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In vivo studies of CRP and LRP in the regulation of *sdaA*, *serA* and *metK* genes in *Escherichia coli* K-12

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Abstract

In vivo studies of CRP and LRP in the regulation of *sdaA*, *serA* and *metK* genes in *Escherichia coli* K-12

Jie Ping Ding

The *sdaA* gene of *Escherichia coli* encodes L-serine deaminase (L-SD), which degrades L-serine to ammonia and pyruvate. LRP represses the expression of both L- SD and *sdaA*. CRP was assumed to affect *sdaA* transcription, but this had not been proven. I demonstrated here that both CRP and LRP were involved in L-SD and *sdaA* expression. LRP repressed L-SD expression and *sdaA* transcription. CRP activated L-SD expression depending on the presence of LRP but did not affect *sdaA* transcription.

The genes for *serA* and *metK* are known to be regulated by LRP and are proposed to be regulated by CRP. CRP activated the *serA* P2 promoter. However, it did not affect *serA* expression overall. CRP was also involved in the regulation of *metK* expression through an unclear mechanism.

By providing *ptsG* expression on a plasmid, I identified one factor causing the slow growth rate of *crp* mutants. The rate of glucose uptake was suggested to affect the growth rate of *crp* mutants.

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Introduction

Catabolite repression has been studied for nearly a century. Cyclic AMP (cAMP), made by adenylate cyclase, encoded by the *cya* gene, and CRP (cAMP receptor protein), a global regulatory protein encoded by the *crp* gene, have been identified and recognized as the principal means of effecting catabolite repression. CRP has been purified and its role as a regulator of the transcription of several promoters related to catabolism has been investigated. It is now recognized that CRP is not only involved in the regulation of several catabolic functions, but also possesses other functions unrelated to catabolism (reviewed in Botsford and Harman, 1992).

The *Escherichia coli (E.coli)* LRP (Leucine-responsive Regulated Protein) regulon was discovered in 1990 (Lin *et al.*, 1990). Since then, more than 30 genes/operons have been identified as members of this regulon family (reviewed in Newman *et al.*, 1996). LRP regulates the transcription of a number of genes by activating their expression including *serA*, *ilvIH*, *gcv*, *gltD*, *ompF*, etc. while repressing the expression of others, for example, *sdaA*, *ompC* and *lysU* (Newman *et al.*, 1995; 1996).

SdaA, an *E.coli* structural gene which encodes L-serine deaminase 1 (L-SD 1), requires LRP for gene repression. LRP is proposed to act at the transcriptional level although more evidence is needed to support this hypothesis. In addition, it has been suggested that CRP is involved in the regulation of *sdaA* gene expression (Su *et al.*, 1991).

This project was intended to answer the following questions: 1). Is CRP involved in the regulation of L-serine deaminase (L-SD) activity and/or *sdaA* gene expression? 2). If so, what is the mechanism involved? 3). Is LRP involved in the regulation of *sdaA* gene expression at the transcriptional level? 4). Is CRP also involved in the regulation of other LRP-regulated regulons (e.g. *metK* and *serA*)?

The project is divided into three sections: 1). Construction of *crp* mutants of *E.coli* and a study of the expression of L-SD and *sdaA* using these mutants. 2). Investigation and comparison of LRP-regulated gene (*sdaA*, *serA*, *metK*) expression in *crp* mutants. 3). Investigation of other characteristics of *crp* mutants, e.g. the effects of *crp* on the growth rate of *E.coli*.

Before presenting my work, I will review the relevant literature. In Part 1, I survey the CRP global regulatory systems and discuss the possible mechanisms of these systems. In Part 2, the LRP regulon is introduced and in Part 3, operons that are regulated by both the cAMP-CRP complex and the LRP regulon are reviewed in more detail. In Part 4, I introduce the regulation of L-SD and the molecular mechanism underlying the regulation of *sdaA* gene expression by the LRP regulon. At the end of the introduction, I describe two other LRP-regulated genes, *serA* and *metK*.

PART 1: The cAMP-CRP (cAMP receptor protein) regulon

1-1. Catabolite repression, cAMP and CRP

When grown in the presence of glucose, many microbes are unable to metabolize a number of other sugars concurrently. This is because during growth on glucose, the synthesis of enzymes that degrade other carbon sources (such as lactose, maltose, mannitol and arabinose) is repressed. This phenomenon of repression was first known as the 'glucose effect'. Further studies showed that this repression is not specific to glucose. The presence of other substrates in the growth medium that can be rapidly metabolized exerts similar but not identical repression. These observations led to the conclusion that under growth conditions that result in more catabolism than anabolism, the synthesis of enzymes involved in other catabolic pathways is repressed. This phenomenon is termed 'catabolite repression' (reviewed in Milton *et al.*, 1996).

The cytoplasmic concentration of cAMP was shown to vary inversely with growth rate when the carbon source was varied. CRP was subsequently shown to be involved in this catabolite repression. Mutants lacking the cAMP biosynthetic enzyme, adenylate cyclase (*cya* mutants), or CRP (*crp* mutants) could not utilize most carbon sources, such as lactose, maltose, and mannitol.

1-2. The role of the cAMP-CRP regulon in the transcription activation

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

E.coli CRP (also known as the catabolite activator protein, CAP) is a classic global regulon. It has become a classic model system for structural and mechanistic studies of transcription activation.

Transcription activation of the simplest CRP-dependent promoters by CRP requires only three macromolecular components: CRP, RNA polymerase (RNAP) and the DNA promoter. CRP-dependent promoters can be organized into two groups: simple CRP-dependent promoters and complex CRP-dependent promoters depending on the number of CRP molecules and/or the presence of other activator molecules required for transcription. Simple CRP-dependent promoters can be sub-grouped into two classes based on their CRP binding sites on promoter DNA (Busby and Ebright, 1999). The components of CRP-dependent promoters and classifications of transcription activation will be reviewed in section 1-2-1 and 1-2-2.

1-2-1. Macromolecular components required for transcription activation of the simplest CRP-dependent promoters

1-2-1-a. CRP

CRP is a dimer of two identical subunits (Kolb *et al.*, 1993a). Each subunit consists of two domains: an N-terminal domain and a C-terminal domain. The N-terminal domain is responsible for dimerization of CRP and for its interaction with the effector cAMP, which binds to CRP and induces a conformational change that results in a

conformation competent for DNA binding. The C-terminal domain is responsible for interaction with the promoter DNA. CRP recognizes a 22-bp. 2-fold-symmetric DNA site with consensus sequence 5'-AAATGTGATCTAGATCACATTT-3' (Botsford and Harman. 1992).

1-2-1-b. RNA polymerase (RNAP)

RNAP contains the subunit composition $\alpha_2\beta\beta'\sigma$ (Chamberlin. 1976). The α subunit is responsible for recognition of the UP element. a supplementary promoter element located 35bp upstream of the translation initiation site in certain promoters (Ross *et al.*. 1993). and responds to a large subset of activators. repressors. elongation factors. and termination factors (Busby and Ebright. 1994). The α subunit N-terminal domain (αNTD) contains the primary determinant for dimerization of the α subunit and interaction of this subunit with the remainder of RNAP and its activators. The α subunit C-terminal domain (αCTD) contains a secondary. weak determinant for dimerization of α and interacts with DNA. activators. repressors. elongation factors. and termination factors. The linker between αNTD and αCTD is flexible. allowing αCTD to occupy different positions relative to αNTD and thus relative to the remainder of RNAP in different transcription complexes (Busby and Ebright. 1994).

The β and β' subunits are responsible for the catalytic activity of RNAP and a subset of activators. repressors. elongation factors. and termination factors (Nechaev and Severinov. 1999).

The σ^{70} subunit is required for recognition of the promoter –10 and –35 promoter elements and a subset of activators (Gross *et al.*, 1998).

1-2-1-c. CRP-dependent promoters

CRP-dependent promoters can be organized into two groups (or three classes) (Uchida & Aiba, 1990; Ebright, 1993).

Group I: simple CRP-dependent promoters (Class I and Class II):

i) Class I: The DNA binding site of CRP is located upstream of the RNAP binding site, which requires only CRP for transcription.

ii) Class II: It contains a single CRP DNA binding site overlapping the RNAP binding sites and requires only CRP for transcription.

Group II: complex CRP-dependent promoters (Class III):

iii) Class III: Multiple activator molecules are required for its full transcription activation, e.g. one or more CRP molecules with regulon-specific activator complexes.

1-2-2. Transcription Activation by CRP

1-2-2-a. Transcription activation at Class I CRP-dependent promoter

CRP alone is sufficient for the initiation of transcription catalyzed by RNAP. The paradigm for this is the lac operon. cAMP-induced binding of one CRP dimer centres between base pairs –61 and –62(61.5) and recruits the binding of RNAP to the promoter. Activation region 1(AR1) of CRP contacts a site located in the C-terminal domain of the α subunit of RNAP. The simplest model suggests that on the lac promoter, CRP recruits the α subunit of RNAP to bind its downstream sequence, and this contact guides the RNAP into place such that contact with both the –10 and –35 regions of the promoter can then be made.

1-2-2-b. Transcription activation at Class II CRP-dependent promoters

CRP can directly activate the Class II CRP promoter. The CRP dimer centres at base pairs –41.5 of the galP1 promoter. The AR1 region of the upstream subunit of the CRP dimer interacts with the αCTD subunit of RNAP, whereas the activation region 2 (AR2) of the downstream subunit of CRP interacts with the αNTD of RNAP. The interaction between AR1 and α-CTD directly increases the affinity of RNAP for the galP1 promoter, while the interaction between AR2 and αNTD functions at a step subsequent to the initial binding of RNAP to the promoter (Niu *et al.*, 1996; Rhodius and Busby, 1998).

1-2-2-c. Transcription activation at Class III CRP-dependent promoters

In certain circumstances, two or more CRP dimers can synergistically activate transcription at some CRP-dependent promoters (Joung *et al.*, 1993). A second transcription activator also regulates many CRP-dependent promoters. A well-studied model is the malK promoter, which requires the binding of maltose-induced MalT to both proximal and distal sites, and CRP binds to a number of sites in between. In the absence of CRP, maltotriose-MalT binds to three upstream promoter sites of malK, where transcription activation cannot take place. CRP binding triggers a repositioning of MalT, and in turn initiates transcription initiation, and ensures that malK expression is co-regulated by CRP and malT (Vidal-Ingigliardi and Raibaud, 1991.)

1-3. cAMP and CRP

The expression of lactose, galactose and arabinose operons requires both cAMP and CRP for transcription initiation. In an *in vitro* purified system of transcription (consisting of lac DNA, RNAP, nucleoside triphophate and ATP), lac mRNA could not be synthesized from lac DNA unless both cAMP and CRP were provided. Similar results were found in gal DNA (Nissley *et al.*, Parks *et al.*, 1971).

The accepted model suggests that when cytoplasmic concentrations of cAMP are high, cAMP associates with CRP to form a complex with a conformation different from that observed for the unliganded form. It can then bind to specific DNA sequences that normally occur near or within the promoters of operons included in the cAMP-CRP modulon.

However. CRP alone can achieve cAMP-independent suppression of the *cya* mutation. In this model. cAMP is no longer needed for CRP function. Therefore. a *cya* mutant carrying such a *crp* mutation (known as *crp**) is able to use lactose and maltose as carbon sources (Melton *et al.*, 1981).

1-4. Deficiencies in *crp* mutants are not limited to carbohydrate utilization: slow growth rate with glucose

1-4-1. Glucose and glucose uptake system

E.coli grown on glucose shows the highest growth rate. Glucose entering the cell is transported by the phosphotransferase system (PTS). This system consists of two common cytoplasmic proteins (enzyme I and HPr). and an array of sugar-specific enzyme II complexes (EIIs). The phosphoryl group from PEP is sequentially transferred to enzyme I. Hpr. EIIs and finally to the substrate as it is translocated across the cell membrane. The glucose-specific EIIs (glucose transporter) consists of the cytoplasmic protein IIAglu and the membrane receptor IICBglu (Figure. 1).

Glucose enters the cells by either of two transporters: the glucose-specific enzyme IIBCglu. a product of the *ptsG* gene located at 25min on the *E.coli* chromosome. or the so-called mannose PTS EIIAB. IIC. IIDman encoded by the *manXYZ* genes, located at 41min. The latter complex shows wide specificity. and transports many sugars including glucose. mannose. fructose. N-acetylglucosamine and glucosamine. Transport of glucose

by either of these systems produces intracellular glucose 6-phosphate, the central compound of intermediary metabolism and the starting point of the trunk route for energy production by glycolysis. Non-phosphorylated glucose can also enter the cell but is converted to Glc-6-P in the cytoplasm (Postma *et al.*, 1996).

1-4-2. cAMP-CRP complex, glucose and regulation of *ptsG* gene

Glucose stimulates transcription of many promoters. For example, the expression of the pts operon, which is composed of *ptsH*, *ptsI*, and *crr*, is known to be stimulated by external glucose (*PtsH* encodes HPr and *ptsI* encodes IIAglu). Glucose also stimulates the expression of the *ptsG* gene. Both the cAMP-CRP complex and Mlc (mlc = make large colonies) proteins are involved in the regulation of the *ptsG* gene at the level of transcription (Kimata *et al.*, 1998; Plumbridge, 1998). cAMP-CRP acts as an activator and Mlc protein as a repressor. cAMP-CRP complex primarily binds to –40.5 (thus defining *ptsG* as a typical type II (class II) CRP-dependent promoter), stimulates RNAP binding to the *ptsG* promoter region, thus activating the expression of *ptsG*. Mlc binds to a region encompassing –20 to +5 of the *ptsG* promoter and inhibits *ptsG* transcription by preventing RNAP binding. The function of Mlc is dominant over that of cAMP-CRP. Glucose induces *ptsG* expression by affecting the function of the Mlc protein. Thus, when glucose is present in the medium, *ptsG* gene expression is increased although the level of cAMP-CRP is limited. The induction of *ptsG* expression requires both glucose

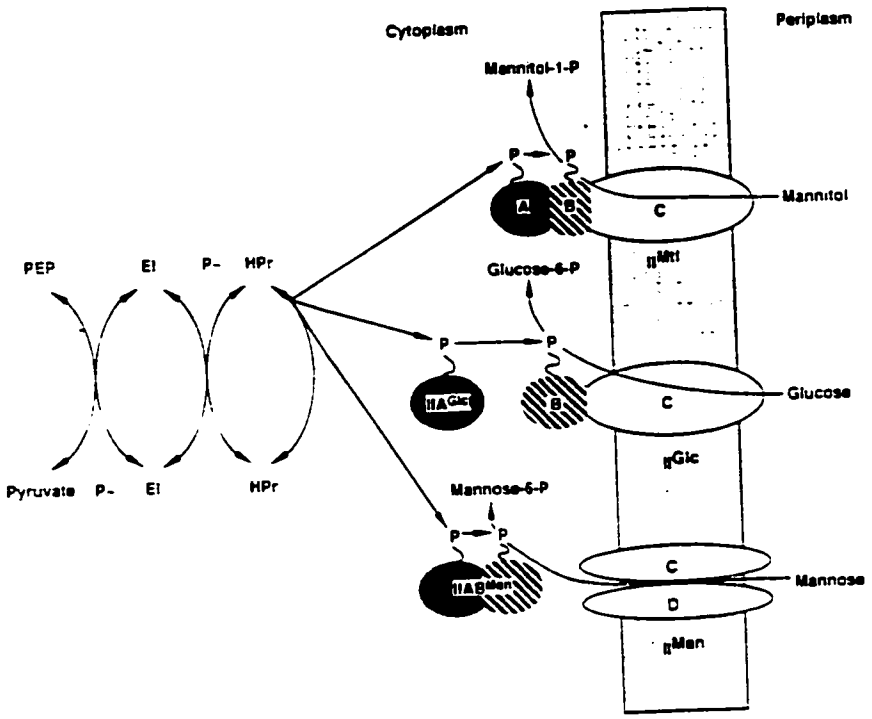

Figure 1: Glucose uptake system in ***Escherichia coli***.

(taken from ***Escherichia coli*** and ***Salmonella***: cellular and molecular biology. 2nd ed. American Society for Microbiology Press. Washington, D.C.)

and cAMP-CRP. In glucose minimal medium, the level of *ptsG* gene expression is proposed to be lower in a *crp*⁻ environment than in a *crp*+ environment.

Part 2: The LRP regulon

LRP (Leucine-responsive Regulatory Protein), encoded by the *lrp* gene located at 20min of the *E.coli* chromosome, is a recently recognized global regulator of metabolism in *E.coli*. It affects the expression of many genes, either negatively or positively. In general, LRP stimulates functions related to biosynthetic pathways, but represses those related to catabolic pathways. L-leucine can potentate or overcome the effects of LRP, and in some *lrp*-regulated promoters it has been shown to have no effect at all (reviewed in Calvo and Matthews 1994; Newman *et al.*, 1996).

LRP is composed of 163 amino acid residues, and exists as a dimer in solution (Platko and Calvo, 1993). The LRP molecule consists of three domains: a DNA binding domain, a transcription activation domain, and a leucine response domain overlapping the transcription activation domain (reviewed in Calvo and Matthews, 1994; Newman *et al.*, 1996).

A potential consensus sequence for LRP binding has been proposed: YAGHAWARRWTDCTR (Y=C/T, H=not G, W=A/T, D=not C, R=A/G) (Cui *et al.*, 1995). This sequence is found upstream of some LRP-regulated genes (e.g. *ilvIH*, *lysU*,

and *tdh*) but not others (e.g. *serA*, *sdaA*, and *livJ/K*). Therefore, it is still under debate whether LRP binds to a specific consensus sequence of DNA, or just to AT rich regions (Calvo *et al.*, 1994). LRP may be a chromosome organizer because large numbers of LRP molecules are present in each cell. It is also a small DNA-binding protein, binding to DNA with no apparent site specificity (Newman and Lin, 1995).

Part 3. Some operons are co-regulated by LRP and cAMP-CRP

Both cAMP-CRP and LRP are global regulatory proteins. Some operons have been reported to be co-regulated by both LRP and cAMP-CRP. These operons include *serC-aroA* multifunctional operon (*serC* encodes phosphoserine aminotransferase and *aroA* encodes enolpyruvolylshikimate 3-phosphate synthase), *osmY* (osmotically induced periplasmic protein), *daa* (F1845 fimbrial adhesin), *dad* (degradative D-amino acid dehydrogenase), *pap* (Pap fimbrae) and *csiD*. However, the involvement of LRP and cAMP-CRP in the co-regulation patterns of these operons is quite different (Table 1. lists all the genes/operons so far identified to be regulated by both LRP and cAMP-CRP). In addition, cAMP-CRP can interact with different operons either directly or indirectly.

Both CRP and LRP repress the expression of *osmY*. The study of the *osmY* promoter region showed that in the presence of cAMP, protection by CRP is centered at -12.5. In contrast to CRP, LRP does not bind to a single site, but binds co-operatively to

the whole promoter region from –90 to +1, suggesting the existence of several LRP binding sites. The region protected by CRP partially overlaps with those regions protected by LRP. Studies using mixtures containing variable concentrations of CRP and LRP suggested that LRP was able to bind to the *osmY* promoter region, while the presence of CRP led to its exclusion from this promoter sequence (Colland *et al.*, 2000; Lange. *et al.*, 1993).

A recent study of *pap pili* showed that CRP was essential for the expression of *pap pili* by uropathogenic *E.coli*. Both *in vitro* and *in vivo* analyses indicated that binding of cAMP-CRP centred at -215.5bp upstream of the *papBA* promoter is essential for the activation of transcription. CRP activation of *papBA* requires binding of LRP to its binding sites that extend from –180 to –149 relative to the transcription start site of *papBA*. CRP and LRP bind independently to *pap* DNA binding sites, and the cAMP-CRP complex is directly involved in regulation of this binding (Weyand *et al.*, 2001).

In the *serC-aroA* operon, LRP serves as an activator, while the cAMP-CRP complex serves as a repressor. Activation by LRP is likely direct, whereas repression by the cAMP-CRP is likely indirect, and possibly via a repressor whose amount or activity is stimulated by cAMP-CRP (Man *et al.*, 1997).

Table 1. *E.coli* operons regulated by both LRP and cAMP-CRP

Genes/operons	Function of cAMP-CRP	Function of LRP	Reference
SerC-aroA	repression	activation	Man *et al.*.1997
OsmY	repression	repression	Lang *et al.*. 1993
			Colland *et al.*.
			2000
Daa	activation	activation	Bilge *et al.*. 1993
Dad	activation	activation and repression	Mathew *et al.*.
			1996
			Zhi *et al.*.
			1999
Pap	activation	activation and repression	Van der Woude *et al.*.
			1995
			Weyand *et al.*. 2001
CsiD	activation	activation	Marschall *et al.*,
			1998
			Germer *et al.*,
			2001

Part 4: Regulation of *sdaA*, *serA* and *metK* in *E.coli*

4-1. L-serine deaminase and the *sdaA* gene in *E.coli*

Multiple enzymes can catalyze the deamination of L-serine to pyruvate and ammonia. In an aerobic environment, *E.coli* contains two L-serine deaminases, which are encoded by the *sdaA* and *sdaB* genes, only the former is expressed in glucose minimal medium (Su *et al.*, 1989,1991).

4-1-1. Regulation of L-serine deaminase in *E.coli*

L-serine degradation, which converts L-serine to pyruvate and ammonia (Figure. 2) involves only one enzymatic step, deamination of L-serine by L-serine deaminase, (McFall and Newman, 1996).

Some environmental factors can regulate L-SD activity. Exposure to DNA damaging agents (like UV irradiation, nalidixic acid and mitomycin), growth at high temperature or in anaerobic conditions, and alcohol shock can all induce L-SD expression (Newman *et al.*, 1982a). This increased expression also can be achieved by addition of glycine and L-leucine (Pardee and Prestidge, 1955), but not L-serine (Pardee and Prestidge, 1955; Isenberg and Newman, 1974) to the growth medium.

Since the activity of L-SD is greatly increased in *lrp* mutants (Lin *et al.*, 1990), LRP is believed to act as a repressor. Addition of exogenous L-leucine releases this repression via the interaction of L-leucine and LRP (Lin *et al.*, 1990; Lin, 1992).

Iron and dithiothreitol (DTT) are required to fully activate the expression of L-SD. A post-translational mechanism could be involved. The newly synthesized enzyme folds into an inactive conformation, but is activated by refolding (Su *et al.*, 1993).

4-1-2. Regulation of expression of the *sdaA* gene

Like L-SD, the expression of *sdaA* is regulated by a number of factors. *SdaA* expression is induced by the amino acids glycine and L-leucine, but not L-serine (Pardee and Prestidge, 1955). It can also be induced anaerobically, at high temperature, and as a response to DNA-damaging agents. Mutations in the membrane-bound protein *Cpx (Ssd)* greatly increase *sdaA* transcription. These mechanisms are still not understood.

Regulation of the *sdaA* gene by LRP is probably at the transcriptional level, although there is still insufficient evidence to support this. It was proposed that LRP binds to at least two sites upstream of the *sdaA* operon, one with high affinity and the other with low affinity. Since the presence of L-leucine greatly reduced LRP binding activity at the *sdaA* promoter (Newman and Lin, 1995), it is likely that LRP represses *sdaA* transcription by binding to its upstream region, while L-leucine activates *sdaA* transcription by releasing the binding of LRP. A putative CRP binding site at the *sdaA* promoter region suggested that CRP might also be involved in the regulation of *sdaA* expression, probably at the transcription level.

4-2. Regulation of expression of the *serA* gene

The *serA* gene in *E.coli*, which encodes 3-phosphoglycerate dehydrogenase, is the first enzyme in the L-serine biosynthesis pathway, as shown in Figure 2.

L-serine, the end product of the serine biosynthesis pathway, does not affect the expression of the *serA* gene. However, a feedback inhibition mechanism represses the enzymatic activity of 3-phosphoglycerate dehydrogenase through a conformational change in the enzyme itself (Stauffer, 1996).

LRP is involved in the regulation of the *serA* gene. Adding LRP causes an up to six-fold increase in *serA* gene expression, while L-leucine causes an up to two-fold reduction (Newman and Lin, 1995). Primer extension experiments (Lin, 1992c) showed that *serA* has two promoters, P1 and P2. Recently, it was confirmed by site directed-mutagenesis experiments that LRP activates P1 but represses P2 in glucose minimal medium (Li *et al.*, 2002). In the absence of LRP, transcription can take place from both P1 and P2 promoters. It was also suggested that the cAMP-CRP complex could positively regulate *serA* by activating P2.

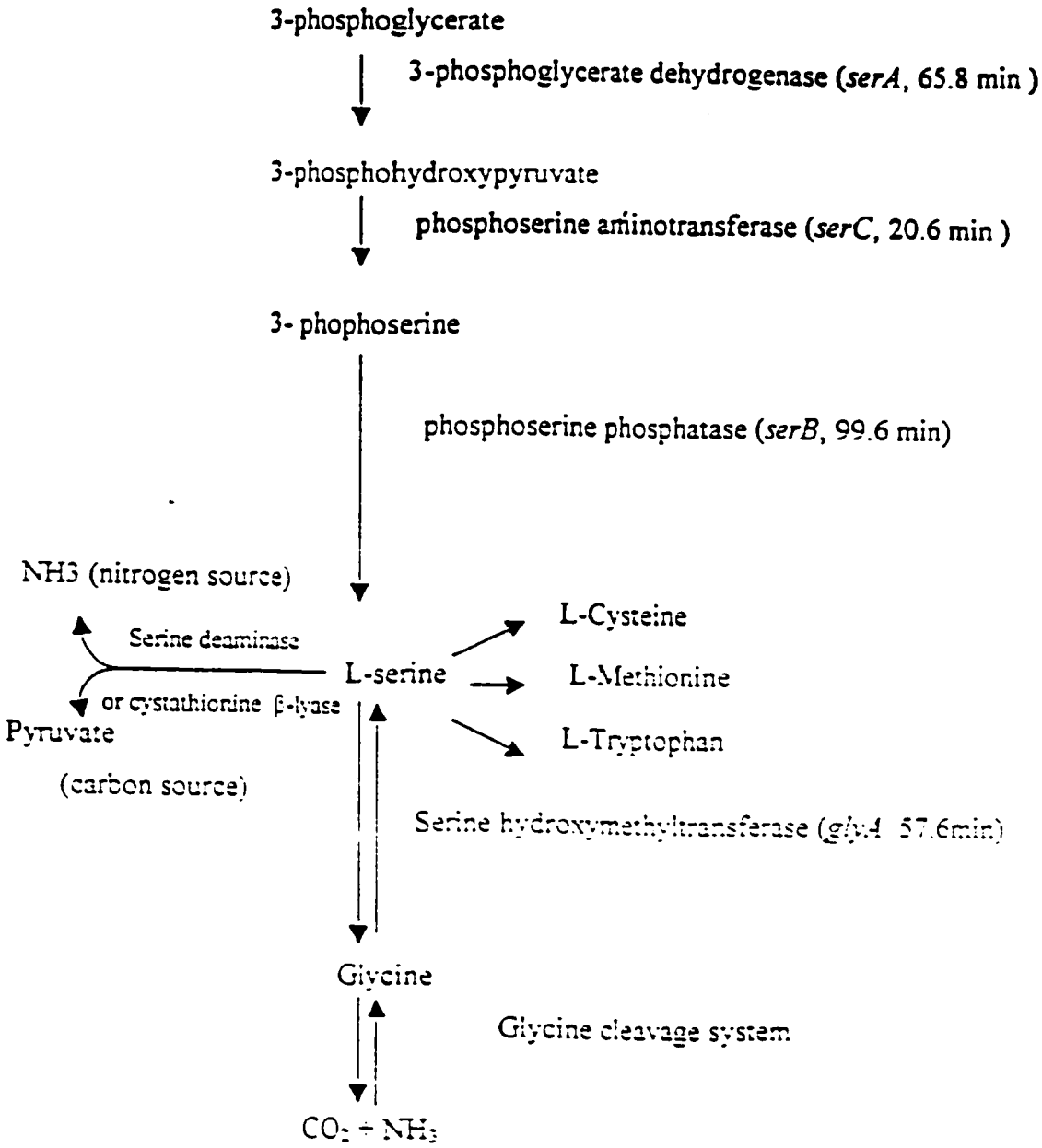

Figure 2: L-serine biosynthesis pathway

(Major pathway was adapted from Stauffer. 1996)

4-3. Regulation of *metK* gene expression

The *metK* gene of *E.coli* encodes S-adenosylmethionine synthetase, which catalyzes the reaction between L-methionine and ATP to form S-adenosylmethionine (SAM), the universal methyl group donor in these cells.

The regulatory mechanisms of *metK* gene expression and the proteins and factors involved are still unknown due to a lack of information about its promoter region.

It has been suggested that LRP can repress *metK* expression, while L-leucine antagonizes LRP to release this repression (Newman *et al.*, 1998). Results from primer extension experiments showed that a single transcription start site located 140 nucleotides upstream of the *metK* ATG start codon was observed for cells grown in LB medium. 12 LRP binding sites have been identified in the *metK* promoter sequence, suggesting a direct role for LRP. A CRP binding site was also found 60bp upstream of the *metK* transcription initiation site, implying that CRP might also be involved in the regulation of *metK* expression (Wei, 2001).

A *metK* mutant strain, *metK*84 had a remarkably low level and activity of SAM, and showed a complex phenotype under certain growth conditions. DNA sequencing analysis identified a single base pair difference, located 150 nucleotides upstream of the *metK* ATG translation start codon as the reason for the complex phenotype of the *metK*84 mutant (Wei, 2001).

Materials and Methods

1. Bacterial strains and plasmids

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

2. Cultures, media, buffers and solutions

2-1. Minimal media

Liquid minimal medium (NIV liquid pH 7.0):

15g K_2HPO_4, 5.25g KH_2PO_4, 2g $(NH_4)_2SO_4$, 0.8g $MgSO_4$, and 0.04g $CaCl_2$ are dissolved in 1 liter distilled water, and the pH adjusted to 7.0.

Liquid minimal medium (NIV liquid pH 6.2):

3.34g K_2HPO_4, 10.88g KH_2PO_4, 2g $(NH_4)_2SO_4$, 0.8g $MgSO_4$, and 0.04g $CaCl_2$ are dissolved in 1 liter distilled water, and adjusted to pH 6.2 (Gerhardt and Murray, 1981).

Solid minimal medium (NIV agar pH 7.0):

NIV solid minimal medium was prepared by adding Bactoagar to liquid minimal medium (NIV liquid pH 7.0) to a final concentration of 2% (w/v).

Table 2: Strains

E.coli K-12 strains	Genotype	Reference/ Source
CU 1008	E.coli K-12 Δ*ilvA*	L.S.Williams
MEW1	CU1008 *ΔlacZ*	Newman *et al.*, 1985b
MEW26	MEW1 *lrp*::Tn10	Lin *et al.*, 1990
JCB_{43}*Δcrp*	whole *crp* gene deleted strain	
	Streptomycin Resistant	*E.coli* genetic stock center
CBK103	*cysG*::Tn5	*E.coli* genetic stock center
SP850	*cyaA*::kan	*E.coli* genetic stock center
CCR	MEW1 Δ*crp*	This study
CCRL	MEW1 Δ*crp lrp*::Tn10	This study
CCY	MEW1 *cyaA*::kan	This study
CCYL	MEW1 *cyaA*::kan *lrp*::Tn10	This study
CuS2	MEW1 *serA*::λplacMu9 kanR	Shao,1992
CuLS	CuS2 *lrp*::Tn10	This study
CuCS	CuS2 Δ*crp*	This study
CuCLS	CuS2 Δ*crp lrp*::Tn10	This study
Cup22	MEW1 *sdaA*::λplacMu9 kanR	Su, 1991

Cup22C	Cup22Δ*crp*	This study
Cup22CL	Cup22Δ*crp lrp*::Tn10	This study
CH22	MEW1*sdaA*::cm	Su, 1991.
CH22C	MEW1Δ*crp sdaA*::cm	This study
CCRG	MEW1Δ*crp* glycerol using strain	This study

Table 3. Plasmids

Plasmids	Genotype / Characteristics	Reference/ Source
pDCRP (pcrp)	wild type *crp* gene cloned into pBR322, containing colE1 origin of replication and encoding ampicillin resistance	Bell *et al.*, 1990b
pMES22(psdaA)	pBR322 carrying 2.6kb fragment of *sdaA* gene	Su *et al.*, 1991
pMC1781	pBR322 with a truncated *lacZ* gene	Gilbert, W
pMES22::lacZ (psdaA::lacZ)	pBR322 carrying truncated *sdaA-lacZ* gene	This study
pserAP1P2	pRS415 carrying the 392bp fragment of the *serA* promoter	Li *et al.*, 2002
pserAP1	pRS415 carrying the 392bp fragment of the *serA* promoter in which only P1 is functional	Li *et al.*, 2002
pserAP2	pRS415 carrying the 392bp fragment of the *serA* promoter in which only P2 is functional	Li *et al.*, 2002
pRS-K (pmetK::lacZ)	pRS415 containing *metK::lacZ* operon fusion	Wei, 2001
pRS-K84 (pmetK84::lacZ)	pRS415 containing *metK84::lacZ* operon fusion	Wei, 2001
pTH111 (pptsG)	derivative of pBR322 containing *ptsG* expressed from the bla promoter	Takahashi *et al.*, 2001

The wild type strain MEW1 (*ΔilvA*) used in our lab and all its derivatives require isoleucine and valine for growth. therefore these amino acids are added to both liquid and solid media at a final concentration of 50μg/ml.

Carbon sources were added to minimal media at a concentration of 0.2% (w/v).

MacConkey plates:

17.0g Peptone, 3.0g Proteose Peptone, 10.0g Lactose, 1.5g Bile salts mixture, 5.0g NaCl, 0.03g Neutral red, 0.001g Crystal violet and 13.5g Agar are dissolved in 1 liter distilled water (Gerhardt and Murray, 1981).

SGL plates:

Minimal medium plates are supplemented with glycine (500μg/ml), L-leucine (200μg/ml) and L-serine (500μg/ml).

2-2. Luria Broth (LB)

10g Bactotryptone, 5g-yeast extract, and 5g NaCl are dissolved in 1 liter of distilled water before autoclaving. Bactoagar to a final concentration of 2% (w/v) was added to the medium before autoclaving when preparing plates.

2-3. NSIV plates

0.2%(w/v) L-serine was added to NIV agar as a sole carbon source.

2-4. R-Top Agar for P1 plate lysate

10g Bacto-tryptone, 1g Bacto-yeast extract, 8g NaCl and 4g Bactoagar are dissolved in 1 liter distilled water. Sterile 2mM $CaCl_2$ and 0.1%(w/v) glucose were added to the medium separately after autoclaving.

2-5. Other additions to the medium

Antibiotics were used at the following concentrations: Ampicillin (Amp) 200μg/ml, Kanamycin (Kan) 50μg/ml, Chloramphenicol (Cm) 25μg/ml, Streptomycin (Str) (50μg/ml), Tetracycline (Tet) 20μg/ml.

Specific antibiotics were added to strains with corresponding antibiotic resistance during culture.

2-6. Buffers and solutions

MC buffer for P1 transduction

MC buffer contained 0.100M $MgSO_4$, and 0.005M $CaCl_2$.

SOC buffer for electro-transformation

SOC buffer for electro-transformation contained 2%(w/v) Bactotryptone, 0.5%(w/v) Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, and 20mM glucose (Sambrook *et al.*, 1989).

TBE (Tris-borate and EDTA) buffer for DNA agarose gel electrophoresis

Concentration of stock solution (5X)

Per 1 liter:

54g Tris-borate

27.5g boric acid

0.01M EDTA (pH 8.0)

(Sambrook *et al.*, 1989).

X-gal solution for selecting lac+ colonies

5-bromo-4-chloro-3-indolyl-β-D-galactoside is dissolved in N-N-dimethylformamide at a concentration of 20mg/ml (Sambrook *et al.*, 1989).

Z buffer for β-galactosidase activity assay

1 liter Z buffer contains 16.1g Na_2HPO_4 H_2O (or 8.52g Na_2HPO_4). 5.3g NaH_2PO_4. H_2O. 0.75g KCl. 0.264g $MgSO_4.7H_2O$ and 2.7ml β-mercaptoethanol (Sambrook *et al.*, 1989).

ONPG for β-galactosidase assay

107g K_2HPO_4. 52.4g KH_2PO_4. and 4g O-Nitropherol-β-D-golactopyraroside (ONPG) were dissolved in 1 liter distilled water and adjusted to pH 7.0 (Sambrook *et al.*, 1989).

Phosphate buffer for whole cell L-SD assay

79.1g K_2HPO_4 and 6.26g KH_2PO_4 are dissolved in 1 liter distilled water (50mM, pH 7.8). (Sambrook *et al.*, 1989).

LB Citrate

147g sodium citrate is dissolved in 1 liter LB liquid (0.5M). (Sambrook *et al.*, 1989).

3. Chemicals

Restriction enzymes, Taq DNA polymerase and DNA modifying enzymes were purchased from MBI Fermentas (Montreal Canada). Oligonucleotides used in this study were purchased from Biocorp Montreal.

Oligonucleotides used in this study:

SdaA-1: 5' GGAAGTCCAGTCACCTTGTC 3'

SdaA-2: 5' GCAGACGAGAAAGCGGGTA 3'

SdaA-1 is located 32bp upstream of the *sdaA* gene start codon. *sdaA*-2 is located 40bp downstream of the *sdaA* stop codon. Both primers are 100% complementary to the DNA sequence.

4. Enzyme Assays

4-1. β-galactosidase assay

Cells were grown to early log-phase in minimal medium. β-galactosidase activity was assayed in whole cells according to the method described by Miller (Miller, 1972) and expressed in Miller units. One unit is the amount of β-galactosidase that produces 1MU-mol/ml O-nitrophenol/min in a standard assay conducted at 28°C, pH 7.0.

4-2. Whole cell L-serine deaminase assay

L-serine deaminase (L-SD) activity was assayed in toluene-treated cells by an assay that detects the pyruvate that is formed from serine. The pyruvate is reacted with 2,4-dinitrophenylhydrazine (DNPH) and the absorbance measured at 540nm (Isenberg and Newman, 1974). The incubation mixture contained 20ul of L-serine (100mg/ml), 180μl of 50mM phosphate buffer (pH 7.8), 300μl of washed cells re-suspended in 50mM phosphate buffer (pH 7.8), and 20μl of toluene. This mixture was incubated for 35 min at 37°C, 900μl of DNPH (250μg/ml in 4.1%HCl) was added, and the mixture was incubated for a further 20 min at room temperature. Following incubation with DNPH, 1.7ml of 10%NaOH was added and the absorbance (OD_{540nm}) was determined. An assay mixture containing no washed cells was included in the assay as a control. Activity is expressed as the difference between the amounts of ketoacid formed in the assay mixture with and without cells.

One unit of L-SD as measured in the whole cell assay is defined as the amount of enzyme that catalyzes the formation of 0.8 μmol of pyruvate in 35 min under whole cell assay conditions.

5. Determination of plasmid maintenance

An appropriate dilution of cells was first plated on LB plates, and the resulting colonies were replicated on LB plates containing 200μg/ml of ampicillin.

6. Transformation and transduction

6-1. Transformation:

Transformation was performed via electro-transformation using a Gene Pulser (Bio-Rad) following the manufacturer's directions.

6-2. Transduction:

P1-mediated transduction was performed as described by Miller (1972) with some modifications. Cells were grown overnight at 37°C and sub-cultured the following day. When the sub-culture reached OD_{600nm} 0.5-0.8, the cells were harvested and re-suspended in MC buffer in half volume. A 0.1ml aliquot of the re-suspended cells was mixed with different dilutions of P1 lysates in room temperature for 30 min. 1ml LB citrate was then added to the reaction mixtures to stop P1 absorption and the mixtures

were incubated at 37°C for one hour to allow the transduced phenotype to be expressed. Appropriate selection plates were then used to select the corresponding transductants.

7. Plasmid isolation and restriction enzyme digestion

7-1. Plasmid isolation

Plasmids were isolated using the QIAgen miniprep kit (QIAGEN, Montreal, Canada) according to the manufacturer's instructions.

7-2. Restriction enzyme digestion

All restriction enzymes were purchased from MBI Fermentas (Montreal, Canada) and digests were carried following the manufacturer's directions.

8. Gel electrophoresis

DNA agarose gel electrophoresis was carried out as described by Sambrook *et al.*, (1989). 0.7% to 1.0% (w/v) agarose gels were generally used.

9. DNA extraction from agarose gels

DNA was extracted from agarose gels using a QIAgen DNA Extraction Kit (QIAGEN, Montreal, Canada) according to the manufacturer's instructions.

10. Determination of growth rates

Growth was measured with a Klett colorimeter equipped with a filter. Cell cultures grown overnight in minimal medium were then sub-cultured in 20ml of the same medium in a side-arm flask to a cell density of Klett unit 20. Cell cultures were then incubated at 37°C. Optical density was determined every 60 min by Klett units.

11.Polymerase Chain Reaction (PCR)

A single colony was picked up from the plate and inoculated in 300µl autoclaved distilled water. After being boiled for 3-5 min at 95°C, the solution was used as a template for PCR. The PCR mixture was made up to a total volume of 50µl containing 5µl 10x Taq buffer (MBI, containing 100mM Tris HCl pH8.8, 500mM KCl, 0.8% Nonidet P40), 3.0µl 25mM $MgCl_2$, 0.8mM each of the four deoxynucleoside triphosphate (dNTPs), 0.6µM each of the primers *sdaA*-1 and *sdaA*-2, 0.5µl template DNA and 2.5U of Taq DNA polymerase (MBI). The reaction mixture was then subjected to the following PCR cycles using an Interscience thermocycler:

Cycle 1:	95°C 5min to denature DNA
Cycles 2-17:	95°C 1 min
	52°C 1 min
	72°C 3 min
Cycle 18:	72°C 10 min

PCR products were visualized by ethidium bromide staining after loading 10μl of the mixture onto a 0.9% agarose gel and electrophoresing.

12. Construction of strains

12-1. Construction of CCR (MEW1 Δ*crp*).

12-1-1. Construction of strain Cu*cysG*::Tn5

The strain CBK103, carrying a defective *cysG* gene, could only grow on minimal media supplemented with cysteine (10μg/ml). It also had a kanamycin insertion (Tn5) in the *cysG* gene. This strain was used to construct Cu*cysG*::Tn5 by transducing P1 phage in CBK103 (*cysG*::Tn5) to the parental strain MEW1. Transductants were selected on LB agar plates containing kanamycin (50μg/ml). In order to verify that the resulting transductants were *cysG* defective, colonies were streaked on glucose minimal media agar plates supplemented with cysteine (10μg/ml) and kanamycin (50μg/ml). The cysteine auxotroph transductants were named Cu*cysG*::Tn5.

12-1-2. Construction of the strain CCR (CuΔ*crp*)

The strain CCR (Cu*cysG*::Tn5) was transduced by P1 phage grown on the strain JCB43Δ*crp* (a *crp* gene deletion strain from the *E.coli* Genetic Stock Centre). Since the *cysG* gene is located at 75.35 min. 0.26 min away from the *crp* gene (75.09 min), the *cysG* gene could be replaced by Δ*crp* with an expected ratio of 79% (formula $N=1-(1-t/k)^3$. (Wu. 1966)). Transductants were selected on glucose minimal media agar plates (cysteine-independent). All resulting colonies were streaked on both glucose minimal and maltose minimal media agar plates to screen for glucose dependence and to ensure that the resulting strain was *crp* defective. Three of one hundred transductants displayed glucose dependence. All three proposed *crp* mutants formed red colonies while the parent strain MEW1 (*crp*+) formed white colonies when streaked on MacConkey plates. These mutants were thus confirmed as *crp*- strains and were named CCR (MEW1 Δcrp). The frequency (3%) of Δ*crp* replacement was much lower than expected (79%) and may be due to the fact that the *cysG* gene carried an insertion (Tn5) that increased the distance between *crp* and *cysG*. In addition, *crp* mutants grow poorly and therefore may be difficult to identify among the *crp*+ transductants on the plate.

12-2. Construction of strain CCRL

The strain CCR (CuΔ*crp*) was transduced with P1 phage grown on the strain MEW26 (*lrp*::Tn10). The transductants were selected on LB plates containing tetracycline at a concentration of 20μg/ml and were named CCRL (MEW1Δ*crp* *lrp*::Tn10). One of these transductants (CCRL) contained the *lrp* defective genotype was confirmed by transducing the CCRL strain with P1 phage grown on the wild type strain

MEW1, and selecting on maltose minimal media agar plates. These new transductants were streaked on NSIV plates supplemented with tetracycline (20μg/ml). Since only *lrp* defective strains can grow on minimal media using L-serine as the sole carbon source and the parental type cannot, the *lrp* defective genotype was thus confirmed. This strain was named CCRL (MEW1Δ*crp lrp*::Tn10).

12-3. Construction of strain CCY (MEW1 *cyaA*::kan)

Phage P1 grown on the strain SP850 (genotype *cyaA*::kan, from *E.coli* Genetic Stock Centre) was used to transduce MEW1 cells. The transductants were selected on LB plates containing kanamycin (50μg/ml). The resulting strain was named CCY, and used glucose but not maltose as sole carbon source.

12-4. Construction of strain CCYL (MEW1 *cyaA*::kan *lrp*::Tn10)

P1 grown on the strain SP850 (*cyaA*::kan) was used to transduce the strain MEW26(MEW1*lrp*::Tn10). The transductants were selected on LB plates containing kanamycin (50μg/ml) and tetracycline (20μg/ml). These transductants were named CCYL (MEW1*cyaA*::kan *lrp*::Tn10). The *cya* defective genotype was confirmed by growing the cells in minimal media supplemented with glucose or maltose. The *lrp* defective genotype was confirmed by transduction of P1 phage in MEW1 to the strain CCYL. The resulting transductants were streaked on NISV plates supplemented with tetracycline (20μg/ml). Only *lrp* defective strains can grow on minimal media using L-

serine as the sole carbon source. This strain was named CCYL (MEW1*cyaA*::kan *lrp*::Tn10).

12-5. Construction of strains CuLS (MEW1*lrp*::Tn10 *serA*::λplacMu9), CuCS(MEW1Δ*crp* *serA*::λplacMu9) and CuLCS(MEW1Δ*crp* *lrp*::Tn10 *serA*::λplacMu9).

12-5-1. Construction of strain CuLS (MEW1 *lrp*::Tn10 *serA*::λplacMu9)

Phage P1 grown on the strain MEW26 (MEW1 *lrp*::Tn10) was used to transduce to strain CuS2 (MEW1 *serA*::λplacMu9). The transductants were selected on LB plates containing tetracycline (20μg/ml) and kanamycin (50μg/ml). The resulting strain was named CuSL, which was confirmed as an L-serine auxotroph by growing the cells in minimal media with and without L-serine.

12-5-2. Construction of strain CuCS (MEW1Δ*crp* *serA*::λplacMu9)

P1 grown on the strain CuS2 (MEW1 *serA*::λplacMu9) was used to transduce the strain CCR (MEW1Δ*crp*). The transductants were selected on LB plates containing kanamycin (50μg/ml). The resulting strain was named CuCS (MEW1Δ*crp* *serA*::λplacMu9), and confirmed as an L-serine auxotroph by growing the cells in minimal media with and as a heterotroph by growing the cells without L-serine. The *Δcrp* genotype was confirmed by streaking on glucose minimal media plates and maltose

minimal media plates supplemented with L-serine (500μg/ml). The *crp* mutants could only grow on minimal media supplemented with glucose and L-serine.

12-5-3. Construction of strain CuCLS (MEW1Δ*crp lrp*::Tn10 *serA*::λplacMu9)

Phage P1 grown on the strain MEW26 (MEW1 *lrp*::Tn10) was used to transduce to strain CuCS (MEW1Δ*crp* *serA*::λplacMu9). The transductants were selected on LB plates containing tetracycline (20μg/ml) and kanamycin (50μg/ml). The resulting strain was named CuCLS. and was confirmed as an L-serine auxotroph by growing the cells in minimal media with and without L-serine.

*12-6. Construction of strain Cup22C and Cup22CL

*12-6-1. Construction of strain Cup22C

P1 phage was grown on strain Cup22 (MEW1 *sdaA*::λplacMu9). then transduced to strain CCR. Transductants were selected on LB X-gal (40μg/ml) containing kanamycin (50μg/ml). Blue colonies that were also kanamycin resistant were named Cup22C.

*12-6-2. Construction of strain Cup22CL

P1 phage was made from strain MEW26 (*lrp*::Tn10), then transduced to strain Cup22C. Transductants were selected on LB plates containing tetracycline (20μg/ml) and named Cup22CL.

12-7. Construction of strain CH22C

P1 grown on the strain CH22 (MEW1 *sdaA*::cm) was used to transduce the strain CCR (MEW1Δ*crp*). Transductants were selected on LB plates containing chloramphenicol (25μg/ml) and the resulting strain was named CH22C (MEW1Δ*crp* *sdaA*::cm) and has been confirmed as a SGL heterotroph. The Δ*crp* genotype was confirmed by streaking on glucose minimal media plates and maltose minimal media plates. C*rp* mutants can grow only on minimal media with glucose.

12-8. Screening for *crp* glycerol using mutants

The strain CCR was grown overnight in glucose minimal medium. 100μl was removed and plated on a minimal medium plate with glycerol (0.2% w/v). After growing for 48 hours. five colonies were detected (ratio: $5/10^7$). These five colonies were further tested and shown to be unable to use maltose. These mutants were named CCRG (MEW1Δ*crp* glycerol+).

13.Construction of plasmids

Construction of plasmid pMES22::lacZ (psdaA::lacZ)

The plasmid pMES22 (psdaA) was cut at its unique HpaI site located within the *sdaA* coding region and the resulting blunt ended linearized DNA was then partially cut with PstI. The resulting 5.4kb DNA fragment containing ampicillin resistance was extracted using a QIAgene DNA extraction kit.

Plasmid pMC1871 causes a *lacZ* gene in which the first 8 codons are missing. This *lacZ* gene was isolated by cutting with SmaI and PstI. The resulting DNA fragment of approximately 3kb and containing a promoterless *lacZ* gene was extracted using a QIAgene DNA extraction kit.

The 5.4kb DNA fragment generated from the plasmid psdaA and the 3kb DNA fragment generated from plasmid pMC1871 were ligated (Figure 3). The strain MEW1 was then transformed with the resulting ligation product. Blue colonies on LB plates containing X-gal (40μg/ml) and ampicillin (200μg/ml) were selected. Plasmids were isolated from these colonies and both the size (8.5kb) and restriction digest pattern were checked.

* The construction and special characteristics will be discussed in the Discussion section.

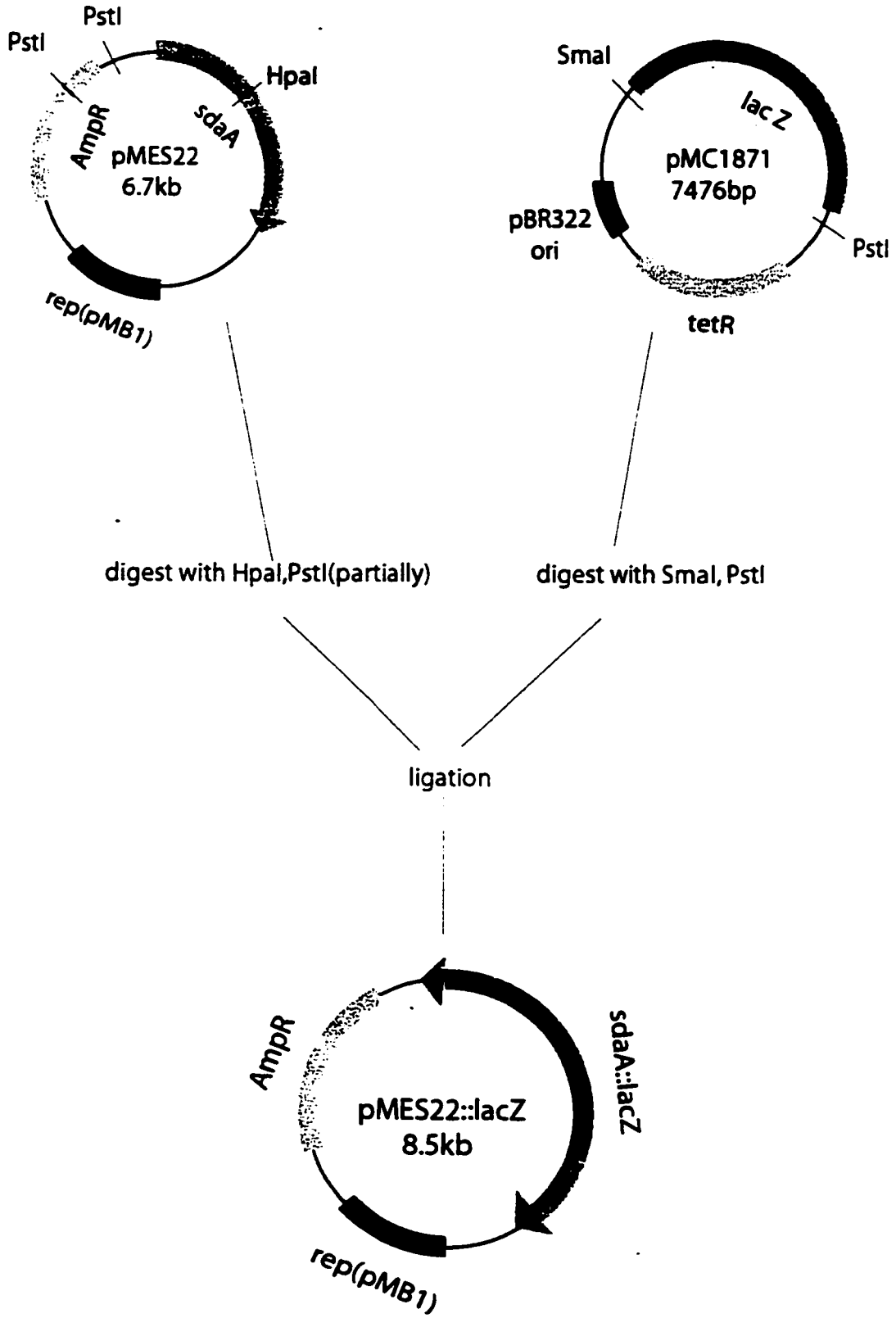

Figure 3: Construction of plasmid pMES22::lacZ (psdaA::lacZ)

Results

Part 1. *In vivo* expression of L-SD and *sdaA::lacZ* in *crp* mutants

Studies of L-serine deaminase (L-SD) expression in the Dr. Newman's lab have indicated that LRP is a major regulator. In a mutant lacking a functional *lrp* gene, L-SD activity is increased 4-fold in cells grown in glucose minimal medium, suggesting that LRP is a repressor of *sdaA* expression.

Previous studies using a fusion of *lacZ* to *sdaA* (*sdaA::lacZ*) indicated that LRP acts on transcription of the *sdaA* gene (Lin, 1990). Lin also showed preliminary evidence for the existence of two LRP binding sites upstream of the *sdaA* binding site.

An examination of the sequence upstream of the *sdaA* coding region reveals a putative CRP binding site, suggesting that CRP could be involved in the regulation of this gene, just as it is involved in *serA* gene expression (Li *et al.*, 2002). To investigate this possibility, I constructed *crp* mutants, and *crp lrp* double mutants and assayed L-SD activity in these strains (Section 1-1-1). I also measured L-SD activity in strains carrying *sdaA* on plasmid pBR322, and compared this with β-galactosidase activity in a strain with a *lacZ* fusion to *sdaA*, carried on the same plasmid (Section 1-1-2).

1-1. *In vivo* activity of L-SD in *crp* mutants

1-1-1. L-SD activity in *crp* mutants (CCR) and *crp lrp* double mutants (CCRL)

I compared the *in vivo* L-SD activity of MEW1 *crp-*, and a *crp lrp* double mutant with that of MEW1 and MEW26 (*lrp-*) using established protocols (Isenberg & Newman 1974: Table 4). As described previously (Lin, 1990), L-SD activity in the *lrp-* strain was approximately three times higher than in the parent strain (0.374 vs. 0.107), confirming that LRP expression results in a decrease in L-SD activity.

The CRP-deficient strain showed much the same activity as the parent (0.108), which suggests CRP has no effect on L-SD activity. However, the absence of CRP had an effect on the *lrp* mutant. This slow-growing double mutant showed only slightly higher activity than either the wild type or the *crp* single mutant (0.140 vs. 0.108). Thus, the absence of CRP prevented the increase in activity usually seen in *lrp* mutants even when grown in glucose minimal medium.

We propose that the increase in L-SD activity in the LRP-deficient strain occurs via a CRP-dependent mechanism since in the absence of CRP, this increase is abolished.

1-1-2. *In vivo* activity of L-SD in *crp* mutants carrying a plasmid that overexpresses *crp*.

If the preceding hypothesis is correct, providing CRP from a plasmid should reverse the effect of CRP deficiency. To test this, I transformed a plasmid carrying the *crp* gene, pDCRP, kindly supplied by Dr. S. Busby, into *crp* and *crp lrp* mutant strains as described in Table 4, and measured L-SD activity in the resulting strains (Column B).

Providing the *crp lrp* mutants with a source of CRP restored the higher level of expression of L-SD activity (0.335 in cells containing the plasmid compared to 0.140 in those cells without the plasmid, Row 4, Columns A and B). This was consistent with the observation that the effects of LRP deficiency are only seen in the presence of CRP (0.118 vs. 0.108, Row 3, Columns A and B).

1-2. Effect of Adenylate Cyclase deficiency on L-SD activity

In wild-type *E.coli*, all known effects of CRP are mediated through its interaction with cyclic AMP (cAMP). Unlike the present phenomenon, these effects are seen in cells grown with carbon sources other than glucose. I therefore tested whether cAMP was needed for the LRP-dependent increase in L-SD activity. To do this I constructed a *cya* mutant, deficient in adenylate cyclase and a *cya lrp* double mutant (see Materials and Methods).

As seen in Table 5, the absence of cAMP had little effect on L-SD expression in the single mutant (0.101 as in the parent strain) but abolished the increase usually seen in LRP-deficient strains (0.113 vs. 0.374). While CRP cannot be provided from outside the

Table 4: *In vivo* activity of L-SD in *crp* mutants

		L-SD activity (units)	
		A	B
Strains	Relevant genotype	No plasmid	With pDCRP(pcrp)
1. MEW1	*crp+lrp+*	0.107±0.019	ND[d]
2. MEW26	*crp+lrp-*	0.374±0.027	ND[d]
3. CCR	*crp-lrp+*	0.108±0.026	0.140±0.026
4. CCRL	*crp-lrp-*	0.140±0.036	0.335±0.1

a). Cells were grown in glucose minimal medium (0.2% glucose), sub-cultured, and assayed in log phase for whole-cell L-SD activity as described in Materials and Methods. Results represent the average ± SD of five experiments.

b). Ampicillin (20μg/ml) was added to strains carrying plasmid.

c). After experiments, plasmid retention was tested by plating cells on LB and LB ampicillin plates for strains CCRpDCRP and CCRLpDCRP. Cells that were able to grow on LB but not on LB ampicillin were considered to have lost plasmid. No significant difference in the number of colonies on LB or LB ampicillin was observed in these experiments.

d). ND: not determined.

Table 5: The effect of Adenylate Cyclase deficiency on L-SD

		L-SD activity (units)	
		A	B
External cAMP(M)		CCY(*cyaA-lrp+*)	CCYL(*cyaA-lrp-*)
1.	0	0.101±0.014	0.113±0.030
2.	10^{-4}	0.104±0.028	0.188±0.013
3.	$5x10^{-4}$	0.097 ±0.004	0.242±0.074
4.	10^{-3}	0.113±0.011	0.341±0.014

a). The concentrations of external cAMP noted were added to the medium and L-SD assayed as in Table 4.

b). Results are the averages ± SD of two or three experiments.

cell. it is possible to provide cAMP. The three concentrations tested restored the increase in the double mutant (0.341 at 10^{-3}M. Table 5, Row 4 Column B), but had little effect on the L-SD activity of the *cya* single mutant which had a functional LRP (0.113 at 10^{-3}M. Table 5. Row 4 Column A).

These results confirm that both CRP and cAMP are involved in the regulation of L-SD activity. This effect is most apparent in cells grown in glucose-minimal medium (in the absence of cAMP synthesis), and is seen only in the absence of LRP.

1-3. CRP probably does not affect transcription of *sdaA*

To determine if the sdaA gene. cloned into pBR322 with its own promoter. was also subject to regulation by the cAMP/CRP system. I took advantage of plasmid pMES22 (psdaA) constructed by Dr. Su (Su *et al.*, 1991). He cloned the coding region of *sdaA* including 643bp upstream and 597bp downstream of the coding region into pBR322.

In order to verify that the psdaA construct was correct. PCR was used to check the size of the coding region using primers outside the coding region. A band of approximately 1.5kb corresponding to the expected 1463bp product was found (data not shown). I then verified that synthesis of L-SD from this plasmid was under the control of the inducers glycine and leucine (Table 6). Addition of glycine and leucine increased L-SD activity three fold as expected (from 0.198 to 0.601, Table 6, Row 1, Columns A and B).

I also transformed psdaA into the other host strains (MEW1 *lrp*::Tn10, MEW1 *crp*- and MEW1*crp*- *lrp*-) used in previous experiments and assayed L-SD activity. Results were very similar to those seen with the chromosomal gene (Table 6, Columns A and B). Enzyme levels were increased three fold in the LRP-deficient strain (0.602 vs. 0.198, Table 6, Row 2) but only slightly when the strain was also CRP-deficient (0.254 vs. 0.198, Table 6, Row 4). Enzyme activity was slightly lower in the mutant lacking only CRP (0.163, Table 6, Row 3 Column B).

Results indicate that the plasmid *sdaA* gene is regulated in the same way as the chromosomal gene. However, while the expression levels in the various strains carrying plasmid *sdaA* follow the same patterns as those strains with a chromosomal *sdaA* gene, levels are considerably lower than one might expect from a multicopy plasmid like pBR322. Levels were often less than twice those seen in strains in which the *sdaA* gene was on the chromosome.

1-3-1. Expression of *lacZ* from the *sdaA* promoter

I created the plasmid pMES22::lacZ (psdaA::lacZ) by fusing a promoterless *lacZ* gene from plasmid pMC1871 to the *sdaA* coding region of psdaA (see Figure 3). Digestion of pMC1871 with restriction enzymes SmaI and PstI generated a fragment of approximately 3kb containing most of the coding region and its own translation stop codon, but lacking the translation start codon and first 8 amino acids.

Plasmid psdaA was cut with HpaI, and partially cut with PstI to generate a fragment containing 813bp of *sdaA* coding region and a 643bp region upstream of the

sdaA gene. The *lacZ* fragment, with one blunt end and one cohesive end was ligated to the linearized psdaA and the ligation mix was transformed into the strain MEW1. Ampicillin-resistant mutants were selected on LB medium containing ampicillin (200μg/ml), and X-gal (40μg/ml). Purified plasmid from one of the blue colonies was the expected size (approximately 8.5kb as determined by agarose gel electrophoresis).

This plasmid had the structure expected of psdaA::lacZ as confirmed in two ways. First, strain MEW1 with plasmid psdaA::lacZ was grown in glucose minimal medium with and without glycine (500μg/ml) and L-leucine(200μg/ml), and its β-galactosidase activity was determined. Cultures grown without inducers generate 900 units of β-galactosidase (Table 7, Row 1, Column A). Cultures grown with glycine (500μg/ml) and L-leucine (200μg/ml) showed two times more β-galactosidase (1665 vs. 900, Table 7, Row 1, Columns A and B), which is similar to the induction of L-SD in strain MEW1 with plasmid psdaA (Table 6), suggesting that the *lacZ* insertion is in a reading frame and its expression is regulated by L-SD inducers. Second, the structure was confirmed by restriction enzyme analysis (data not shown).

1-3-2. The effect of *crp* deficiency on the expression of *sdaA::lacZ*

To determine whether CRP affects β-galactosidase activity, I transformed the plasmid into the four hosts used in earlier experiments, and assayed β-galactosidase activity in each of them (Table 7). The β-galactosidase activity in the LRP-deficient strain MEW26 carrying the plasmid psdaA::lacZ was approximately 2-fold higher than in the wild type strain MEW1 (1,815 vs. 900, Column A, Rows 1 and 2). β-galactosidase

activity in the *lrp crp* double mutant strain CCRL with the plasmid psdaA::lacZ was approximately 2-fold higher than in the *crp-* strain (1,910 vs. 1,108, Table 7, Rows 4 and 2). These results demonstrate that LRP serves as a repressor of *sdaA* gene transcription, regardless of CRP. There was no significant difference in β-galactosidase activity between the wild type strain MEW1psdaA::lacZ and the *crp* mutant carrying the plasmid psdaA::lacZ (CCRpsdaA::lacZ).

Table 6: Expression of L-SD in strains with plasmid psdaA

Strains	Relevant genotype	L-SD activity (units)		
		No plasmid	With psdaA	
			No inducers	With inducers
		A	B	C
1. MEW1	*crp+lrp+*	0.107 ±0.019	0.198 ±0.076	0.601±0.076
2. CT4	*crp+lrp-*	0.374 ±0.027	0.602±0.060	ND[b]
3. CCR	*crp-lrp+*	0.108 ±0.026	0.163±0.019	ND[b]
4. CCRL	*crp-lrp-*	0.140±0.036	0.254±0.050	ND[b]

a). Experiments were carried out as described in Table 4.

b). ND: Not determined.

c). Results are the averages ± SD of two or three experiments.

d). Plasmid retention was tested by plating the cells on LB and LB ampicillin plates. Cells that grow on LB but not on LB ampicillin were considered to have lost plasmid. No significant difference in the number of colonies on LB or LB ampicillin was observed.

e). The inducers in this experiment are glycine (500μg/ml) and L-leucine (200μg/ml), both given together.

Table 7: β-galactosidase activity in strains with psdaA::lacZ

Strains		β-galactosidase Activity (units)	
With psdaA::lacZ	Relevant genotype	No inducers	With inducers
		A	B
MEW1psdaA::lacZ	*crp*+*lrp*+psdaA::lacZ	900±187	1,665±98
CT4psdaA::lacZ	*crp*+*lrp*-psdaA::lacZ	1.815±202	ND[d]
CCRpsdaA::lacZ	*crp-lrp*+psdaA::lacZ	1.108±128	ND[d]
CCRLpsdaA::lacZ	*crp-lrp*-psdaA::lacZ	1.910±289	ND[d]

a). β-galactosidase activity was assayed as described in Materials and Methods.

b). The inducers in this experiment are glycine (500μg/ml) and L-leucine (200μg/ml).

c). Results are the averages ± SD of three experiments.

d). ND: Not determined.

e). Plasmid retention was tested by plating the cells on LB and LB ampicillin plates after experiments using strains with plasmid psdaA::lacZ. Cells that grow on LB but not on LB ampicillin were considered to have lost plasmid. No significant difference in the number of colonies on LB or LB ampicillin was observed.

Part 2. *In vivo* study of the expression of *serA* gene in *crp* mutants

Since CRP interacts with LRP in the regulation of *sdaA*, I wondered if it might also have a role in the regulation of other *lrp*-regulated genes. I therefore investigated the effect of *crp* mutations on the *serA* promoter, which has been reported to be affected by CRP under some growth conditions (Li *et al.*, 2002).

2-1. Expression of the chromosomal *serA* gene

I compared the expression of β-galactosidase from a *serA::lacZ* chromosomal insertion in the same set of host strains used previously: wild-type, *lrp-*, *crp-*, and *crp-lrp-* (Table 8). As discussed earlier, the LRP-deficient strain shows a greatly decreased level of β-galactosidase synthesis, in these experiments 265 vs. 1,475 units. The results were almost identical when CRP-deficient strains were compared, 250 units in *crp-lrp-* vs. 1,375 units in *crp-lrp+* mutants.

It seems then that LRP is needed to activate *serA* transcription. In the absence of LRP, transcription was low with and without CRP (265 and 250 units respectively). In the presence of LRP, transcription was high in both cases (1475 and 1375 units respectively). These results are similar to those reported by Li *et al.*, 2002.

2-2. Expression of plasmid-carried *serA* promoters

The *serA* gene has two promoters, only one of which (P2) appears to be regulated by CRP (Li *et al.*, 2002). I transferred plasmid constructs (made by Li) carrying *serA::lacZ* with either one or both promoters active, to *crp+* and *crp-* hosts, and studied the expression of β-galactosidase (results are summarized in Table 9).

The *crp+lrp+* carrying pBR322 *serA::lacZ* with both promoters showed 12,150 units of β-galactosidase activity (Table 9, Row 1), about 9-fold higher than with the chromosomal gene, no doubt due to the high copy number. Most of this activity (14,400 units) can be attributed to the P1 promoter (Table 9, Row 2). These results were relatively unaffected by CRP-deficiency (Table 9, Rows 4 and 5).

Expression from P2 was much lower, as described previously (Li *et al.*, 2002) and was reduced to 50% in the *crp-* strain (Table 9, Rows 3 and 6). It thus seems that CRP activates P2 but that this has very little effect on the intact promoter - indeed there is a somewhat higher level of transcription in the *crp-* host (Table 9, Rows 1 and 4).

Results from these experiments cannot be interpreted with great confidence because of the considerable degree of plasmid loss, which resulted in lower values than might have been seen otherwise. These particular plasmids are easily lost as described previously (Li *et al.*, 2002).

In cells grown on glucose minimal media, LRP is a major regulator of *serA* expression, and is unaffected by CRP. This is consistent with Li's finding that wild type strain had a measurable effect when grown in glycerol minimal medium. This could not be tested directly because the *crp* mutant does not grow in glycerol.

Table 8: Expression of *serA::lacZ* in *E.coli* chromosome

Strains	Relevant genotype	β-galactosidase activity (units)
CuS2	*crp+lrp+ serA::lacZ*	1.478±167
CuSL	*crp+lrp- serA::lacZ*	265±67
CuSC	*crp-lrp+ serA::lacZ*	1.379±236
CuSCL	*crp-lrp- serA::lacZ*	250±106

a). Experiments were carried out as described in Table 7.

b). Results are the averages ± SD of three experiments.

Table 9:The expression of *lacZ* from individual *serA* promoters

Strains		Active promoter	β-galactosidase activity(units)
MEW1(*crp+lrp+*)	1.	P1P2	12,160±581
	2.	P1	14,400±1250
	3.	P2	1,070±200
CCR(*crp-lrp+*)	4.	P1P2	15,723±1979
	5.	P1	14,963±1017
	6.	P2	408±117

a). Experiments were carried out as described in Table 7.

b). Results are the averages ± SD of three experiments.

c). Plasmid retention was tested by plating cells on LB and LB ampicillin plates after each experiment. . Cells that grow on LB but not on LB ampicillin were considered to have lost plasmid. Plasmid retention in these experiments was 75%-80%.

Part 3: Studies on the effect of CRP on *metK* expression

Apparently CRP had an effect on L-SD activity, and no effect on *serA* transcription (Table 8). The assay for the *serA* gene product is so insensitive that a comparison between strains was prohibitive (McKitrick and Prizer, 1980). I therefore decided to investigate transcription of one other gene that is known to be regulated by LRP, namely the essential gene *metK* (Wei and Newman, 2002). I investigated this using plasmid-carried fusions of the wild-type *metK* promoter and a well-known mutant promoter to *lacZ*. Because chromosomal insertion could not be used in this series of experiments, results constitute an imperfect comparison with the *sdaA* gene.

As seen in Table 10, loss of either LRP or CRP, or indeed both, reduced transcription of *lacZ* by 30%-50% (8417, 6409, 8191 vs. 12030). Since LRP is known to be a repressor of the *metK* gene (Lin *et al.*, 1990), it is quite surprising that expression of *lacZ* from this plasmid should be reduced in the LRP-deficient cell. However, experiments like this are fraught with difficulties in interpretation, as we have already seen. Plasmid copy number may vary. The effects of normal levels of CRP on multiple copies of the *metK* promoter may be different than its effects on a single copy of *metK*. Differences in plasmid retention may also affect the results.

This data is therefore not clearly interpretable. Expression of the mutant *metK* gene, *metK84*, is equally difficult. Expression of *metK84*::lacZ in the wild-type strain is

Table 10:

Expression of *metK::lacZ* and *metK84::lacZ* operon fusion

Strains	Relevant genotype	β-galactosidase activity(units) Plasmid	
		pmetK	pmetK84
MEW1	*crp+lrp+*	12.030 ±1540	1.684±353
CT4	*crp+lrp-*	8.417 ±2208	5.619±89
CCR	*crp-lrp+*	6.409 ±0	2.720±496
CCRL	*crp-lrp-*	8.191±820	5.835±825

a). The β-galactosidase assay was carried out as described in Materials and Methods.

b). Results are the averages ± SD of two or three experiments.

c). The retention of plasmid was tested by plating the cells on LB and LB ampicillin plates after each experiment. Cells that grow on LB but not on LB ampicillin were considered to have lost plasmid. Plasmid retention in these experiments was 75%-80%.

about 1/7 of that seen from *metK*, which is what one might expect from earlier assays showing decreased SAM synthetase in this mutant (Green *et al.*,1973; Budman, 1998). The β-galactosidase level of 1684 units is increased in the *lrp*- host strain (5619 units), and only slightly increased in the *crp*- host.

Part 4. Unexpected Growth Deficiencies of *crp* mutants

4-1. Slow use of Glucose

CRP is well known as a regulator of expression of a wide variety of genes whose products are involved in energy provision. This phenomenon is known as catabolite repression. *Crp* mutants are therefore unable to use a wide variety of carbon sources and rely on glucose and related compounds for growth.

However, mutations in *crp* result in a variety of metabolic effects which are not obviously related to catabolite repression. For example, the pH sensitivity of *crp* mutants has been studied (Ahmad and Newman, 1988). Another is their very slow growth rate in glucose minimal medium. The fact that *crp*-deficient mutants retain other catabolite-related features probably explains why these characteristics are rarely mentioned.

I therefore studied the growth rate of freshly transduced *crp* mutants, which grew in glucose minimal medium pH 6.2 with a doubling time of 180 min (Figure 5, Table 11) at 37°C. This slow growth rate of the *crp* mutant on glucose is quite remarkable since this is the one carbon source one might expect it to use readily. I therefore characterized the growth rate of the *crp* mutant and derivatives of interest.

4-2. Effect of glucosephosphotransferase enzymeII (*ptsG* gene) expression on the growth rate in glucose minimal medium

The *crp* mutant grew more slowly on glucose than the parental strain, which is shown in Figures 4 and 5. The doubling time (Table 11) for the *crp* mutant when grown on glucose was 180 minutes, as compared to 72 minutes for the parental strain. Plasmid pTH111 (pptsG) (kindly supplied by Dr. Aiba) transformed into these *crp* mutants dramatically reduced the doubling time (108 minutes), but had only a minor effect on the parental strain (84 minutes). These results demonstrated that the growth rate of the *crp* mutant strain is limited by the rate of entry of glucose into the cell since the transcription of *ptsG* requires CRP and cAMP. Unknown to us, Dr. Aiba had earlier reported similar results (Plumbridge, 1998).

4- 3. Glycerol cannot be used as a carbon source by *crp* mutants

Glycerol is a carbon source that enters the cytoplasm by facilitated diffusion across the cytoplasmic membrane. It is dependent on the functions of the glycerol facilitator as well as on levels of enzymes needed for glycerol degradation (the *glp* operon). The *glpFK* operon, the common operon for both *glpK* and *glpF* genes, is located near minute 88 on the linkage map of *E.coli* with *glpF* promoter proximal. *GlpF* codes for the facilitator, whereas glycerol kinase, encoded by *glpK*, converts glycerol to G3P. It was suggested that the expression of the *glpFK* operon is sensitive to glucose repression since glycerol kinase expression requires cAMP. One would therefore not expect a *crp* mutant to grow in glycerol minimal medium. Indeed newly transduced *crp* mutants were

unable to use glycerol (Weissenborn *et al.*, 1992). I selected glycerol-using *crp* mutants by growing the *crp* mutants in glucose minimal medium and plating 100ul of the culture on plates containing 0.2% glycerol. After 48 hours incubation, five colonies were observed on the plate. These colonies, like their parent strain still could not use maltose as the sole carbon source. These mutants were named CCRG (MEW1Δ*crp* glycerol+). One of them was carried on for further study.

4-4. Growth rates of the glycerol-using strain

The doubling time of the glycerol-using strain in glucose minimal medium was 180 min (Figure 6, Table 11). Growth was much faster when the cell also carried *ptsG* (108 min). Thus growth on glucose was not affected by selecting a glycerol-using mutant.

In glycerol minimal medium, the glycerol-using *crp* mutant strain grew at the same rate as wild-type *E.coli*. 120 min (Figure 8, Table 11). The *ptsG* plasmid did not improve growth in either case.

Figure 4: Growth of strains MEW1 and MEW1pptsG in glucose minimal medium

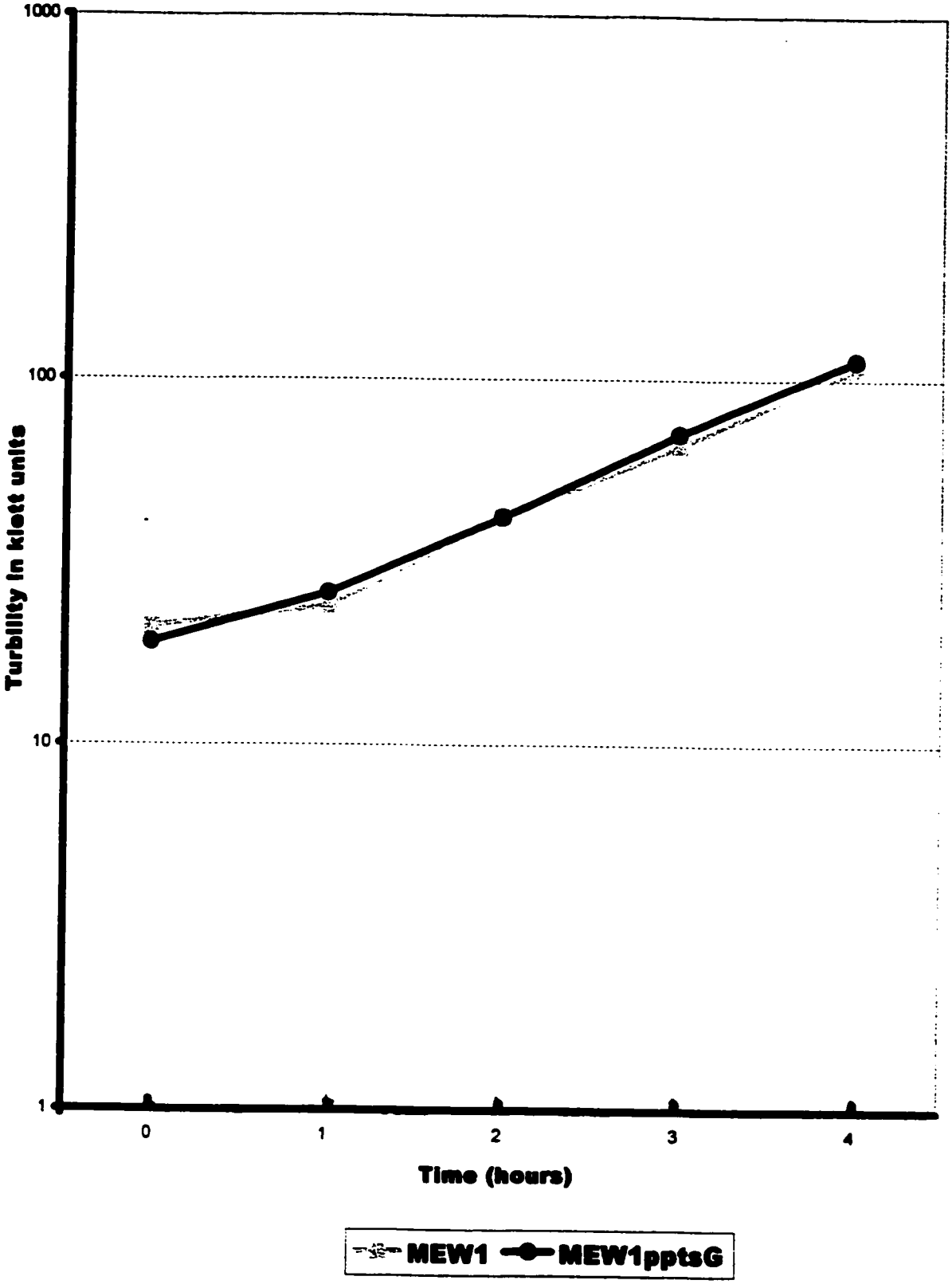

Figure 5: Growth of strains CCR and CCRpptsG in glucose minimal medium

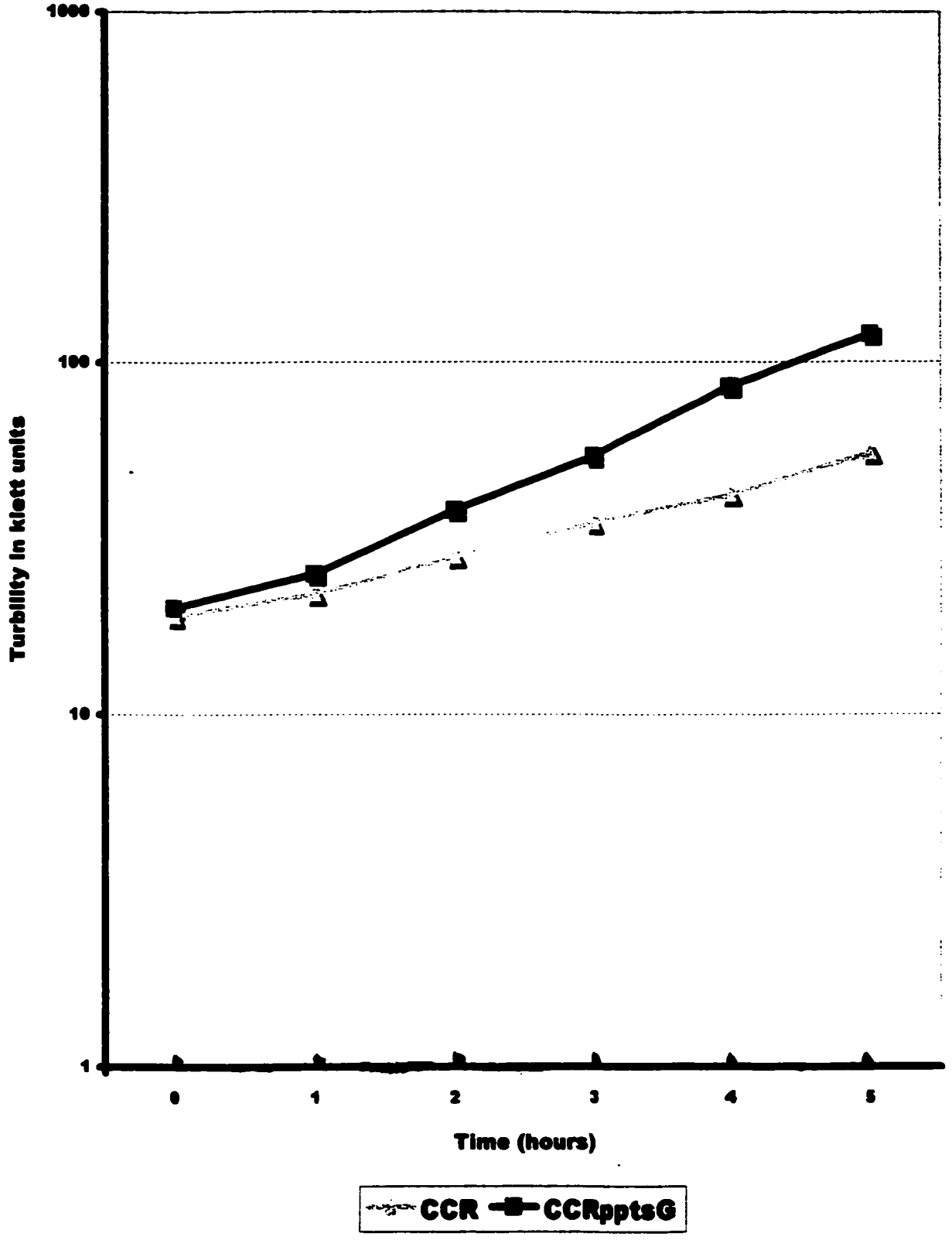

Figure 6: Growth of strains CCRG and CCRGpptsG in glucose minimal medium

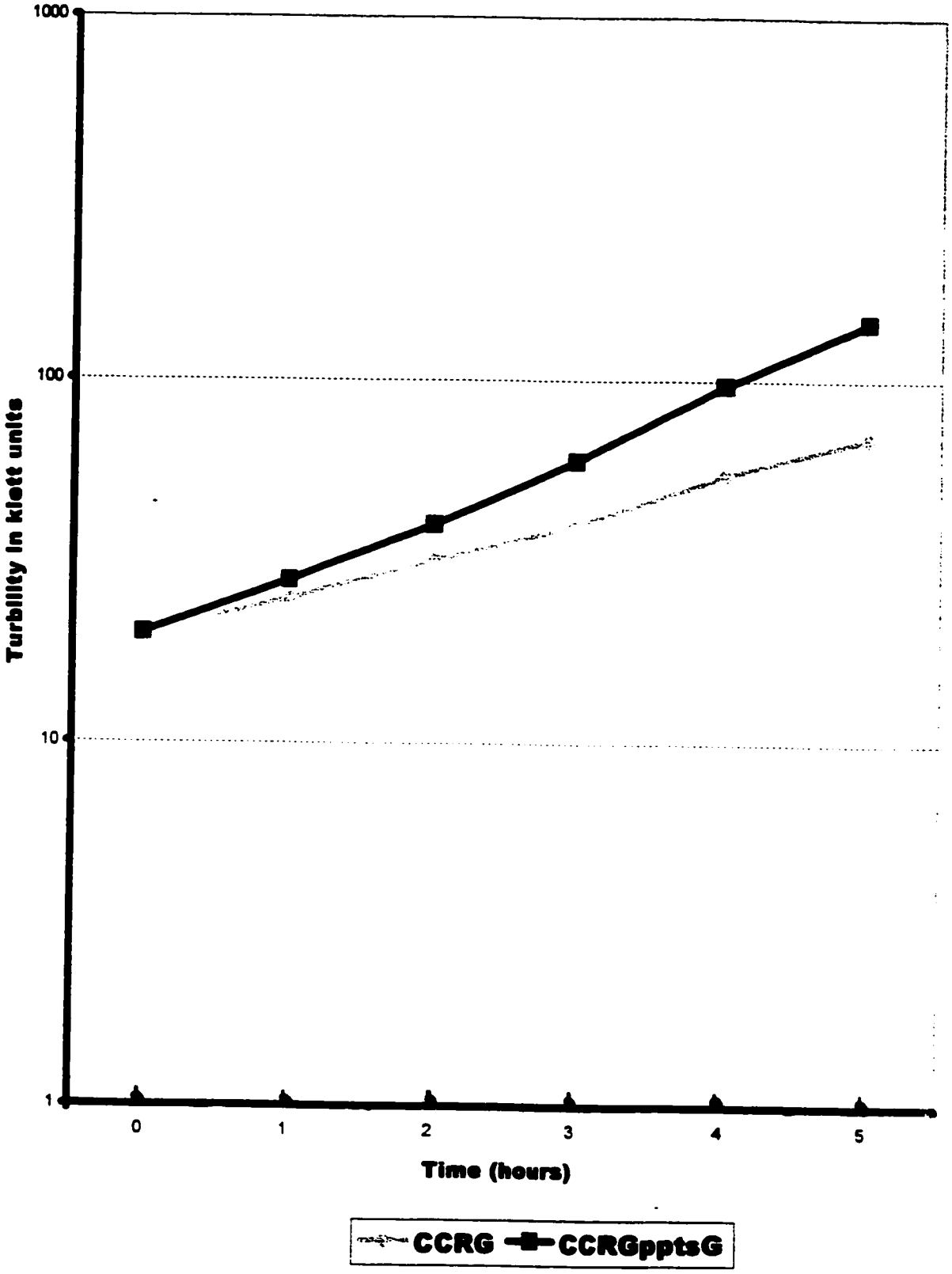

Figure 7: Growth of strains MEW1 and MEW1pptsG in glycerol minimal medium

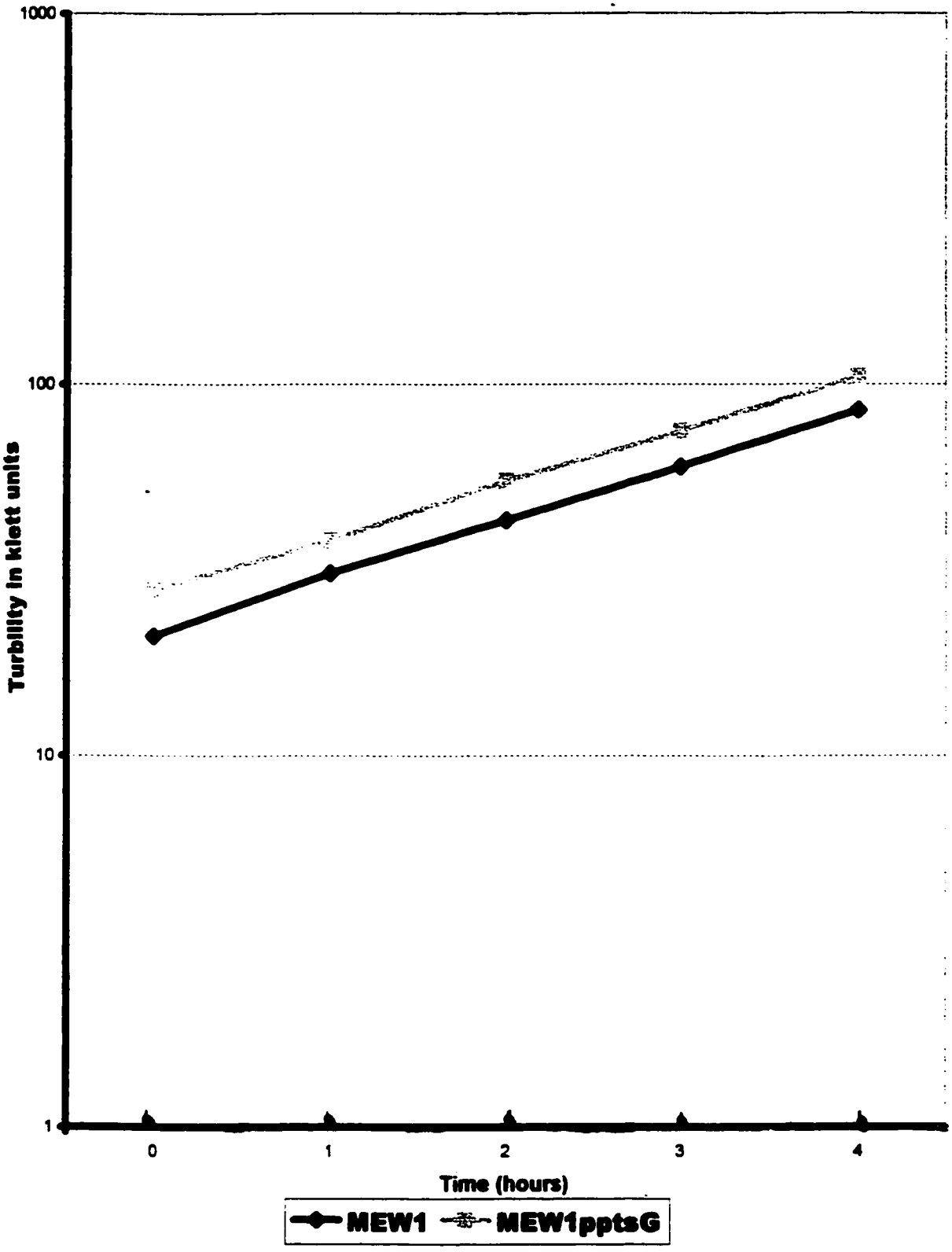

Figure 8: Growth of strains CCRG and CCRGpptsG in Glycerol minimal medium

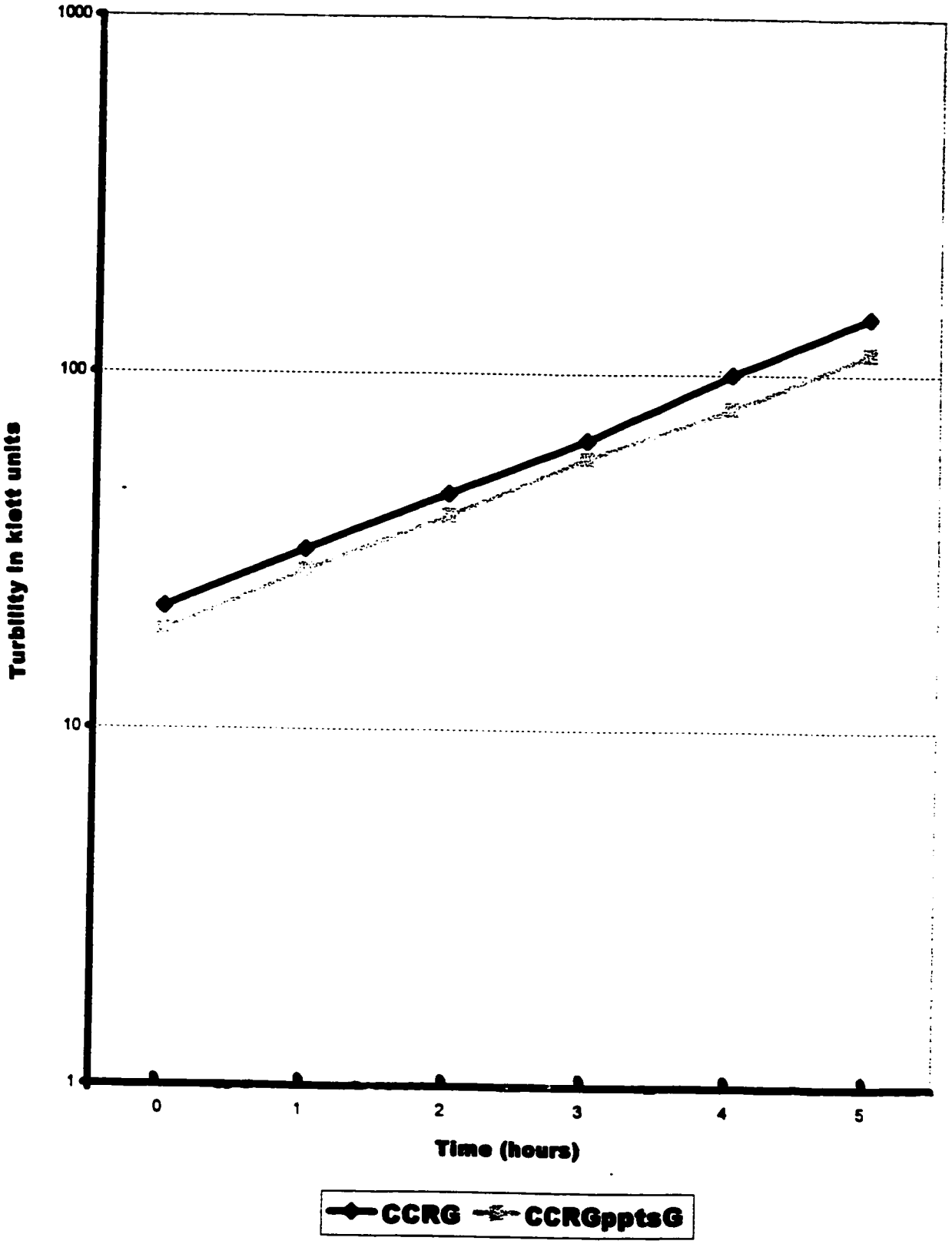

Table 11: Doubling time for *crp* mutants

Strains	relevant genotype	plasmid pTH111 (pptsG)	doubling time (minutes) in GMM	doubling time (minutes) in glycerol MM
MEW1	crp+glycerol+	----	72	120
MEW1	crp+glycerol+	pptsG	84	120
CCR	crp-glycerol-	---	180	--
CCR	crp-glycerol-	pptsG	108	--
CCRG	crp-glycerol+	--	180	120
CCRG	crp-glycerol+	pptsG	108	120

Discussion

Overview and Organization

This project was intended to determine the effects of two major transcription regulators, CRP and LRP, on the expression of the gene coding for L-serine deaminase, *sdaA*, by comparing its expression in a set of strains differing only in the presence and absence of these regulators. Expression was to be compared in four strains, the wild type parental strain, and three mutants derived from it, a *crp-*, an *lrp-* and a *crp lrp* double mutant.

Although this approach has been used many times in microbial physiology, it proved to be impossible to study the effects of CRP and LRP in this way. The reasons for this will be discussed in the text. First, the *crp* mutant itself is so slow-growing in glucose minimal medium that partial revertants were easily selected. One could therefore never be absolutely sure that one was comparing the correct strains. This was an even greater problem with the *crp lrp* double mutant which is exceedingly slow growing. For reasons that are not clear, it proved even more difficult to make the *crp- lrp- sdaA::lacZ* mutant needed for the intended comparisons. I instead tried to use plasmid-carried *sdaA::lacZ* fusions only to find that the assay used for many years to measure *sdaA* activity was not reliable at high expression levels.

The experiments described in this work were intended to elucidate the ways in which CRP and LRP affect the transcription of some *E.coli* genes, *sdaA* in particular. This proved exceedingly complex. In Part 1 of this discussion, I summarize my results on

sdaA expression and make an exceedingly tentative proposal that although CRP is involved in establishing the amount of L-SD activity in the cell, this is not achieved through a direct effect on transcription. In Part 2, I survey the evidence that CRP activates the *serA* P2 promoter though it is not the major effector of *serA* expression. In Part 3, I survey the influence of CRP on *metK* expression, though again without a clear answer. In Part 4, I compare the three promoters, *serA*, *sdaA* and *metK*. In Part 5, I discuss the reasons for the slow growth on glucose and for the failure to grow on glycerol seen in freshly transduced *crp* mutants.

Part 1. The involvement of *crp* in the regulation of L-SD and *sdaA* gene expression

LRP is known to be involved in the regulation of L-SD. It was also proposed to repress *sdaA* gene expression at the level of transcription (Lin, 1992) based on the fact that transcription of an *sdaA::lacZ* fusion is greatly increased in the LRP-deficient mutant. The presence of a putative CRP binding-site in the *sdaA* promoter region suggested that CRP could be directly involved in the regulation of *sdaA* gene expression at the level of transcription.

1-1. The roles of LRP and CRP in the regulation of L-SD in *E.coli*

In the work presented here, I confirmed that L-SD activity is about four times higher in the *lrp* null mutant MEW26 (*lrp::Tn10*) than in the wild type strain MEW1 (Table 4.). I was surprised to find that CRP appeared to be needed for the increase in L-SD activity in the absence of LRP, based on results obtained with the *crp* mutant. This result is interesting but subject to doubt for at least two reasons. It could be due simply to the very slow growth rate of the *crp* mutant strains. This explanation is unlikely however since the *crp* mutant itself grows slowly and has low levels of *sdaA* expression, whereas the double mutant grows only slightly more slowly and has much more activity. However, if the effect of adding a plasmid carrying *ptsG* were known at the beginning of this project, it would have been a good idea to test all four of the strains in Table 4 with a *ptsG* plasmid transformed into them.

The second problem is that the *lrp crp* double mutant is a 'sick' strain. Although the *crp*- phenotype is always verified by plating strains on maltose, *lrp*- is confirmed by transducing MEW1 to *crp- lrp-*, and selecting maltose+ and NSIV autotrophs (see Materials and Methods). However, since both *crp* and *lrp* collect partial suppressors, the construct can not be completely verified. That is, prolonged growth of either strain selects for a faster and more efficient growth rate, due to as yet unknown factors. Thus, except for very recent transductants of single mutations, one cannot be sure that the observed phenotypes are the result of the known introduced mutations, or the result of an

unwittingly introduced suppressor(s). Introduction of a plasmid carrying *crp* could have partially compensated for this problem.

To check this further, I studied the effect of LRP on transcription of chromosomal *sdaA::lacZ*. This gave clear results for the *lrp* mutant strain (4x higher, Table 12). However, the *crp* mutant and the *crp, lrp* double mutant proved to be very difficult to construct, suggesting that double mutant may not be viable (see Section 1-3).

I therefore decided to try the same experiments with *sdaA* and *sdaA::lacZ* carried on a plasmid. Whole cell L-serine deaminase assays gave results quite similar to those seen with the chromosomal *sdaA* gene. However, assay results for genes carried on a moderately high copy plasmid showed an L-SD activity only slightly higher than that with the chromosomal *sdaA* gene, suggesting that we might not be measuring *sdaA* activity accurately.

The L-SD assay was devised in 1955 by Pardee and Prestidge, and modified by Isenberg and Newman in 1974. It calls for washing log phase cells, re-suspending in phosphate buffer pH 7.8, and assaying for 35 minutes. It was used to measure the low expression levels seen in chromosomal genes and was checked exhaustively at that time for linearity with time, cell volume, and substrate. When others in the lab and I realized that these conditions might not be optimum for high expressing strains, we checked whether the assay was linear for 35 minutes and found that linearity stopped at around 15 minutes for reasons not yet known. YeMan Tang re-suspended cells in glycylglycine, used fewer cells (20ul instead of 100-300ul), and showed that the assay was reliable under these conditions for at least 30 minutes.

The assay for plasmid carrying *lacZ* was problem-free due to the extraordinary range of *lacZ* expression that can be assayed, and the amazing stability of the enzyme itself. Table 7 showed that the values measured in strains carrying the plasmid were approximately nine times higher than from the chromosomal gene (Table 12) and were inducible by glycine and leucine, as expected for the *sdaA* promoter. Once again *lacZ* activity was increased by LRP-deficiency with or without CRP suggesting that transcription of *sdaA* is independent of CRP.

1-2. Post-translational regulation of *sdaA*

Given the uncertainties as discussed in the previous section, it is not possible to come to a clear conclusion as to the relationship of LRP and CRP in the regulation of *sdaA* gene expression. Nonetheless, since both the chromosomal and the plasmid-carried *sdaA::lacZ* fusions are transcribed at a higher level in the absence of LRP than in its presence, whether CRP is available or not, it seems likely that LRP influences transcription of *sdaA* and CRP does not.

Suppose then that the *crp lrp* double mutant really has much less L-SD activity than the *lrp* mutant – what could cause this? We know that a loss of CRP does not affect transcription (see Table 7). If the same amount of transcription takes place in the double mutant, then less *sdaA* mRNA must be translated into active gene product. This would occur if the mRNA were unstable in the absence of CRP, although nothing is currently known about a possible mechanism that might account for this. However, since it is

known that the gene product requires activation (Newman *et al.*, 1985), it is possible that the activation step(s) involve a gene product of CRP-dependent transcription.

1-3. Can an unsuppressed *sdaA::lacZ*, *Δcrp* mutant exist?

One of the strains that was important for this work is one with both Δ*crp* and *sdaA::lacZ* on the chromosome. One would not expect this to present any particular difficulty in construction. Since our *sdaA::lacZ* was constructed by inserting λplacMu (Su *et al.*, 1991) and thus has the structure *sdaA*::λplacMu9 and is kanamycin-resistant, I attempted to make this strain by growing the phage P1 on *sdaA::lacZ* and transducing to the *crp*- host and selecting for kanamycin resistance. However, the resulting transductants all had unexpected phenotypes, including serine requirement for growth, ability to use serine as carbon source (which the parent strain does not) and relatively high L-SD activity as seen in Table 12.

These results seemed extremely interesting. However when I used PCR to see if the strain still had *sdaA*, I unexpectedly found a 1.5kb band which has the equal size of *sdaA* gene, and the restriction enzyme digestion analysis showed the same patterns as *sdaA* gene (Figures 9 and 10). Since the primers were in the upstream and downstream regions of *sdaA*, they could not amplify *sdaB* or *tdcG*, the homologues of *sdaA* in *E.coli*. It seems then that the Mu9 insert moved during transduction as it often does. However this does not account for the odd phenotypes just described.

Figure 9: PCR amplification of the *sdaA* gene in different strains

PCR mixes and program are described in Materials and Methods. Different template DNAs were used for PCR as described below. 10ul of amplified product was loaded onto the agarose gel.

Lane M: 1kb DNA marker. The corresponding DNA sizes from the top to the bottom of the gel are: 10kb, 8kb, 6kb, 5kb, 4kb, 3.5kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb, 750bp, and 500bp.

Lane 1: no template DNA was added to the PCR

Lane 2: template DNA from strain MEW1

Lane 3: template DNA isolated from strain Cup22 (MEW1 *sdaA*::λplacMu9)

Lane 4: template DNA isolated from strain Cup22L (MEW1 *sdaA*::λplacMu9 *lrp*::Tn10)

Lane 5: template DNA isolated from strain Cup22C (MEW1 *sdaA*::λplacMu9 Δ*crp*)

Lane 6: template DNA isolated from strain Cup22CL (MEW1 *sdaA*::λplacMu9 *lrp*::Tn10Δ*crp*)

Lane 7: template DNA isolated from strain CH22 (MEW1 *sdaA*::cm)

Lane 8: template DNA isolated from strain CH22C (MEW1 *sdaA*::cm Δ*crp*)

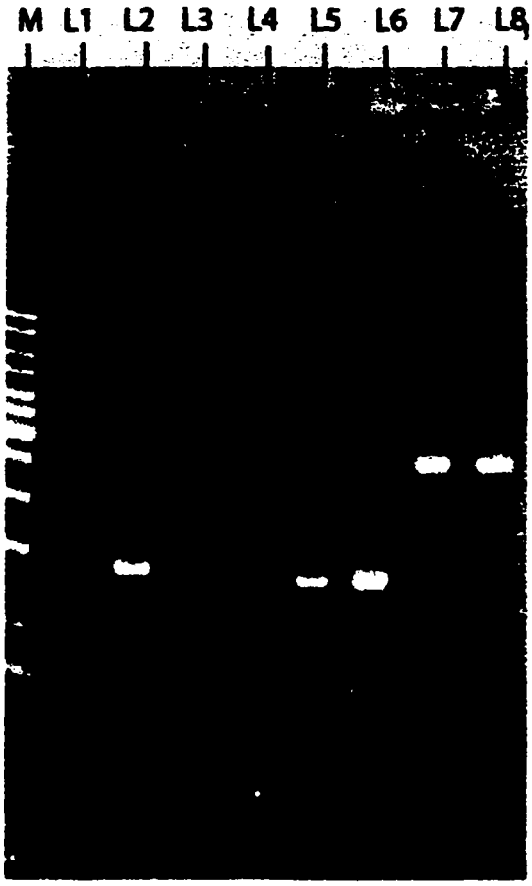

Figure 9: PCR amplification of the ***sdaA*** gene in different strains

Figure 10: Restriction Enzyme Digestion Analysis of Amplified '*sdaA* gene'

A. PCR products digested with HpaI

Lane M: 1kb DNA marker. (Corresponding sizes are described in Figure 9.)

Lanes 1, 3, 5: undigested PCR products (using DNA isolated from strain MEW1, Cup22C, Cup22CL as template respectively)

Lanes 2, 4, 6: PCR products (strain MEW1, Cup22C, Cup22CL) digested with HpaI

B. PCR products digested with ClaI

Lane M: 1kb DNA marker. (Corresponding sizes are described in Figure 9.)

Lane 1, 2, 3: PCR products (strain MEW1, Cup22C, Cup22CL) digested with ClaI

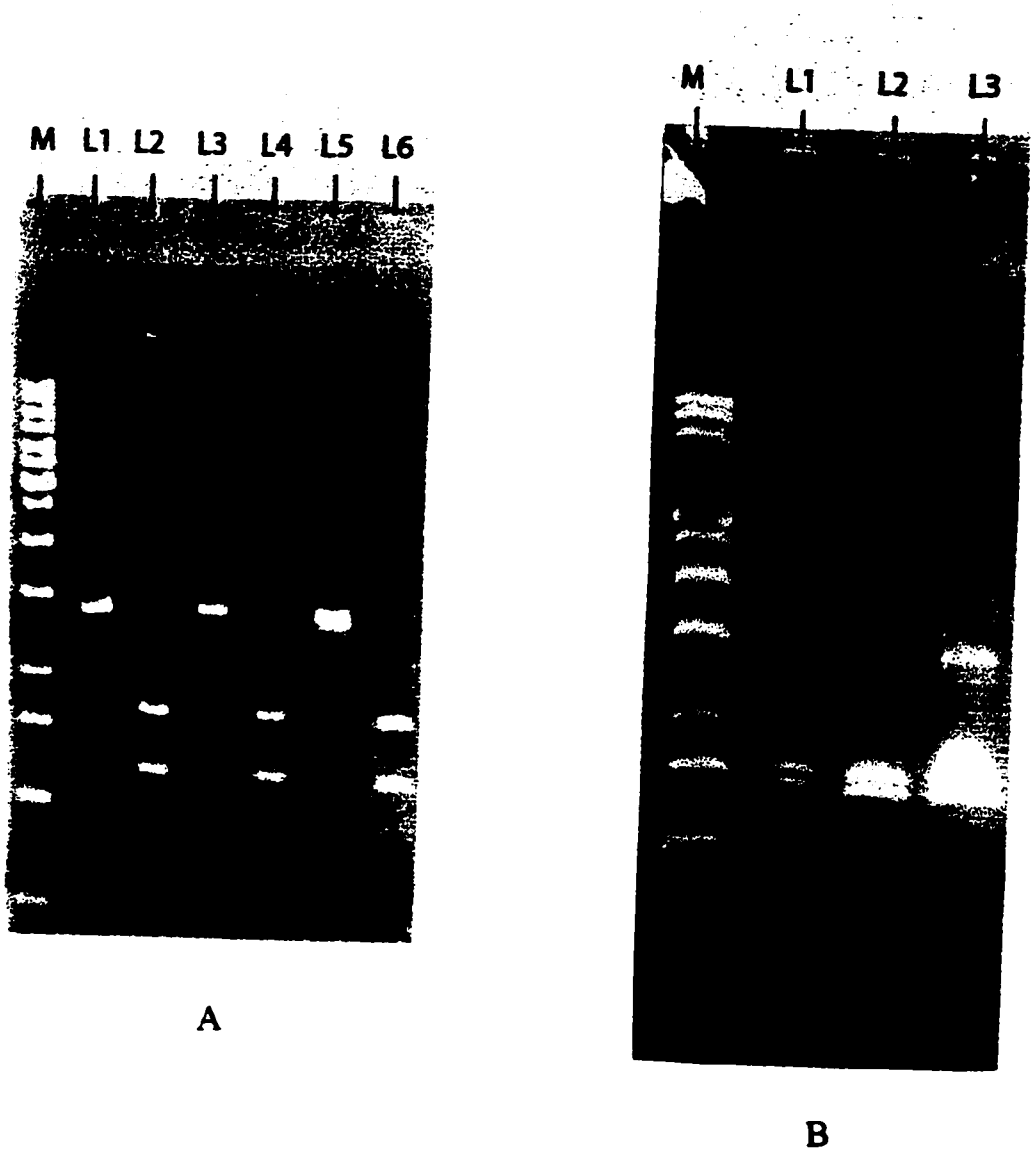

Figure 10: Restriction Enzyme Digestion Analysis of Amplified '*sdaA* gene'

Table 12. Expression of L-SD and β-galactosidase activity in *sdaA* deficient strains

Strains	Relevant genotype	L-SD (units)	β-gal
Cup22	*sdaA*::λplacMu9	0.059±0.020	90±29
Cup22L	*sdaA*::λplacMu9 *lrp*::Tn10	0.069±0.020	311±26
Cup22C	*sdaA*::λplacMu9 Δ*crp*	0.183±0.060	ND[3]
Cup22CL	*sdaA*::λplacMu9 Δ*crp lrp*::Tn10	0.175±0.018	ND[3]
CH22	*sdaA*::cm	0.088±0.006	----
CH22C	*sdaA*::cm Δ*crp*	0.083±0.016	----

1). Whole cell L-SD and β-galactosidase assays are described in Materials and Methods.

2). Results are the averages ± SD of two or three independent experiments.

3). ND: not determined.

I therefore tried to do the transduction in the opposite direction, making phage on JCB43Δ*crp* and selecting a closely linked streptomycin-resistance gene *rpsL*. No transductant lacking both L-SD activity and CRP was found on the many occasions that this transduction was attempted.

I tried to make the same double mutant using strain CH22 as *sdaA::cm* donor. This was constructed by transducing *sdaA::cm* into *crp-* host strain. It proved relatively easy to make this transduction into *Δcrp* selecting chloramphenicol resistance. This produced a strain with the same low, almost negligible level of L-SD activity as the original CH22 (Table 12), and gave a PCR product of the correct size (Figure 9, Lanes 7 and 8).

1-4. How might one study this problem more effectively?

The *crp* mutant is a fragile strain (Ahdmad and Newman, 1988). Many odd characteristics have been described over the years, but none affected the results of experiments on the interactions of CRP and RNA polymerase at various promoters (Ebright and Busy, 1999).

Nonetheless even if the molecular mechanisms of CRP function in catabolite repression are clear, the physiological roles of the cAMP/CRP complex are not. The slow growth of the *crp* mutant on glucose has been partially explained here and elsewhere, but

it is not clear why the addition of pptsG increases the growth rate to only about half the wild-type rate. Conflicting reports in the early literature as to whether a *crp* mutant can use glycerol have also been resolved by the demonstration that suppressor mutations can restore glycerol use.

The *E.coli* chromosome has many putative CRP binding sites according to the Church Web site (http://www.arep.med.harvard.edu). Indeed it has been proposed that CRP might influence chromosome structure in *E.coli* by virtue of these numerous sites. Problems in constructing strains as described earlier could be avoided if there were a way to construct the double mutant in the presence of CRP and then rapidly lose the CRP from the cell.

One way to do this would be to have a plasmid carrying *crp* so that *crp* was continuously synthesized. i.e. pcrp would replace chromosomal *crp*. One would then add the desired mutations in the presence of CRP and screen for strains that lose plasmid. The only drawback to this approach is that the time required to plate the strain to select for plasmid loss would allow too much time to select suppressors.

It would be clearer if *crp* were cloned into an expression vector that could be completely turned off. This was difficult in the past, but Wei has constructed a low copy number, regulatable vector called p15A using elements of the Lutz and Bujard plasmid system. This vector has been shown to be sufficiently regulated to demonstrate that a total loss of *metK* was lethal to *E.coli* (Wei and Newman, 2002). By cloning the *crp* gene into this plasmid with the tet promoter, I could make expression of *crp* dependent on the presence of the inducer anhydrotetracycline, make the double or triple mutants desired, and then assay either L-SD or β-galactosidase in the generations following

withdrawal of inducer, a short period in which suppression is not a factor. Attempts to do this are now underway.

Part 2. Possible molecular regulatory mechanism of *serA* gene by CRP in *E.coli*

The molecular mechanism of *serA* gene expression regulation was studied in more detail in Dr. Newman's lab. LRP can decrease *serA* transcription in glucose minimal medium. Two functional promoters were present in the *serA* gene promoter region. A site-directed mutagenesis study showed that LRP represses *serA* transcription at the P1 promoter, while activating *serA* transcription at the P2 promoter, as also reported in this thesis.

Li suggested that CRP is involved in the regulation of the *serA* P2 promoter by testing β-galactosidase activity in liquid minimal media with different carbon sources. Relatively high expression from MEW26pserAP2 in glycerol minimal medium suggested that CRP might be involved in the regulation of the *serA* P2 promoter in the absence of LRP (Li *et al.*, 2002). Moreover, a putative CRP-binding site was found near the P2 promoter (Figure 11). It was therefore suggested that CRP activates *serA* expression at the *serA* P2 promoter in the absence of LRP.

My results (Table 8) confirmed that CRP activates the *serA* P2 promoter *in vivo* even in the presence of LRP. Since expression of *serA* from the P2 promoter accounts for

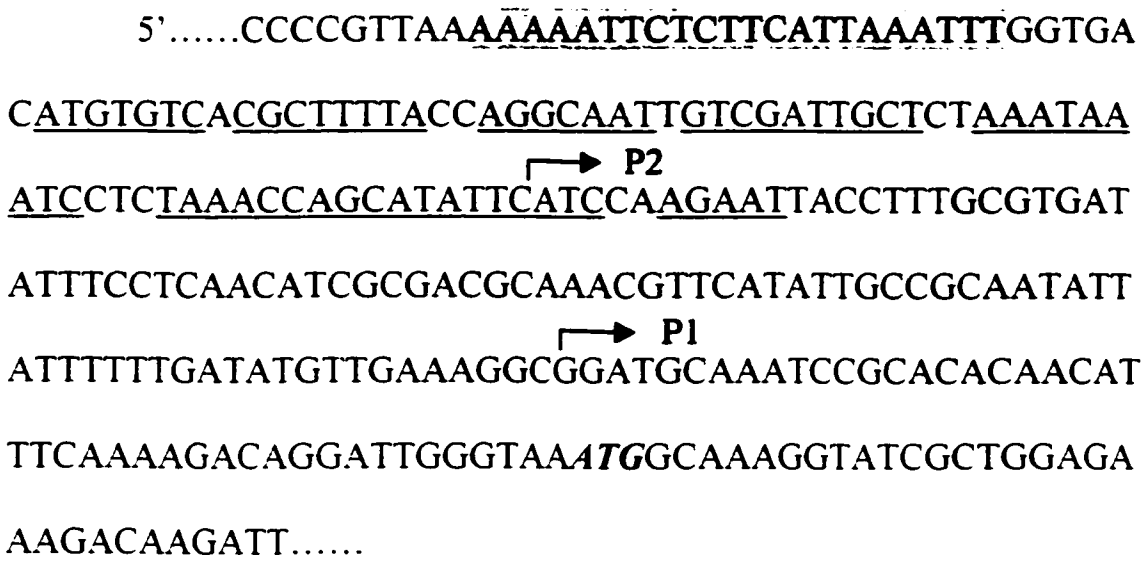

Figure 11: Structure of the *serA* promoter

serA has two transcription initiation sites corresponding to the promoters P1 and P2. The P1 initiation site is a G residue. LRP-protected nucleotides are underlined. Highlighted nucleotides indicate a putative cAMP-CRP binding site (Li *et al.*, 2002 and (http://www.arep.med.harvard.edu).

only 1/10 to 1/40 of full *serA* expression. it follows that CRP is not required for full *serA* expression.

To further confirm this result. the putative CRP binding site (Figure 11) could be defined using *in vitro* site-directed mutagenesis.

Part 3. Possible molecular regulatory mechanism of *metK* by CRP

The promoter region and transcriptional regulation of the *metK* gene have not yet been well studied. Therefore. the regulatory mechanism of *metK* gene expression and the proteins and factors involved are still not clear. although LRP has been suggested to repress *metK* expression.

The *metK84* mutant strain grown in glucose minimal medium could be avoided by the presence of high concentrations of L-leucine. or by loss of LRP. Therefore. it has been suggested that LRP represses *metK* expression while L-leucine antagonizes LRP to release this repression (Newman *et al.*, 1998). Recently, Wei observed a point mutation at the −10 region of the *metK* promoter in the *metK*84 mutant. *metK* would be affected if this site was altered (Wei and Newman. 2002). The results of my β-galactosidase assay are consistent with the phenomenon as I mentioned earlier. i.e. the *metK* mutant (*metK*84) can drastically decrease *metK* expression (Table 10).

Only one functional promoter was defined in the rich minimal medium LB. However. the existence of other possible functional promoter(s) in glucose minimal

media remains unknown (Wei. 2001). Based on my results, the regulation of *metK* gene expression at the transcriptional level cannot be explained by the presence of only one functional promoter when the cells are grown in rich medium. Two or more functional promoters were probably present in the glucose minimal medium.

Further studies are required to understand the fundamental role of LRP and CRP in the regulation the *metK* promoter. For examples, DNA footprinting can be used to test whether LRP or/and CRP directly binds to the *metK* promoter. Gel retardation assays can be performed to investigate binding specificity of LRP or CRP on wild type or mutant *metK* promoters.

Part 4. Different patterns of *crp* involved in the regulation of *sdaA*, *serA* and *metK* genes

As discussed above, different regulation patterns in the *E.coli sdaA*, *serA* and *metK* genes suggest that CRP is involved in the regulation of these three genes with different mechanisms.

Part 5. Glucose uptake is one of the factors that affect slow growth of the *crp* mutants.

The fact that the *crp* mutant grows extremely slowly in glucose minimal medium led us to study the possible mechanisms involved. Results indicating that CRP was involved in the regulation of the gene that encodes the main glucose transporter, *ptsG*, suggested that the slow growth of the *crp* mutant may be related to its role in *ptsG* gene regulation. However, the doubling time for *crp* mutant strains carrying plasmid *ptsG* was still longer than the wild type strain with or without plasmid pptsG, although much shorter than the 180 minutes for *crp* mutant without plasmid pptsG. This suggests that besides CRP-affected glucose uptake, CRP must be involved in other mechanisms that regulate cell growth rate.

During this work, I made a glycerol usage *crp* mutant. Some phenotype studies of this mutant suggested that the mutation is located near the *glpK* promoter region. Further study of the structure of the *glpK* promoter region could provide more information about the binding site of CRP on the promoter, and the role of CRP in the regulation of *glpK* expression.

Summary

Studies of L-serine deaminase (L-SD) expression in Dr. Newman's laboratory have suggested that LRP is a major regulator affecting *sdaA* transcription. A putative CRP binding site at the *sdaA* promoter region suggested that CRP might also be involved in the regulation of *sdaA* expression, probably at the level of transcription. The intent of this work was to determine the effect of two major transcription regulators, CRP and LRP, on the expression of the gene *sdaA*.

In order to test whether CRP really affects *sdaA* expression, I constructed *crp* and *crp, lrp* double mutants. I assayed and compared the *in vivo* L-SD activity in these two mutants (MEW1*crp*-, MEW1*crp-lrp*-) with that of MEW1 and MEW26*lrp*-. The results demonstrated that the increase in L-SD activity in the LRP-deficient strain occurs via a CRP-dependent mechanism. This result was confirmed by providing CRP carried on a plasmid. I also showed that this mechanism was true for *sdaA* expression. LRP regulates *sdaA* transcription while CRP is not involved and does not affect transcription.

I also studied two other LRP-regulated genes: *serA* and *metK*. Investigation of the *serA* gene P1 and P2 promoters suggested that CRP activated the *serA* P2 promoter but did not affect the *serA* P1 promoter. However, for full *serA* expression, CRP was not affected. The study of the role of CRP in the regulation of *metK* expression did not give a clear answer due to the unknown character of the *metK* promoter.

Comparison of the regulation patterns of the *serA*, *sdaA* and *metK* promoters showed that LRP and CRP played quite different roles in their expression.

In another part of this project, I studied the reasons for the slow growth of *crp* mutants on glucose. Results indicated that the slow growth rate was partially due to the effect of the *crp* gene on glucose uptake. Results also demonstrated that cells take up glucose and glycerol in different ways. A glycerol using *crp* mutant was also selected and results suggested that the mutation was positioned at the glpK promoter region.

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