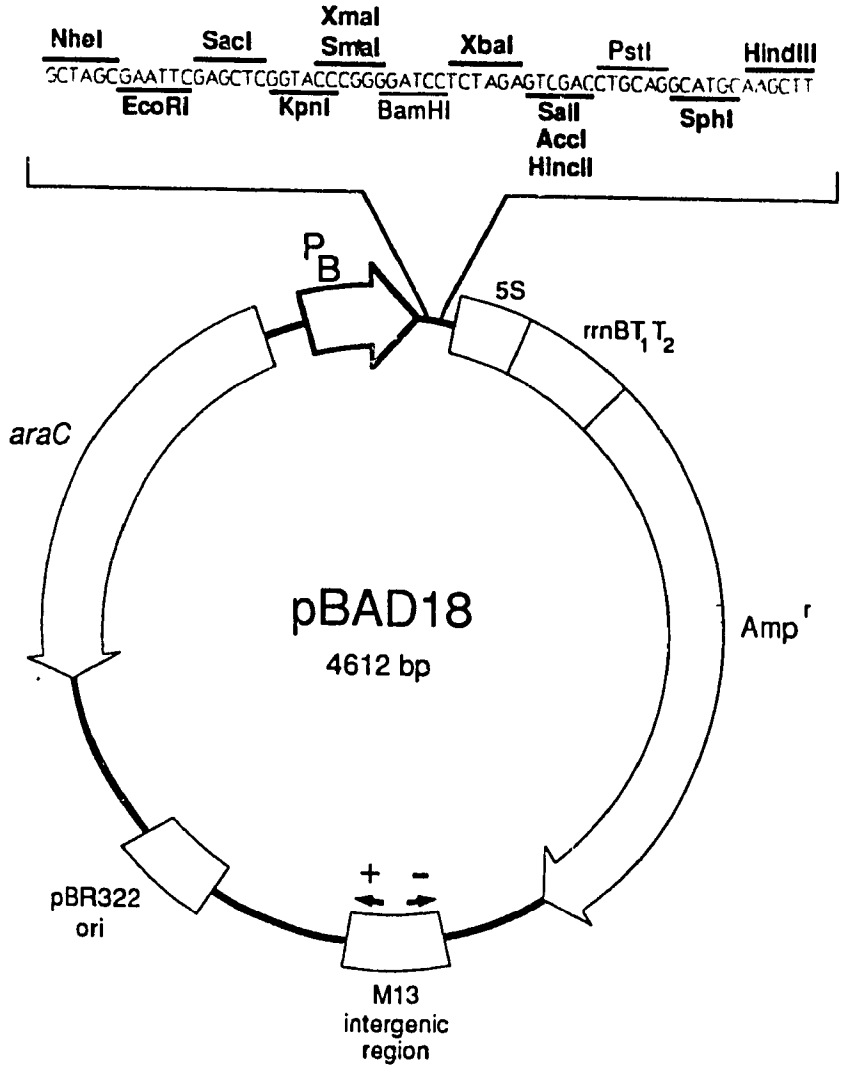



**Fig. III-2 Map of plasmid pBAD18**. This map shows locations of the arabinose promoter $p_{BAD}$, of the *araC* gene and other less relevant genes, as well as the details of the polylinker region (Guzman, 1995).

The sequence of the promoter $p_{BAD}$ and partial polylinker on pBAD18 was provided by John Beckwith's lab (Fig. III-3). The regulation mechanism of the *araBAD* promoter above has been described in detail in the Introduction to this thesis. Here I summarize the principal characteristics of the $p_{BAD}$ promoter.

Expression from the *araBAD* promoter is governed both by glucose and arabinose. In the presence of glucose, the arabinose promoter cannot be expressed, an effect mediated by cyclic AMP and Crp protein, and by inducer exclusion. In the presence of glycerol, a much less efficient catabolite repressor, the catabolite effect is minimal, but the arabinose operon is still not expressed. The arabinose operon is useful in this work because the ratio of fully induced (as in cells grown with arabinose, or in this work, with glycerol and arabinose) to fully repressed (as in glucose-grown cells) is large- about 250-fold in rich medium and about 1000-fold in minimal medium (Guzman et al, 1995). Moreover expression is very sensitive to the arabinose concentration and can be modulated over a wide range of inducer concentrations, no induction at 1.33 uM arabinose (0.00002% w/v), and full induction at 13,300 uM arabinose (0.2% w/v).

```
                                   981  3'-GTATGAGGGC GGTAAGTCTC

1001  TTCTTTGGTT AACAGGTATA ACGTAGTCTG TAACGGCAGT GACGCAGAAA
        |-> operator O2 <-|
        1003              1020

1051  ATGACCGAGA AGAGCGATTG GTTTGGCCAT TGGGGCGAAT AATTTTCGTA


1101  AGACATTGTT TCGCCCTGGT TTCGGTACTG TTTTTGCGCA TTGTTTTCAC
                                |->     promoter Pc
                                1125

1151  AGATATTAGT GCCGTCTTTT CAGGTGTAAC TAATAAACGT GCCGCAGTGT
      <-|         |->       O1         <-|
        1153      1161                    1182


1201  GAAACGATAC GGTATCGTAA AAATAGGTAT TCTAATCGCC TAGGATGGAC
         |->   CRP | <-|                                   |->
         1204       |   1217                                1250
                    |->              I2 + I1

1251  TGCGAAAAAT AGCGTTGAGA GATGACAAAG AGGTATGGGC AAAAAAACCC
        |  PBAD                       <-|                  |->
        |                                1277               1300
     <-|

1301  GATCGCTTAA GCTCGAGCCA TGGGCCC-5' 1327 ............
            polylinker       SmaI                    <-|
                                                       1360
```

**Fig. III-3 Sequence of the ara*BAD* regulatory region**. This sequence, retrieved from Genbank, shows certain regulatory elements, and part of the polylinker sequence.

### 1-2-2. Construction of plasmid pBAD*lrp*

The lrp gene was cloned into the polylinker site downstream of $p_{BAD}$ on pBAD18 by Z.Q. Shao. PCR mutagenesis was used to convert the AATAAT sequence one base upstream of the *lrp* start codon ATG (see Fig. III-4) to AATATT which can be cut by the endonuclease SspI.

```
...CAATATTAatg GT........CTAAGCTT
   SspI   Start          HindIII
```

After PCR, the *lrp* gene carrying an SspI site and a HindIII site could be subcloned readily. Therefore, the SspI and HindIII cut 761 bp *lrp* gene was subcloned into the SmaI and HindIII sites of the polylinker on pBAD18 (4577 bp, Fig. III-2), forming a 5338 bp recombinant plasmid, pBAD*lrp*. Agarose gel electrophoresis showed that the size of the resulting plasmid was 5.3 kb (Fig. III-5). This plasmid is under the name pBAD*lrp*.

### 1-2-3. Transformation of pBAD*lrp* into the strain deficient in *ara* and *lrp* genes

The plasmid pBADlrp was isolated, and transferred into strain LT20 (*araΔ lrp*$^-$) using a selection based on the following principle. Lrp is needed for activation of the

....ACAA

TAATAatgGTAGATAGCAAGAAGCGCCCTGGCAAAGATCTCGACCGTATCGATCGTAACATTCTT
Start

AATGAGTTGCAAAAGGATGGGCGTATTTCTAACGTCGAGCTTTCTAAACGTGTGGGACTTTCCCC

AACGCCGTGCCTTGAGCGTGTGCGTCGGCTGGAAAGACAAGGGTTTATTCAGGGCTATACGGCGC

TGCAAAACCCCCATTATCTGGATGCATCACTTCTGGTATTCGTTGAGATTACTCTGAATCGTGGC

GCACCGGATGTGTTTGAACAATTCAATACCGCTGTACAAAAACTTGAAGAAATTCAGGAGTGTCA

TTTAGTATCCGGTGATTTCGACTACCTGTTGAAAACACGCGTGCCGGATATGGCAGCCTACCGTA

AGTTGCTGGGGGAAACCCTGCTGCGTCTGCCTGGCGTGAATGACACACGGACATACGTCGTTATG

GAAGAAGTCAAGCAGAGTAATCGTCTGGTTATTAAGACGCGCTAACACGGAACAGGTGCAAAATC

GGCGTATTTTGATTACACTCCTGTTAATCCATACAGCAACAGTACTGGGGTAACCTGGTACTGTT

GTCCGTTTTTAGCATCGGGCAGGAAAAGCCTGTAACCTGGAGAGCCTTTCTTGAGCCAGGAATAC

ATTGAAGACAAAGAAGTCACATTGACAAAGTTAAGTAGCGGCCGCGCCCTTCTGGATCGGTGCTG

ATCCTTATTGTCCTGTTTGCCGTCTGGTTGATGGCTGCCTTACTAAGCTT
HindIII

**Fig. III-4 Sequence of the *lrp* coding region.** This sequence was retrieved from Genbank.

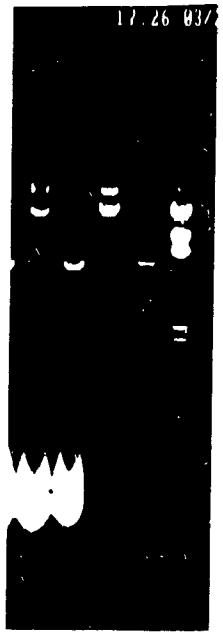



**Fig. III-5 Determination of the size of plasmid pBAD*lrp*.** Cells of strain LT32 (*ara*Δ *lrp*⁻ *gcv::lacZ* pBAD*lrp*) were grown in LB with appropriate antibiotics and the plasmid was isolated. The plasmid was subjected to 1% agarose gel electrophoresis. Lane 1 contains uncut plasmid. Lane 2 contains the same plasmid cut with HindIII. Lanes 3 and 4 contain the original plasmid constructed by Shao, uncut and cut with HindIII respectively. Lane 5 contains lambda DNA cut with HindIII. The samples in lane 3 and 4 were treated with RNase.

glycine cleavage operon, *gcvTHP*, which codes for the enzymes for glycine cleavage. The *lrp*$^-$ mutant is deficient in expression of this gene, and therefore unable to cleave glycine in order to use it as nitrogen source (Lin et al, 1990).

An *lrp*- mutant therefore cannot grow in minimal medium with glycine as nitrogen source. However if it were provided with arabinose to induce Lrp production from the plasmid, it should be able to grow. Since arabinose cannot enter glucose-grown cells, we carried out the transformation on glycerol/arabinose minimal medium with ampicillin and with glycine as nitrogen source. The transformants which appeared on such plates were tested on the same medium, with and without arabinose. Without arabinose, they grew slowly or not at all. With arabinose, they grew as E. coli usually does on glycerol with glycine as nitrogen source.

This indicates that arabinose could be taken up by glycerol-grown cells, and could induce the expression of the plasmid-carried lrp gene. This selected transformant is under the name LT30 (*ara*Δ *lrp*$^-$ pBAD*lrp*).

**1-3. Modulated expression of *lrp* in strain carrying pBAD*lrp***

Before using this system for regulated Lrp production, I

wished to characterize it further.

### 1-3-1. Plasmid sizes and plasmid maintenance

I first wished to verify that the plasmid isolated from strain LT30 (*ara*Δ *lrp*⁻ pBAD*lrp*) had the same size (5300 bp) as the original plasmid. I therefore reisolated the plasmid from LT30 using the plasmid mini-prep method, then linearized plasmid by digestion with HindIII. All of the linear plasmid isolated from individual transformants had the same size, that is 5.3 kb, the size as the original plasmid (Fig. III-5).

If the cells lost the plasmid readily, the arabinose induction would not give reliable results. However in fact the plasmid was rarely lost. To test this, I grew a culture in ampicillin-containing medium, and then diluted and plated on LB without antibiotic. The colonies which grew on LB without antibiotic were tested with and without antibiotic, only 1-2% lost the ability to grow in the presence of ampicillin. It seems then that the plasmid is rarely lost, though this was verified for all the promoter reaction experiments described in this thesis.

### 1-3-2. Verification of the effect of arabinose on Lrp production by direct assay of L-serine deaminase

L-serine deaminase I (L-SD I) encoded by *sdaA* converts L-serine to pyruvate and ammonium in *E. coli*. Synthesis of this enzyme is repressed by Lrp (Lin et al, 1990). Therefore L-SD activity is high in an *lrp*$^-$ mutant, and much lower in the *lrp*$^+$ parent. Because L-SD can be assayed easily, I used these assays to further characterize the system.

To do this, I inoculated cells of strain LT30 pBADlrp. from a recently streaked LB ampicillin tetracycline plate into 0.5% glycerol minimal medium, with and without arabinose, and with and without leucine, shaken overnight at 37$^{o}$C and subcultured in the same medium for 6-7 hours. The cells were then assayed for L-SD activity, which is expressed in these preliminary experiments as absorbance in Klett units for a standard suspension of cells. The results of such an experiment are given in Table III-1. In order to be sure that the strains without the plasmid did not respond to arabinose in the same way, the strains LT10 (*ara*$\Delta$) and LT20 (*ara*$\Delta$ *lrp*$^-$) both with and without leucine (Table III-2).

The result of this experiment demonstrates that the modulated system is satisfactory in its essential characteristics. This conclusion depends on the following

**Table III-1 Effect of arabinose on the L-SD 1 activity of cells carrying pBAD*lrp*.**

| arabinose | L-SD 1 activity | | ratio |
|---|---|---|---|
| (μg/ml) | +leu (A) | -leu (B) | +leu/-leu (C) |
| 0 | 705 | 648 | 1.1 |
| 0.5 | 525 | 359 | 1.5 |
| 1 | 494 | 347 | 1.4 |
| 5 | 450 | 330 | 1.4 |
| 10 | 375 | 225 | 1.7 |
| 50 | 258 | 72 | 3.6 |
| 100 | 353 | 65 | 5.4 |

Cells of strain LT30 (*ara*Δ *lrp*⁻ pBAD*lrp*) were grown in glycerol minimal medium, with leucine (column A) and witout (column B) with the concentrations of arabinose noted. L-serine deaminase was assayed in cells harvested in exponential phase, and expressed in Klett units without further conversion. Column C gives the ratio of column A to column B.

**Table III-2 Demonstration that L-SD 1 activity is not affected by arabinose in strains devoid of plasmid pBAD*lrp***

| strain | arabinose (μg/ml) (A) | leucine (μg/ml) (B) | L-SD 1 activity (C) |
|---|---|---|---|
| LT10 | 0 | 0 | 68 |
| | 100 | 0 | 58 |
| | 0 | 100 | 263 |
| | 100 | 100 | 312 |
| LT20 | 0 | 0 | 432 |
| | 100 | 0 | 465 |
| | 0 | 100 | 515 |
| | 100 | 100 | 568 |

Strains LT10 (*ara*Δ) and LT20 (*ara*Δ *lrp*⁻) were grown, harvested and assayed as in Table III-1.

considerations.

i) Without arabinose, there is no synthesis of Lrp from the plasmid. Thus the L-SD activity of LT30 grown in minimal medium without arabinose (648, Table III-1, (B)) showed like that of LT20 (432, Table III-2, (C)). Synthesis from the plasmid is thus entirely arabinose-dependent.

ii) Addition of arabinose induced synthesis of Lrp when the plasmid was present (Table III-1) but not in isogenic strains lacking the plasmid (Table III-2). This is deduced from the fact that expression of *sdaA* of LT30 in minimal medium greatly decreased in cells grown with as little as 1μg/ml arabinose. Cells grown with 50 μg/ml of arabino showed levels of L-SD equal to those of an *lrp*+ cell (72, Table 1, (B) vs. 68, Table III-2 (C)). Further addition of arabinose did not reduce synthesis further, indicating that there is some expression from *sdaA* which is resistant to Lrp repression.

iii) Leucine regulated *sdaA* expression when arabinose was present just as it does in the parent strain. Leucine is one of the inducers of L-SD synthesis (Lin et al, 1992), perhaps by counteracting Lrp repression. Leucine had no effect when there was no Lrp synthesized (Table III-1 col A/B 1.1), and also had no effect on the control strains (Tables III-2).

However when arabinose was added and Lrp production induced, leucine induced L-SD production. Quantitative details of this will be discussed in a later section.

### 1-3-3. Verification of the sequence near the *ara-lrp* junction

The plasmid pBADlrp seems to work well. To be sure that it had the same sequence as the original plasmid, I wanted to sequence the area of the plasmid from within the *lrp* coding region back into the *araC* gene, that is all the part of the plasmid that might be relevant to the regulation of the *araBAD* promoter.

I determined the sequence in the laboratory of Dr. F. Lang at the University of Montreal, and compared to the sequence provided by John Beckwith and retrieved from Genbank for plasmid pBAD18. No difference between our sequence and Beckwith's was seen, in the regulatory region from the multiple cloning site up to 320 bp, nor was there any change in the first 6 codons of the *lrp* gene (Fig. III-3, 4).

I conclude that the construct I made is the one desired, and that it works well enough to permit the intended experiments. Lrp production is almost nonexistent in the absence of arabinose and is saturated at about 50 μg/ml arabinose.

## 2. Growth characteristics at different Lrp levels

### 2-1. Expression of *lrp* carried on the plasmid pBAD*lrp*

The preceding results indicates that arabinose acts as an inducer of Lrp production in cells carrying the plasmid pBAD*lrp*. To determine the details of Lrp production, I wished to insert a *lacZ* reporter into the beginning of the *lrp* coding region on pBAD*lrp*, and use ß-galactosidase activity as a measure of Lrp production. The details of these experiments are given in the next sections.

#### 2-1-1. Fusion of reporter gene *lacZ* after the start codon ATG of *lrp* on pBAD*lrp*

Since the BamHI GGATCC is compatible with a BglII site, it was possible to cut out *lacZ* with BamH1, ligate it into the unique BglIII site on pBAD*lrp*, and have *lacZ* in the correct reading frame after it is inserted into BglII site. Therefore the ß-galactosidase should be expressed under the control of the $p_{BAD}$ promoter. Since *lacZ* carries a stop codon, so the part of the *lrp* after BglII site will not be expressed. In other words, the inserted plasmid pBAD*lrp::lacZ* expresses ß-gal only.

The recombined plasmid pBAD*lrp::lacZ* was reisolated, cut

by HindIII and checked on agarose gel (Fig III-6). The linear plasmid shows 7.6 kb, which is the right size. The Strain carrying pBAD*lrp::lacZ* is under name LT31 (*araΔ lrp*⁻ pBAD*lrp::lacZ*).

**2-1-2. ß-galactosidase assays of strain carrying pBAD*lrp::lacZ* grown with various levels of arabinose**

To estimate Lrp production from the original pBAD*lrp* plasmid, the production of ß-galactosidase from cells carrying pBAD*lrp::lac* was measured, using cells grown in glycerol with a variety of arabinose concentrations. Table III-3 and Fig. III-7 show the results from such experiments. Each culture tested was plated on LB, then replicated on LB with and without ampicillin to check for plasmid retention. No significant loss of plasmid was seen.

The ß-galactosidase level of cells grown without arabinose (Table III-4) was almost as low as the background level of host MEW1, so it can be concluded that the promoter is almost totally shut off without arabinose. Fig. III-7 shows that the expression of *lrp::lacZ* on the plasmid was approximately proportional to arabinose in concentrations from 0 to 20 μg/ml, and changed very little thereafter, even at arabinose concentrations as high as 1000 or 2000 μg/ml. It seems that about 50 μg/ml arabinose is sufficient to activate

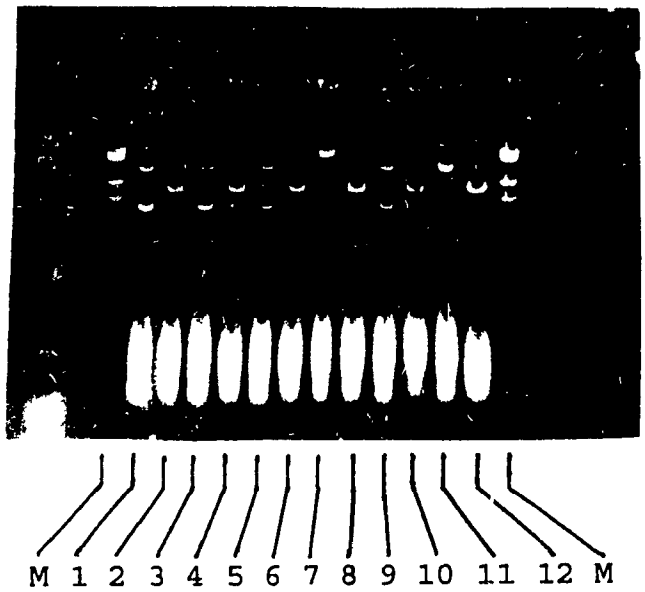



**Fig. III-6 Determination of the size of plasmid pBAD*lrp::lacZ*.** The *lacZ* gene was isolated from plasmid pMC1871 cut with BamH1 and ligated to plasmid pBAD*lrp* cut with BglII. The ligation mixture was transformed into strain LT10 (*ara*Δ) and strain LT20 (*ara*Δ *lrp*$^-$). Plasmid was isolated from 4 transformants of LT20 (lanes 1-8) and 2 transformants of strain LT10 (lanes 9-12) and subjected to electrophoresis on 1% agarose. Odd-numbered lanes represent uncut DNA; even-numbered, DNA cut with HindIII. Lane M contains lambda DNA digested with HindIII.

**Table III-3 Expression of *lrp* under the control of the $p_{BAD}$ promoter**

| arabinose (μg/ml) | ß-galactosidase activity (Miller unit) | SEM |
|---|---|---|
| 0 | 20 | 7.2 |
| 1 | 225 | 60.3 |
| 5 | 573 | 190.8 |
| 10 | 1138 | 162.3 |
| 20 | 2258 | 388.0 |
| 30 | 2301 | 400.4 |
| 40 | 2550 | 695.1 |
| 50 | 2642 | 617.8 |
| 1000 | 2426 | 500.8 |
| 2000 | 2557 | 496.1 |

ß-galactosidase produced by *lacZ* inserted into the *lrp* gene carried on the pBAD plasmid was assayed in exponential-phase cells of strain LT31 (*ara*Δ *lrp*$^-$ pBAD*lrp::lacZ*) grown in glycerol minimal medium with the concentrations of arabinose noted. The result is the average of the data from 3 replicates.

Fig. III-7 Expression of lrp::lacZ

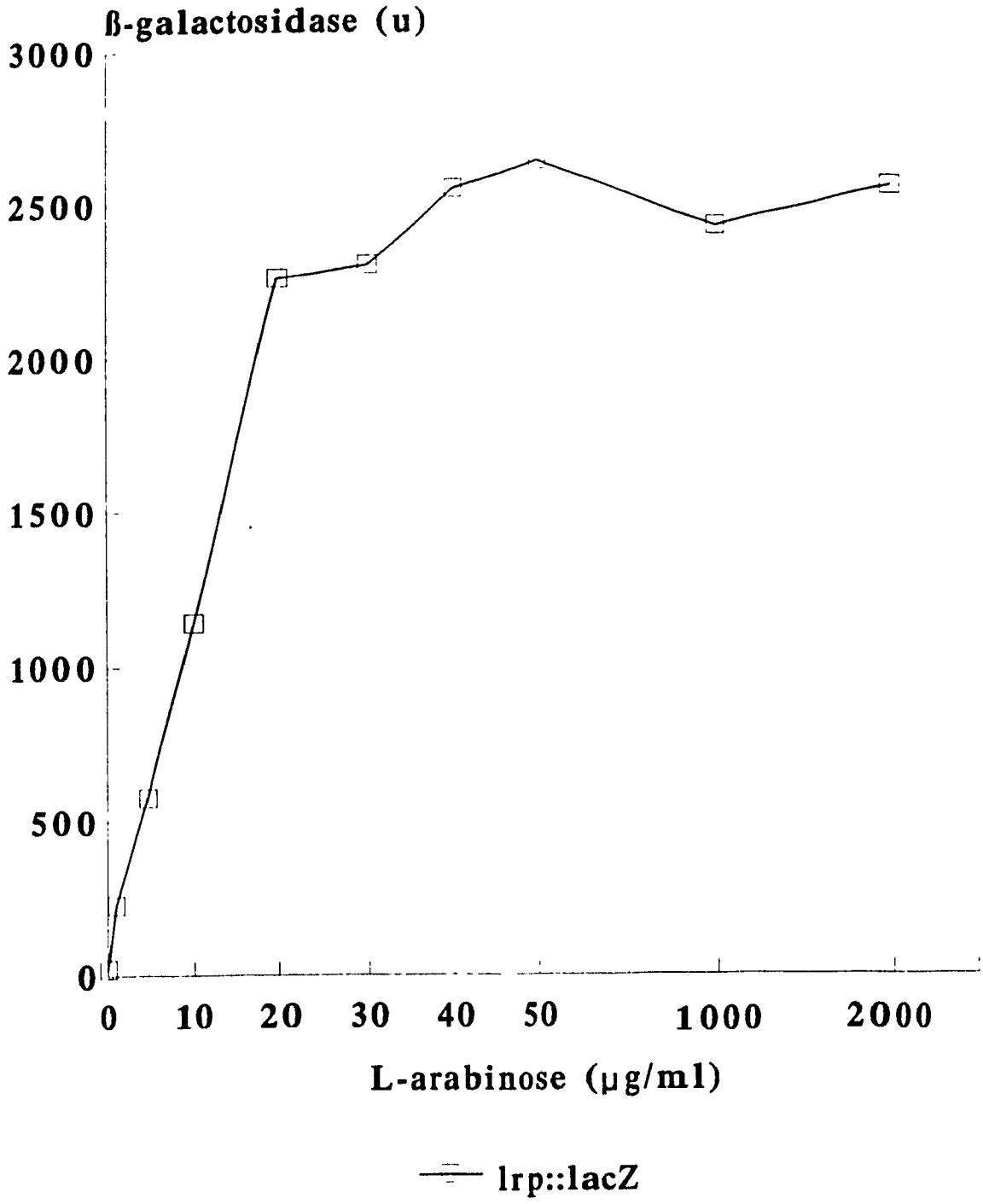


**Fig. III-7 Expression of *lrp* under the control of the $p_{BAD}$ promoter.** Graphical representation of the data presented on Table III-3.

**Table III-4 Expression of pBAD*lrp::lacZ* in the absence of arabinose**

| Expt. No. | ß-galactosidase activity |
|---|---|
| 1 | 15 |
| 2 | 12 |
| 3 | 28 |
| 4 | 19 |
| 5 | 18 |
| 6 | 19 |
| 7 | 19 |
| 8 | 19 |
| 9 | 16 |
| 10 | 24 |
| 11 | 25 |
| average | 20 |
| SEM | 4.6 |

ß-galactosidase from all assays in the absence of arabinose are grouped in this table.

the promoters on all the plasmids in the cell, and that addition of further arabinose therefore had no effect.

### 2-1-3. Induction of expression of *lrp* on pBAD*lrp* in rich medium

The expression of *lrp* on the plasmid was also tested in rich medium with a series of concentrations of arabinose. The induction of the ß-galactosidase level of LT31 in LB depended on the culture density as can be seen in Table III-5. At low turbidity, cultures showed very little activity, 48 units at 50 μg/ml arabinose, compared to 2650 in minimal medium with the same arabinose concentration. The activity increased about four-fold later in the growth phase. However this was still only 10% of the value seen in minimal medium. Use of this system in LB will rarely be useful- perhaps because arabinose does not enter the cell, or because something in LB inhibits induction by arabinose.

## 2-2. Growth at different Lrp concentrations

### 2-2-1. Assessment of arabinose toxicity

The physiological importance of Lrp, governing as it does expression of so many operons, is reflected in the fact that the *lrp*$^-$ mutant grows considerably more slowly than its wild

**Table III-5 Expression of *lrp* from pBAD*lrp* in cells grown in rich medium**

| arabinose (μg/ml) | OD600 (A) | ß-gal | OD600 (B) | ß-gal |
|---|---|---|---|---|
| 0 | .300 | 10 | 1.353 | 10 |
| 1 | .322 | 8 | 1.402 | 6 |
| 5 | .365 | 11 | 1.416 | 50 |
| 10 | .322 | 22 | 1.308 | 140 |
| 30 | .314 | 39 | 1.214 | 217 |
| 50 | .298 | 48 | 1.157 | 205 |
| 2000 | .330 | 52 | 1.106 | 187 |
| 5000 | .298 | 55 | 1.137 | 153 |
| 50000 | .132 | 32 | .738 | 112 |

This experiment was done as in Table III-3 but the cells grown in LB. In this experiment, cells were assayed in cultures at low turbidity (column A) and later, at high turbidity (column B).

type parent in minimal medium (Lin, et al, 1992). A deficiency of Lrp thus slows growth. However, an excess of Lrp might also interfere with growth. At high Lrp concentration, the cells might shut off necessary genes or turn on deleterious ones, causing physiological chaos in the cells.

To investigate this, I grew cells of strain LT30 (*ara*Δ *lrp*$^-$ pBAD*lrp*) in glycerol minimal medium, and subcultured them in the same medium but with various concentrations of arabinose, following turbidity as a function of time. Cells with 50 μg/ml arabinose grew as well as cells without arabinose. This represents the highest level of *lrp* expression obtained in the assay of LT31 (see Fig. III-7). It seems then that neither arabinose nor Lrp is toxic to these cells, at least at the levels tested. This was verified in the same experiments for the strains from which LT30 is derived, i.e. LT10 (*ara*Δ) and LT20 (*ara*Δ *lrp*$^-$).

According to the growth curves the doubling times of LT30 with different arabinose concentrations was determined (Table III-6). The growth of strain LT30 with arabinose was a little faster than it was without arabinose, but not close to that of the parent stain LT10. The interpretation of these results is however a little less than straightforward. One would normally expect that arabinose would increase the growth rate, since it increases the production of Lrp, and the Lrp-producing wild-

**Table III-6 Assessment of arabinose/Lrp toxicity tc strain carrying pBAD*lrp***

| arabinose (μg/ml) | doubling time |
|---|---|
| 0 | 120 |
| 1 | 120 |
| 5 | 105 |
| 10 | 105 |
| 20 | 105 |
| 20 | 105 |
| 30 | 105 |
| 50 | 120 |
| 1000 | 110 |
| 2000 | 150 |

Strain LT30 (*ara*Δ *lrp*$^-$ pBAD*lrp*) was grown in glycerol minimal medium and subcultured into the same medium with a variety of concentrations of arabinose. Turbidity was measured hourly and the apparent doubling time calculated for the exponential phase of the curve. These may be compared with the apparent doubling times for the parent strains without plasmid LT10 (*ara*Δ), LT20 (*ara*Δ *lrp*$^-$) and LT20 grown with glycine and leucine: 80, 140 and 105 min.

type cell normally grows faster. The slow growth of the mutant with arabinose is not clear.

### 3. Regulatory patterns of the expression of three genes of the leucine/Lrp regulon

The effect of Lrp on expression of various genes of the leucine/Lrp regulon, such as *gcv*, *gltD* and *sdaA*, has been well documented by comparing levels of expression in the *lrp*$^-$ mutant and its parent. However that shows expression only with and without Lrp. In these experiments, I investigate the effects of intermediate levels of Lrp.

#### 3-1. Construction of the strains

P1 transduction were employed to transduce chromosomal fusions of *lacZ* to *gcv*, *gltD* and *sdaA* into the chromosome of LT30, selecting for kanamycin-resistance conferred by the λp*lac*Mu insert. Transductants were selected on glycerol minimal medium with antibiotics and Xgal, with 50 μg/ml arabinose for the Lrp activated genes and without arabinose for the Lrp repressed genes, *sdaA*. Blue colonies were purified and checked for the phenotypes expected for each of the target genes.

### 3-2-1 Expression of *gltD* as affected by arabinose/Lrp concentration

*gltD* is one of the genes in *gltBDF* operon, it encodes one of the subunits of glutamate synthase. The expression of *gltD* is activated by Lrp, and this involves direct binding of Lrp to the upstream region as judged by gel retardation. Externally supplied leucine decreases the affinity of Lrp for the promoter (Ernsting et al, 1993)

As expected from this in vitro characterization of the *gltD* operon,when tested in my experimental system, *gltD::lacZ* expression increased with arabinose concentration from 0 to 20 μg/ml arabinose and levelled off thereafter. That is, *lrp* production from pBAD*lrp* increased with arabinose concentration and this resulted in increased expression of the chromosomal *gltD::lacZ* fusion.

Three such experiments are represented in Figure III-8, 9 and 10. There is a considerable difference in the level of expression between experiment C and A (or B). However the trend is the same in all cases and the expression saturates at around the same arabinose concentration (Fig. III-8). The reason for the variability in total expression is not clear.

These experiments indicate that variation of Lrp in vivo

**Fig. III-8 Expression of gltD::lacZ without leucine**

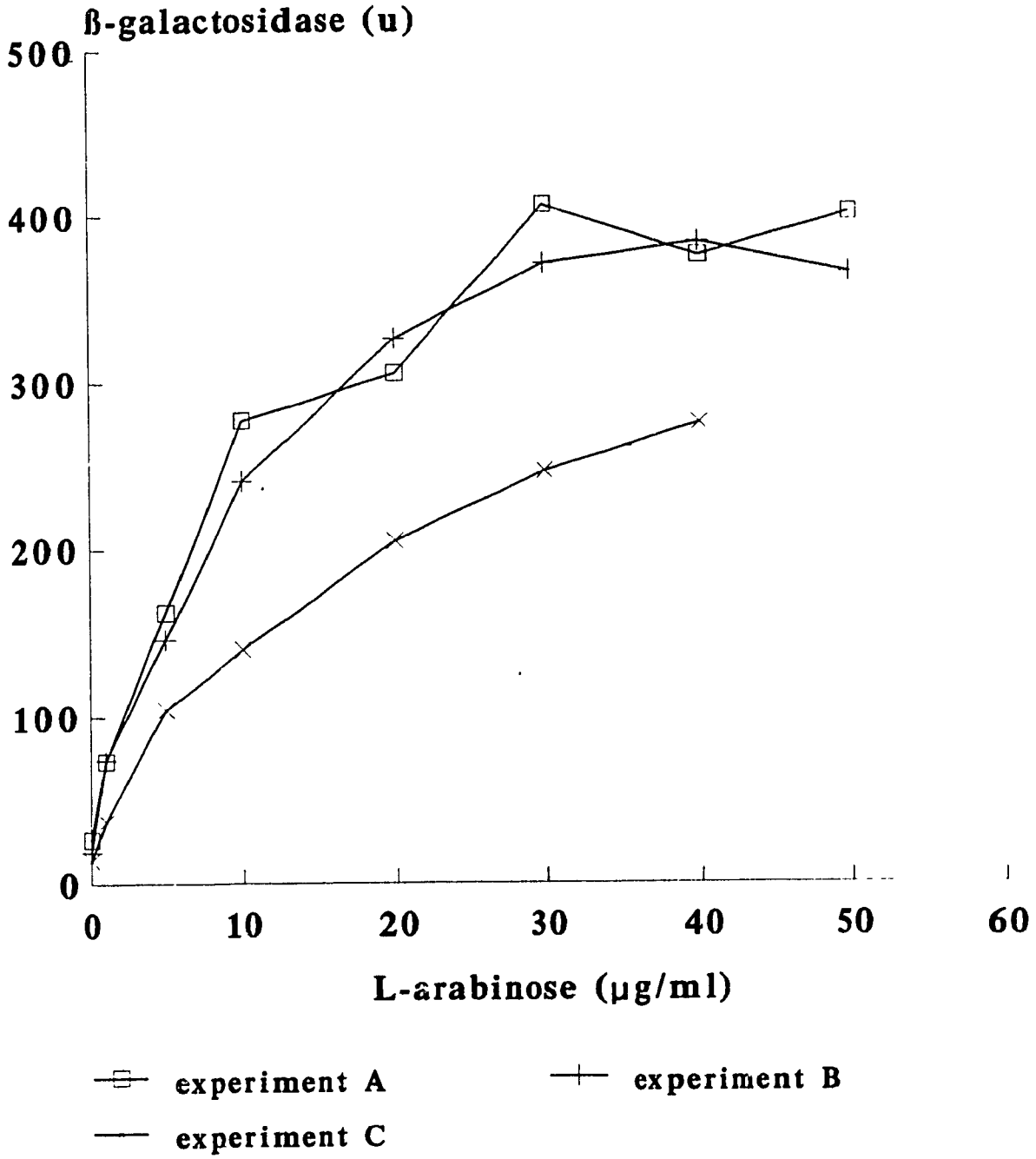


**Fig. III-8 Effect of arabinose/Lrp on the expression of** ***gltD::lacZ***. Graphical representation of the experiment (A), (B) and (C) in which strain LT33 (*ara*Δ *lrp*$^-$ *gltD::lacZ* pBAD*lrp*) was grown in glycerol minimal medium without leucine and ß-galactosidase was assayed as in table III-3.

Fig. III-9 Expression of gltD::lacZ
(Expt. A)

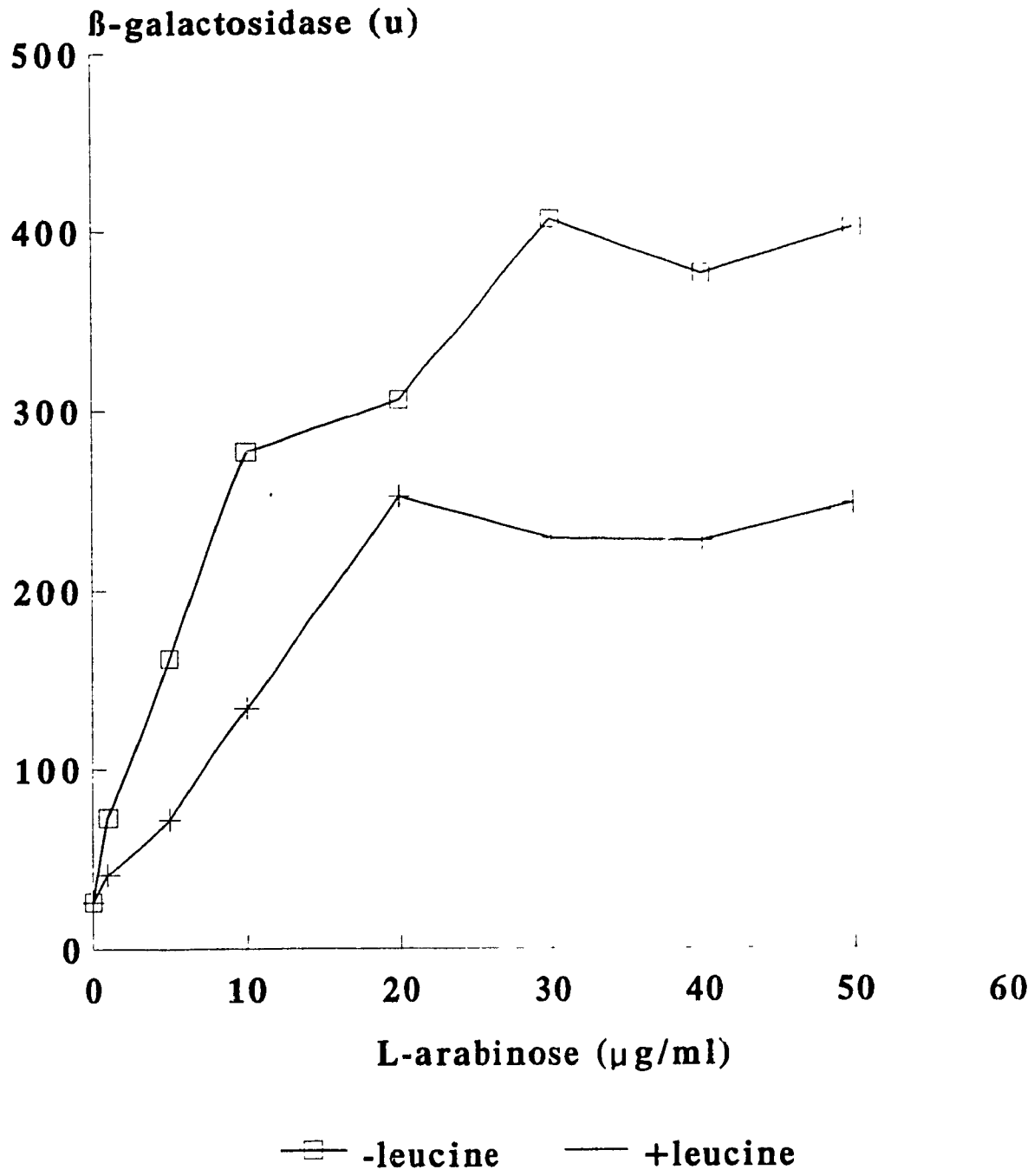


Fig. III-10 Expression of gltD::lacZ
(Expt. B)

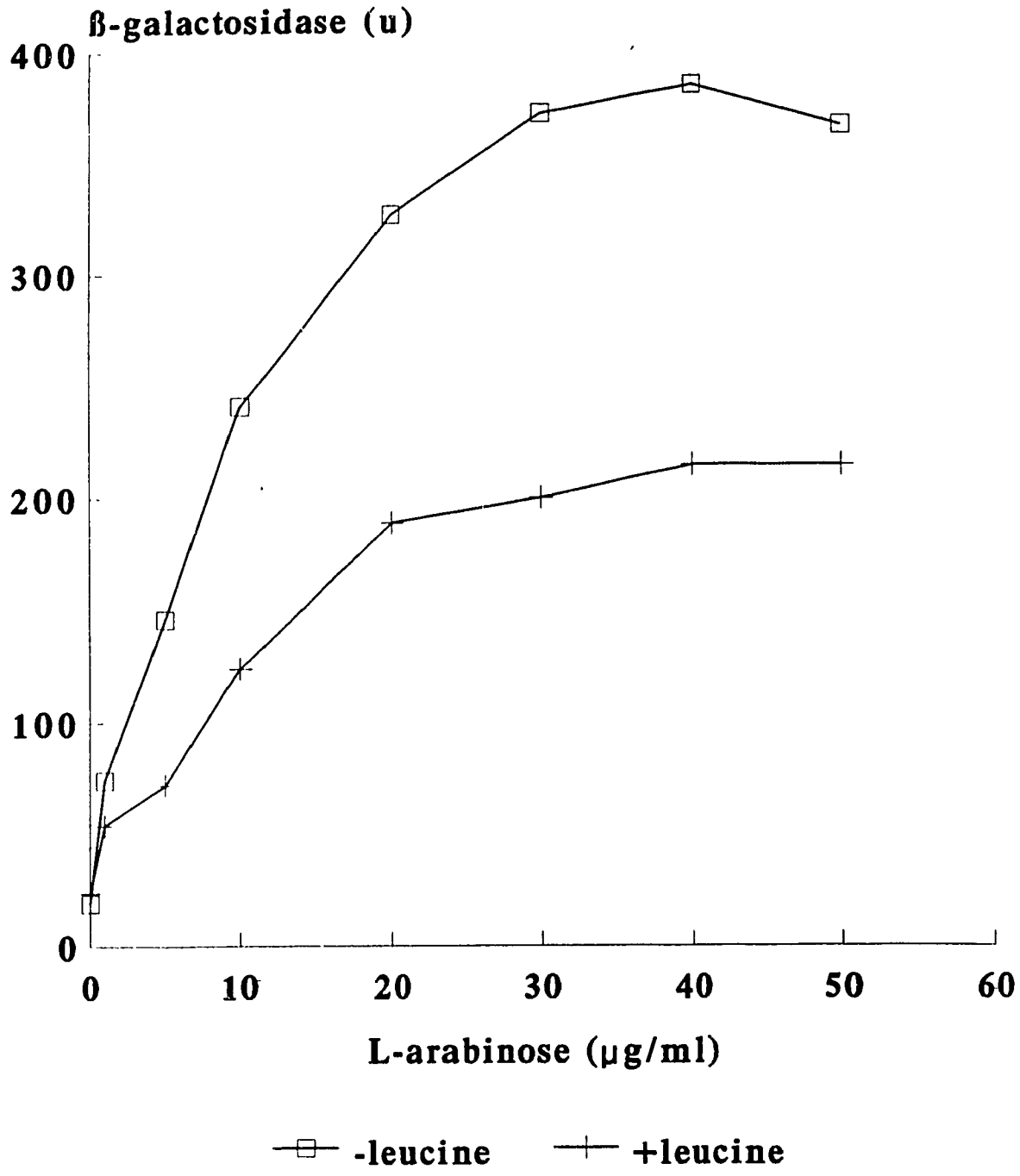


**Fig. III-9, 10 Effect of leucine on the expression of** *gltD:lacZ*. Graphical representation of experiment (A) and (B) with and without addition of leucine.

**Table III-7 Expression of gltD::lacZ in strains not carrying pBAD*lrp***

| Strain | ß-galactosidase activity | | |
|---|---|---|---|
| | (A) | (B) | (C) |
| Cp8 | 298 | 282 | 225 |
| Cp8+leu | 294 | 174 | 131 |
| Ca8 | 26 | 17 | 16 |
| Cp8 in LB | 29 | 41 | 25 |

Strain Cp8 (*gltD::lacZ*) and its *lrp*$^-$ derivative strain Ca8 were grown in glycerol minimal medium and ß-galactosidase measured as in the preceding table. Three replicates are given for each in order to estimate the variability of the assay in non-plasmid carrying strains.

**Table III-8 Effect of culture density on the expression of *gltD::lacZ*.**

| $OD_{600}$ (1) | ß-gal activity (2) | $OD_{600}$ (3) | ß-gal activity (4) |
|---|---|---|---|
| 0.370 | 22 | 0.187 | 362 |
| 0.587 | 23 | 0.331 | 396 |
| 0.889 | 22 | 0.405 | 383 |
| 1.243 | 23 | 0.784 | 396 |
| 1.542 | 26 | 1.065 | 414 |

Cells of strain Ca8 (*lrp*$^-$ *gltD::lacZ*) (column 1, 2) and LT33 (*ara*Δ *lrp*$^-$ *gltD:lacZ* pBAD*lrp*) (column 3, 4) were grown in glycerol minimal medium and samples taken at the optical densities noted (column 1, 3) and assayed for ß-galactosidase (column 2, 4).

causes a variation in *gltD* expression. In two cases a parallel series of flasks was grown in the presence of 100 μg/ml leucine. As seen in Fig. III-9 and 10, this decreased *gltD* expression by about 50%. This is similar to the effect of leucine on *lrp*$^+$ cells (Ernsting et al, 1993) and occurs at all Lrp concentrations, which may indicate a direct interaction between leucine and Lrp. As controls, Cp8 and Ca8 were also tested (Table III-7), and culture density had no effect on the expression of *gltD::lacZ* (Table III-8).

### 3-2-2. Expression of *gcv* as affected by arabinose/Lrp concentration

The *gcvTHP* operon, mapped at 62 min on *E. coli* chromosome, encodes genes which code for the enzymes responsible for glycine cleavage, GcvT, GcvH and GcvP. This operon is negatively regulated by PurR, and positively directly regulated by Lrp. Lrp is required for expression of *gcv*.

The level of expression of *lacZ* in strain LT32 (*ara*Δ *lrp*$^-$ *gcv::lacZ* pBAD*lrp*) increased in the same manner as did that of *gltD*, but at somewhat lower arabinose concentration (Tables III-9, 10 and Fig. III-11). Some expression was seen even without Lrp, about 80 units of ß-galactosidase. Addition of 1 μg/ml arabinose raised this to between 610 and 793 units. Again there was considerable variability between replicates,

**Table III-9 Effect of arabinose/Lrp on the expression of *gcv::lacZ***

| arabinose | -leu | | +leu | |
|---|---|---|---|---|
| (μg/ml) | ß-gal | SEM | ß-gal | SEM |
| 0 | 78 | 7.8 | 89 | 10.2 |
| 1 | 707 | 9.2 | 527 | 207.8 |
| 5 | 888 | 4.6 | 759 | 100.6 |
| 10 | 1165 | 126.7 | 887 | 213.8 |
| 20 | 1306 | 124.0 | 953 | 282.9 |
| 30 | 1229 | 118.7 | 833 | 2.8 |
| 40 | 1363 | 250.1 | 1107 | 244.1 |
| 50 | 1269 | 219.7 | 1065 | 253.2 |
| 100 | - | - | 1117 | 200.5 |

Strain LT32 (*ara*Δ *lrp*$^-$ *gcv::lacZ* pBAD*lrp*) was grown and treated as in Fig III-8, 9, 10. The result is the average of the data from 3 replicates of the experiment with and without leucine.

Fig. III-11 Expression of gcv::lacZ

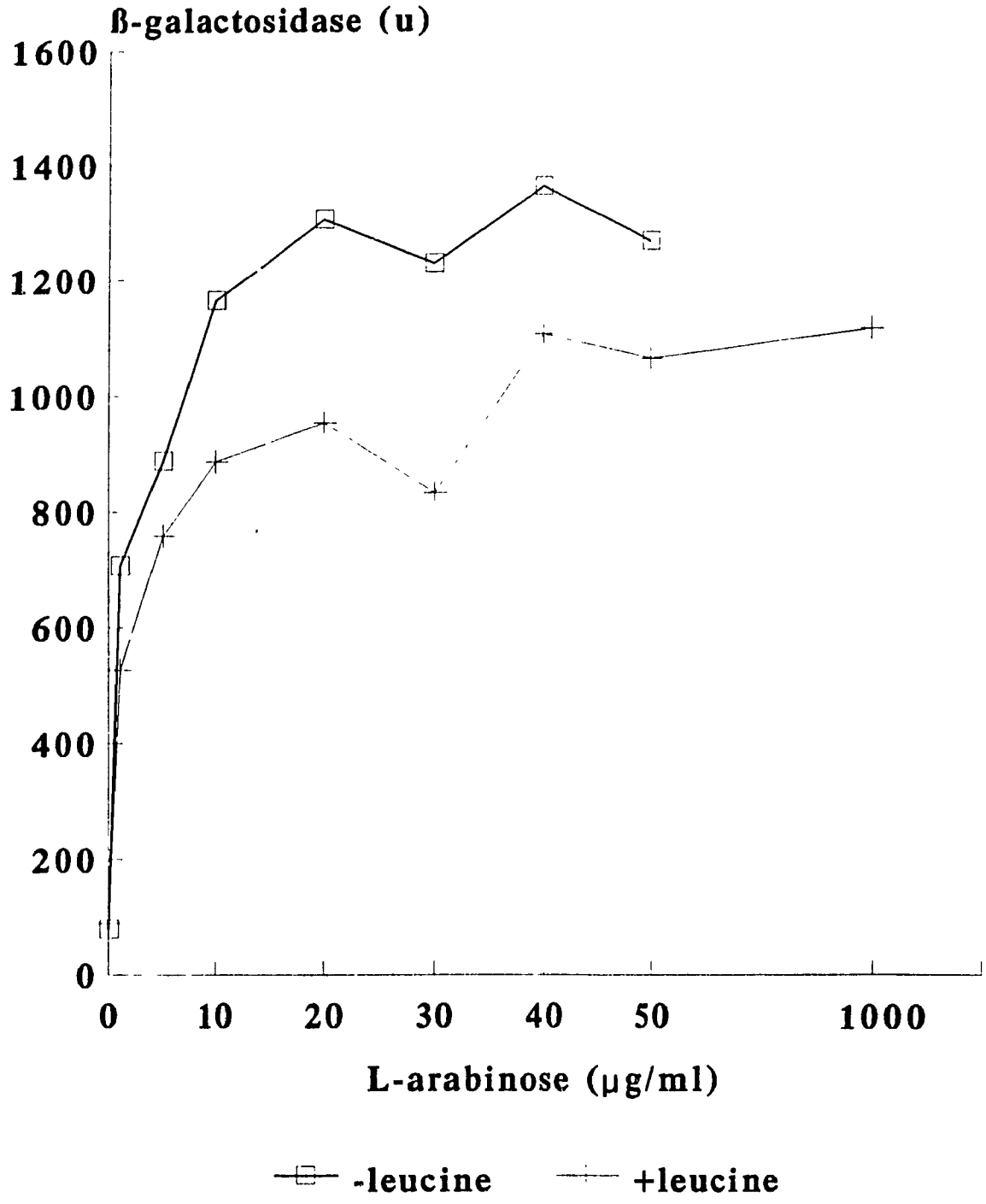


**Fig. III-11 Effect of arabinose/Lrp on the expression of** ***gcv:lacZ*****.** Graphical representation of data on Table III-9.

**Table III-10 Expression of *gcv::lacZ* in strains not carrying pBAD*lrp***

| strain | ß-galactosidase activity | SEM |
|---|---|---|
| Cp67 | 1478 | 650.0 |
| Cp67+leucine | 1447 | 439.0 |
| Ca67 | 81 | 20.6 |
| Cp67 in LB | 317 | 156.9 |

Strain Cp67 (*gcv:lacZ*) and its *lrp*$^-$ derivative strain Ca67
were grown in glycerol minimal medium, ß-galactosidase measured and the data presented as in Table III-7.

**Table III-11 Effect of culture density on the expression of *gcv::lacZ***

| $OD_{600}$ (1) | ß-gal (2) | $OD_{600}$ (3) | ß-gal (4) | $OD_{600}$ (5) | ß-gal (6) |
|---|---|---|---|---|---|
| 0.182 | 96 | 0.201 | 677 | 0.241 | 1872 |
| 0.272 | 96 | 0.346 | 604 | 0.424 | 1684 |
| 0.396 | 93 | 0.442 | 878 | 0.642 | 1826 |
| 0.542 | 116 | 0.679 | 928 | 0.933 | 1760 |
| 0.722 | 111 | 0.887 | 920 | 1.197 | 1976 |

Cells of strain LT21 (*ara*Δ *lrp*$^-$ *gcv::lacZ*) (column 1, 2) and its derivative carrying plasmid pBAD*lrp* (column 3, 4, 5, 6) were grown in glycerol minimal medium with arabinose 0 μg/ml (column 1, 2), 1 μg/ml (column 3, 4) and 20 μg/ml (column 5, 6) and assayed as in Table III-8.

but the trend was always the same, as was the increased sensitivity. Moreover, Fig. III-11 demonstrates that leucine lowered the expression of *gcv* by about 20%.

It is clear that the expression of *gcv* increases with increased arabinose/Lrp. However the analysis is complicated by the fact that gcv expression also varies during growth of a culture, increasing as the culture becomes more dense (Lin et al, 1992). This effect could also be seen in my experiments when the data was analyzed as a function of density of the culture at the time of harvesting. This was not however a large enough effect to obscure the general trend of a response to arabinose.

I assessed the effect of growth phase by growing strain LT32 with 1 μg/ml and 20 μg/ml arabinose and assaying ß-galactosidase as a function of culture turbidity (Table III-9). This experiment was done only once, and so has not been used to correct the data presented earlier. However it is clear that at low arabinose, the gene is not fully expressed, and expression increases up to 50% by the time the culture grows from 0.201 to 0.887. However at a saturating level of arabinose, no dependence on turbidity was seen.

I investigated this further by growing the parent strain without the plasmid- i.e. a totally Lrp deficient strain LT21

(*ara*Δ *lrp*⁻ *gcv::lacZ*) and assayed ß-galactosidase as a function of turbidity. When the culture was assayed at an $OD_{600}$ of 0.182, it showed 95 units of ß-galactosidase (Table III-9). This increased to 110 at O.D. 0.722. There may then be a very minimal effect independent of Lrp, and it would be worthwhile assaying this at higher turbidity as was done in the original study. In any case, Lrp is essential for almost all transcription from *gcv*, though its effect may be modified by other factors.

I examined whether expression from *gltD* varies with turbidity. Though this experiment was done only once, it is clear that it does not (Table III-8).

### 3-2-3. Expression of *sdaA* as affected by arabinose/Lrp concentration

Gene *sdaA* encodes L-serine deaminase I (L-SD I) for converting L-serine to pyruvate and ammonium. This gene is repressed by Lrp so that its level is increased about 10-fold in the Lrp mutant in glucose minimal medium, allowing the mutant to use serine as carbon source. Many factors intervene in the regulation of L-SD production including heat shock, anaerobiosis and UV irradiation (Su et al, 1989). Leucine decreases the effect of Lrp, and thus appears as an inducer in vivo.

**Table III-12 Effect of arabinose/Lrp on the expression of *sadA::lacZ***

| arabinose | -leu | | +leu | |
|---|---|---|---|---|
| (μg/ml) | ß-gal | SEM | ß-gal | SEM |
| 0 | 337 | 33.8 | 361 | 36.8 |
| 1 | 311 | 36.9 | 345 | 39.6 |
| 5 | 317 | 31.9 | 310 | 5.0 |
| 10 | 255 | 33.7 | 258 | 1.4 |
| 20 | 154 | 39.1 | 153 | 17.0 |
| 30 | 94 | 23.6 | 116 | 40.3 |
| 40 | 77 | 18.7 | 133 | 21.9 |
| 50 | 115 | 79.8 | 143 | 74.2 |
| 1000 | 55 | 16.3 | 122 | 2.1 |
| 2000 | 62 | 17.0 | 110 | 6.4 |

Strain LT34 (*ara*Δ *lrp*$^-$ *sdaA::lacZ* pBAD*lrp*) was grown in glycerol minimal medium with arabinose with and without L-leucine and ß-galactosidase assayed as in Fig. III-8, 9, 10. The result is the average of the data from 3 replicates of the experiment without leucine and 2 replicates of the experiments with leucine.

Fig. III-12 Expression of sdaA::lacZ

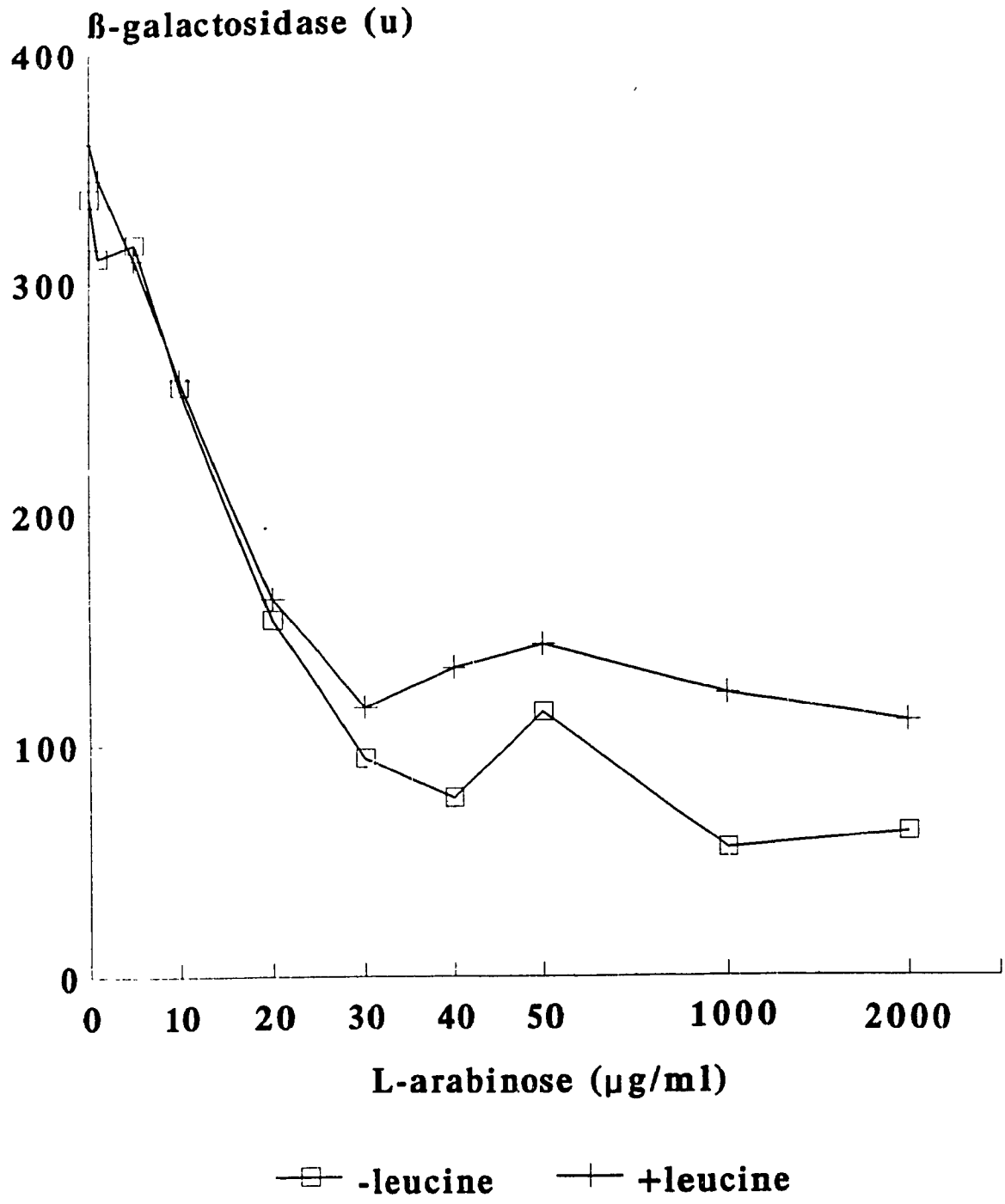


**Fig. III-12 Effect of arabinose/Lrp and Leucine on the expression of *sdaA:lacZ*.** Graphical representation of the data on table III-12.

**Table III-13 Expression of *sdaA:lacZ* in strains not carrying pBAD*lrp***

| Strain | ß-galactosidase activity | SEM |
|---|---|---|
| Cup22 | 110 | 96.3 |
| Cup22+leucine | 86 | 19.8 |
| Cap22 | 299 | 96.4 |

Strains Cup22 (*sdaA:lacZ*) and its *lrp*$^-$ derivative strain Cap22 were tested as on Table III-7.

As the arabinose concentration was increased, the expression of *lacZ* by strain LT34 (*araΔ lrp⁻ sdaA::lacZ* pBAD*lrp*) decreased, as would be expected for a repressed gene(Table III-12 and III-13 and Fig. III-12). Repression was not seen at low concentrations of arabinose which were sufficient for almost complete expression from *gcv*. The reproducibility of these experiments was rather better than that of the other two genes as seen on Table III-12.

Leucine increased *lacZ* expression somewhat. In glucose-minimal medium, leucine has a stronger effect on *sdaA* expression. However it is not known whether this is also true in glycerol minimal medium.

### 3-3. Comparison of the regulation of *gcv*, *gltD* and *sdaA*

It is clear that expression or repression of these 3 genes is affected by externally provided arabinose, and it is also clear that the sensitivity of the three genes varies a great deal. Variability of the data makes comparison difficult. However I made an average of the data to plot them together in figure Fig. III-13.

Those curves before Fig. III-13 are plotted against arabinose concentration. However Lrp concentration is not a linear function of arabinose concentration. In Figure III-13

Fig. III-13 Comparison of expression of gcv::lacZ, gltD::lacZ and sdaA::lacZ

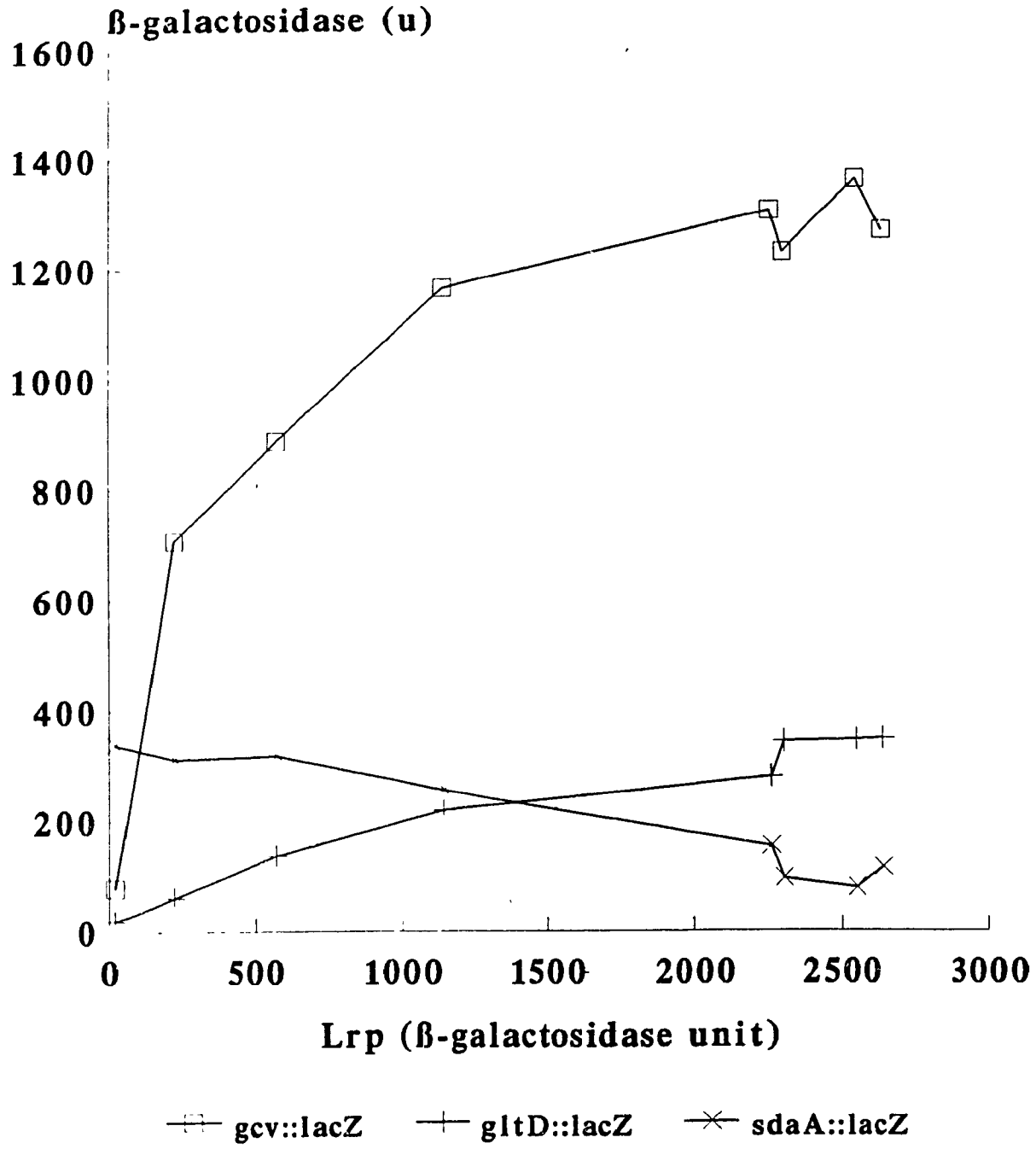


**Fig. III-13 A comparison of the effect of Lrp on expression of 3 genes.** In this graphical transformation of preceding data, the arabinose concentrations are converted into ß-galactosidase units of Lrp amounts according to the data on Table III-3.

Fig. III-14 Percentage of expression of gcv::lacZ, gltD::lacZ and sdaA::lacZ

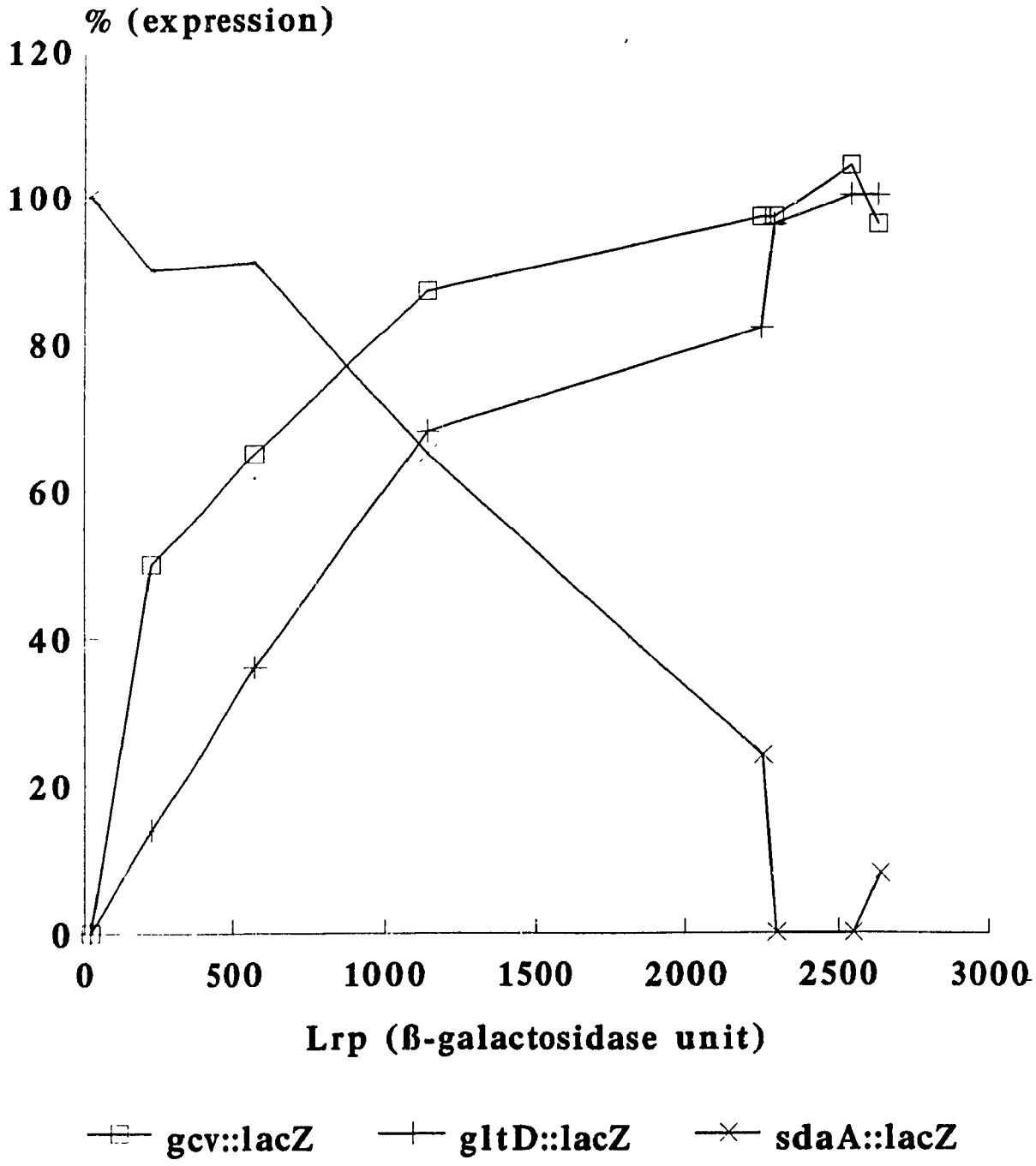


**Fig. III-14 A comparison of the effect of *lrp* on the degree of regulation of 3 genes.** In this graphical transformation of Fig. III-13, the data for each determination is expressed as the percentage change in expression seen at saturation.

I have plotted the data against Lrp concentration, using the data of Table III-3 to make the conversion. I have then made this somewhat clearer by expressing the data for each gene as a per cent of the total Lrp effect, as judged by taking an average of the readings at 40 and 50 μg/ml arabinose as full expression (Fig. III-14).

From this curve it is possible to make an estimate of the amount of Lrp needed for 50% expression of each gene. Admitting the variability in the data, the insufficient number of data points, and the approximation of the calculation, it seems that it requires much less Lrp to activate *gcv* than *gltD*, and less to activate *gltD* than to repress *sdaA* (more in Discussion section).

**4. A method to search for new gene regulated by Lrp**

The number of Lrp regulated genes has been estimated at between 30 and 100 (Newman, 1992) of which most have not been identified. Lrp-regulated genes can be discovered readily by making λp*lac*Mu insertions in a strain deficient in Lrp production, but carrying the pBAD*lrp* plasmid, and screening for arabinose/Lrp regulated genes.

I made a preliminary study to demonstrate the validity of this approach. Strain LT30 (*ara*Δ *lrp*$^-$ pBAD*lrp*) was infected

with λ*plac*Mu9 with the help of λ*plac*507, the phage used in earlier studies (Bremer et al, 1985, Lin et al, 1992). The infected cells were plated on glycerol minimal medium with Xgal with and without arabinose, and colonies tested on both media. Blue colonies were purified, and cells from each of the purified strains were suspended in minimal medium and plated on glycerol Xgal plates with and without arabinose. After 24 hours, the Lrp regulated genes inserted with λ*plac*Mu could be identified in comparison of the colours between the two plates with and without arabinose.

3 genes out 550 inserts with Kan resistance have been readily screened as the Lrp regulated gene using this method. These strains could be further tested for expression of ß-galalactosidase in liquid medium, partially characterized, and identified with the use of inverse PCR as described by Tchetina (1995).

This method identified genes in which a deficiency does not prevent growth in minimal medium. One could do similar studies to identify genes in which a deficiency does prevent growth in minimal medium if one could find a gratuitous inducer analogous to IPTG which was efficient in LB.

# IV. DISCUSSION

## 1. Construction of Lrp modulation system

### 1-1. Indications that pBAD*lrp* serves the propose intended

The intent of this work was to clone the *lrp* gene on a well-controlled variable expression vector, and study the effects of different intracellular concentrations of Lrp on the expression of target genes, and on cell function. For this work, the transcription of the vector-carried gene must be turned off under one set of conditions, expressed well in another, expressed variably as a function of some external, controllable signal, and the vector must be stably maintained. These aspects are discussed in the following sections.

#### 1-1-1. Is the $p_{BAD}$-carried *lrp* gene transcribed in the absence of arabinose?

Guzman et al (1995) reported that transcription from the *araBAD* promoter is completely shut off in glucose-minimal medium and close to zero in glycerol minimal medium without arabinose. This is in keeping with the descriptions of the function of the *ara* operon in vitro. Indeed this vector was chosen for this work because it was thought to be one of the least leaky (reviewed in introduction).

In my work some slight expression from this promoter could be detected. Patches of strain LT31 (*araΔ lrp*⁻ pBAD*lrp::lacZ*) appeared very pale blue on glycerol minimal medium with X-gal even without arabinose, though only after more than 3 days incubation at 37°C.

This low-level leakage is acceptable for this work. Strain MEW1, from which the *lacZ* gene has been deleted, shows 12-15 units of ß-galactosidase activity. Strain LT31 grown without arabinose gave values from 12 to 28, with an average of 20 (Table III-4). So the system without arabinose is very close to being totally shut off, and it can be seen that this level can be practically regarded as zero on Fig. III-7.

This leakage is sufficiently insignificant that the Lrp-regulated genes are not affected by it. In the absence of arabinose, the expression of *lacZ* fused to any of *gcv*, *gltD* and *sdaA* was very similar in strains devoid of chromosomal *lrp* gene function, whether they contained pBAD*lrp* or not. That is, any transcription of pBADlrp in the absence of arabinose was insufficient to alter transcription of the target genes (Table IV-1).

**Table IV-1 An estimate of the expression of pBAD*lrp* in the absence of arabinose**

| Strain | | ß-galactosidase activity |
|---|---|---|
| Cp67 | (A) | 82 |
| LT32 | (B) | 89 |
| Ca8 | (A) | 22 |
| LT33 | (B) | 25 |
| Cap22 | (A) | 299 |
| LT34 | (B) | 337 |

ß-galactosidase activity is listed for strains carrying *gcv::lacZ*, *gltD::lacZ* and *sdaA:lacZ* without (A) and with (B) pBAD*lrp*. The figures reported are the average of the determinations listed in earlier tables.

**1-1-2. Is the expression of *lrp* on the plasmid high enough when the promoter $p_{BAD}$ was fully turned on?**

Transcription of *lrp::lacZ* from the plasmid looks saturated at an external arabinose concentration around 50 μg/ml (Fig. III-7). The cells grow as well with 50 μg/ml or higher arabinose as with less- but transcription is not increased. The rate of Lrp production at this external arabinose concentration may represent the limit of the capacity of this promoter.

However, some indication of arabinose toxicity was observed during the construction and purification of the system carrying the plasmid pBAD*lrp*, suggesting that some copies of the plasmid might mutate to counteract that. After purification the toxicity disappeared, possibly because the mutated plasmid with higher expression of *lrp* caused the toxicity, could not be maintained in the cell. The promoter region of the plasmid pBAD*lrp* isolated from the stable cells was sequenced and no change was seen in the sequenced region in comparison with that from the literature.

It might be interesting to determine the effect of still

higher levels of Lrp on the target gene. To do that, one would need to strengthen the promoter by mutation, or to use another vector. Since the target genes saturated at close to their in vivo levels in wild-type cells, this is not a pressing problem. However one could do this by plating. One can plate LT33 (*ara*Δ *lrp*$^-$ *gltD::lacZ* pBAD*lrp*) on glycerol minimal medium with X-gal and a lower arabinose concentrations, then try to select very dark colonies, which might carry a plasmid with higher expression of *lrp*.

**1-1-3. Is the system stable and reproducible?**

Variability was a major problem in this work. The absolute values varied a great deal. However, the response to increasing arabinose was always clear. Some factors which may affect the variation are discussed bellow.

i) Loss of the plasmid from host cells

If some fraction of the host cells in a population lose their plasmid, expression of the plasmid-carried gene will appear low. This can happen particularly with plasmids carrying ampicillin resistance, because the fraction of the cells which still carry the plasmid will degrade the ampicillin via ß-lactamase, and allow the plasmid-free cells

to grow.

If a proportion of the cells lose the plasmid pBAD*lrp*, the apparent expression level of the Lrp activated genes will be decreased in proportion. I tested culture samples to assess plasmid loss, and in general there was little loss, 2-4%. However in experiments where the ß-galactosidase level was lower than expected, I often found 20-40% plasmid loss. Plasmid loss is the main reason for the variation of the measurement in this system. However it is not clear what governs the extent of plasmid loss.

ii) Multiple factors in the regulation of genes of the Lrp regulon.

Genes are frequently regulated by several effectors- e.g. regulation of the maltose operon by both MalT and Crp. If Lrp is not the only factor affecting a given gene, then variation in measurements may be due to variation in a second factor. This is clear for the case of the *gcv* gene which is regulated by a number of factors. As described earlier, and also demonstrated here, Lrp is required for activation (Lin et al, 1992). A second factor, GcvA activates *gcv* expression in the presence of exogenous glycine and repressed *gcv* in the presence of exogenous purines without glycine(Wilsom et al, 1993). A third regulator, PurR, a repressor protein involved

in negatively regulating purine nucleotide synthesis, also is involved in negative regulation of the Gcv enzyme system (Wilson et al, 1993).

In experiments of the type reported here, it is usually assumed that conditions are kept constant so that only the factor of interest, here Lrp concentration, varies. The earlier study showed that *gcv* activity in *lrp* wild type had a significantly increased level at higher culture density (Lin et al, 1992).

I tested *gcv* activities at different culture densities at various Lrp concentrations, and found that the *gcv* activity levels was considerably affected. This may also be the part of the reason for the variability. (Table III-9).

**1-2. Problems in using pBAD*lrp***

The pBAD vector is useful in that it is not leaky, it is easily modulated according to the external arabinose concentration and therefore convenient to use, and usually not extensively lost from the cells.

It is unfortunate that the system cannot be induced during growth in glucose minimal medium, because so many other physiological experiments are carried out with glucose as

carbon source. However glucose excludes arabinose efficiently from the cell (Miyada et al, 1984). No gratuitous inducer has been described.

I did not pursue the use of this plasmid in LB in detail. Production of Lrp from pBAD*lrp* was not seen early in exponential phase, but did appear later. This may be due to factors in LB which exclude arabinose or prevent activation. In any case, there are too many other factors in LB to allow a simple analysis of promoter function by Lrp.

Measurements in glycerol minimal medium were quite satisfactory. However the growth rate of the cells in glycerol minimal medium is slower than in glucose, and therefore the experiments are longer.

**1-3. A comparison between out experiments with pBAD*lrp* and those of the original investigators**

A series of $p_{BAD}$ vectors were constructed in Dr. Beckwith's laboratory at the Harvard Medical School (Guzman et al, 1995). All these vectors use the promoter $p_{BAD}$ and *araC* gene, but with different antibiotic resistance genes and different cloning sites for different purposes. In their work, they showed that 1) repression $p_{BAD}$ was rapid and efficient, 2) the promoter $p_{BAD}$ had a very fast induction rate, 3) the

ratio of repression /induction from $p_{BAD}$ vectors was high, and 4) the *araC*-$p_{BAD}$ system could be modulated. They also reported that this system was used to study the null mutations of essential genes, such as *gtsQ*, *ftsL*, *ffh*, *ftsI* and *secEDF*.

Here, I compare my results with theirs concerning the modulation controlled by inducer arabinose. They use *phoA* as a reporter gene to monitor the expression controlled by promoter $p_{BAD}$. The arabinose concentrations they used ranged from 0 to 2000 μg/ml, which is the same as mine. The expression controlled by promoter $p_{BAD}$ increased greatly from 0 to 20 μg/ml, slightly with the arabinose concentrations higher than 20 μg/ml. The results about the expression induced by arabinose are very close between theirs and mine. But I showed that the expression reached saturation in presence of 50 μg/ml arabinose (Fig. III-7), while they reported that the expression increased at even high arabinose concentrations

## 1-4. Other possible experiments using promoter $p_{BAD}$

### 1-4-1. Characterization of other Lrp-like proteins

Lrp proteins with as high as 87-91% conservation have been described in other microorganisms *S. typhimurium*, *K. aerogenes*, *S. marcesens* and *E. aerogense* (Calvo, 1994). It might be interesting to compare their efficiency in *in vivo*

regulation and relate this to structural difference between the proteins by cloning them into pBAD vectors.

### 1-4-2. Studies of possible relationships between the global response regulators

i) Lrp and H-NS

H-NS, encoded by *hns* at 27 min, is a histone-like protein in *E. coli*. It is a neutral protein with strong DNA binding affinity and a well-conserved amino acid sequence between the *E. coli* and *S. typhimurium* protein. (Schmid, 1990). H-NS plays an important role in DNA compaction and transcription. It functions directly as a transcriptional repressor for some promoters, so it is also a global response regulator, the controlling factor of the H-NS regulon (Ueguchi et al, 1993).

I tried to construct a double mutant *lrp*$^-$*hns*$^-$, but could not. It seems that *lrp*$^-$ and *hns*$^-$ are not compatible. It should however be possible to transduce a *hns* mutation into an *lrp* mutant carrying pBAD*lrp* as long as arabinose is provided to the cell. One could then study the effects of withdrawing arabinose- i.e. the effects of the double mutation.

One could moreover put the two regulators into the same cell each on its own variable promoter, and study effects of

variation in each factor. Indeed, pTRPhns has been constructed in Mizuno's lab (Ueguchi et al, 1993).

ii) Lrp and Crp

As mentioned in Intrduction section, Crp is a crucial global responsive transcriptional regulator. So it will be very interesting to observe how the cell behaves after modulations of the two important regulators Lrp and Crp.

## 2. Effects of modulation of intracellular Lrp concentration on expression of genes regulated by Lrp

### 2-1. Reactions of the different promoters to Lrp

The Lrp molecule regulates expression of a large number of genes. These genes do not all necessarily have the same response to Lrp, so that at any particular concentration of Lrp, they may not be equally affected. This is, of course, true of all regulators. For example, the affinity for Crp of *lac* promoter is one of the strongest, and some functional sites exhibit a 50-fold lower affinity than does lac (Kolb, et al, 1993).

The expression from a given promoter, then, will depend on

the Lrp concentration, the concentration of effectors of Lrp like leucine, and the concentrations of other regulatory proteins and their effectors. Thus, even if Lrp regulates 30 or more genes, a change in Lrp concentration would not have equal effects on all the genes. Therefore it becomes very interesting to understand how each individual promoter reacts to different concentrations of the global regulator, in as quantitative a manner as is possible.

Preliminary experiments indicated that the curve of gene expression against Lrp concentration might have two or more slopes. The genes seem to respond to very low levels of Lrp with great sensitivity. As the concentration of Lrp increases, the effect on gene expression is much smaller. However the reproducibility of experiments at low arabinose concentrations was much less than that at higher concentrations- for reasons that are not at all clear.

I estimated the amount of Lrp needed for 50% of maximal expression or repression, $A_{Lrp(50)}$

I define $A_{Lrp(50)}$ as:

$$A_{Lrp(50)} = U_{\beta\text{-gal}}(Lrp) \text{ at } (U_{\beta\text{-gal}(100)} - U_{\beta\text{-gal}(0)}) / 2.$$

Here, $U_{\beta\text{-gal}(100)}$ is the ß-gal units of full expression or

repression of the tested gene, $U_{ß\text{-}gal(0)}$ is the ß-gal units of the tested genes in the absence of Lrp.

According to Fig. III-14, $A_{Lrp(50)}$ can be calculated graphically by extrapolating from the horizontal phase of the curve, noting the gene expression that corresponds, halving that number and noting the Lrp concentration that corresponds, therefore,

$A_{Lrp(50)}$ (*gcv*) = 250 ß-gal units

$A_{Lrp(50)}$ (*gltD*) = 800 ß-gal units

$A_{Lrp(50)}$ (*sdaA*) = 1500 ß-gal units

$A_{Lrp(50)}$ presents the characteristic of the promoter tested. The lower the $A_{Lrp(50)}$ is, the more sensitive is the promoter to the Lrp. The $A_{Lrp(50)}$ (*gcv*) is the lowest one of the promoters tested, it shows that *gcv* promoter is the most sensitive to Lrp.

**2-2. Regulatory patterns of the Lrp regulated genes**

As stated earlier, the pBAD promoter is not leaky, and the expression of target genes in a lrp$^-$ strain carrying pBADlrp but grown without arabinose is very similar to the expression in an lrp mutant without plasmid. (Table III-3, IV-1). For

Lrp activated genes, there are some basic levels, *gltD* with 20 and *gcv* with 80.

As showed by the regulatory pattern curve of *gcv*, *gcv* expression rises up sharply in the presence of a few Lrp molecules, and continue to increase the expression with more Lrp. This demonstrates how important the *gcv* products is while the cell is at the state of insufficient Lrp. However, the expression of *gltD* goes up gently with the increases of Lrp. Lrp controls the lower level of *gltD* product in the cells

For *sdaA*, the Lrp repressed gene, a high level of the product of this gene presents in cell without Lrp. And *sdaA* activity decreases slowly while Lrp increases in the cell, leaving some basic level when it is fully repressed.

**2-3. Leucine effect on the expressions of Lrp regulated genes**

The expression levels, differences, and leucine effects of the operons *gcv*, *gltD* and *sdaA* in presence of Lrp, with and without leucine are listed on Table IV-2. The data on the table indicates that leucine has different effects on the expression of different operons. Although the difference of the expression of *gcv* without and with leucine is large (-250), the leucine effect is not so huge (-19.%) in consideration of its expression level without leucine (1300).

Therefore, this work clearly proves the leucine effect on *gcv* operon at least in glycerol minimal medium. However, Table IV-2 shows that leucine effect on *gltD* operon is significant (-41%) on the lower basis of expression level without leucine (390). Similarly, leucine has great effects on the expression on *sdaA* (83%). Although the available regulatory pattern curves are limited now, the results presented here suggest that leucine has significant effect on the lower expression operons.

**Table IV-2 Effect of leucine on the expression of 3 genes**

| operon effect | ß-galactosidase | | difference | leucine |
|---|---|---|---|---|
| | -leu | +leu | -leu and +leu | % |
| *gcv* | 1300 | 1050 | -250 | -19.2 |
| *gltD* | 390 | 230 | -160 | -41.0 |
| *sdaA* | 60 | 110 | 50 | 83.3 |

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