

## ABSTRACT

Title of dissertation:           **AUTOINDUCER-2 (AI-2) MEDIATED QUORUM SENSING IN *ESCHERICHIA COLI***

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Bacteria have evolved complex genetic circuits to regulate their physiological activities and behaviors in response to extracellular signals. In a process termed “quorum sensing”, or density-dependent gene regulation, bacteria produce, release and respond to certain signaling molecules termed autoinducers. The bacterial autoinducer-2 (AI-2) has received intense interest recently because the gene for its synthesis, *luxS*, is common in a large number of Gram-negative and Gram-positive bacterial species. In this study, the *luxS* controlled genes were identified in *Escherichia coli* K12 strain under two different growth conditions using DNA microarrays. Deletion of the *luxS* was shown to affect expression of genes involved in AI-2 transport (the *lsr* operon) and methionine biosynthesis (*metE*), and to a lesser degree those involved in methyl transfer, iron uptake, resistance to oxidative stress, utilization of various carbon sources, and virulence. The effects of glucose on extracellular AI-2 level were investigated further. It

was shown that both AI-2 synthesis and uptake in *Escherichia coli* are subject to catabolite repression through the cAMP-CRP complex. This complex directly stimulates transcription of the *lsr* (*luxS*-regulated) operon and indirectly represses *luxS* expression. Specifically, cAMP-CRP is shown to bind to a CRP binding site located in the upstream region of the *lsr* promoter and works with LsrR repressor to regulate AI-2 uptake. This study, for the first time, has shown that quorum sensing regulates specific activities in *E. coli* K12, and has elucidated regulatory mechanisms for AI-2 biosynthesis and transport in this organism. With a better understanding of AI-2/*luxS* mediated gene regulation, we may be able to develop strategies for harnessing AI-2 quorum sensing for our advantage in bioreactor studies and ultimately in control of the bacterial pathogenicity.

AUTOINDUCER-2 (AI-2) MEDIATED QUORUM SENSING IN  
*ESCHERICHIA COLI*

by

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Doctor of Philosophy

## DEDICATION

I would like to dedicate this work to my wife, Min Zhou, without whom none of the following would have been possible.

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## Chapter 1 General Introduction

### What is Quorum Sensing?

Bacteria are capable of responding to a variety of chemical and physical changes in the environment by regulating their gene expression. These changes could be environmental stresses, such as heat shock, nutrient limitation, high osmolarity, which cause cells to have multigenic responses in transcription and translation. In addition, these changes could be caused by the metabolic activities of the bacteria themselves. For example, they produce some chemical molecules that are released into the environment as the cell density increases. In certain situations, some metabolic products evolve into signaling molecules, which can be perceived by the cells to control gene expression. This type of regulation (autoinduction) gives bacteria the capability to communicate with each other and coordinate their activities, and has been termed quorum sensing (118). In quorum sensing, the concentration of the chemical molecules or autoinducers reflects the number of bacterial cells. When a threshold stimulatory level of autoinducers is achieved, a signal transduction cascade is initiated that ultimately results in a change in the behavior of the bacteria. Many Gram-positive bacteria use small peptides as signal molecules (86, 131). Gram-negative bacteria appear to use small molecules (eg. Acyl-homoserine lactone) for this purpose (203, 206). The cellular processes regulated by quorum sensing are diverse. Among these are bioluminescence (51, 96), spore formation (95), motility (50, 64), competence (102), conjugation (60), antibiotic synthesis (7, 41), virulence (119, 138, 172), and biofilm maturation (36, 147).

### Acyl-Homoserine Lactone (AHL) Mediated Quorum Sensing

AHL autoinducers have been found in more than 70 species of Gram-negative bacteria (37, 59, 118, 136). These molecules share a common homoserine lactone moiety with differences only in the lengths of the acyl side-chain and the substitutions on the side chain (37, 59). In all cases of this type of quorum sensing, the bacteria use similar LuxI/LuxR type protein pairs to control different cellular activities. The LuxI-like proteins are AHL synthases, which through their products determine the specificity of the autoinducers. The LuxR-like proteins are responsible for binding to specific DNA promoter regions to regulate the expression of the target genes upon interaction with the AHL autoinducers (61). Due to the highly specific interaction between AHL and LuxR, Gram-negative bacteria use the mechanism to conduct intraspecies cell-cell communication without interference from the other species.

### The *Vibrio fischeri* LuxI/LuxR System

Quorum sensing in the marine bacteria, *Vibrio fischeri* and *Vibrio harveyi*, was first described in the early 1970's (47, 127). *Vibrio fischeri* is found free-living in the ocean as well as in the light organs of certain marine fishes and squids(157) . *Vibrio fischeri* synthesizes one specific autoinducer, *N*-3-(oxohexanoyl)homoserine lactone (48), which can freely diffuse across the cell membrane down a concentration gradient. At low cell densities, the autoinducer is at low concentration. When the cell density increases, the autoinducer can accumulate to a concentration sufficient for the activation of luminescence. In the light organs where *Vibrio fischeri* cells can achieve a density of

$10^{10}$  to  $10^{11}$  cells per ml, the autoinducers can reach a critical concentration (5 to 10 nM) required for *lux* operon stimulation; while in the sea water, the density of these bacteria is less than  $10^2$  cells per ml, and the autoinducers diffuse out of the cells, resulting in no observable luminescence (157). Therefore, the autoinduction system allows *Vibrio fischeri* cells to distinguish between the free-living state (low cell density) and the symbiotic state (high cell density).

The symbiotic relationship between squid *Euprymna scolopes* and *Vibrio fischeri* provides a remarkable example of specific cooperativity during the development and growth of both organisms (157, 158, 191). Once the juvenile squid is infected by the *Vibrio fischeri* cells, maturation of its light organ begins, while growth in sterile sea water prevents the full development of the organ (111). The luminescing bacteria provide benefits to the squid, a nocturnal forager, by erasing the shadow that would normally be cast when the moon's rays strike the squid from above, thus protecting it from predators below (98, 157). The squid, in turn, rewards the bacteria by providing a sheltering haven with a stable source of nutrients (98, 157). The cell density dependent control of luminescence ensures that the bacteria waste little energy on light production until they reach a high cell density, when the illumination provided for the squid is likely to be well repaid in food and protection (98).

The light production in *Vibrio fischeri* is controlled by the *lux* genes, which are organized into two different transcription units. One unit contains *luxR*, the gene product of which is thought to be an AHL-dependent transcriptional activator of luminescence. The other unit is the *luxICDABEG* operon, which is activated by LuxR in the presence of AHL. The *luxI* gene encodes the autoinducer synthase that directs the

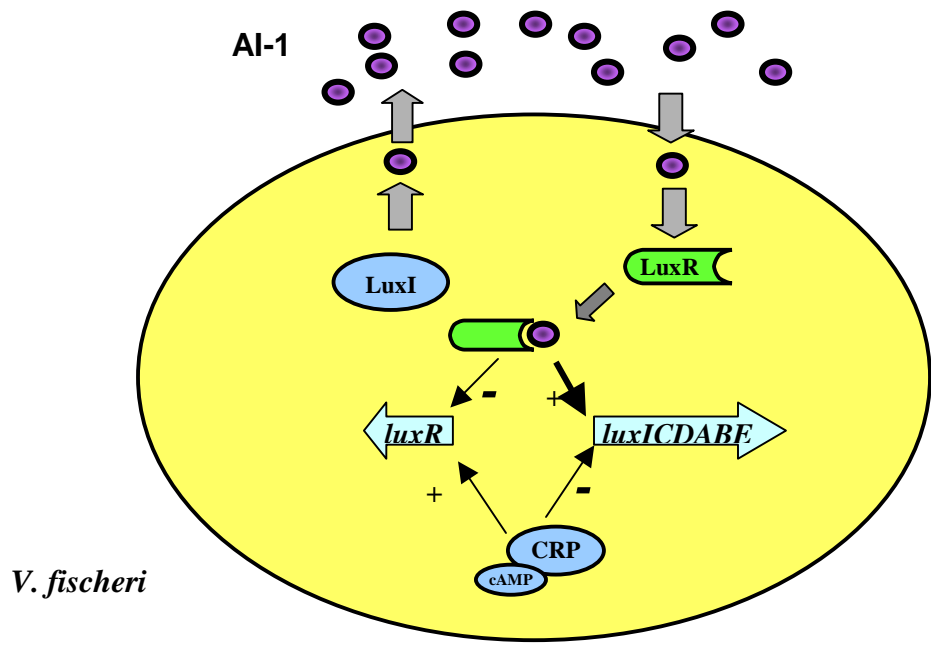
bacteria to synthesize the AHL, *N*-3-(oxohexanoyl)homoserine lactone (48, 52). The other genes play mechanistic roles in light production. The *luxA* and *luxB* genes encode the  $\alpha$  and  $\beta$  subunits of luciferase, respectively. The *luxC*, *luxD*, and *luxE* genes encode components of the fatty acid reductase complex required for the synthesis of the aldehyde substrate for luciferase (52). The *luxG* gene might code for a flavin mononucleotide reductase that generates reduced flavin mononucleotide as a substrate for luciferase (4).

LuxR and LuxI play central roles in the cell density-dependent control of *lux* gene expression (Figure 1-1). At a low cell density, *luxICDABEG* is transcribed at a basal level. The concentration of the autoinducer is low, and only a low level of light is produced (51). As population of the bacterium increases, autoinducer accumulates slowly in the growth medium and inside the cells. At a sufficiently high AHL concentration, this signal molecule interacts with LuxR protein to form a positive transcriptional complex, which then activates transcription of the *luxICDABEG* operon by approximately a thousand fold. This results in the induction of luminescence and in the positive autoregulation of *luxI* (61, 70). The transcriptional complex of the LuxR-AHL also negatively regulates the expression of *luxR* itself, which reduces transcription of the *luxICDABEG* operon in a feedback loop (51).

Luminescence in *Vibrio fischeri* is inhibited by growth in the presence of glucose (57). The concentration LuxR is very low when the cells are grown in a high glucose condition, suggestive of catabolite repression, a process dependent upon CRP and cAMP. A consensus CRP binding site is located in the center region between *luxR*

**Figure 1-1 The LuxR/LuxI-based quorum sensing in *Vibrio fischeri***

In *Vibrio fischeri*, LuxI is responsible for the production of autoinducer AI-1, an acyl-homoserine lactone (AHL) signaling molecule, which can move freely across the cell membrane. The concentration of AI-1 increases as a function of cell density. When a threshold concentration is reached, the AI-1 binds to a transcriptional regulator, LuxR, which then activates transcription of the bioluminescence genes *luxICDABE*, and represses its own expression. In addition, cAMP-CRP stimulates expression of the *luxR* genes, but inhibits *lux* operon expression.





and *luxI* genes. The cAMP and CRP stimulate *luxR* expression, but inhibit *luxICDABEG* expression. However, when the region between *luxR* and *luxI* is intact, the luminescence is stimulated by cAMP and CRP probably due to higher levels of *luxR* expression(113, 114).

Interestingly, Miyamoto *et al.*(2000) reported that *V. fischeri* uses LuxO and LuxU to regulate luminescence in a second signal transduction system (34). These two proteins were first discovered in *V. harveyi* in its control of luminescence (14, 55, 56, 75). Their presence in *V. fischeri* is unexpected because LuxI/LuxR based autoinduction of luminescence has been well established in *V. fischeri*. Therefore, it is likely that the *V. fischeri* possesses similar parallel signal pathways.

#### *LuxI/LuxR Type Family and AHL Biosynthesis*

Many Gram-negative bacteria have been reported to produce protein pairs similar to LuxR and LuxI (Table 1-1) (118). Furthermore, most bacteria with LuxR/LuxI type proteins also produce AHL autoinducers. Some examples of these LuxR/LuxI homologues are the ExpR/ExpI proteins of *Erwinia carotovora*, which regulate synthesis of exoenzymes and carbapenem antibiotic (125, 153); TraR/TraI proteins of *Agrobacterium tumefaciens*, which control the Ti plasmid conjugal transfer (143, 207); the YpsI/YpsR and YtbI/YtbR proteins in *Yersinia pseudotuberculosis*, which regulate bacterial motility and aggregation (5); and the LasR/LasI and RhII/RhIR proteins of *Pseudomonas aeruginosa*, which control a number of virulence factors (36, 37, 91, 141). Surprisingly, the proteins in LuxR/LuxI family show very low sequence conservation, with 18-25% identity between LuxR homologues and 28-35% identity for

Table 1-1 LuxI/LuxR Type Family

Organism	LuxI/LuxR Homologue(s)	Autoinducer Identity	Target Genes and Functions	References
<i>Vibrio fischeri</i>	LuxI/LuxR	<i>N</i> -(3-oxohexanoyl)-HSL	<i>luxICDABE</i> (bioluminescence)	(48, 51)
<i>Aeromonas hydrophila</i>	AhyI/AhyR	<i>N</i> -butanoyl-HSL	Serine protease and metalloprotease production	(182)
<i>Aeromonas salmonicida</i>	AsaI/AsaR	<i>N</i> -butanoyl-HSL	<i>aspA</i> (exoprotease)	(183)
<i>Agrobacterium tumefaciens</i>	TraI/TraR	<i>N</i> -(3-oxooctanoyl)-HSL	<i>tra</i> , <i>trb</i> (Ti plasmid conjugal transfer)	(143, 207)
<i>Burkholderia cepacia</i>	CepI/CepR	<i>N</i> -octanoyl-HSL	Protease and siderophore production	(93)
<i>Chromobacterium violaceum</i>	CviI/CviR	<i>N</i> -hexanoyl-HSL	Production of violacein pigment, hydrogen cyanide, antibiotics, exoproteases and chitinolytic enzymes	(29, 110)
<i>Enterobacter agglomerans</i>	EagI/EagR	<i>N</i> -(3-oxohexanoyl)-HSL	Unknown	(184)
<i>Erwinia carotovora</i>	(a) ExpI/ExpR	<i>N</i> -(3-oxohexanoyl)-HSL	(a) Exoenzyme synthesis,	(80, 144)
	(b) CarI/CarR		(b) Carbapenem antibiotic synthesis	(8)
<i>Erwinia chrysanthemi</i>	ExpI/ExpR	<i>N</i> -(3-oxohexanoyl)-HSL	<i>pecS</i> (regulator of pectinase synthesis)	(125, 153)
<i>Erwinia stewartii</i>	EsaI/EsaR	<i>N</i> -(3-oxohexanoyl)-HSL	Capsular polysaccharide biosynthesis, virulence	(15)
<i>Escherichia coli</i>	?/SdiA	?	<i>ftsQAZ</i> (cell division), chromosome replication	(62, 167, 204)
<i>Pseudomonas aereofaciens</i>	PhzI/PhzR	<i>N</i> -hexanoyl-HSL	phz (phenazine antibiotic biosynthesis)	(142, 205)
<i>Pseudomonas aeruginosa</i>	(a) LasI/LasR	(a) <i>N</i> -(3-oxododecanoyl)-HSL	(a) <i>lasA</i> , <i>lasB</i> , <i>aprA</i> , <i>toxA</i> (exoprotease virulence factors),	(36, 37, 140)

Organism	LuxI/LuxR Homologue(s)	Autoinducer Identity	Target Genes and Functions	References
	(b) RhlI/RhlR	(b) <i>N</i> -butyryl-HSL	biofilm formation and references therein; (b) <i>lasB</i> , <i>rhlAB</i> (rhamnoli-pid), <i>rpoS</i> (stationary phase) and references therein;	(37, 91, 141)
<i>Ralstonia solanacearum</i>	SolI/SolR	<i>N</i> -hexanoyl-HSL, <i>N</i> -octanoyl-HSL	Unknown	(53)
<i>Rhizobium etli</i>	RaiI/RaiR	Multiple, unconfirmed	Restriction of nodule number	(156)
<i>Rhizobium leguminosarum</i>	(a) RhiI/RhiR	(a) <i>N</i> -hexanoyl-HSL	(a) <i>rhiABC</i> (rhizosphere genes) and stationary phase	(34, 66, 155)
	(b) CinI/CinR	(b) <i>N</i> -(3-hydroxy-7- <i>cis</i> -tetradecenoyl)-HSL	(b) Quorum sensing regulatory cascade	(97)
<i>Rhodobacter sphaeroides</i>	CerI/CerR	7,8- <i>cis</i> - <i>N</i> -(tetradecanoyl)-HSL	Prevents bacterial aggregation	(148)
<i>Salmonella typhimurium</i>	?/SdiA	?	<i>rck</i> (resistance to competence killing), ORF on <i>Salmonella</i> virulence plasmid	(2)
<i>Serratia liquefaciens</i>	SwrI/?	<i>N</i> -butanoyl-HSL	Swarmer cell differentiation, exoprotease	(49, 65)
<i>Vibrio anguillarum</i>	VanI/VanR	<i>N</i> -(3-oxodecanoyl)-HSL	Unknown	(121)
<i>Yersinia enterocolitica</i>	YenI/YenR	<i>N</i> -hexanoyl-HSL, <i>N</i> -(3-oxohexanoyl)-HSL	Unknown	(187)
<i>Yersinia pseudotuberculosis</i>	(a) YpsI/YpsR	(a) <i>N</i> -(3-oxohexanoyl)-HSL	Hierarchical quorum sensing cascade	(5)
	(b) YtbI/YtbR	(b) <i>N</i> -octanoyl-HSL	regulating bacterial aggregation and motility	

<sup>a</sup> Information from Miller *et al* (118).

LuxI homologues (59). This partly explains the diversity of cell activities controlled by quorum sensing.

Genetic analysis of LuxR suggests that it is composed of two functional domains: an amino-terminal domain with an AHL binding region and a carboxy terminal transcription regulation domain, which includes a helix-turn-helix DNA binding motif. The DNA-binding domains share sequence similarity with a much larger group of transcription factors (the so-called LuxR or FixJ superfamily) (83). LuxR molecules consisting of amino-terminal deletion polypeptides show AHL independent activation of *lux* genes (30). These results suggested that in the absence of AHL, the amino-terminal half of the protein blocks the function of the DNA-binding domain. Interaction with the acyl-HSL abolishes the inhibition and allows transcriptional activation (30). In addition, some observations suggest that the transcriptional activation requires formation of multimers, and the residues in the carboxy-terminal are important in multimerization of LuxR(175 -177). In the autoinduction of the *lux* operon, LuxR is considered to bind a 20 base pair inverted repeat, known as the *lux* box (42). Similar sequences to *lux* box are found upstream of at least some promoters regulated by LuxR-type proteins in other bacteria (58, 159). The LuxR-type proteins contain relatively conserved sequence in their DNA binding domains. The specificity in gene regulation comes from the unique structures of the AHL autoinducers. In addition to the role in activating gene expression, there are studies suggesting that some LuxR homologues such as EsaR, YenR, and ExpR, appear to act as repressors, and binding by the autoinducers inhibits their functions (59, 122).

The biochemical mechanism of AHL biosynthesis by the LuxI-type enzymes has been studied in several Gram-negative bacteria (71, 124, 137, 190). The substrates for the reaction are *S*-adenosyl-methionine (SAM) and acylated acyl carrier protein (acyl-ACP). Recent advance in the study of *Pseudomonas aeruginosa* RhlI suggested a bi ter (two substrate, three product) sequential ordered reaction mechanism, in which the SAM and the acyl-ACP bind to the enzyme sequentially (137). During catalysis by the LuxI-type protein, the acyl side chain of the acyl-ACP is donated to the amine part of the SAM with the release of apo-ACP. Subsequent lactonization results in the formation of the acylated homoserine lactone and release of methylthioadenosine (MTA) (Figure 1-2). The acyl side chain of the acyl-ACP determines specificity of the AHLs produced in different bacteria. The AHLs may have 4 to 18 carbon acyl chain with possible change in the oxidation state at C3, or saturation state of the chain (Figure 1-3) (135).

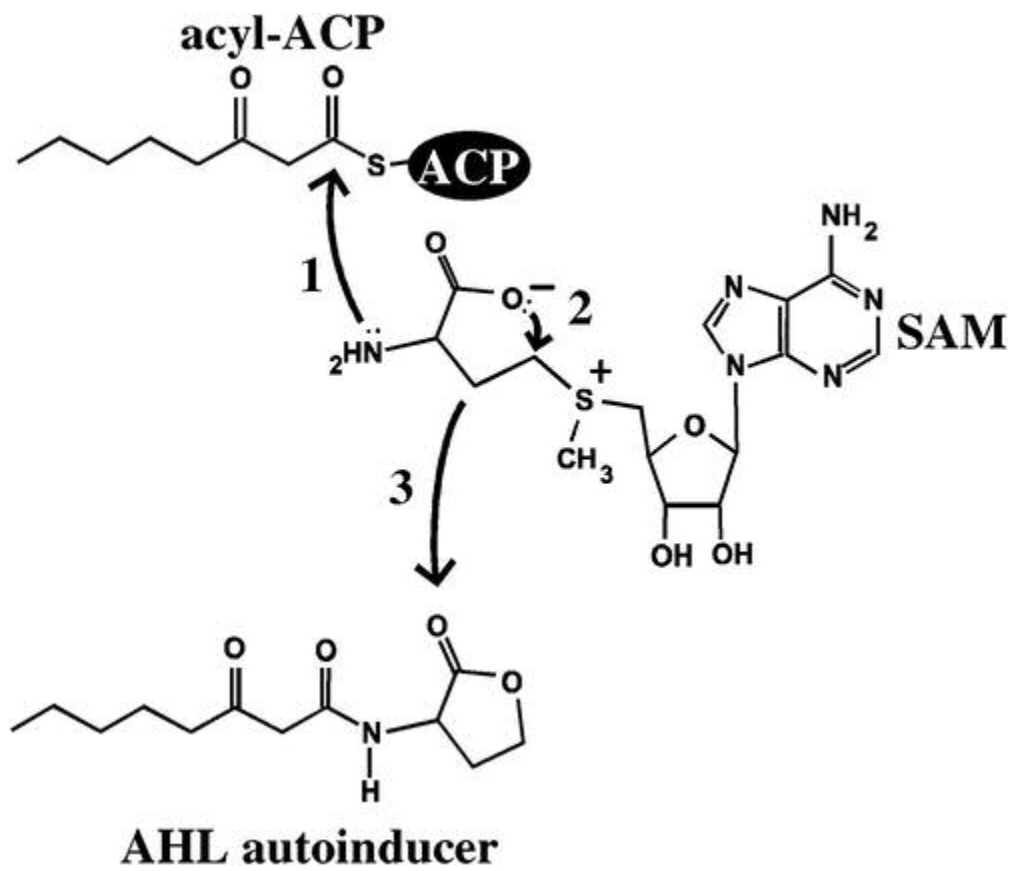
There are some reports suggesting that the AHLs may be synthesized by the enzymes that do not belong to the LuxI family. LuxM and Ains, probably a second family of AHL synthases, are responsible for the production of AHL in *Vibrio harveyi* and *Vibrio fischeri*, respectively (12, 72). HdtS in *Pseudomonas fluorescens* F113, is likely a third type of AHL synthase with no homology to either the LuxI or LuxM families (72).

#### *SdiA, the LuxR homologue in E. coli*

To identify genes that were capable of preventing the MinC/MinD-induced division block *E. coli*, a library of chromosome DNA in the low copy plasmid pGB2 was introduced into strain PB114( $\lambda$ PB173)[ $\Delta$ *minCDE*(Plac::*minCD*)], in which

**Figure 1-2 Biosynthesis of the acyl-homoserine lactone (AI-1)**

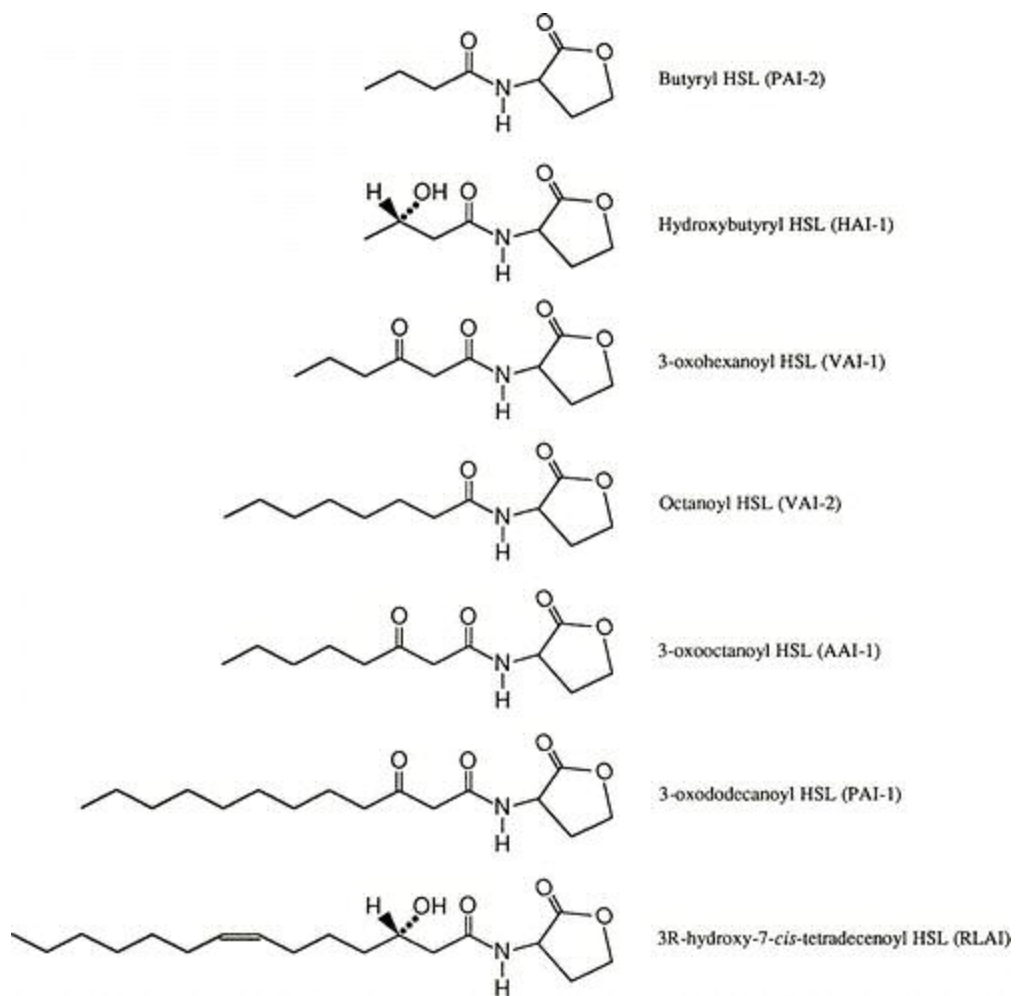
*S*-adenosyl-methionine (SAM) and acylated acyl carrier protein (acyl-ACP) are the two substrates for the biosynthesis of the acyl-homoserine lactone molecule. The SAM and the acyl-ACP bind to the enzyme protein of the LuxI-type family sequentially. During catalysis by the enzyme, the acyl side chain of the acyl-ACP is donated to the amine part of the SAM with the release of apo-ACP. Subsequent of lactonization results in the formation of the acylated homoserine lactone and release of the methylthioadenosine (MTA). Shown in the figure is the biosynthesis of the *N*-(3-oxooctanoyl)-homoserine lactone adapted from (118).



### **Figure 1-3 Structures of several *N*-acyl homoserine lactone molecules**

In a large number of Gram-negative bacteria, *N*-acyl homoserine lactone molecules (AI-1) are used as quorum signals to regulate a variety of cell activities. All of the AI-1 molecules have common homoserine lactone moiety, but with difference in their acyl side-chains, which decide specificity of the signaling molecules. The acyl side-chain varies from four to eighteen carbons in its length, with changes in the oxidation state at C3, and the saturation state of the chain. Shown are structures of several AI-1 molecules as reported in (59).



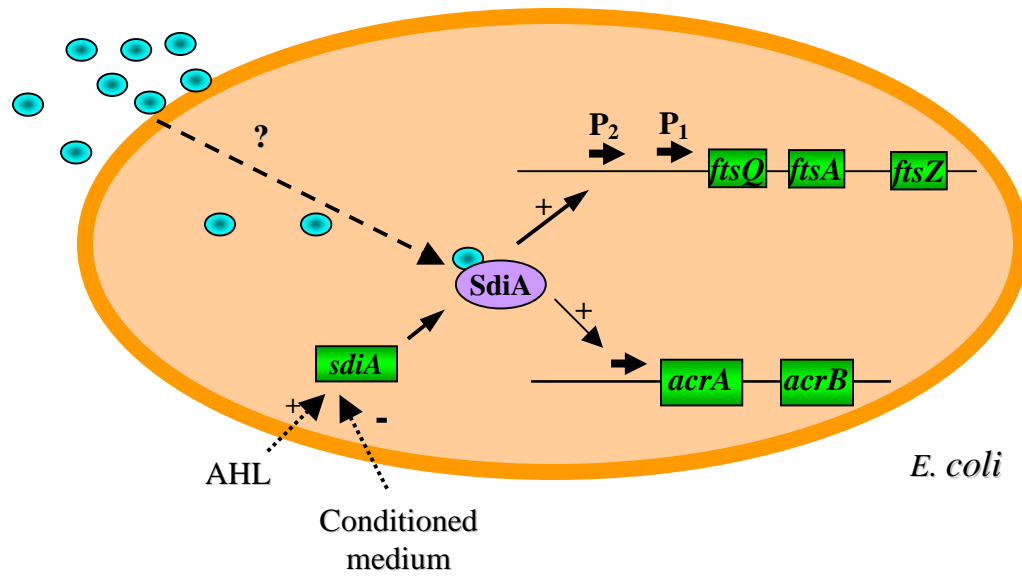


expression of *minCD* was induced by IPTG (195). Through this screen, *sdiA* gene was identified. SdiA protein shows sequence similarity to the LuxR of *Vibrio fischeri*. The homology between SdiA and LuxR suggested the possibility that SdiA might function by a mechanism similar to that of LuxR. It has been shown that SdiA increased the transcription of the plasmid-based *ftsQAZ* gene cluster 5-13 fold when *sdiA* was expressed from a low copy plasmid, but only 1.6 fold when it was expressed from its original chromosome location. This activation results from enhanced activity of the PQ2 promoter of the *ftsQAZ* operon (Figure 1-4) (195). To see whether SdiA is regulated by a factor excreted into the medium, conditioned medium was used to test for an effect on PQ2-*lacZ* expression. One group reported a four- to fivefold stimulation early in the growth curve (167), whereas another found such activation resulted from the change of growth rate caused by the experimental method (62). In addition, the expression from the *sdiA* promoter is reduced about 2-fold by an extracellular factor during the stationary phase (62). Although AHL of *V. fischeri* was also shown to stimulate the PQ2-*lacZ* expression through plasmid-encoded *sdiA* slightly (167), *E. coli* has no gene homologous to *luxI* of *V. fischeri*. If SdiA indeed functions as a type 1 AHL transcriptional regulator, it appears that *E. coli* cells use SdiA to detect the AHLs produced by the other bacteria. The locus responsible for the biosynthesis of the hypothesized factor in *E. coli* remains to be determined.

To identify genes involved in quinolone resistance in *E. coli*, an overexpression screen with a pBR322-based *E. coli* genomic library was performed (150). Rahmati, S. *et al* reported that overexpression of SdiA confers multidrug resistance to ciprofloxacin,

#### **Figure 1-4 SdiA mediated gene regulation in *E. coli***

SdiA is a LuxR homologue in *E. coli*. SdiA stimulates transcription of a cell division gene cluster *ftsQAZ* through its P2 promoter, and also activates expression of the *acrAB* operon, which encodes a multigrug efflux pump. The conditioned medium (cell free fluids) represses the *sdiA* expression, while AHL of *V. fischeri* activates its expression. *E. coli* does not have a LuxI homologue to produce the AHL, although some evidence indicated that SdiA protein binds a factor from the conditioned medium.



norfloxacin, nalidixic acid and chloramphenicol through positive control of the AcrAB efflux pump, but the SdiA mediated resistance of quinolone, ofloxacin, tetracycline and kanamycin was not abolished in *acrAB* mutant strains. In addition, a  $\Delta sdiA$  strain was shown to be 2-3 fold more sensitive to the fluoroquinolones than the wild type, but was not significantly more sensitive to chloramphenicol, nalidixic acid, or tetracycline (150). These results suggested that not only AcrAB efflux pump but also other AcrAB independent mechanisms are responsible for the SdiA-mediated drug resistance (150).

Similar results were obtained for the effect of SdiA on mitomycin C (MMC) resistance (199). Wei *et al.* showed that overexpression of *sdiA* increases resistance to MMC, however, a null mutant of *sdiA* did not show hypersensitivity to MMC (199). Consistent with the above observance, a microarray analysis to study the cell's response to *sdiA* overexpression caused increased expression of the *ftsQAZ*, *acrAB*, *acrD* and *acrEF* genes (198). Of the 62 genes that were downregulated by more than three fold, 41 were involved in motility and chemotaxis. Of the 75 genes upregulated by more than three fold, almost half have unknown function (198). Although this study linked the SdiA functions to a variety of cell activities, it is not clear whether the chromosomal *sdiA* gene has similar regulatory roles, since overexpression of protein may cause some irrelevant response from the cells.

In another experiment with enterohaemorrhagic *E. coli* O157:H7, Kanamaru *et al.* reported that overexpression of *sdiA* inhibits expression of virulence factors EspD and intimin (84). In addition, a gel mobility shift assay showed the GST-SdiA fusion protein binds to the promoter regions of the *esp* and *eae* genes. They also found the N-terminal part of SdiA can bind an extracellular factor, which accumulates in stationary

phase and inhibits the synthesis of EspD and intimin (84). Whether this factor is a specific AHL or a different ligand needs further investigation since the EHEC strains appeared to lack the AHL production (116).

### Quorum Quenching

Recently, Dong *et al.* reported an approach to attenuate bacterial virulence by quenching its quorum signaling (44, 45). *Erwinia carotovora* is a plant pathogen that is able to produce and secrete plant cell wall degrading enzymes (89). Its virulence is dependent on its AHL-mediated quorum sensing (80). To block its quorum signal, Dong *et al.* harnessed an AHL lactonase encoded by *aiiA* from *Bacillus* sp. 24081 (45), which can hydrolyse the lactone bond of AHLs (44). When expressed in *Erwinia carotovora*, the AiiA reduced the AHL production and the bacterial pectolytic enzyme activities, thus attenuating its virulence (45). Furthermore, the *aiiA* transgenic plants successfully quench the pathogen quorum sensing and showed increased resistance to *Erwinia carotovora* (44). Similarly, Manfield *et al.* harnessed structurally related halogenated furanones from the red alga *Delisa pulchra* to displace AHL from its LuxR receptor and prevented bacterial quorum signaling (104-106). The above experiments provided us with two very promising methods to potentially combat the bacterial infection by quenching their quorum signaling.

### Peptide Mediated Quorum Sensing

Unlike the AHL-mediated quorum sensing, Gram-positive bacteria typically use peptides as quorum signals in the control of some cell density dependent activities

(Figure 1-5). Some examples are: the ComP/ComA competence and sporulation system in *Bacillus subtilis* (102, 170, 171); the AgrC/AgrA virulence system in *Staphylococcus aureus* (79, 100, 109). These bacteria produce peptides and secrete them via an ABC transporter. With increase in the cell density, the concentration of the secreted peptide autoinducers accumulates to a certain level, which can then be detected by the sensor kinase of a two-component system. Through phosphorylation/dephosphorylation, the signal is transduced from the sensor to a transcriptional regulator. The phosphorylated regulatory protein then stimulates expression of target genes.

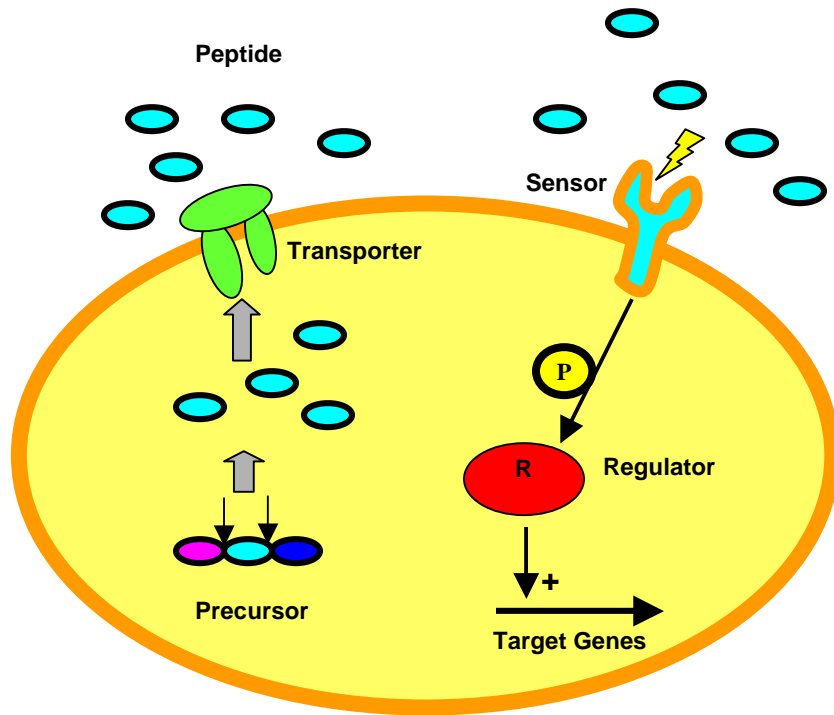
#### Parallel Quorum Sensing Systems in *Vibrio harveyi*

*Vibrio harveyi* is another marine luminous bacterium, which has density-dependent autoinduction of luminescence. However, the mechanism of autoinduction in *Vibrio harveyi* appears to be more complicated. *Vibrio harveyi* possesses three autoinducer-responsive systems that function in parallel to control the expression of the *luxCDABEGH* operon and other genes (Figure 1-6) (12, 13). Like other Gram-negative quorum sensing bacteria, *Vibrio harveyi* produces and responds to an AHL autoinducer (AI-1, *N*-(3-hydroxybutanoyl)-homoserine lactone)(12, 24) . It is interesting that *Vibrio harveyi* has no LuxI/LuxR homologues. Instead, the *luxL* and *luxM* genes are required for synthesis of the autoinducer AI-1 (12, 24). AI-1 is recognized by a two-component-type sensor kinase LuxN (12, 13). Besides the AI-1 signal-response system (system 1), *Vibrio harveyi* has a second regulatory system (system 2) which functions through another signal molecule AI-2 (*S*-THMF-borate) (13, 27, 120). AI-2 synthesis requires

### **Figure 1-5 Peptide mediated quorum sensing in Gram-positive bacteria**

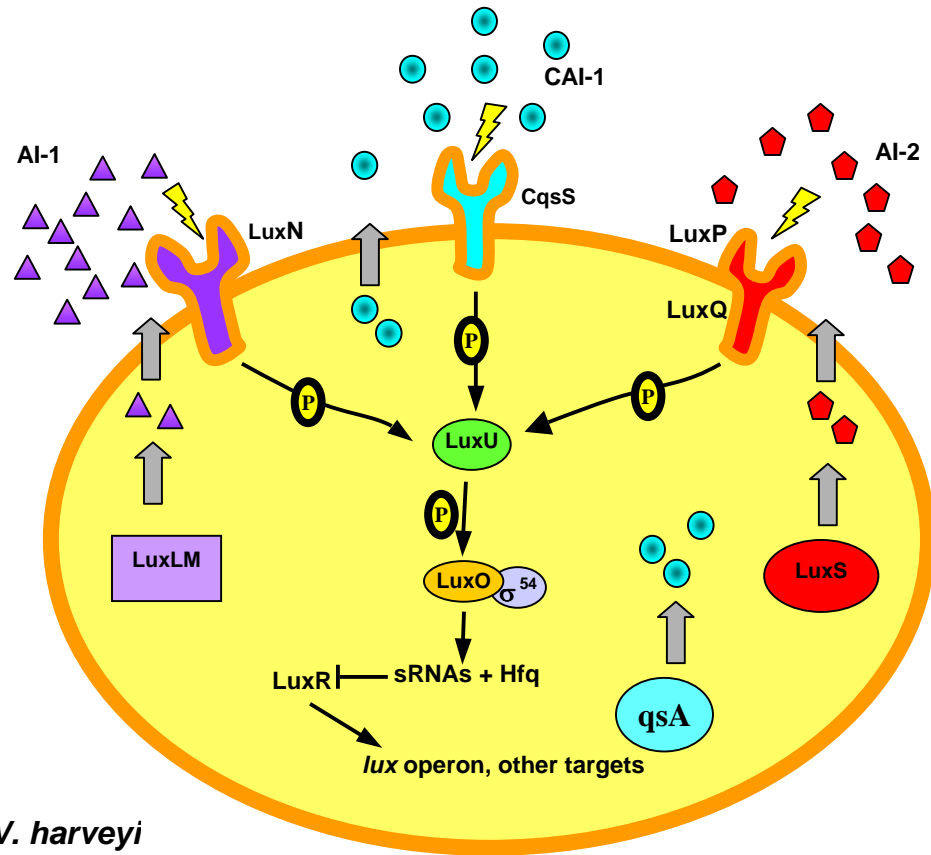
Instead of the AHLs, the Gram positive bacteria use certain small peptide as a quorum signal, which is produced through cleaving a precursor protein. These peptides are secreted out of the cells by an ABC transporter. With an increase in the cell density, the peptide can accumulate up to a threshold concentration, when it is recognized by a kinase sensor protein. Through phosphorylation/dephosphorylation, the signal is transferred from the sensor to a transcriptional regulator, which then stimulates expression of the target genes.





**Figure 1-6 Three parallel quorum sensing systems in *Vibrio harveyi***

The *Vibrio harveyi* has three sensor kinases, LuxN, LuxQ, and CqsS, which respond to the three autoinducers, AI-1, AI-2, and CAI-1, respectively. The proteins of LuxLM, LuxS, and CqsA are responsible for the production of the three autoinducers respectively. At low concentration of the autoinducers, phosphate flows toward LuxO and phosphorylated LuxO works with  $\sigma^{54}$  to activate the expression of the loci encoding four sRNAs. These sRNA interact with a chaperone protein Hfq to reduce the mRNA stability of the *luxR* gene, which encodes a transcriptional activator of the *lux* operon. As a result, low luminescence is produced. At high cell density, when autoinducers are present, phosphate flows away from LuxO and repression of *lux* operon is eliminated. Differing from system 1 and 2, the CAI-1-CqsS system responds to CAI-1 at much lower cell densities.



*V. harveyi*

the *luxS* gene (181). Perception of AI-2 depends on another sensor kinase LuxQ and a periplasm-binding protein LuxP. The LuxP, which binds AI-2, interacts with LuxQ to recognize AI-2 (13). Henke *et al.* (2004) showed that in *V. harveyi*, there exists a third quorum sensing systems, the CAI-1-CqsS system, which acts in parallel with system 1 and 2 (75). The CAI-1 autoinducer is produced by CqsA, and the CqsS is a sensor kinase (75). Sensory information from the three systems is transduced by phosphorylation/dephosphorylation to a shared signal integrator protein, LuxU, which subsequently conveys the signal to the response regulator protein, LuxO (Figure 1-6) (14, 55, 56, 75). At low cell density, phosphate flows toward LuxO and phosphorylated LuxO works with  $\sigma^{54}$  to activate the expression of a negative regulator that controls *lux* operon (96). At high cell density, when autoinducers are present, phosphate flows away from LuxO and repression of *lux* operon is eliminated (96). Differing from system 1 and 2, the CAI-1-CqsS system responds to CAI-1 at much lower cell densities (75).

Recent advances indicate that multiple small RNAs (sRNAs) are also involved in regulation of quorum sensing in *Vibrio harveyi* (92). It was shown that the phospho-LuxO, together with  $\sigma^{54}$ , stimulates the expression of loci encoding four sRNAs (92). These sRNA interact with a chaperone protein Hfq to control the mRNA stability of the *luxR* gene, which encodes a transcriptional activator of the *lux* operon (92, 107, 123, 165). It was proposed that *Vibrio harveyi* harnesses the Hfq and sRNA to form an ultrasensitive switch to regulate quorum sensing (92) .

### AI-2 system and LuxS family

To investigate whether other bacteria can produce stimulatory signaling molecules that work through *V. harveyi* signaling system 1, system 2, or both, Bassler *et al.* (1997) prepared and tested cell-free fluids from a variety of bacterial species (11). Three reporting *V. harveyi* strains with different autoinducer response phenotypes were used in the study (11). BB120 is a wild type strain. It is “sensor 1<sup>+</sup> sensor 2<sup>+</sup>”, and responds to both AI-1 and AI-2. BB886 is “sensor 1<sup>+</sup> sensor 2<sup>-</sup>” and responds only to AI-1, while BB170 is “sensor 1<sup>-</sup> sensor 2<sup>+</sup>” and responds only to AI-2. Through their experiments, a number of bacterial species have been found to induce light production in *V. harveyi*. Most of the species tested make substances that mimic the action of AI-2 but not AI-1, indicating that *V. harveyi* system 1 is specific, while *V. harveyi* system 2 is less specific. It was suggested that the function of *V. harveyi* system 1 is to monitor the environment of *V. harveyi* organisms, while the function of system 2 is to monitor the environment of other species of bacteria.

Database searches have revealed that more than 55 bacterial species possess genes homologous to the *V. harveyi luxS* and therefore are likely to make AI-2 (181, 206). Both Gram-negative and Gram-positive bacterial species are included in this list, such as *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, and *Yersinia pestis* (181). Most of the bacteria possessing a *luxS* gene have been shown to produce AI-2 activity. The structures of the LuxS proteins from three different species have been solved using an X-ray crystallographic structural genomics approach (94). LuxS is seen to have a new alpha-

beta fold and display a homodimer interaction in its asymmetric unit. The zinc ion is found bound to a Cys-His-His triad at the dimer interface.

### Biosynthesis and formation of AI-2 molecules

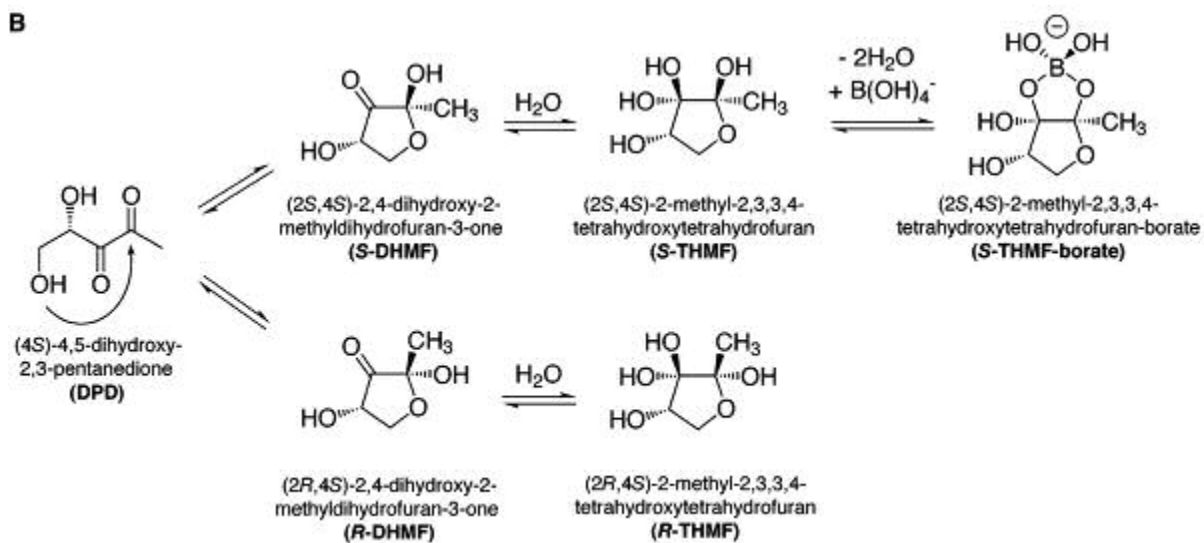
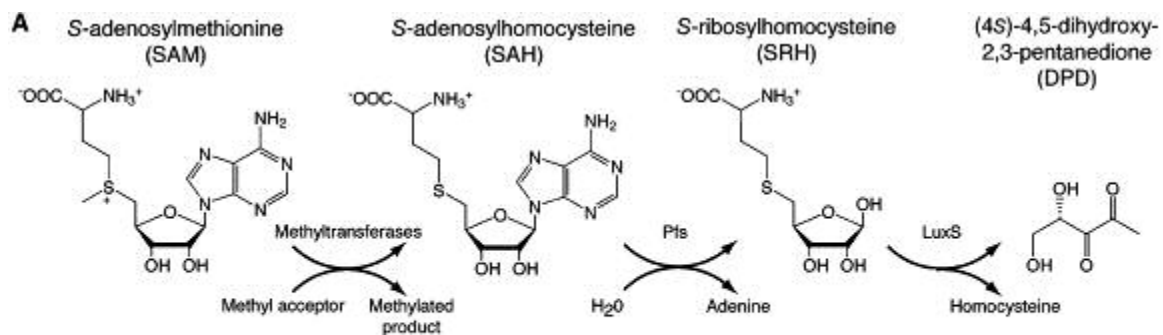
Using *V. harveyi* reporter strain BB170, Surette and Bassler investigated the conditions under which *E. coli* and *S. e. typhimurium* synthesize, secrete and degrade the AI-2 signaling factor (179). They found that this factor is produced in LB medium only when it is supplemented with glucose (0.5%). The inducing activity was not detected in cultures grown in the absence of glucose, although other sugars transported by the phosphotransferase system (PTS) also stimulate production (179). Maximal secretion of the signaling molecule occurs in the mid-exponential phase, and the extracellular activity is reduced as the glucose is depleted from the medium or by the onset of stationary phase (179). Moreover, some environmental factors affect the activity of the signal (180). An increase in signaling activity was observed when *S. e. typhimurium* was transferred to a high-osmolarity (0.4M NaCl) or to a low-pH (pH 5.0) environment after growth in the presence of glucose (180). Conditions of low osmolarity (0.1M NaCl) stimulate the degradation of this signal.

An important step toward understanding quorum sensing is the clarification of the AI-2 biosynthetic pathway and biochemical intermediates (Figure 1-7A) (163). They demonstrated that AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps with LuxS serving as the putative terminal AI-2 synthase (163). The substrate for LuxS is *S*-ribosylhomocysteine (SRH), which is cleaved to form homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). The DPD is a highly

### **Figure 1-7 Biosynthesis and formation of AI-2 signaling molecules**

A. Biosynthesis of AI-2 precursor, DPD (120). The DPD is produced from SAM in three enzymatic steps. With catalysis of several methyltransferases, the SAM donates its methyl group to a methyl acceptor, resulting in production of the SAH, which is degraded by Pfs into adenine and SRH. LuxS acts on SRH to produce homocysteine and DPD.

B. Formation of the AI-2 signaling molecules (proposed by Miller S. T. *et al.* (2004) (120)). The DPD molecules undergo self-cyclization and arrangement to form *S*-DHMF and *R*-DHMF, which hydrate to yield *S*-THMF and *R*-THMF (the *S. e. typhimurium* AI-2). *S*-THMF may undergo further reaction with borate to form *S*-THMF-borate, the *V. harveyi* AI-2. The different DPD derived molecules are in an equilibrium, which can be shifted toward *S*-THMF-borate by the presence of borate.





reactive molecule that readily undergoes nucleophilic attack. The spontaneous cyclization and further rearrangement of this molecule likely result in formation of AI-2. It was shown that the biosynthetic pathway of AI-2 is identical in *E. coli*, *S. e. typhimurium*, *V. harveyi*, *Vibrio cholerae* and *Enterococcus faecalis* (163), although DPD derived AI-2 molecules in various bacteria may have different forms (see below) .

Chen *et al.* (2002) reported identification of the *V. harveyi* AI-2 as a boron-containing molecule – a furanosyl borate diester – by determining the crystal structure of the AI-2 sensor protein LuxP in a complex with the autoinducer (27). These findings suggest, for the first time, a potential biological role for Boron. Recently, using similar X-ray crystallographic structural approach, Miller *et al.* (2004) determined the crystal structure of another AI-2 binding protein, LsrB from *S. e. typhimurium* (120). LsrB binds to a different AI-2. This molecule is (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), which does not contain boron (120). It was proposed that DPD, one of the products of LuxS reaction, undergoes cyclization and arrangement to form *S*-DHMF and *R*-DHMF, which hydrate to yield *S*-THMF and *R*-THMF (Figure 1-7B)(120) . *S*-THMF may undergo further reaction with borate to form *S*-THMF-borate, the *Vibrio harveyi* AI-2. It was further hypothesized that DPD, *R*-THMF, and *S*-THMF-borate are in equilibrium, which can be shifted toward *S*-THMF-borate by the presence of borate (120). Therefore, AI-2 molecules from various bacterial species can differ in structure, although all of them are derived from DPD.

### AI-2 and luxS controlled behaviors

As mentioned earlier, a number of bacterial species possess a *luxS* gene and produce AI-2 that can be detected by *Vibrio harveyi* reporter strains. Whether AI-2 is used as a signal or just a metabolic waste product has been argued by different research groups. As a matter of fact, mutation of the *luxS* gene has been found to cause changes in certain cell activities and gene expression in some bacteria. Lyon *et al.* (101) showed that disruption of the *luxS* gene in *Streptococcus pyogenes* results in enhanced haemolytic activity and reduction in secreted proteolytic activity, which are linked respectively with increased expression of the haemolytic S-associated *sagA* and decreased expression of the SpeB cysteine protease. In addition, they found the *luxS* mutant has a media-dependent growth defect, with reduced growth rate in Todd-Hewitt yeast extract media (101), suggesting that the phenotype of *luxS* mutant can be observed only in some specific conditions.

The effects of *luxS* mutation were also reported in several other bacteria. For example, in *Porphyromonas gingivalis*, inactivation of *luxS* causes induction of a hemin acquisition protein and an arginine-specific protein (RgpA), but causes repression of a hemin-regulated protein and an exonuclease (31). Another group found that, although the *luxS* mutant of this bacterium has lower expression of *rgp* and *kgp* and reduced haemagglutinin activities, the strain does not have attenuated virulence as shown in a murine lesion model of infection (21). Interestingly, McNab *et al.* reported that the *luxS* gene in *Streptococcus gordonii* is indispensable for formation of the mixed biofilm with a *luxS* null strain of *Porphyromonas gingivalis*, although *luxS* mutation does not inhibit biofilm formation in these two strains separately (112). In addition, the *luxS* mutant of

*Streptococcus gordonii* has downregulated expression of several genes involved in carbohydrate metabolism (112). It was suggested that the LuxS dependent cell-to-cell communication is necessary between *Streptococcus gordonii* and *Porphyromonas gingivalis* cells to form a mixed biofilm (112).

*Clostridium perfringens* is a Gram-positive anaerobic pathogen, which causes clostridial myonecrosis or gas gangrene in humans (133). Ohtani *et al.* showed mutation of the *luxS* gene in this bacterium results in reduced production of the alpha-, kappa-, and theta-toxins (133). However, the toxin production is stimulated by conditioned medium (cell free fluids) from wild type cells but not from *luxS* mutant, suggesting AI-2 may be involved in regulation of the toxin production (133). They further showed that the expression of the theta-toxin gene *pfoA* (but not alpha- and kappa-toxin genes) is lower in *luxS* mutant during mid-exponential phase, and is also activated by the conditioned medium from wild type strain (133). These results indicated that the AI-2/LuxS mediated quorum sensing is important in controlling toxin production in *Clostridium perfringens*.

Sperandio *et al.* (1999) reported that mutation of the *luxS* gene reduces expression of type III protein secretion system in enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). A DNA microarray study showed the *luxS* inactivation in EHEC causes expression changes of a large number of genes, although faster growth rate in the *luxS* mutant may have muddled the effects of quorum sensing (173). More recently, it was shown that AI-2 synthesized *in vitro* does not restore expression of type III protein secretion system of EHEC, suggesting that AI-2 is not involved in regulation of the EHEC signaling (174). Instead, another unidentified *luxS*-

dependent molecule, AI-3, has been shown to be the actual signal to stimulate expression of the LEE and flagella genes (174). In the previous microarray study from our lab with *E. coli* K12 W3110, conditioned media from the wild type and the *luxS* mutant was compared regarding their effects on the *luxS* mutant. Expression of the 242 genes was significantly affected, but the complexity of the conditioned medium made it harder to identify the AI-2 controlled genes (40).

To identify *luxS*-regulated genes in *S. e. typhimurium*, Taga *et al.* (186) screened 11000 isogenic wild type and *luxS* null strains harbouring random MudJ ( *lacZ*) reporter insertions in the *S. e. typhimurium* chromosome. The *lsrACDBFGE* operon and the methionine synthase gene *metE* were identified. The *lsr* (*luxS* regulated) operon encodes an AI-2 uptake and modification system that has similarity to the ribose transport system of *E. coli* and *S. e. typhimurium* (186). The AI 2 transport apparatus is encoded by *lsrA*, *lsrC*, *lsrD*, and *lsrB*. Upon entry into the cells, AI-2 is phosphorylated by a kinase encoded by *lsrK* (185). The phospho-AI-2 appears to be the inducer that releases LsrR-mediated repression of the *lsr* transcription (185). LsrR is homologous to SorC, a DNA-binding transcriptional regulator involved in sorbose metabolism in *Klebsiella pneumoniae* (197). At this time, LsrR has not been demonstrated to bind to the promoter region of the *lsr* operon, and it is not clear whether the effect of phospho-AI-2 on LsrR is direct, although these are likely based on the available data. LsrF and LsrG are responsible for further processing of phospho-AI-2 (185). LsrE is homologous to Rpe, a ribulose phosphate epimerase. In *E. coli*, there exists a similar set of genes including an *lsr* operon (b1513 operon) and homologues to *lsrK* (*ydeV*) and *lsrR* (*ydeW*); there is no

*lsrE* homolog. To date, there have been no studies regarding the function and/or regulation of the *E. coli lsr* operon.

The accumulated evidence suggests that AI-2 could be used as a signal by many bacterial species. The link between SAM utilization and AI-2 production makes it a good candidate for measuring cell's metabolic potential. However, outside of the *Vibrio* species, the *lsr* operon in *S. typhimurium* is the only target shown to date that is controlled directly through AI-2 (or phospho-AI-2). It is not clear whether AI-2 also regulates additional genes through the transcriptional regulator LsrR, or whether there exists additional sensor systems as in *Vibrio harveyi*.

### Statement of Purpose

The study of quorum sensing has attracted much attention. Elucidation of several bacterial quorum sensing systems has opened a window for us to gain significant knowledge about how bacteria conduct inter or intra-species cell-to-cell communication. These signaling systems are important for bacteria as they respond to dynamically evolving environments at a multicellular level. The AI-2 mediated quorum sensing has received intense interest recently because more than 55 bacterial species possess a gene homologous to the AI-2 synthase gene, *luxS*. It has also been shown that AI-2/*luxS* are involved in the control of some important activities such as virulence factor expression (119, 133), antibiotics biosynthesis (41), biofilm formation (147), and the expression of recombinant proteins (39). Given such widespread importance, it is therefore important for us to understand the regulation of AI-2 production and response of cells to this potential signal molecule.

It has repeatedly been observed that in many bacterial species, AI-2 is produced in exponential phase but removed at later phases of growth (54, 112, 179, 200). Addition of glucose greatly increases AI-2 levels (38, 179, 180). However, it is not clear how the AI-2 activity is affected by glucose or by the host physiology, and how its production and removal are regulated at the molecular level. In addition, there has remained a question about what cell processes are regulated by AI-2 in *E. coli* K12 and what is the mechanism responsible for the AI-2 mediated gene regulation. This work was undertaken in an endeavor to answer some of these questions.

Specifically, we undertook this investigation to understand how glucose affects AI-2 biosynthesis and to study the expression of genes involved in the AI-2 biosynthesis and uptake pathways (chapter 2 & 4). Having initially found that glucose affected AI-2 level, we were interested in a potential role of cAMP and CRP in regulation of AI-2 production and removal (chapter 2). We were also interested in understanding potential mechanism of AI-2-mediated gene regulation, and importantly, its impact on the rest of the cell's metabolic processes (chapter 2 & 3). Thus, we wanted to know what genes and cell activities are controlled by AI-2/*luxS* in *E. coli* K12 (chapter 3). Answering these questions would lead to a better understanding of the AI-2 mediated autoinduction and the cell-to-cell communication in *E. coli*. In general, we may develop strategies for harnessing AI-2 quorum sensing for our advantage in bioreactor studies and in the control of bacterial pathogenicity.

## **Chapter 2 cAMP and cAMP Receptor (CRP) Influence Both Synthesis and Uptake of Extracellular Autoinducer-2 in *Escherichia coli***

### Abstract

Bacterial autoinducer-2 (AI-2) is proposed to be an interspecies mediator of cell-cell communication that enables cells to function at the multicellular level. Many environmental stimuli have been shown to affect the extracellular AI-2 levels, carbon sources being among the most important. In this report, we show that both AI-2 synthesis and uptake in *Escherichia coli* are subject to catabolite repression through the cAMP-CRP complex, which directly stimulates transcription of the *lsr* (*luxS* regulated) operon and indirectly represses *luxS* expression. Specifically, cAMP-CRP is shown to bind to a CRP binding site located in the upstream region of the *lsr* promoter and works with LsrR repressor to regulate AI-2 uptake. The functions of the *lsr* operon and its regulators, LsrR and LsrK, previously reported in *S. e. typhimurium* (185, 186) are confirmed here for *Escherichia coli*. The elucidation of cAMP-CRP involvement in *E. coli* autoinduction impacts many areas, including the growth of *E. coli* in fermentation processes.

### Introduction

Bacteria have evolved complex genetic circuits to modulate their physiological states and behaviors in response to a variety of extracellular signals. In a process termed “quorum sensing”, or density-dependent gene regulation, bacteria produce, release and respond to signaling molecules (autoinducers), which accumulate as a

function of cell density. Quorum sensing allows bacteria to communicate with each other and coordinate their activities at a multicellular level. The autoinducers of many Gram-positive bacteria are secreted peptides (86, 131), while Gram-negative bacteria use small chemical molecules (203). Among Gram-negative bacteria, the LuxI/LuxR signal synthase / signal receptor system is the most studied at the molecular level, with the signaling species being a family of N-acylhomoserine lactones (AHLs). However, the cross-species autoinducer, AI-2, has received intense interest recently because the gene for its terminal synthase, *luxS*, is present in over 55 bacteria and its activity can be readily assayed biologically (206). It is known that quorum sensing regulates diverse cellular processes including: bioluminescence (51, 96), spore formation (95), motility (50, 64), competence (102), conjugation (60), antibiotic synthesis (7, 41), virulence (119, 138, 172), and biofilm maturation (36, 147).

Our laboratory is interested in understanding and controlling microbial behavior in bioreactors in order to enhance recombinant protein synthesis and yield. Since quorum sensing is emerging as a global regulator of many intracellular processes, including those that influence protein synthesis, efforts to understand this “tunable” controller are essential. In our previous work using chemostat cultures (38), many stimuli were found to affect the level of the AI-2. Among these, the pulsed addition of glucose, a common carbon source for recombinant *E. coli* fermentations, resulted in increased AI-2 levels, but with the dynamic response dependent on the steady-state growth rate (e.g., dilution rate) of the culture. Indeed, AI-2 production on a per cell basis, was linearly proportional to the growth rate of the cells (38). Also, the level of AI-2 in extracellular fluids was reduced relative to controls in direct response to protein



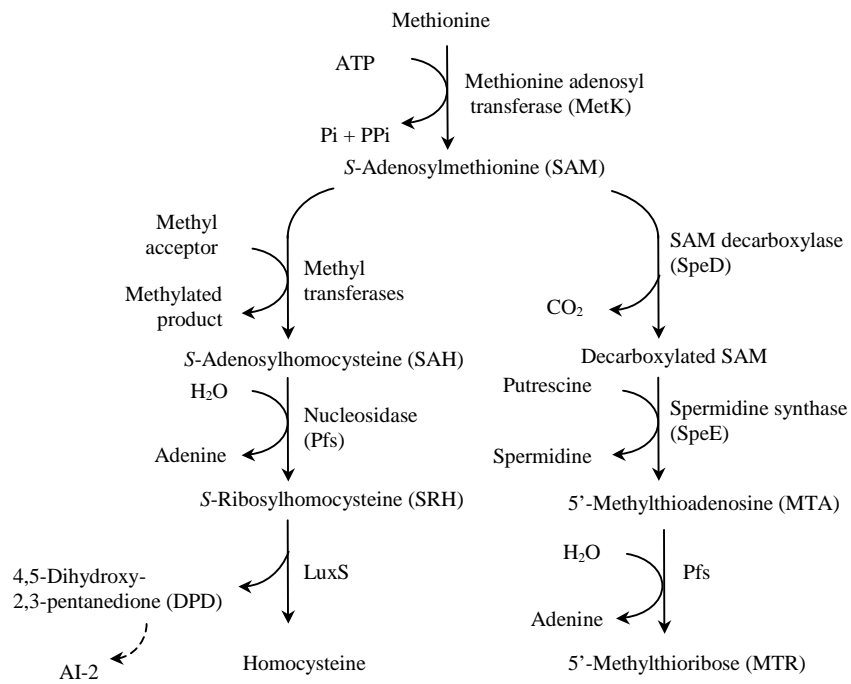
overexpression. This was attributed to the “metabolic burden” commonly associated with the requisite redirection of metabolites and resources (39).

The level and possible role of AI-2 as a mediator of intra- and intercellular coordination has been the subject of varied interpretation, as recently reviewed by Ahmer (1). Outside of the *Vibrio* genus, only the *lsr* operon of *Salmonella* has been found to directly respond to AI-2, while mutation of its synthase, LuxS, has far-reaching effects (40, 173); although the evidence is muddled by the coincident effects on cell growth (1). Hence, it is important to delineate pleiotropic effects of *luxS* gene knockout and of varying glucose level and growth rate from molecular events directly attributed to AI-2. In the present work, we investigated the mechanistic effects of glucose on the synthesis and uptake of AI-2 in *E. coli* W3110.

Schauder *et al.* demonstrated that AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps and that LuxS is an AI-2 synthase (163) (Figure 2-1). SAM serves as methyl donor in a variety of methylation reactions and as a propylamino donor in polyamine biosynthesis (68). The methyl group of SAM is transferred by several methyltransferases to its acceptors, resulting in production of S-adenosylhomocysteine (SAH). Accumulation of SAH is toxic to the cell, and it is rapidly degraded by a nucleosidase, Pfs, into adenine and S-ribosylhomocysteine (SRH). LuxS acts on SRH to produce homocysteine, which can be recycled to methionine (68) and 4,5-dihydroxy-2,3-pentanedione, that likely undergoes cyclization and further rearrangement to yield AI-2 (27, 120, 163). Interestingly, the same nucleosidase, Pfs, participates in the SpeD-directed SAM decarboxylation pathway that ultimately produces methylthioribose

## Figure 2-1 Pathways for AI-2 biosynthesis and SAM utilization

See text for details. Modified from (68, 163)



during synthesis of polyamines. In some bacteria, Pfs is also involved in the pathway responsible for AHL production (124, 137, 190).

The uptake of AI-2 was recently elucidated by Taga *et al*, in *S. e. typhimurium* (185, 186). They found that the *lsrACDBFGE* operon encodes an AI-2 uptake and modification system. Upon entry into the cells, AI-2 is phosphorylated by a kinase encoded by *lsrK* (185). The phospho-AI-2 appears to be an inducer that releases LsrR-mediated repression of *lsr* transcription. LsrF and LsrG are responsible for further processing of phospho-AI-2 (185). In *E. coli*, there exists a similar *lsr* operon (b1513 operon), except that it does not have the *lsrE* homolog. To date, there have been no studies regarding the function and/or regulation of the *E. coli lsr* operon.

It has long been known that the presence of glucose (or other phosphotransferase system (PTS) sugars) in the growth medium of *E. coli* and *S. e. typhimurium* cultures affects the level of extracellular AI-2 (73, 179). Moreover, maximal AI-2 activity is typically observed during mid to late exponential phase and this extracellular activity is removed when glucose becomes depleted (179). These findings suggest a linkage between catabolite repression and AI-2 production and transport. In this study, we show that catabolite repression influences AI-2 accumulation through the cAMP-CRP complex, which directly stimulates transcription of the *lsr* operon and indirectly represses *luxS* expression. cAMP-CRP is shown to bind to the upstream region of the promoter of the *lsr* operon and works with LsrR repressor to regulate AI-2 uptake. A working model describing the appearance and disappearance of AI-2 in *E. coli* cultures is presented, along with our interpretation of AI-2 regulation.

## Materials and Methods

### **Bacterial strains and growth media.**

The bacterial strains used in this study are listed in Table 1. Luria-Bertani broth (LB) contains 5 g L<sup>-1</sup> yeast extract (Sigma), 10 g L<sup>-1</sup> bacto tryptone (Difco), and 10 g L<sup>-1</sup> NaCl. Glucose and cAMP, when present, were added at 0.8%, and 10 mM, respectively. The autoinducer bioassay (AB) and LM media are described in detail elsewhere (13, 67). When necessary, media were supplemented by antibiotics at the following concentrations: ampicillin, 60 µg ml<sup>-1</sup>; kanamycin, 50 µg ml<sup>-1</sup>.

### **Plasmid construction.**

The plasmids used in this study are listed in Table 1, and were generated using standard procedures (162). Restriction enzymes, T4 DNA ligase and Vent DNA polymerase were used as specified by the manufacturer (New England Biolabs, Beverly, MA). *E. coli* W3110 chromosomal DNA preparation was performed using the Qiagen Dneasy Tissue Kit (Qiagen, Valencia, CA). Extractions of DNA from agarose gels were performed using the Qiagen Qiaquick Gel Extraction Kit. Oligonucleotides were from Gene Probe Technologies (Gaithersburg, MD). DNA sequencing was performed at the DNA core facility of the Center of Biosystems Research (University of Maryland Biotechnology Institute). All constructs made by PCR were sequenced to verify their integrity.

Plasmid pFZY1 is a mini-F derivative (average copy number, 1 to 2 per cell) with a polycloning site upstream of a promoterless *galk'*-*lacZYA* reporter segment (88).

Table 2-1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and property	Source or reference
Strains		
<i>E. coli</i>		
W3110	Wild type	Laboratory stock
LW1	W3110 $\Delta crp::Kan$	This study
LW5	W3110 $\Delta(lsrACDBFG)::Kan$	This study
LW6	W3110 $\Delta lsrR::Kan$	This study
LW10	W3110 $\Delta lsrK::Kan$	This study
HT28	W3110 $\Delta cya::Kan$	(85)
ZK126	W3110 $\Delta lacU169 tna-2$	(33)
ZK1000	ZK126 $\Delta rpoS::Kan$	(18)
LW2	ZK126 $\Delta crp::Kan$	This study
LW7	ZK126 $\Delta luxS::Kan$	This study
LW8	ZK126 $\Delta lsrR::Kan$	This study
LW9	ZK126 $\Delta(lsrACDBFG)::Kan$	This study
LW11	ZK126 $\Delta lsrK::Kan$	This study
<i>V. harveyi</i>		
BB152	BB120 $luxL::Tn5$ (AI-1 <sup>-</sup> , AI-2 <sup>+</sup> )	(179)
BB170	BB120 $luxN::Tn5$ (sensor 1 <sup>-</sup> , sensor 2 <sup>+</sup> )	(12)
Plasmids		
pFZY1	<i>galk'-lacZYA</i> transcriptional fusion vector, Ap <sup>r</sup>	(88)
pLW10	pFZY1 derivative, containing <i>luxS</i> promoter region, Ap <sup>r</sup>	This study
pLW11	pFZY1 derivative, containing <i>lsrACDBFG</i> promoter region, Ap <sup>r</sup>	This study
pLW12	pFZY1 derivative, containing mutated <i>lsrACDBFG</i> promoter region, Ap <sup>r</sup>	This study
pYH10	pFZY1 derivative, containing <i>pfs</i> promoter region, Ap <sup>r</sup>	This study
pLW9	pCR-Blunt (invitrogen) derivative, containing <i>luxS</i> promoter region, Kan <sup>r</sup>	This study
pHA7E	pBR322 derivative, <i>crp</i> <sup>+</sup> Ap <sup>r</sup>	(81)
pIT302	pACYC184 derivative, <i>cya</i> <sup>+</sup> Cm <sup>r</sup>	(85)

It allows for the insertion of a variety of restriction fragments containing potential regulatory regions for the study of gene modulation. To create pLW10, the *luxS* promoter region was amplified by PCR using primers luxSpF and luxSpR (Table 2). This DNA fragment was cloned into the pCR-Blunt vector (Invitrogen). The resulting plasmid, pLW9, was digested with *EcoRI*; the fragment containing the *luxS* promoter was inserted into the *EcoRI* site of pFZY1, producing plasmid pLW10. The *luxS* promoter region (-104 to +36 relative to the *luxS* start codon) contains the native sequence up to the transcriptional terminator of the upstream gene, *gshA*. The pYH10, which contains the *pfs* promoter region (-337 to +113 relative to the *pfs* start codon) was constructed by Dr. Hashimoto. To create pLW11, the promoter region of *lsrACDBFG* operon (-307 to +92 relative to the start codon of *lsrA* (b1513)) was amplified by PCR using primers lsrpF and lsrpR (Table 2). The purified PCR product was digested with *EcoRI*-*BamHI*, and was inserted into *EcoRI* *BamHI* digested pFZY1.

To create pLW12, the method of site-directed mutagenesis by PCR was used and modified as follows. Two subsequent PCR steps were carried out. In the first round of PCR, lsrPM and lsrPR were used as primers with pLW11 as template. The PCR products were purified with Qiagen MinElute PCR Purification Kit and used as a megaprimer in the second round of PCR with pLW11 as the template and lsrPF as another primer. The products in the second round of PCR were purified using Qiagen Qiaquick Gel Extraction Kit and cloned into pCR-Blunt II TOPO vector (Invitrogen), which was transformed into Top 10 competent cells (Invitrogen). The plasmids were prepared from the transformants and sequenced to confirm the mutation of the *lsr* promoter region. The plasmid with correct insertion was digested with *EcoRI*-*BamHI*,

Table 2-2 Oligonucleotide primers used in this study

Name	Sequence	Relevant description
luxSpF	CCACTCGTGAGTGGCCAA	Upstream primer for cloning <i>luxS</i> promoter
luxSpR	GGTATGATCGACTGTGAAGCTATCTAA	Downstream primer for cloning <i>luxS</i> promoter
pfspF	CCGGAATCAAAAATTTCTTTGGCGATGTAGCG	Upstream primer for cloning <i>pfs</i> promoter
pfspR	CGCGGATCCAGTTGGCCGGTATAGATTTCCG	Downstream primer for cloning <i>pfs</i> promoter
lsrpF	CCGGAATTCGCGACCTGTTCTTCTTCACACATT	Upstream primer for cloning <i>lsr</i> operon promoter
lsrpR	CTCGGATCCTCGATGCCTTTCAGGACATTG	Downstream primer for cloning <i>lsr</i> operon promoter
lsrpM	ATAGCATAAATCGATCTCTATTTCGTCGGAAAT ATGTGCAATG	Primer for making mutation of <i>lsr</i> operon promoter
crpHP1	ACTCTCGAATGGTTCCTTGTCTCATTGCCACATT CATAAGTGTAGGCTGGAGCTGCTTC	Primer for deletion of <i>crp</i> gene
crpHP2	AAACGACGATGGTTTTACCGTGTGCGGAGATC AGGTTCTGCATATGAATATCCTCCTTAG	Primer for deletion of <i>crp</i> gene
luxSHP1	ATGCCGTTGTTAGATAGCTTCACAGTCGATCAT ACCCGGA GTGTAGGCTGGAGCTGCTTC	Primer for deletion of <i>luxS</i> gene
luxSHP2	CTAGATGTGCAGTTCCTGCAACTTCTTTCGG CAGTGCC CATATGAATATCCTCCTTAG	Primer for deletion of <i>luxS</i> gene
lsrRHP1	ATGACAATCAACGATTCGGCAATTCAGAACA GGGAATGT GTGTAGGCTGGAGCTGCTTC	Primer for deletion of <i>lsrR</i> gene
lsrRHP2	TGATCGGTAACCAAGTGCCTTGATATAACCGCC TTTCATTG CATATGAATATCCTCCTTAG	Primer for deletion of <i>lsrR</i> gene
lsrKHP1	GCGTGAAGGCATTGTTTTATATAACAATGAAG GAACAC C GTGTAGGCTGGAGCTGCTTCG	Primer for deletion of <i>lsrK</i> gene
lsrKHP2	TCCGCCTGCAAAGACTAACGATGAAGGATGAA TAGTCGAATTCCGGGGATCCGTCGACC	Primer for deletion of <i>lsrK</i> gene
lsrHP1	CGTCCGTTTATAAACAGTATTCAGGGGTCAA TGCC TGA GTGTAGGCTGGAGCTGCTTC	Primer for deletion of <i>lsrACDBFG</i> operon
lsrHP2	AGATTCCAGTTTCGCGACACAGGTTTTGTAGT GGGGCGTG CATATGAATATCCTCCTTAG	Primer for deletion of <i>lsrACDBFG</i> operon

and the DNA fragment containing mutated *lsr* promoter was inserted into *EcoRI* *Bam*HI digested pFZY1.

### **Chromosomal deletions of *crp*, *luxS*, *lsrR*, *lsrK*, and *lsrACDBFG* operon.**

The one-step replacement method described by Datsenko and Wanner (35) was used to construct a *crp* deletion in *E. coli* W3110 and ZK126. We used the phage  $\lambda$  Red recombination system to replace *crp* with a *crp::kan* PCR fragment. PCR was done using pKD4 as template and the primers crpHP1 and crpHP2 (Table 2). The underlined sequences anneal to the template plasmid, while the remaining sequences correspond to the ends of the *crp* gene. The PCR products were treated with *Dpn*I and introduced by electroporation into *E. coli* W3110 or ZK126 containing plasmid pKD46, which expresses the Red recombinase and was cured later by growth at 37°C. Recombinants were selected on LB supplemented with kanamycin. Deletions of *luxS*, *lsrR* and the *lsrACDBFG* operon were constructed similarly by PCR amplification of pKD4 with primers luxSHP1/luxSHP2, lsrRHP1/lsrRHP2, and lsrHP1/lsrHP2 respectively except that in making deletion of *lsrK*, pKD13 was used as template with primers lsrKHP1/lsrKHP2 for PCR (Table 2). The deletion of genes was verified by PCR tests.

### **Preparation of Cell-free Fluids.**

Unless otherwise stated, an overnight culture grown in LB or LB+0.8% glucose was diluted 100-fold to OD<sub>600</sub> below 0.03 in LB or LB+0.8% glucose. Cells were



incubated at 30°C with shaking at 250 rpm in Erlenmeyer flasks. Samples were removed at regular intervals. Cell-free culture fluids were prepared by centrifugation of the *E. coli* culture at 12,000 rpm for 10 min in a microcentrifuge. Cleared supernatants were filtered (0.2 µm HT Tuffryn filters, Pall Corp., Ann Arbor, MI) and stored at -20 °C.

### **AI-2 Activity Assay.**

*E. coli* cell-free culture fluids were tested for the presence of AI-2 by inducing luminescence in *V. harveyi* reporter strain BB170. The assays were performed as outlined by Surette and Bassler (180). Briefly, BB170 was grown for 16 h with shaking at 30°C in AB medium, diluted 1:5000 in fresh AB medium and aliquoted to sterile 12x75 mm tubes (Fisher Scientific Co., Inc., Pittsburgh, PA). Cell-free culture fluids were added to a final concentration of 10% (v/v) to these tubes. Positive controls contained 10% (v/v) cell-free culture fluid from BB152 while negative controls contained 10% (v/v) sterile LB or LB plus 0.8% glucose. Tubes were shaken at 175 rpm and 30°C in an air shaker (New Brunswick Scientific) and hourly measurements of luminescence were taken. Luminescence was measured as a function of *V. harveyi* cell density by quantitating light production with a luminometer (EG&G Berthold, Gaithersburg, MD). *V. harveyi* cell density was determined by spreading identical dilutions used for luminescence measurements onto solid LM medium and counting colonies after overnight growth. Relative light units (RLU) were defined as (counts min<sup>-1</sup> ml<sup>-1</sup> x 10<sup>3</sup>)/(colony forming units ml<sup>-1</sup>). AI-2 activities were obtained by dividing the RLU produced by the reporter after addition of *E. coli* culture fluid by the RLU of

the reporter when growth medium alone was added (179, 180). The obtained values were in a linear range.

### **$\beta$ -galactosidase assays.**

Cultures of *E. coli* were grown overnight in LB, diluted 100-fold into fresh LB and grown to mid-exponential phase, then diluted into different medium with OD<sub>600</sub> below 0.03. The cultures were incubated at 30°C with shaking at 250 rpm in flasks. Samples were removed at intervals for determination of OD<sub>600</sub> and  $\beta$ -galactosidase activity. Briefly, 0.1 ml cell cultures of known OD<sub>600</sub> was mixed with 0.9 ml Z buffer (100 mM sodium phosphate / 10 mM KCl / 1 mM MgSO<sub>4</sub> / 50 mM 2-mercaptoethanol, pH 7.0), 100  $\mu$ l chloroform, and 50  $\mu$ l 0.1% SDS; vortexed for 10 sec; and equilibrated for 5 min at 28°C. The substrate (0.2 ml of 4 mg/ml *o*-nitrophenyl  $\beta$ -D-galactopyranside (ONPG)) was added (record the time) and the reaction mixture was incubated at 28°C until yellow color developed. The reaction was stopped by addition of 0.5 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> (record the time), vortexed briefly, and centrifuged 5 min at maximum to remove debris and chloroform. The supernatant was removed for determination OD<sub>550</sub> and OD<sub>420</sub>, and the specific activity of  $\beta$ -galactosidase is calculated in Miller units (117) as  $1000 \cdot [OD_{420} - (1.75 \cdot OD_{550})] / (\text{time in minutes}) \cdot 0.1 \cdot OD_{600}$ .

### **Gel mobility shift assay.**

The 140 bp *Eco*RI fragment containing the promoter region of the *luxS* gene was prepared from pLW9. The 42 bp, 89 bp, and 120 bp DNA fragments containing the wild type or mutated promoter regions of the *lsr* operon were synthesized by Integrated DNA

Technologies (Coralville, IA). The synthetic 108 bp *syncon* promoter (90) was used as positive control. A digoxigenin (DIG) gel shift kit (Boehringer Mannheim) was used for labeling of DNA fragments and detection of signals according to the manufacturer's instructions. Binding reactions were performed by incubating the labeled DNA fragments with various amount of purified CRP (generously provided by Dr. Fred Schwarz, UMBI) in 20  $\mu$ l of binding buffer (10 mM Tris-HCl [pH8.0], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 50  $\mu$ g ml<sup>-1</sup> bovine serum albumin, 15  $\mu$ g ml<sup>-1</sup> sonicated salmon sperm DNA, 100  $\mu$ M cAMP). Following incubation at 37°C for 10 min, 5  $\mu$ l of gel loading buffer (0.25 x TBE, 60%; glycerol, 40%; bromphenol, 0.2% [w/v]) was added and mixtures were electrophoresed in a 6% native polyacrylamide gel in 0.5 x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) containing 100  $\mu$ M cAMP. DNA bands were detected according to the manufacturer's instructions.

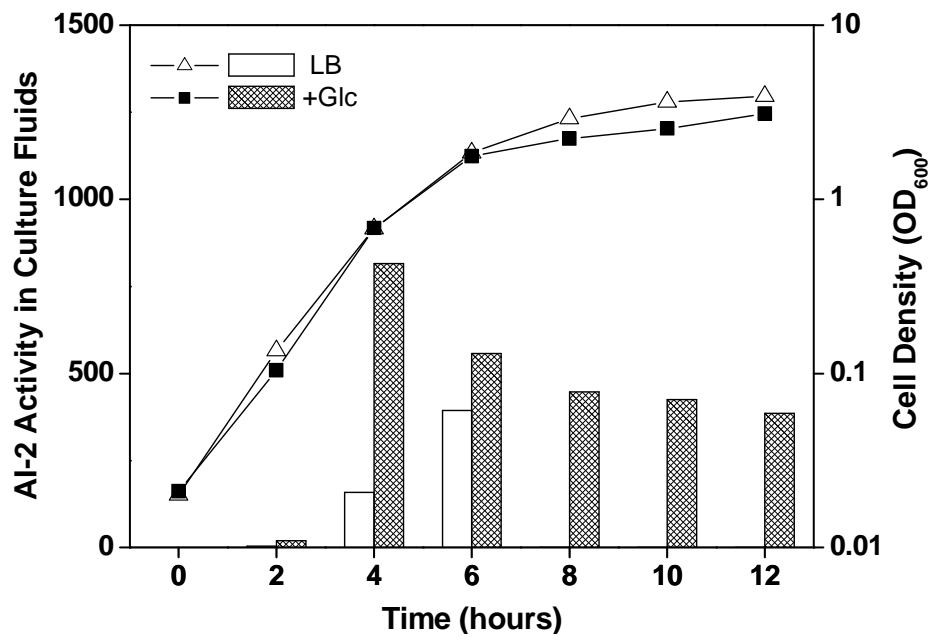
## Results

### **Extracellular AI-2 activity is increased by deletion of *crp* or *cya*.**

Extracellular AI-2 activity is produced when *E. coli* is grown with glucose or other PTS saccharides (179). Levels of extracellular AI-2 in the cell-free culture fluids of *E. coli* W3110 grown in LB and LB containing 0.8% glucose are shown in Fig. 2-2. AI-2 was produced in both growth conditions but at different levels. In the presence of glucose, AI-2 activity increased during the exponential phase, and reached the maximum at 4 h, then declined slowly. This result is consistent with a previous report (179). However, we also found that when grown in LB in the absence of glucose, the

### **Figure 2-2 Effects of glucose on extracellular AI-2 activity**

Overnight cultures of *E. coli* W3110 were diluted in LB or LB + 0.8% glucose to OD<sub>600</sub> below 0.03. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> (Triangle and square) and AI-2 activity (Bar). AI-2 activity in the culture fluids was measured using the *V. harveyi* BB170 AI-2 bioassay, and the values shown are representative of three independent experiments (some values are very small, but measured, as indicated). Replicate assays agreed to within 10%.



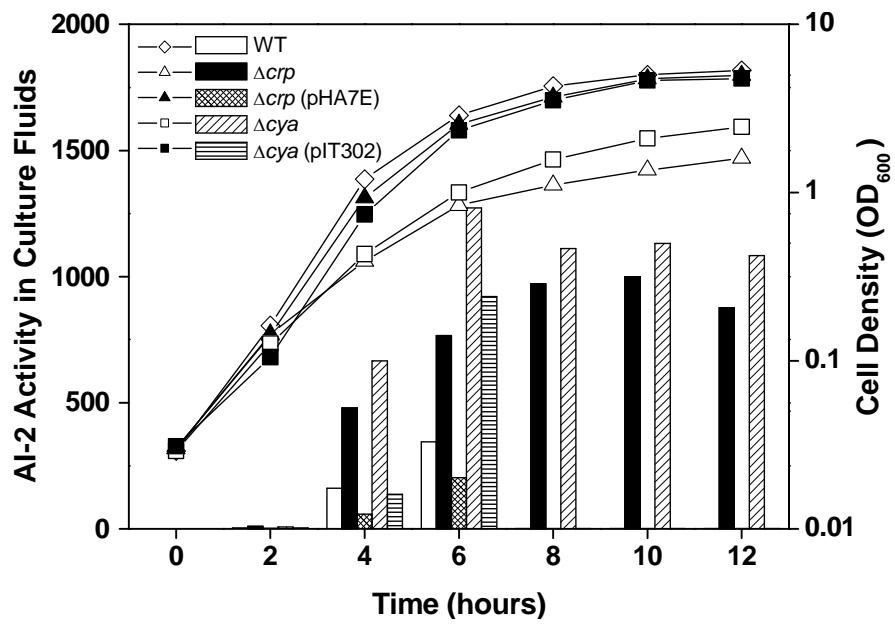
bacteria still produced AI-2 activity during exponential phase, but at a much lower level. Furthermore, no AI-2 activity in cell-free culture fluids was detected after the cells entered stationary phase.

Glucose is known to affect gene expression through the cAMP-CRP complex, and the presence of glucose in growth medium results in decreased levels of cAMP and CRP (78, 103). To check whether cAMP and CRP are involved in the production of extracellular AI-2 activity, we compared the extracellular AI-2 activity produced by W3110 and isogenic *crp* and *cya* null mutants grown in LB (Figure 2-3). Deletion of the *crp* gene or the *cya* gene in W3110 resulted in much higher AI-2 activity throughout the cell cultures (peak levels increased ~4 fold). In addition, the AI-2 activity remained high during stationary phase, suggesting the involvement of cAMP and CRP in regulating biosynthesis and/or removal of AI-2 from the media. The differences of AI-2 activities in cell-free fluids from wt and *crp/cya* null mutants grown in presence of glucose were not as marked as observed without glucose (data not shown), probably due to the already very low level of cAMP and CRP in the cells.

The introduction of plasmid-borne *crp* or *cya* reduced the AI-2 activity to levels closer to W3110. In fact, the *crp* null mutant LW1 transformed with *crp*<sup>+</sup> plasmid pHA7E resulted in even lower AI-2 activity than that produced by wt. This may be due to the use of multicopy pHA7E, which increased the concentration of CRP above that in W3110.

**Figure 2-3 *crp* and *cya* deletions increase extracellular AI-2 activity**

Overnight cultures of *E. coli* W3110 (wild type) and strains containing deletion of *crp* and *cya* were diluted in LB to OD<sub>600</sub> below 0.03. At different time points, aliquots were collected for measurement at OD<sub>600</sub> (Diamond, triangle and square) and AI-2 activity (Bar). Plasmid pHA7E and pIT302 carry wild type *crp* and *cya* genes, respectively. AI-2 activities shown are representative of three independent experiments. Replicate assays agreed to within 10%.





### **cAMP and CRP negatively regulate the expression of *luxS* but not *pfs***

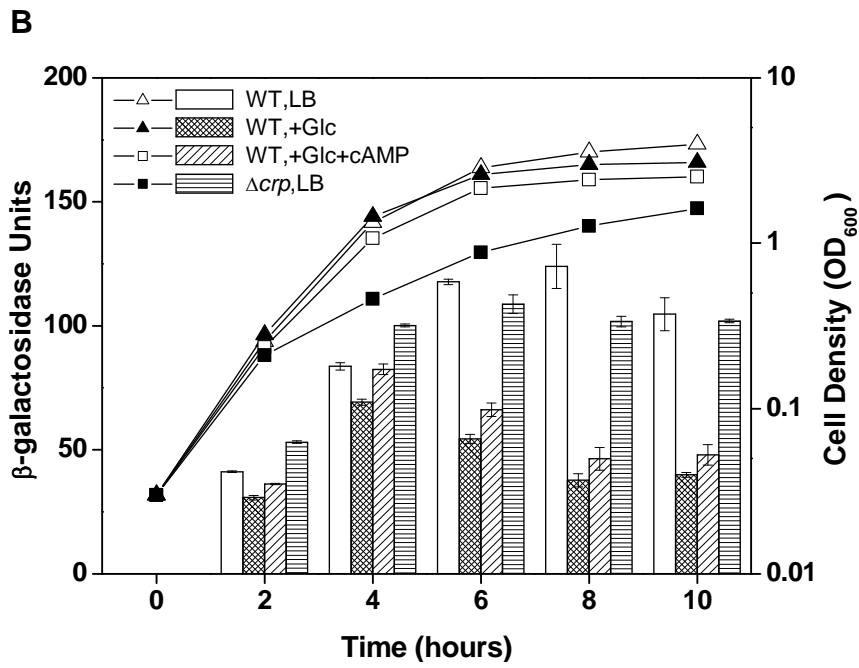
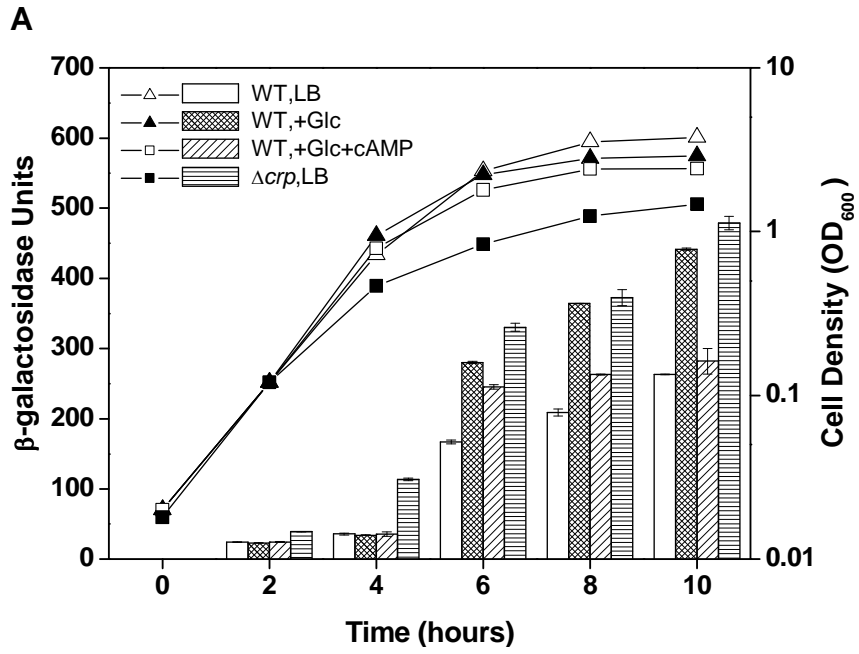
Since deletion of either *crp* or *cya* greatly enhanced extracellular AI-2 activity, we tested whether this was due to modulation of some genes involved in AI-2 biosynthesis. *lacZ* fusions were constructed to check the regulation of *luxS* and *pfs*, which encode enzymes responsible for AI-2 synthesis. We found that addition of 0.8% glucose to the growth medium slightly increased the  $\beta$ -galactosidase activity from the *luxS* promoter (<2-fold), while addition of 10 mM cAMP partly offset this glucose effect (Figure 2-4A). Moreover, deletion of the *crp* gene increased the expression of *luxS* (Figure 2-4A). These results suggest that the expression of *luxS* gene is negatively regulated by cAMP and CRP.

In contrast to the effect of glucose on *luxS* transcription, the presence of glucose in the growth medium lowered the level of  $\beta$ -galactosidase activity expressed from the *pfs* promoter, principally in the stationary phase (Figure 2-4B). The addition of cAMP to the cells grown in LB w/glucose did not restore  $\beta$ -galactosidase activity to the level when the cells were grown in LB alone. Furthermore, deletion of *crp* had no effect on  $\beta$ -galactosidase expression from the *pfs* promoter relative to the wild type control. While these results suggest that glucose plays a role in *pfs* expression, the control is likely to be through a mechanism other than the cAMP-CRP complex.

We tested whether the action of CRP and cAMP on *luxS* transcription was mediated by cAMP-CRP binding near the promoter of *luxS*. CRP binding sites contain a palindromic sequence, in which two conserved motifs, TGTGA and TCACA, are separated by a spacer (87). The length of the spacer is usually 6 bp; but, those spacers of 7 or 8, and possibly, 9 bp have been observed (9, 10, 87, 149). Although a potential

**Figure 2-4 Effects of cAMP and CRP on the transcription of *luxS* and *pfs***

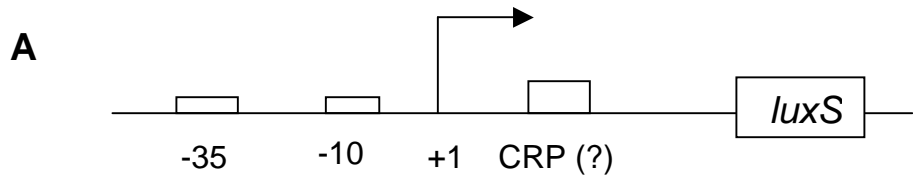
Conditions for cell growth and  $\beta$ -galactosidase activity are described in Materials and Methods. *E. coli* ZK126 (wild type) and isogenic *crp* null mutant carrying (A) plasmid pLW10 (*luxS-lacZ*), and (B) pYH10 (*pfs-lacZ*) were grown in LB, LB + 0.8% glucose or LB + 0.8% glucose + 10 mM cAMP. At different time points during cell growth, aliquots were collected for measurement at OD<sub>600</sub> (Triangle and square) and  $\beta$ -galactosidase activity (Bar).



**Figure 2-5 No binding of cAMP-CRP to *luxS* promoter region.**

A. A cartoon to show potential CRP binding site in the *luxS* promoter region. Also shown are CRP consensus sequence and DNA fragments used for CRP binding assay. Consensus and potential CRP recognition site is shown in bold letter. The numbers indicate nucleotide position relative to the predicted *luxS* transcription start site.

B. Gel mobility shift assay was done as in Materials and Methods. Digoxigenin labeled fragments of *luxS* promoter and *syncon* promoter (positive control containing consensus CRP binding site) (90) were incubated with 0 to 80 nM of purified CRP as indicated at the top. cAMP was included in all reaction mixtures at a final concentration of 100  $\mu$ M. The arrow denotes CRP-DNA complex.



Consensus  
CRP

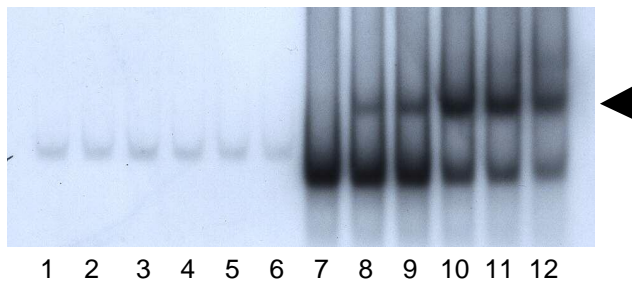
**TGTGA** Spacer **TCACA**

*luxS* promoter  
seq (141 bp):      -49 ----**TGGGA** AGAAAGAGT **TCAGA** ---- +92

*syncon* promoter  
seq (108 bp):      -----**TGTGATCTAGA** **TCACA**-----

**B**

	<i>luxS</i> promoter						<i>syncon</i> promoter					
CRP (nM)	0	5	10	20	40	80	0	5	10	20	40	80



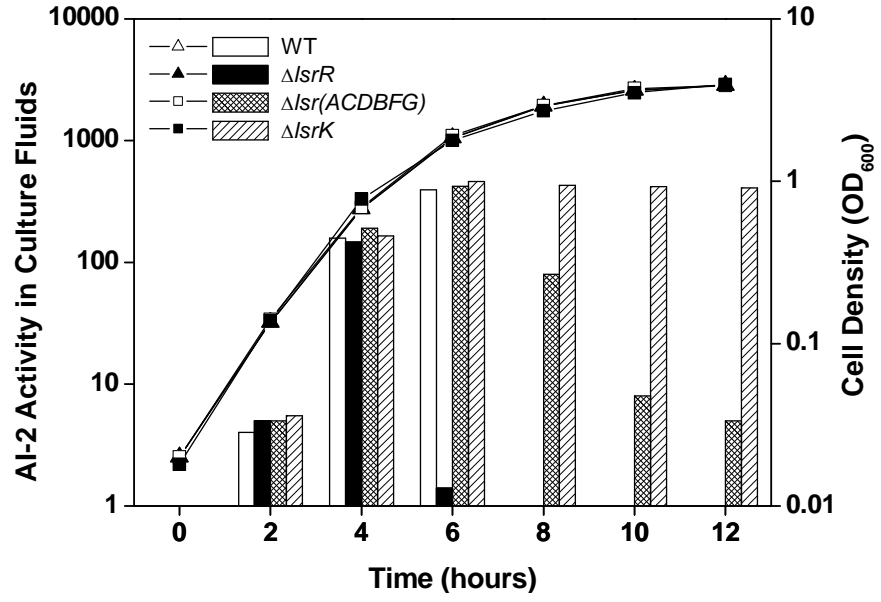
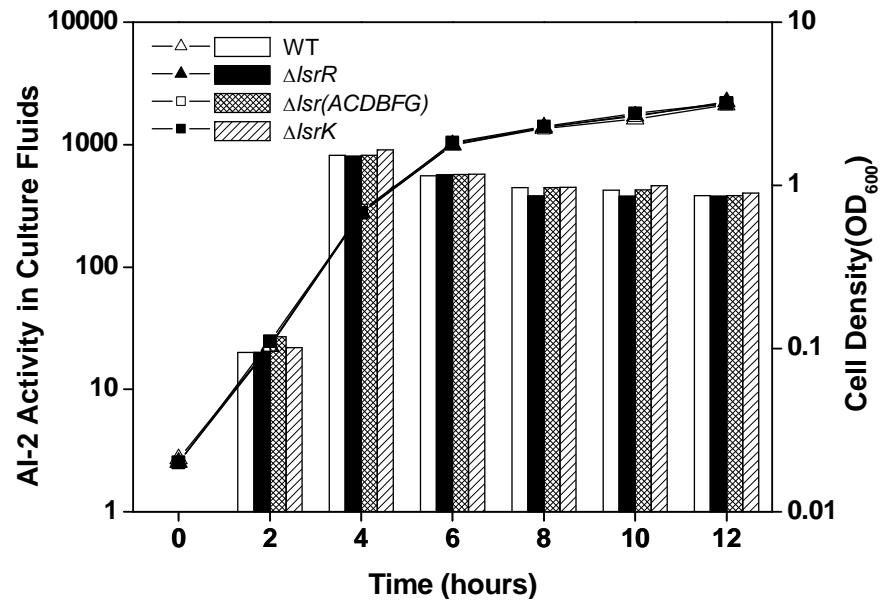
CRP-binding site: 5'-TGGGAagaaagagTCAGA-3' was present (at an atypical location), a gel mobility shift assay showed no binding of the cAMP-CRP complex to the *luxS* promoter region (Figure 2-5), suggesting that the effect of CRP and cAMP on *luxS* transcription was probably indirect or additional conditions were needed for this binding.

**cAMP-CRP stimulates expression of *lsr* operon by directly binding to upstream region of its promoter.**

Although deletion of the *crp* gene resulted in an increase of *luxS* expression (< 2-fold) in the absence of glucose (Figure 2-4A), this seemed insufficient to explain the dramatic differences in extracellular AI-2 activities in the wild type and *crp* mutant, particularly during the stationary phase (Figure 2-3). Thus, we investigated the role of cAMP and CRP in AI-2 uptake. First, to confirm the role of the *lsr* operon in *E. coli*, we made a deletion of the entire *lsrACDBFG*<sub>*E.c.*</sub> operon (see Methods). The  $\Delta$ *lsr(ACDBFG)*<sub>*E.c.*</sub> mutant showed much slower removal of AI-2 from extracellular fluids relative to the wild type when grown in absence of glucose (Figure 2-6A), although the removal of AI-2 was not completely blocked. It is likely that there is an alternative mechanism for AI-2 removal from extracellular medium in *E. coli*. Taga *et al.* suggested that there may exist another low affinity transporter for AI-2 uptake in *S. e. typhimurium* (185). Deletion of *lsrR*<sub>*E.c.*</sub> resulted in accelerated removal of AI-2 from extracellular fluids (Figure 2-6A) relative to the wild type when grown in LB, similar to an *S. e. typhimurium lsrR*<sub>*S.t.*</sub> mutant (185). Finally, deletion of *lsrK*<sub>*E.c.*</sub> caused a severe

### **Figure 2-6 AI-2 activity profiles of *E. coli* *lsr* mutants**

Overnight cultures of *E. coli* W3110 (wild type) and strains containing deletion of *lsrR*, *lsrK*, and *lsrACDBFG* were diluted in LB (A) or LB + 0.8% glucose (B) to OD<sub>600</sub> below 0.03. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> (Triangle and square) and AI-2 activity (Bar). AI-2 activities shown are representative of three independent experiments. Replicate assays agreed to within 10%.

**A****B**



defect in AI-2 removal (Figure 2-6A). This is also consistent with the *S. e. typhimurium* *lsrK<sub>S.t.</sub>* mutant, where it was suggested that the absence of LsrK prevents sequestration of AI-2 in cytoplasm in the form of phospho-AI-2 (185). In summary, the function and control of the *lsr<sub>E.c.</sub>* operon seems to operate similarly as in *S. e. typhimurium* in its role as an AI-2 autoregulated transporter and processing system.

Although the  $\Delta$ *lsrR*,  $\Delta$ *lsrK*, or  $\Delta$ *lsr(ACDBFG)* mutants and the wild type cells displayed different rates of AI-2 removal when grown in LB without glucose, all of the mutants and the wild type showed very similar extracellular AI-2 levels when glucose was present in the growth medium. The levels of AI-2 were relatively high throughout the stationary phase (Figure 2-6B). These results suggest the presence of glucose may affect the regulation of AI-2 uptake.

To investigate involvement of glucose-mediated catabolite repression in *lsr* regulation, we constructed a *lacZ* translational fusion under control of the promoter region of *lsrACDBFG* operon. When the wild type cells (ZK126) were grown in LB medium, *lsr* expression remained very low until the cells entered the stationary phase, consistent with the accumulation of AI-2 as observed earlier (Figures 2-3, 2-7, and Table 2-3). The addition of 0.8% glucose to the growth medium strongly decreased transcription from the *lsr* promoter in the wild type and in all of the *lsr* mutants (Table 2-3). These results are consistent with the significantly higher extracellular AI-2 activities of these cells (Figure 2-6B).

Deletion of the *crp* gene decreased *lsr* expression (Table 2-3), indicating that CRP is needed to activate transcription from the *lsr* promoter. Deletion of *lsrR* caused a significant increase in *lsr* expression (Figure 2-7 & Table 2-3), confirming the role of

**Figure 2-7 Regulation of the transcription of *E. coli lsr* operon**

*E. coli* ZK126 (wild type) and strains containing deletion of *luxS*, *lsrK*, *lsrR* and *lsrACDBFG* carry plasmid pLW11 (*lacZ* fusion containing *lsrA* promoter region). ZK126 (WT\*) carries plasmid pLW12 (*lacZ* fusion containing mutated *lsrA* promoter region with base substitutions in CRP-binding motif). Cells were grown in LB medium. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> (Triangle, square and diamond) and β-galactosidase activity (Bar).

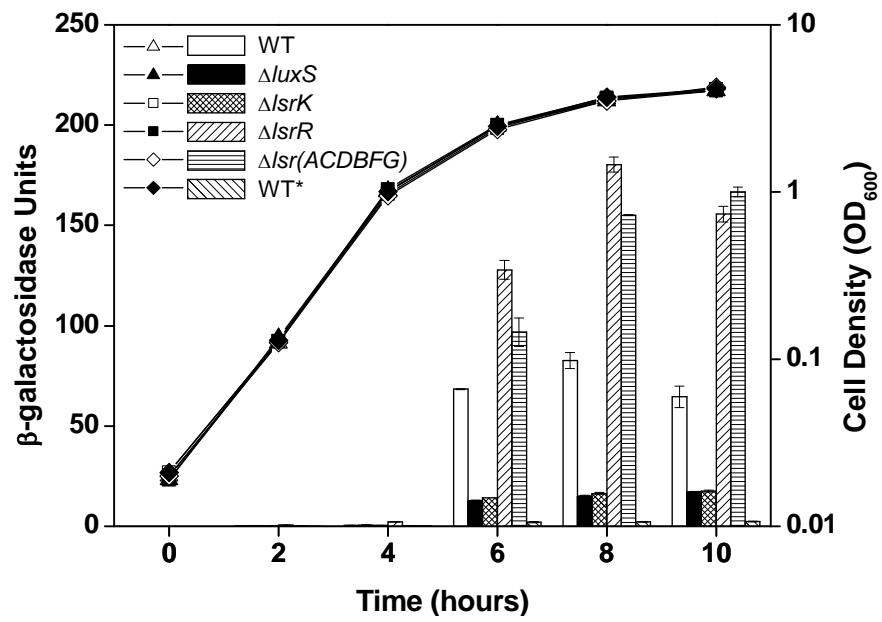


Table 2-3 Regulation of the expression of *lsrACDBFG* operon

Strain genotype	Plasmid genotype	$\beta$ -Galactosidase activity (Miller units)			
		Late exponential (4h)		Early stationary (6h)	
		LB	LB + Glc	LB	LB + Glc
Wild type	pLW11	0.5	0.1	68	0.4
$\Delta crp$	pLW11	0.2	–	0.4	–
$\Delta luxS$	pLW11	0.6	0.2	13	0.4
$\Delta lsrK$	pLW11	0.5	0.2	14	0.4
$\Delta lsrR$	pLW11	2.4	0.3	128	0.7
$\Delta lsr(ACDBFG)$	pLW11	0.4	0.2	97	0.5
$\Delta rpoS$	pLW11	4.0	–	119	–
Wild type	pLW12	0.3	–	2	–

a. *E. coli* ZK126 (wild type), strains containing deletion of *crp*, *luxS*, *lsrK*, *lsrR*, *lsr(ACDBFG)* and *rpoS*, carry plasmid pLW11 (*lacZ* fusion containing *lsrA* promoter region), or plasmid pLW12 (*lacZ* fusion containing mutated *lsrA* promoter region with base substitutions in CRP-binding motif). Cells were grown in LB or LB plus 0.8% glucose. Growth conditions were the same as in Fig. 2-6.

b. Late exponential-growth phase is at 4 hr growth (Fig. 2-6); Early stationary phase is at 6 hr growth (Fig. 2-6).

c. All measurements were within +/-10% std.dev. –, not determined.

the LsrR protein as a repressor protein. These transcription results are consistent with the rapid AI-2 removal observed in the  $\Delta lsrR$  mutant (Figure 2-6). The much lower *lsr* expression in the exponential phase in the  $\Delta lsrR$  mutant and other strains are likely caused by the levels of glucose and other PTS sugars present in LB medium (194). It is also noteworthy that deletion of either *luxS* or *lsrK* resulted in a much lower level of *lsr* expression (Figure 2-7 & Table 2-3), supporting the AI-2/phospho-AI-2-dependent regulation as shown in *S. e. typhimurium* (185). It is further shown here that LsrR-mediated repression of *lsr* expression is not complete, as noted by significant expression levels in the  $\Delta luxS$  or  $\Delta lsrK$  mutants. Finally, deletion of the entire *lsrACDBFG* operon resulted in a significant increase in transcription from the *lsr* promoter (Figure 2-7 & Table 2-3). This was, at first, unexpected since the absence of the Lsr transporter decreases uptake of AI-2, which is recruited to enhance *lsr* transcription. Thus, in the absence of AI-2 we would expect effective LsrR-mediated repression. We suggest that LsrK phosphorylates endogenous AI-2 and/or AI-2 imported by the alternative transporter, which then derepresses transcription from the *lsr* promoter through LsrR (see Discussion). Moreover, since LsrF and LsrG are not present, which are reported to promote AI-2 degradation (185), the inducer phospho-AI-2 may persist.

To evaluate whether cAMP-CRP directly modulates *lsr* transcription, we performed gel mobility shift assays. The promoter region of the *lsr* operon contains two potential CRP binding sites (Figure 2-8A). Site 1 is located 19 to 34 bp upstream of the predicted transcription start site, while Site 2 is located at residues -60 to -77. Our results revealed that cAMP-CRP binds to the DNA fragment containing both Site 1 and Site 2, but not the fragment containing only Site 2 (Figure 2-8B). A shorter DNA

fragment containing only Site 1 was bound by cAMP-CRP (Figure 2-8C), but there was no binding of cAMP-CRP to a DNA fragment that contains only Site 1 with substitutions in four base pairs in one of the CRP-binding motifs (Figure 2-8C). These data confirm that cAMP-CRP specifically recognizes and binds to the CRP binding site located in Site 1, and not Site 2. Interestingly, the spacer for this binding site is 8-bp while most of the known CRP binding sites contain a conventional 6-bp spacer (9, 10, 87). It has been demonstrated that cAMP-CRP binds to many sites with an 8-bp spacer *in vitro* (9, 10, 25, 164), although we have found no reports showing such CRP binding sites are functional *in vivo*. In this study, *in vivo* experiments were performed with wild type ZK126 cells carrying pLW12, which contains the identical *lsr* promoter region as pLW11 but with the four base substitutions in the CRP binding site as used in the gel shift assay (seq4). Significantly decreased transcription from the mutated *lsr* promoter was found (Figure 2-7 & Table 3), indicating a requirement for this site in cAMP-CRP mediated activation. These results demonstrate that cAMP-CRP stimulates transcription of the *lsrACDBFG* operon by directly binding to the upstream region of the *lsr* promoter, and that mutation in the CRP binding site abolishes this stimulation.

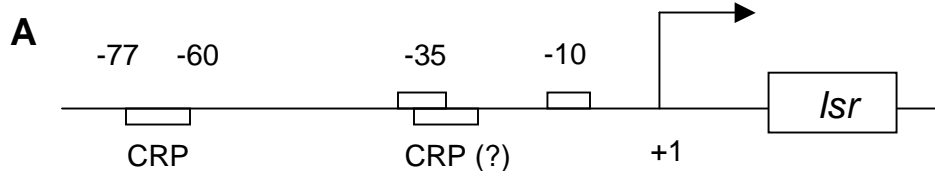
#### **A role of $\sigma^s$ in AI-2 uptake.**

As much of this transcriptional regulation occurs late in the exponential phase, we investigated the possibility that transcription factor,  $\sigma^s$ , plays an additional role. Recent reports have indicated  $\sigma^s$  is important in sensing stress via changes in growth rate (1). Interestingly, we found that deletion of *rpoS* resulted in an 8-fold increase in *lsr* expression during the late exponential phase, although the transcription differences

**Figure 2-8 cAMP-CRP binds to upstream region of the *lsr* promoter.**

(A) A cartoon to show potential CRP binding sites in the *lsr* promoter region. Also shown are CRP consensus sequence and DNA fragments used for CRP binding assay. Consensus and potential CRP recognition sites are shown in **bold** font. The underlined bases in seq4 show substitutions ablating CRP binding. The numbers indicate nucleotide position relative to the predicted *lsrA* transcription start site.

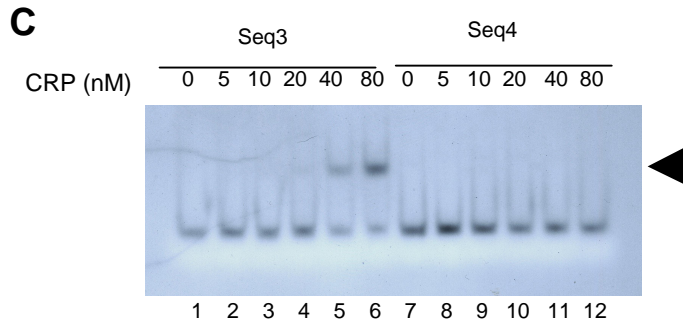
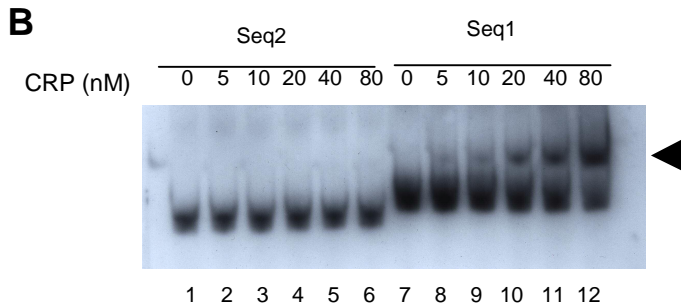
(B) & (C) Gel mobility shift assays were performed as in Materials and Methods. Digoxigenin labeled DNA fragments of seq1, seq2, seq3 and seq4 were incubated with 0 to 80 nM of purified CRP, as indicated. cAMP was included in all reaction mixtures at a final concentration of 100  $\mu$ M. The arrow denotes the CRP-DNA complex.



Consensus CRP binding site:

TGTGA spacer TCACA

		<b>Site 1</b>	-60 -34	<b>Site 2</b>	
<i>lsr</i> promoter seq1:	-88	---TGTGA	TCTATTTCG	TCGGA---	TATGA ACAAAT TAAAA--- +32
<i>lsr</i> promoter seq2:			<b>Site 2</b>		
			-34		
	-57	----TATGA	ACAAAT	TAAAA----	+32
<i>lsr</i> promoter seq3:			<b>Site 1</b>	-60	
	-88	---TGTGA	TCTATTTCG	TCGGA----	-47
<i>lsr</i> promoter* seq4:			<b>Site 1</b>	-60	
	-88	---CGATC	TCTATTTCG	TCGGA----	-47

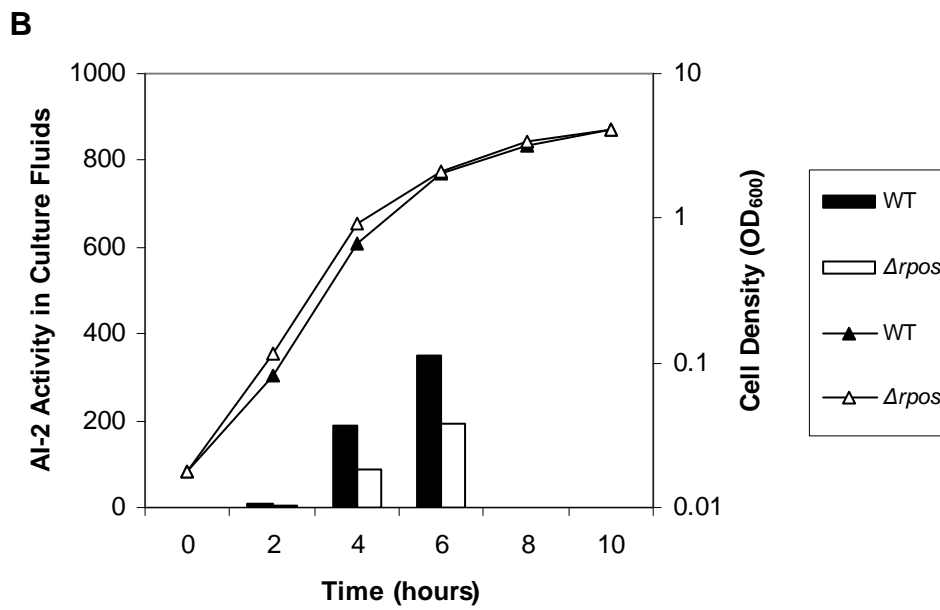
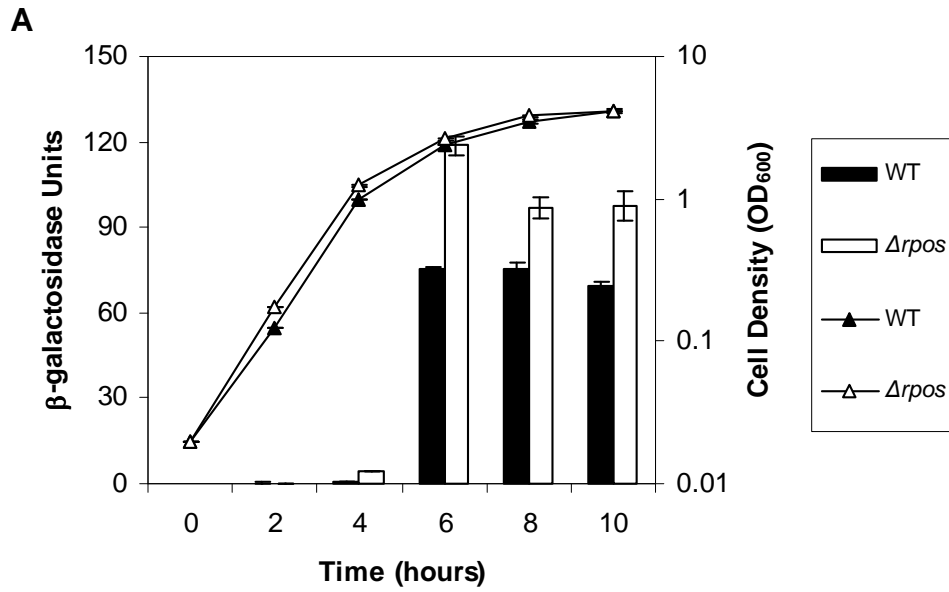




**Figure 2-9  $\sigma^S$  negatively regulates expression of the *lsr* operon.**

A. *E. coli* ZK126 (wild type) and the  $\Delta rpoS$  mutant carry plasmid pLW11 (*lacZ* fusion containing *lsrA* promoter region). Cells were grown in LB medium. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> (Triangle) and  $\beta$ -galactosidase activity (Bar).

B. *E. coli* ZK126 (wild type) and the  $\Delta rpoS$  mutant were grown in LB medium. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> (Triangle) and extracellular AI-2 activity (Bar). AI-2 activities shown are representative of three independent experiments. Replicate assays agreed to within 10%.



between the wild type and  $\Delta rpoS$  mutant were decreased during stationary phase (Figure 2-9A & Table 2-3). Correspondingly, extracellular AI-2 activity in  $\Delta rpoS$  mutant was reduced relative to the wild type (Figure 2-9B). While confirming a role, exactly how  $\sigma^S$  affects *lsr* expression is under further examination.

### Discussion

AI-2 was first discovered in *V. harveyi* as a quorum signal to regulate bioluminescence (12, 13). In addition to communicating cell density, it is suggested that AI-2 may relay information pertaining to the growth phase and metabolic potential of the bacterial cells (38, 206). Since growth on glucose results in production of the highest level of extracellular AI-2 activity in *E. coli* and *S. e. typhimurium* (179), we were interested in whether cAMP-CRP mediates regulation of AI-2 production. We found that deletion of either *cya* or *crp* genes in *E. coli* results in a significant increase in extracellular AI-2 activity. We further showed that cAMP and CRP negatively regulate *luxS* transcription. However, the action of CRP and cAMP on *luxS* transcription is not mediated by cAMP-CRP binding to the promoter region of *luxS*, as indicated by the gel mobility shift assay (Figure 2-5). It is possible that cAMP-CRP may control another transcriptional regulator(s) that modulates the *luxS* expression. Interestingly, we found that the expression of *pfs* is reduced by the presence of glucose, however, not through the cAMP-CRP complex. Considering that Pfs is involved in both AI-2 synthesis and polyamine formation pathways, while LuxS is involved only in AI-2 synthesis (Fig. 2-1), regulation of AI-2 production through LuxS appears more practical and efficient, although the other factors, such as the concentrations of precursors, may

also affect AI-2 synthesis. Beeston and Surette found that the extracellular AI-2 activities were correlated with the transcription profile of *pfs* in *S. e. typhimurium* (16), which is similar to our result in the presence of glucose (Fig. 2-4B). Despite the correlation, the earlier (185, 186) and current work suggest that the extracellular AI-2 activities are controlled by a dynamic regulatory process involving many factors that affect both its synthesis and transport. In the stationary phase the AI-2 levels in culture medium appear to be decided by the rate of its import rather than the rate of synthesis, as suggested by this study.

Since *E. coli* possesses almost all genes homologous to those in the *lsr* operon of *S. e. typhimurium*, we were curious whether the function and regulation of the *E. coli* Lsr transporter was similar to that in *S. e. typhimurium*, and whether cAMP and CRP were involved in regulation of AI-2 transport. Our data are consistent with both, although some differences were noted. For example, *lsrE* is apparently absent in *E. coli*. This gene is homologous to *rpe*, which encodes the ribulose phosphate epimerase. It is possible that additional step(s) are required for AI-2 modification in *S. e. typhimurium*, which somehow is lost or not gained in *E. coli* during evolution. The gene is probably useful for *S. e. typhimurium*, but maybe not important for *E. coli* to live. Also, examination of the upstream region of the *S. e. typhimurium lsr* operon reveals one potential CRP binding site: 5'-TGAGAGttttTGACC-3'(-36 to -51 relative to the predicted transcriptional start site of the *lsr* operon). This site has a 6-bp spacer and its function has yet to be confirmed. Promoters where CRP behaves as a transcription activator usually have the CRP site located around -41, -61, -70 (87). We have shown in this study that CRP binds to the *lsr<sub>E. c.</sub>* promoter region of -60 to -77. While the

molecular basis for the cAMP-CRP mediated regulation may be slightly different, the function and control of the Lsr transporter are similar. In a genetic screen for the regulator of the *lsr* operon in *S. e. typhimurium*, mutation of either *cya* or *ptsI* caused reduced transcription of the *lsrC-lacZ* reporter (185). Since Cya is directly involved in the production of cAMP, and since PtsI is Enzyme I of the PTS and is required for the activation of Cya, it is not surprising that mutation of *cya* or *ptsI* results in lower expression of the *lsr* operon.

We noted that deletion of the whole *lsr* operon in *E. coli* does not completely block AI-2 import (Fig. 2-6A), which was also the case for the Lsr transport mutants of *S. e. typhimurium* (185, 186). Taga *et al.* proposed the existence of other low -affinity Lsr-independent transport mechanism(s) (185). Interestingly, while the transport of AI-2 is slower in this mutant, there is much higher transcription from the *lsr* promoter relative to the both wild type and the  $\Delta luxS$  mutant (Fig. 2-7) (all in LB medium). In other words, transcription from the *lsr* promoter can still be induced even without AI-2 import by the Lsr transporter. It is possible that AI-2 imported from an alternative low affinity transporter can serve as a substrate for the LsrK kinase to produce phospho-AI-2. It is also possible that the endogenous AI-2 can be phosphorylated directly by LsrK. In *S. e. typhimurium*, *lsr* expression in the  $\Delta lsrB$  mutant with defective Lsr transporter is higher than that in a  $\Delta luxS$  mutant, although lower than that in the wild type (185). The reduced *lsr* expression in the  $\Delta lsrB$  mutant relative to the wild type probably results from a lower rate of AI-2 phosphorylation due to the absence of AI-2 uptake from a functional Lsr transporter. The higher induction from the *lsr* promoter in *E. coli*  $\Delta lsrACDBFG$  mutants is probably also influenced by the loss of the phospho -AI-2

degradation by LsrF and LsrG. Hence, LsrF and LsrG may function as a signal terminator in the wild type *E. coli* cells, as shown in *S. e. typhimurium* (185).

There are, therefore, several regulators that influence the expression level of the Lsr transporter. Since  $\sigma^s$  is very important in sensing stress during the transition to stationary phase (1), we investigated the possibility that  $\sigma^s$  plays an additional role in controlling AI-2 transport. Our results showed that deletion of *rpoS* (encoding  $\sigma^s$ ), causes an 8-fold increase in the transcription of the *E. coli lsr* operon during the late exponential growth phase when cells are grown in absence of glucose (Figure 2-9A & Table 2-3), and extracellular AI-2 activities in  $\Delta rpoS$  mutant are significantly decreased (Figure 2-9B). Notley-McRobb *et al.* (130) reported that the mutation of *rpoS* induces expression of certain transporter genes under glucose limitation. It was suggested that absence of *rpoS* results in loss of competition between  $\sigma^s$  and  $\sigma^{70}$  for core RNA polymerase (130), since recognition sequences of  $\sigma^s$  and  $\sigma^{70}$  are similar. Also, it may be possible that  $\sigma^s$  controls other regulators (eg. *lsrR* and *lsrK*) that affect the *lsr* expression. In addition to  $\sigma^s$ , Taga *et al.* reported that transcription of the *lsrC-lacZ* reporter in *S. e. typhimurium* is lowered eight-fold by mutation of the *lon* gene, which encodes the Lon protease (185). How Lon is related to the regulation of the *lsr* expression is unclear.

Our working model for AI-2 synthesis and transport in *E. coli* (Figure 2-10) is that cAMP-CRP acts as a global controller, while LsrR functions as a specific controller. When glucose or other PTS sugars are present in the growth medium, low intracellular levels of cAMP and CRP result, and there is almost no transcription of the *lsr* operon. Instead, the level of *luxS* transcription increases, and it is likely that the metabolic flux of precursors (SAM, SAH, etc.) also increases concomitant with an increased demand

for biosynthesis. It is also likely that other cellular activities requiring methylation (through SAM) will impact the level of the substrate for AI-2 synthesis. Thus, with sufficient glucose, increased carbon and energy storage may result and the net effect is an acceleration in AI-2 synthesis and export. This is consistent with the exponential phase results of Figures 2-6 & 2-7, where there is minimal *lsr* transcription and the AI-2 levels were significantly higher (~3x) in both the wild type and the  $\Delta$ *lsrACDBFG* mutant (having drastically impaired AI-2 uptake) in the presence of glucose.

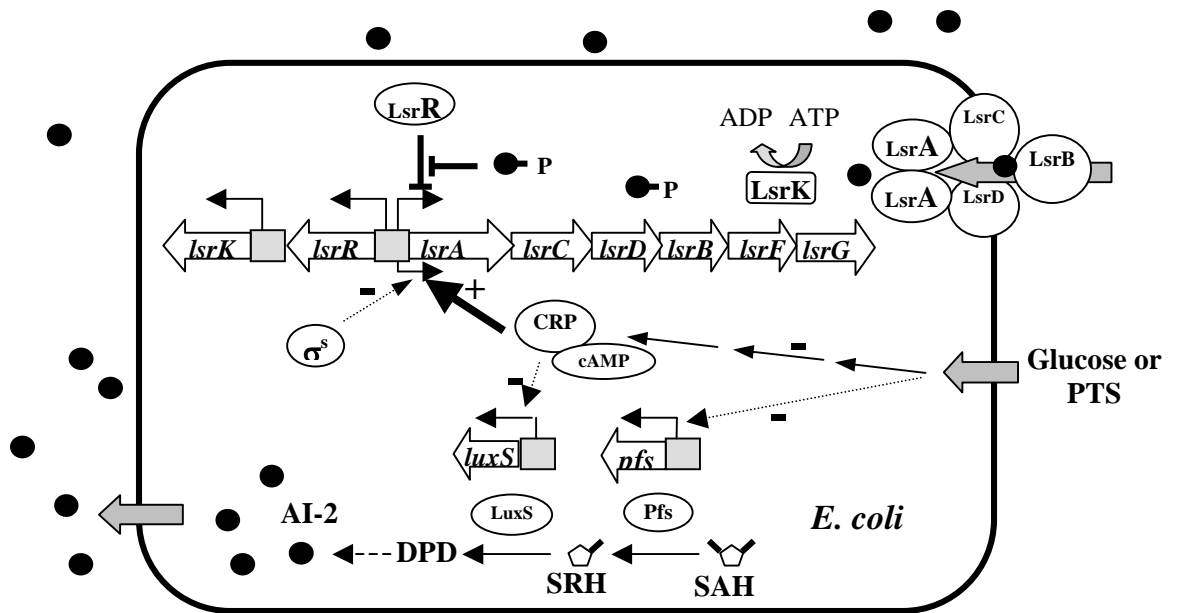
However, when glucose or other PTS sugars are absent, the cAMP-CRP complex binds to the upstream region of the *lsr* promoter and stimulates its transcription. It is in this condition that LsrR, LsrK, LsrF, and LsrG, play a greater role in regulating and “tuning” AI-2 uptake. If there is no inducer (phospho-AI-2), LsrR represses transcription of the *lsr* operon. During the late exponential phase, a basal level of *lsr* expression likely results in uptake and phosphorylation of a small amount of AI-2. Endogenous AI-2 and AI-2 imported by a low-affinity transporter may also provide substrate for LsrK. The phospho-AI-2 inactivates the repressor LsrR and increases transcription of the *lsr* operon, as shown in *S. e. typhimurium* (185). Then, more AI-2 is transported into the cells and the cycle continues until LsrF and LsrG provide feedback control (185). Thus the cAMP-CRP-mediated regulatory mechanism of the *lsr* operon is very similar to the well-described control of the lactose (*lac*), arabinose (*ara*) and galactose (*gal*) operons of *E. coli*.

In summary, we have shown that *E. coli* cells synthesize and secrete AI-2 in the early growth phase and take it up during the stationary phase under glucose limitation. The simplest interpretation is that the bacterial cells use AI 2 as a carbon source in the

**Figure 2-10 Conceptual model AI-2 synthesis and uptake in *E. coli*.**

In the presence of glucose, low levels of cAMP and CRP result in almost no expression of the *lsr* operon. Indirect upregulation of *luxS*, and likely increased precursor flux, increases AI-2 synthesis. Both enable rapid accumulation of AI-2 in the extracellular media. In the absence of glucose, cAMP-CRP is needed to stimulate the *lsr* expression, while LsrR represses its expression in the absence of inducer, phospho-AI-2. As AI-2 accumulates, the *lsr* transcription is de-repressed enabling more AI-2 uptake. In addition,  $\sigma^s$  negatively affects *lsr* expression, especially during the late exponential phase. As noted above, the expression of *pfs* is negatively influenced by presence of glucose; the effects of this are unclear, but might be complicated by the polyamine pathways also utilizing Pfs and SAM. Transcriptional regulation is shown by solid arrows (direct) or dashed arrows (indirect or under unclear mechanisms). Plus and minus signs indicate positive and negative regulations, respectively. SAH: S-Adenosylhomocysteine; SRH: S-Ribosylhomocysteine; DPD: 4,5-Dihydroxy-2,3-pentanedione. See text for additional details.





absence of the preferred glucose, just like they use lactose, arabinose, and galactose, which are similarly regulated. However, Taga *et al.* reported that *S. e. typhimurium* can not grow in minimal media containing AI-2 as the sole carbon source (186). Winzer *et al.* suggested that utilization of AI-2 as the sole carbon source might require additional conditions (202). They suggested further that AI-2 may be a toxic byproduct of SAM metabolism, which is excluded during early growth and taken up and metabolized at a later stage (for detoxifying and recycling the energetically expensive ‘ribose equivalent’ unit (201, 202)). We found that the  $\Delta luxS$  mutant of *E. coli* ZK126 grows as well as the wild type in LB medium (Fig. 2-7), but Sperandio and co-workers found that mutation of *luxS* in enterohemorrhagic *E. coli* (EHEC) O157:H7 resulted in a faster growth of the mutant when grown in DMEM and that there was a global effect on gene expression (173). It is therefore possible that the specific growth condition and/or specific genotype might affect or determine the role of AI-2 as a signal molecule. Indeed, there are many other reports suggesting that AI-2 may participate in the control of certain genes and physiological activities (reviewed in (206)). For example, LuxP of *Vibrio harveyi* is known to be a specific receptor for AI-2 in its role as a quorum signal (13, 96), while LsrB in *E. coli* and *S. e. typhimurium* can bind AI-2, but may function as a part of the Lsr transporter complex. The transcriptional regulator, LsrR, acts as an internal sensor for the AI-2/phospho-AI-2 and works with phospho-AI-2 to de-repress *lsr* transcription. Whether LsrR is also involved in modulation of other genes and/or cellular activities remains an open question. Thus, while the role of AI-2 as a signal molecule remains unclear in *E. coli* W3110, it is clear that these cells have a complex hierarchical

regulatory system for its control, suggesting that the recruitment of AI-2 in the regulation of many cellular processes will continue to emerge.

## Chapter 3 Genomic Expression Profiling Revealed Growth Condition Dependent Gene Regulation by *luxS* in *Escherichia coli* K12

### Abstract

Bacteria have evolved complex genetic circuits to regulate their physiological activities and behaviors in response to a variety of extracellular signals. In a process termed “quorum sensing”, or density-dependent gene regulation, bacteria produce, release and respond to certain signaling molecules (autoinducers). The bacterial autoinducer-2 (AI-2), has received intense interest recently because the gene for its synthase, *luxS*, is common in a large number of Gram-negative and Gram-positive bacterial species. In this study, we have identified the *luxS*-controlled genes in *E. coli* under two different growth conditions using DNA microarrays. Using a fold change cutoff of +/- 1.8, we found that 24 genes were affected by *luxS* deletion in the presence of glucose, and 67 genes influenced by *luxS* deletion in the absence of glucose. The *lsrACDBFG* operon, *lsrK* and *metE* were among the most significantly induced genes in the latter condition. To a lesser degree, the deletion of *luxS* was shown to affect the expression of genes involved in diverse cell activities such as methionine biosynthesis, methyltransfer reactions, iron uptake, resistance to oxidative stress, utilization of various carbon sources, and potentially, virulence. Most important in this chapter, we showed that the impact of *luxS* was found to depend on the growth phase. This contributes to the understanding of AI-2/*luxS* mediated gene regulation and suggests the importance of carefully selected experimental conditions in identifying AI-2/*luxS* controlled genes in various bacteria.

## Introduction

Bacteria are capable of responding to a variety of chemical and physical changes in the environment by regulating their gene expression. These changes could be environmental stresses, such as heat shock, nutrient limitation, high osmolarity, which cause cells to have multigenic responses in transcription and translation. In addition, these changes could be caused by the metabolic activities of bacterial cells. For example, some bacteria produce certain metabolites that are released into the environment as the cell density increases. These molecules could be just metabolic wastes, which are toxic to the normal physiological activities of the cells and are therefore secreted outside. However, in certain situations, some metabolic products may serve as signaling molecules, which can be perceived by the cells to control specific gene expression. This type of regulation (autoinduction) confers upon bacteria the capability to communicate with each other and coordinate their activities, and has been termed quorum sensing.

For example, many bacteria produce and secrete a hormone-like signaling molecule, acyl-homoserine lactone (AHL). With an increase in cell density, the concentration of AHL can increase and reach a threshold stimulatory level, at which the signal molecule binds to a LuxR-like protein, the transcriptional regulator, to control varied gene expression and cell activities. The AHL-mediated quorum sensing is well documented in Gram-negative bacteria (118, 203).

Besides AHLs, a large number of Gram-negative and Gram-positive bacteria are found to produce and release another type of signaling molecule, AI-2, which can stimulate production of bioluminescence in the *Vibrio harveyi* reporter strain. Schauder

*et al.* showed that AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps and LuxS is the enzyme directly involved in AI-2 production (163). More than 55 bacterial species possess a gene homologous to *luxS* and many have been shown to produce AI-2-like activities by using a *V. harveyi*BB170 reporter strain (181, 206). Recent advances have indicated that the AI-2 molecules from various bacterial species may differ in their structure, although all of them are derived from the product of the LuxS reaction, 4,5-dihydroxy-2,3-pentanedione (DPD) (120). The DPD is a highly reactive molecule, which likely undergoes cyclization and further arrangements to form a mixture of varied chemical molecules (163). The AI-2 molecules from *Vibrio harveyi* and *Salmonella trphimurium* have been reported to be (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF-borate) and (2*R*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) respectively (27, 120). It was also suggested that DPD, *R*-THMF, and *S*-THMF-borate are in equilibrium, which is affected by the presence of borate (120).

Evidence accumulated during the last several years suggests that AI-2 may be used as a signal by a variety of bacterial species (206). For example, Ohtani *et al.* showed that mutation of the *luxS* gene in the *Clostridium perfringens* results in reduced production of the alpha-, kappa-, and theta-toxins (133). However, toxin production is stimulated by conditioned medium (cell free fluids) from wild type cells but not from the *luxS* mutant, suggesting AI-2 may be involved in regulation of the toxin production (133). They further showed that the expression of the theta-toxin gene, *pfoA*, (but not alpha- and kappa-toxin genes) is lower in the *luxS* mutant during mid-exponential phase, and is also activated by the conditioned medium from wild type strain (133). These

results indicate that the AI-2/*luxS*-mediated signaling is important in controlling toxin production in *Clostridium perfringens*.

In a search for *luxS* -regulated genes in *S. e. typhimurium*, the *lsrACDBFGE* operon and the methionine synthase gene *metE* were identified by Taga *et al.* (186). They found that the *lsrACDBFGE* operon encodes an AI-2 uptake and modification system. In *E. coli*, there exists a similar *lsr* operon (b1513 operon), except that it does not have the *lsrE* homolog. In chapter 2, we have shown that the functions of the *E. coli* *lsr* operon and its regulators, LsrR and LsrK, are similar to those in *S. e. typhimurium*, and cAMP-CRP are involved in regulation of the *lsr* operon (193).

In this study, we have attempted to identify the *luxS* controlled genes by comparing the wild type and  $\Delta luxS$  mutant under two different growth conditions using DNA microarrays. In the first case, we examined cells in the presence of glucose at low cell density (mid exponential phase). Then, we examined cells in the absence of glucose at high density. This is the condition where *lsr* operon was induced in the wild type cells, and was hypothesized to be the case where AI-2-mediated signaling will have more of an impact (chapter 2). It turned out that many more genes are significantly affected by *luxS* deletion at this condition. Specifically, with a fold change cutoff of +/- 1.8, there are 24 genes affected by *luxS* deletion in the presence of glucose with OD<sub>600</sub> of 1.0, but 67 genes influenced in the absence of glucose with OD<sub>600</sub> of 2.4. The deletion of *luxS* affects expression of genes which are involved in diverse cell activities such as methionine biosynthesis, methyltransfer reactions, iron uptake, AI- 2 uptake, resistance to oxidative stress, utilization of various carbon sources, and potentially, virulence. This study serves to enhance our understanding about the growth condition

dependent gene modulation by AI-2/*luxS* and has opened a window for us to view the *luxS* controlled cell activities in *E. coli* K12 strain.

### Materials and Methods

#### **Bacterial strains and growth conditions.**

*E. coli* K-12 strain W3110 (F,  $\lambda$ , in(*rrnD-rrnE*)) was obtained from Genetic Stock Center (New Haven, Connecticut). The  $\Delta$ *luxS::kan* was moved into W3110 from LW7 (ZK126,  $\Delta$ *luxS::kan*) (193) via P1<sub>vir</sub> transduction. Luria Bertani broth (LB) contains 5 g L<sup>-1</sup> yeast extract (Difco), 10 g L<sup>-1</sup> bacto tryptone (Difco), and 10 g L<sup>-1</sup> NaCl. Cultures of *E. coli* (wild type and the  $\Delta$ *luxS* mutant) that had been grown overnight in LB or LB plus 0.8% glucose, were diluted to OD<sub>600</sub> about 0.02 in LB or LB plus 0.8% glucose. The cultures were then incubated at 30°C with shaking at 250 rpm in 50 ml flasks. When the cultures reached the appropriate OD<sub>600</sub> (1.0 or 2.4), the cells were harvested for RNA extraction.

#### **RNA isolation.**

Total RNA was isolated from the cultures using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The procedure for RNA isolation combines the selective RNA binding properties of a silica-gel-based membrane with the speed of microspin technology. RNeasy Protect Bacteria Reagent (containing tetradecyltrimethylammonium bromide) (Qiagen, Inc., Valencia, CA) was added to the cultures to stabilize RNA before isolation. The RNeasy-Free DNase Set (Qiagen, Inc., Valencia, CA) was used for on-column DNase digestion to remove



residue DNA during RNA purification. Finally, RNA was eluted with nuclease-free water.

### **cDNA synthesis and labeling.**

cDNA was synthesized and labeled according to the manufacturer's suggestions for the Affymetrix *E. coli* Antisense Genome Array (Affymetrix, Inc., Santa Clara, CA). Briefly, in 60  $\mu$ l of reaction mixture, 10  $\mu$ g of total RNA was used for cDNA synthesis by random primers (12.5 ng/ $\mu$ l) and SuperScript II reverse transcriptase (25 U/ $\mu$ l) (both from Invitrogen Corp., Carlsbad, CA). RNA was removed by addition of 20  $\mu$ l of 1N NaOH and incubation at 65°C for 30 minutes. cDNA was purified with Qiaquick PCR Purification Kit (Qiagen, Inc., Valencia, CA), then fragmented using DNase I (0.6 U/ $\mu$ g of DNA) (Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C for 10 minutes. The Enzo BioArray Terminal Labeling Kit with Biotin-ddUTP (Affymetrix, Inc., Santa Clara, CA) was used to label the 3' termini of the fragmented cDNA by terminal deoxynucleotide transferase. A gel-shift assay with NeutrAvidin (Pierce Biothehnology, Inc. Rockford, IL) was performed to estimate the labeling efficiency based on the instructions from Affymetrix.

### **Microarray hybridization, washing and scanning.**

Hybridization solution mix was made with the labeled cDNA according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA), and the mixture was hybridized to the *E. coli* Antisense Genome Arrays at 45°C for 16 hours. GeneChip Fluidics Station (Affymetrix, Inc., Santa Clara, CA) was used to automate the washing

and staining of the arrays. Sequentially, the arrays were stained with ImmunoPure streptavidin (Pierce Biotechnology, Inc., Rockford, IL), Anti-streptavidin goat antibody (Vector Laboratories, Inc., Burlingame, CA), and R-Phycoerythrin streptavidin (Molecular probes, Inc., Eugene, OR). Finally, the probe arrays were scanned using the Affymetrix GeneArray scanner.

### **Data analysis.**

Microarray Data was analyzed with the Affymetrix Microarray Suite Software 5.1 (Affymetrix, Inc., Santa Clara, CA) and the four-comparison survival method (28). The fluorescence of each array was normalized by scaling total chip fluorescence intensities to a common value of 500. For each growth condition, two independent experimental cell cultures (wild type) were compared with two independent control groups ( $\Delta luxS$  mutant) and four comparisons were made. The fold change for each gene was calculated as division of signal intensity for the wild type by the signal intensity for the  $\Delta luxS$  mutant. The reported value for the fold change is the average of the four comparisons. Genes with consistent increase or decrease in all comparisons were determined and used for the analysis. However, the induced genes with absent calls of the array signal in the experimental groups, and the repressed genes with absent calls of the array signal in the control groups are eliminated for the analysis. The determination of gene functional categories was based on the *E. coli* K12-MG1655 role category database from TIGR ([http://www.tigr.org/tigr-scripts/CMR2/gene\\_table.spl?db=ntec01](http://www.tigr.org/tigr-scripts/CMR2/gene_table.spl?db=ntec01)).

### **Motility assays.**

Media used for motility swimming assay is tryptone broth (10g/liter tryptone (Difco), 5g/liter NaCl) that contained 0.3% Difco agar. Cultures of *E. coli* were grown overnight in liquid tryptone broth, diluted 100-fold into the same fresh medium and grown to mid-exponential phase. Swim plates were inoculated at the center with 5  $\mu$ l of cell culture, and incubated at 30°C in humid environment for 11h.

### **Biofilm assays.**

The biofilm assays were performed as described previously (146) with modifications. *E. coli* cells were grown in polypropylene tubes in LB with or without 0.8% glucose at room temperature without shaking for 24 h, and subcultured at a 1:100 dilution into LB or LB plus 0.8% glucose. These cultures were grown for 48 h at room temperature without shaking, then rinsed with distilled water and stained with 1.0% crystal violet. After 20 min, the tubes were rinsed. The biofilm associated crystal violet was solubilized by DMSO, and the OD<sub>570</sub> of suspension was measured.

## Results and Discussion

### **Deletion of the *E. coli* W3110 *luxS* does not affect growth, motility, and biofilm formation.**

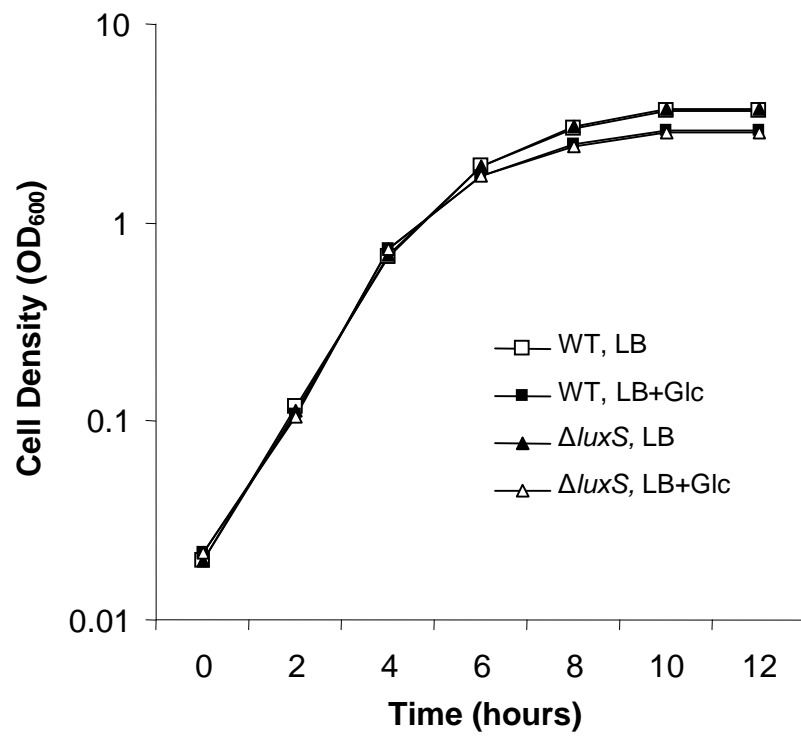
Previous reports showed that deletion of *luxS* resulted in increased growth rate, and reduced motility in enterohemorrhagic *E. coli* (EHEC) (173). Similarly, a *luxS* mutant of *Campylobacter jejuni* had reduced motility (50). In addition, it was shown that the *luxS* mutant of *S. e. typhimurium* has a defective ability to form biofilms (147).

We investigated whether the mutation of *luxS* of *E. coli* K12 strain W3110 has similar effects on these phenotypes. Figure 3-1 shows that the  $\Delta luxS$  mutant grows as well as its isogenic parent when the cells are grown in LB or in LB plus glucose. We further tested the motility of the  $\Delta luxS$  mutant. On the 0.3% agar swim plate of tryptone broth, there was no apparent difference of the swimming, measured by the ability to form halos, between the mutant and the wild type (Figure 3-2). Finally, biofilm formation was not affected by the mutation of the *luxS* gene, although we found the presence of glucose inhibited biofilm formation in both the *luxS* mutant and the wild type (Figure 3-3).

In summary, we have shown that deletion of *luxS* did not affect cell growth, swimming motility, and biofilm formation of W3110 under the investigated conditions. However, these experiments did not rule out the possibility that these phenotypes might be influenced by the conditions we have not tested. For example, Lyon *et al.* (101) reported that the  $\Delta luxS$  mutant of *Streptococcus pyogenes* has a media-dependent growth defect with reduced growth rate in Todd-Hewitt yeast extract media, however, there were similar doubling times for the wild type and mutant in the C-media (a peptide-rich and carbohydrate-poor media that is used routinely to support high level expression of the *S. pyogenes* cysteine protease.) (101). Belhert *et al.* reported that the  $\Delta luxS$  mutant of *Streptococcus gordonii* exhibits an altered biofilm structure when grown in flow cells with saliva as the growth medium (17). We are investigating additional conditions under which AI-2/*luxS* may be important for the quorum sensing.

**Figure 3-1 Growth of wild type and  $\Delta luxS$  mutant of the *E. coli* W3110**

Overnight cultures of *E. coli* W3110 and the  $\Delta luxS$  mutant were diluted in LB or LB plus 0.8% glucose to OD<sub>600</sub> about 0.02. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub>.



**Figure 3-2 Swimming motility is not affected by *luxS* deletion**

Media used for motility swimming assay is tryptone broth (10g/liter tryptone (Difco), 5g/liter NaCl) that contained 0.3% Difco agar. Cultures of *E. coli* W3110 and the  $\Delta luxS$  mutant were grown overnight in liquid tryptone broth, diluted 100-fold into the same fresh medium and grown to mid-exponential phase. Swim plates were inoculated at the center with 5  $\mu$ l of cell culture, and incubated at 30°C in humid environment for 11h.



*$\Delta luxS$*

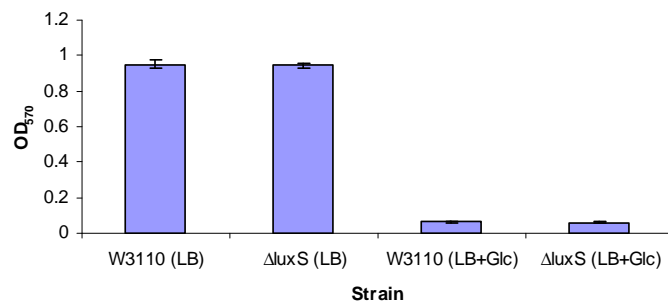
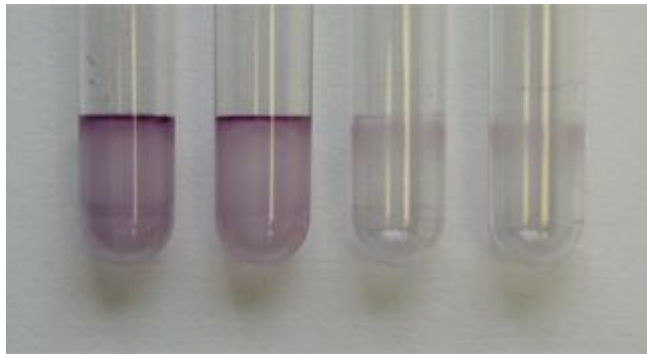


**WT**



### **Figure 3-3 Biofilm formation is not affected by *luxS* deletion**

The W3110 wild type and  $\Delta luxS$  mutant were grown in polypropylene tubes in LB with or without 0.8% glucose at room temperature without shaking for 24 h, and subcultured at a 1:100 dilution into LB or LB plus 0.8% glucose. These cultures were grown for 48 h at room temperature without shaking, then rinsed with distilled water and stained with 1.0% crystal violet. After 20 min, the tubes were rinsed. The biofilm associated crystal violet was solubilized by DMSO, and the OD<sub>570</sub> of suspension was measured.



### **Genomic transcriptional analyses of the *luxS* deletion.**

Using DNA microarrays, we compared genomic transcript levels of wild type and  $\Delta luxS$  mutant of the *E. coli* W3110 under two different growth conditions. One condition was in LB plus 0.8% glucose when the cells reach OD<sub>600</sub> of 1.0 with shaking at 30°C. This stage of culture has very high extracellular AI-2 activity in the wild type, but the  $\Delta luxS$  mutant does not have any AI-2 activity(193) . To calculate the number of genes differentially expressed, we initially used 1.5-fold as cutoff for the fold change. Although a 2-fold cutoff is commonly used for analysis of microarray data (e.g., (77)), the previous studies have indicated that a 1.5-fold difference in transcript level can be biologically significant (20, 76, 168). Table 3-1 shows that there were 45 and 27 genes that were up and down regulated at least by 1.5-fold (wt /  $\Delta luxS$ ), respectively, by the presence of the *luxS* gene. If the cutoff for the fold change was elevated to +/- 1.8 (Table 3-3), the numbers of induced and repressed genes were reduced to 16 and 8 respectively, indicating expression of most of the genes (4346 genes assayed in the chips) were not affected markedly by the *luxS* deletion. In fact, it is remarkable that expression of most of the genes was so similar in the *luxS*<sup>+</sup>/*luxS*<sup>-</sup> cells of W3110. Surprisingly, Sperandio *et al.* (173) reported that 404 genes were regulated by *luxS* at least 5-fold in the EHEC strain. The genes identified by our experiment are different from those identified in their work, in which the flagella and motility genes were highly induced (173). In both studies, the cells were harvested at OD<sub>600</sub> of 1.0, but the EHEC cells were grown in DMEM at 37°C.

The comparison between genomic sequences of K12 MG1655 and EHEC revealed that there is 1.34 Mb of EHEC genomic DNA not found in MG1655, and there

is 0.53 Mb of MG1655 genomic DNA not found in EHEC (173). One phenotypic difference between the *luxS* mutants of the W3110 and EHEC is that the *luxS* mutant of EHEC grows much faster than its parent strain (173), while the growth of the W3110 *luxS* mutant and its parent strain are indistinguishable under the investigated conditions. Although we do not understand the reason for the *luxS*-mediated growth stimulation in EHEC, we suspect that the faster growth rate in the EHEC *luxS* mutant may have distorted the effects reported for the *luxS* mutation. A second phenotypic difference between the two strains is the EHEC *luxS* mutant has reduced motility relative to the wild type (173), while the W3110 *luxS* mutant does not. The differences in the genetic background and the growth conditions are probably reasons that both account for identification of the different *luxS* controlled genes in W3110 and EHEC.

In Chapter 2, we showed that the AI-2/*luxS* controlled *lsrACDBFG* operon of *E. coli* is induced in LB when the cells enter the early stationary phase, and the CRP is required for the activation of the *lsr* operon (193). This observation prompts us to ask whether there exist growth conditions (e.g., in stationary phase) more appropriate for finding genes regulated by *luxS*, and AI-2 may have different roles in gene regulation during this phase. We performed another set of DNA array experiments with the RNA isolated from cells harvested at OD<sub>600</sub> of 2.4 (stationary phase) when they were grown in LB without glucose addition. Table 3-1 showed that there are 28 and 112 genes that are induced and repressed at least 1.5-fold by the *luxS* respectively. Most of the controlled genes are different from those observed in first condition and the total number of the regulated genes under this condition is almost double of the number of the affected genes found in first condition. If the +/- 1.8 cutoff for the fold change is

Table 3-1 Effects of *luxS* deletion on gene expression patterns under two growth conditions

Functional categories	Total	OD 1.0 in LB + Glc			OD 2.4 in LB		
		higher in WT <sup>a</sup>	higher in $\Delta luxS$	% <sup>b</sup>	higher in WT	higher in $\Delta luxS$	%
Whole genome	4346	45	27	1.7	28	112	3.2
Amino acid biosynthesis	115	2	1	2.6	1	3	3.5
Biosynthesis of cofactors, prosthetic groups and carriers	120	0	0	0.0	0	1	0.8
Cell envelope	176	0	1	0.6	1	3	2.3
Cellular processes	189	0	1	0.5	2	4	3.2
Central intermediary metabolism	73	2	0	2.7	0	5	6.8
DNA metabolism	107	1	0	0.9	0	1	0.9
Energy metabolism	399	2	1	0.8	4	11	3.8
Fatty acid and phospholipid metabolism	70	1	1	2.9	0	1	1.4
Mobile and extrachromosomal element	39	0	0	0.0	0	1	2.6
Protein fate	117	0	0	0.0	0	8	6.8
Protein synthesis	123	0	1	0.8	0	0	0.0
Purines, Pyrimidines, nucleosides, nucleotides	82	1	0	1.2	0	0	0.0
Regulatory functions	177	1	0	0.6	2	5	4.0
Transcription	45	0	0	0.0	0	1	2.2
Transport and binding proteins	322	3	4	2.2	5	4	2.8
Hypothetical, unclassified, unknown	2244	34	18	2.3	14	71	3.8

<sup>a</sup> only the genes with not less than +/-1.5 fold changes are calculated. Several genes with multiple functions appear in different groups, but are counted once in total item.

<sup>b</sup> Percentage of differentially expressed genes in the functional group.

used (Table 3-2), the numbers of induced and repressed genes by *luxS* is reduced to 19 and 48 respectively. Importantly, the only genes to have been shown affected specifically by *luxS*/AI-2 in *E. coli*, the *lsr* operon (chapter 2), were not altered at low cell density, but instead were among the most dramatically influenced genes found at high cell density. These results indicated that the AI-2/*luxS* controlled gene expression varies from one condition to another, suggesting careful experimental designs are required to identify the AI-2/*luxS* controlled genes in various bacteria.

#### **Genes controlled by *luxS* in the absence of glucose at OD 2.4.**

There are more genes down-regulated than those up-regulated by *luxS* when cells were grown to OD 2.4 in the absence of glucose (48 to 19 genes with fold change cutoff of +/-1.8) (Table 3-2). The most induced genes by *luxS* belong to the *lsrACDBFG* operon (Figure 3-4). This result is consistent with our previous *lsr-lacZ* fusion study performed in *E. coli*, which showed the *lsr* operon is differentially expressed between the wild type and the *luxS* mutant mainly in stationary phase (193). The relatively lower fold change of the *lsrC* compared to the other genes in the *lsr* operon probably resulted from the interfering effects of certain cDNA fragments, which masked the hybridization of *lsrC* to its probes. Surprisingly, transcription of *lsrR*, *lsrK*, *tam*, and *yneE*, which flank the *lsrACDBFG* operon, are also significantly induced by *luxS* (Figure 3-4). Expression of *lsrR* and *lsrK*, which encode the transcriptional regulator for the *lsr* operon and the AI-2 kinase respectively, is increased by 2.2 and 5.5-fold in the wild type cells. It seems reasonable that the *lsrK* expression is induced by *luxS* because the cells need more LsrK to phosphorylate newly uptaken AI-2. It is interesting, however,

### **Figure 3-4 Transcriptional organization of the *lsr* operon region**

Genes of the *E. coli* K12 strain are represented by the broad arrows. Direction of the arrows indicates the direction of the transcription. The promoter regions for the genes are represented by the grey boxes. The number above the genes indicates the fold change (WT /  $\Delta luxS$ ). Tam: trans-aconitate 2-methyltransferase (SAM dependent); YneE: protein with unknown function.

Fold Change  
(WT /  $\Delta luxS$ )

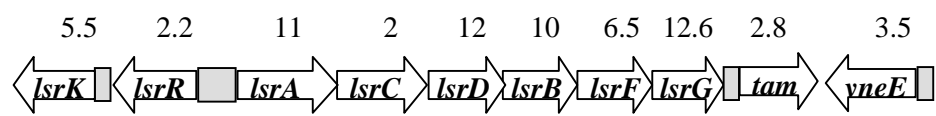




Table 3-2 Genes regulated by *luxS* at OD 2.4 in LB

Functional group	B number	Gene	Gene product	Fold change <sup>a</sup> (WT / $\Delta luxS$ )
Amino acids	b3829	<i>metE</i>	tetrahydropteroyltriL-glutamate methyltransferase	9.74
Cell envelope	b2060	<i>wzc</i>	putative tyrosine-protein kinase	-2.49
Central intermediary metabolism	b0339	<i>cynT</i>	carbonic anhydrase	-3.53
	b2993	<i>hybD</i>	probable processing element for hydrogenase-2	-2.59
	b3945	<i>gldA</i>	glycerol dehydrogenase, (NAD)	-1.95
Cellular processes	b1558	<i>cspF</i>	cold shock protein	3.21
Energy metabolism	b0339	<i>cynT</i>	carbonic anhydrase	-3.53
	b0974	<i>hyaC</i>	probable Ni/Fe-hydrogenase 1 b-type cytochrome subunit	-1.83
	b1511	<i>lsrK</i>	putative kinase	5.52
	b2797	<i>sdaB</i>	L-serine dehydratase (deaminase), L-SD2	-2.57
	b4196	<i>sgaH</i>	probable hexulose-6-phosphate synthase	-1.87
	b4395	<i>gpmB</i>	phosphoglyceromutase 2	1.99
Fatty acid and phospholipid metabolism	b2919	<i>ygfG</i>	putative enzyme	-2.4
Protein fate	b0630	<i>lipB</i>	protein of lipoate biosynthesis	-1.87
	b0823	<i>ybiW</i>	putative formate acetyltransferase	-2
	b1127	<i>pepT</i>	putative peptidase T	-1.85
	b2968	<i>yghD</i>	putative secretion pathway protein	-2.33
	b3103	<i>yhaH</i>	putative cytochrome	-2.07
	Regulatory functions	b1022	<i>ycdQ</i>	orf, hypothetical protein
b1512		<i>lsrR</i>	putative transcriptional regulator, SorC family	2.22
b3906		<i>rhaR</i>	positive regulator for <i>rhaRS</i> operon	-2.47
Transport and binding proteins	b1513	<i>lsrA</i>	putative ATP-binding component of a transport system	11.16
	b1514	<i>lsrC</i>	putative transport system permease protein	2
	b1515	<i>lsrD</i>	putative transport system permease protein	11.9
	b1516	<i>lsrB</i>	putative LACI-type transcriptional regulator	10.14
	b3683	<i>glvC</i>	PTS system, arbutin-like IIC component	-2.2
Hypothetical, unclassified,	b0042	<i>fixB</i>	probable flavoprotein subunit, carnitine metabolism	-2.7

unknown	b0076	<i>leuO</i>	probable transcriptional activator for <i>leuABCD</i> operon	-2.14
			putative amino acid/amine transport protein	
	b0260	<i>ykfD</i>		-2.38
	b0579	<i>ybdF</i>	orf, hypothetical protein	-2.34
	b0621	<i>dcuC</i>	transport of dicarboxylates	-2.09
	b0648	<i>ybeU</i>	putative tRNA ligase	-1.98
	b0790	<i>ybhP</i>	orf, hypothetical protein	-2.51
	b1001	<i>yccE</i>	orf, hypothetical protein	-2.87
	b1010	<i>ycdK</i>	orf, hypothetical protein	-2.53
	b1012	<i>ycdM</i>	orf, hypothetical protein	-3.24
	b1019	<i>ycdB</i>	orf, hypothetical protein	-1.85
	b1112	<i>ycfR</i>	orf, hypothetical protein	-2
			putative binding-protein dependent transport protein	
	b1311	<i>ycjO</i>		-3.06
	b1407	<i>ydbD</i>	orf, hypothetical protein	-2.11
	b1437	<i>b1437</i>	orf, hypothetical protein	-1.88
	b1517	<i>lsrF</i>	putative aldolase	6.54
	b1518	<i>lsrG</i>	orf, hypothetical protein	12.64
	b1519	<i>tam</i>	trans-aconitate 2-methyltransferase	2.82
	b1520	<i>yneE</i>	orf, hypothetical protein	3.49
	b1550	<i>gnsB</i>	orf, hypothetical protein, GnsB protein	-1.86
	b1571	<i>ydfA</i>	orf, hypothetical protein	-6.85
	b1680	<i>sufS</i>	selenocysteine lyase, PLP-dependent	-1.98
	b1720	<i>b1720</i>	orf, hypothetical protein	-2.52
	b2087	<i>gatR</i>	split galactitol utilization operon repressor, interrupted	-1.81
	b2236	<i>yfaE</i>	orf, hypothetical protein	3.19
	b2406	<i>xapB</i>	xanthosine permease	-1.93
	b2549	<i>yphG</i>	orf, hypothetical protein	-2.97
	b2687	<i>luxS</i>	autoinducer 2 synthase	8.19
	b2723	<i>hycC</i>	membrane-spanning protein of hydrogenase 3 (part of FHL complex)	-2.14
	b3028	<i>mdaB</i>	NADPH-quinone reductase (modulator of drug activity B)	-2.22
	b3046	<i>yqiG</i>	putative membrane protein	-2.39
			putative galactosamine-6-phosphate isomerase	
	b3141	<i>agal</i>		-3.15
	b3220	<i>yhcG</i>	orf, hypothetical protein	-2.35
	b3796	<i>argX</i>	Arginine tRNA3	1.97
	b3852	<i>ileT</i>	Isoleucine tRNA1, triplicate	1.91
	b3939	<i>metB</i>	cystathionine gamma-synthase	-1.93
			putative regulator of acetyl CoA synthetase	
	b4017	<i>arpA</i>		1.89
	b4186	<i>yjfC</i>	putative synthetase/amidase	-1.92
	b4288	<i>fecD</i>	citrate-dependent iron transport, membrane-bound protein	-2.03
	b4308	<i>yjhR</i>	putative frameshift suppressor	2.46
	b4310	<i>yjhT</i>	orf, hypothetical protein	-2.05

<sup>a</sup> only the genes with not less than +/-1.8 fold changes are shown. Genes with multiple functions may appear in different groups.

that the *lsrR* expression is also induced in the wild type cells. This was unexpected because the produced LsrR can not repress the *lsr* operon (or *lsr* regulon) in the presence of the phospho-AI-2. However, the increased level of the LsrR may provide a mechanism for quickly shutting down expression of the *lsr* regulon (perhaps many genes) when the phospho-AI-2 is degraded by LsrF and LsrG, and AI-2 is no longer taken up into the cells.

Expression of *tam* and *yneE* was also increased by *luxS* (2.8 and 3.5-fold respectively). The *tam* gene encodes an *S*-adenosyl-L-methionine-dependent methyltransferase, which catalyzes the methyl esterification of *trans*-aconitate(22) . The *trans*-aconitate appears to be formed spontaneously from the citric acid cycle intermediate *cis*-aconitate (22). The benefit of methylation of the *trans*-aconitate to the *E. coli* cells is not clear, but it is linked to the AI-2 biosynthesis pathway by the reaction of the SAM dependent methyl transfer. This reaction appears to be inhibited in the *luxS* mutant due to the much lower level of the Tam expression. Cai *et al.* showed *tam* is expressed in early stationary phase under the RpoS control (22). The other *luxS* - dependent gene, *yneE*, is within close proximity to the *lsr* operon, and encodes a protein with unknown function. This gene is transcribed in the opposite direction as the *tam* gene and the *lsr* operon. It is not clear whether these four genes belong to the same regulon as the *lsrACDBFG* operon, which are regulated by the LsrR repressor and the phospho-AI-2 inducer, but this would seem feasible.

### **Methionine metabolism.**

The *metE*, which encodes tetrahydropteroyltriglutamate methyltransferase (or methionine synthase), has much lower expression in the  $\Delta luxS$  mutant than in the wild

type strain (9.7-fold down). This enzyme catalyses the last step of methionine synthesis in a cobalamin-independent way from the homocysteine, which can be recycled back to methionine after the LuxS-catalyzed reaction with *S*-ribosylhomocysteine (Figure 3-5). It was previously reported that homocysteine is required for the full induction of *metE* expression (23, 108, 189). The reason that the  $\Delta luxS$  mutant has lower transcription of *metE* is probably due to lack of homocysteine. The *metE* gene was also identified before by Taga *et al.* in their search for *luxS* controlled genes in *S. e. typhimurium* (186). In order to meet the normal requirement of the cells for methionine and SAM, it is necessary for the  $\Delta luxS$  cells to increase the levels of the homocysteine. This is probably the reason that expression of *metA*, *metB*, and *metC*, which are involved in homocysteine biosynthesis from  $L$ -homoserine (rather than from the AI-2 biosynthesis pathway), is increased in the  $\Delta luxS$  mutant (1.43, 1.93, and 1.2-fold respectively) (Figure 3-5). The effects of the *luxS* deletion on the expression of genes involved in methionine biosynthesis are consistent with a metabolic disturbance caused by of the *luxS* deletion rather than the AI-2 signaling.

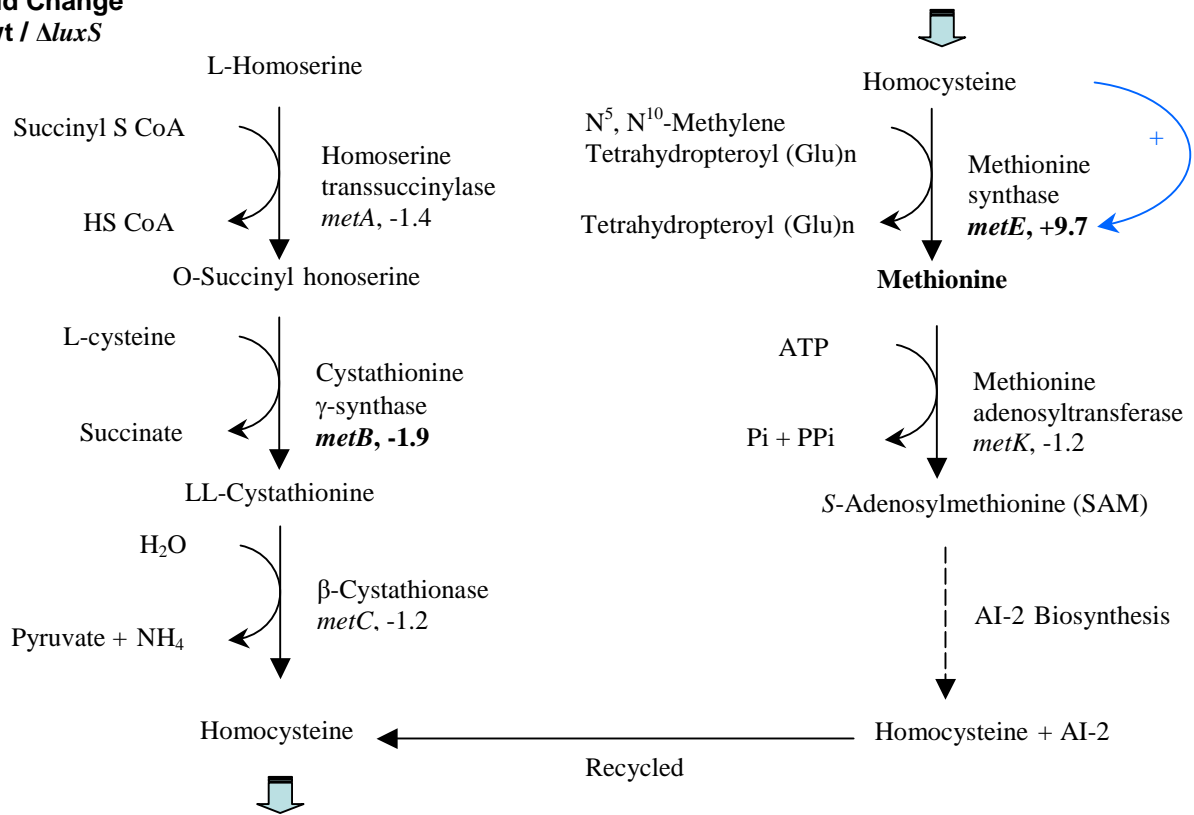
### **Virulence.**

Our results also showed that expression of *arpA* (or *yjiC*), which encodes a putative regulator of the acetyl CoA synthase, is repressed in  $\Delta luxS$  mutant (1.89-fold lower). In the *E. coli* chromosome, this gene is located between *aceBAK* operon and the *iclR* gene, which encodes a transcriptional repressor for *aceBAK* operon. Analysis of the amino acid sequence of the ArpA revealed seven tandem repeats similar to ankyrin motifs in eukaryotic proteins (43, 129). Expression of the ankyrin repeats from human endoribonuclease RNase L, an enzyme induced by interferons, inhibits growth of *E. coli*

**Figure 3-5 Deletion of *luxS* affects methionine metabolism.**

See text for details. Modified from (68).

**Fold Change  
wt /  $\Delta luxS$**



grown on acetate as a sole carbon source, correlating a severely reduced induction of the enzymes (AceA and AceB) involved in the glyoxylate bypass (43). This result suggested a possible function of the ArpA in regulation of the acetate metabolism. Also, a recent report suggested a potential role of the *arpA* in the control of virulence in the pathogenic *E. coli* (32). The *arpA* was found to be absent from all group B2 and most group D (highly virulent) of the *E. coli* neonatal meningitis (ECNM) strains, but present in all group A and B1 (less pathogenic) of the ECNM strains and the nonpathogenic *E. coli* K12 strains (32). This suggests that *arpA* may be incompatible with full expression of the virulence of extraintestinal pathogenic *E. coli* (32). It is interesting that deletion of the *luxS* in *E. coli* K12 strain W3110 represses expression of the *arpA* gene. Whether reduced expression of the *arpA* gene in pathogenic or nonpathogenic *E. coli* strains stimulates virulence expression in those strains needs further investigations.

Expression of the *mdaB* gene is also increased in the *luxS* mutant (2.2-fold). MdaB was first identified as a modulator of drug activity (26), and later was found to be the NADPH-quinone reductase that catalyzes reduction of quinone to quinols (74). The reduced ubiquinone (ubiquinol) is an important antioxidant in *E. coli* cells (169). The resistance to oxidative stress is known to be one of the key capabilities of many pathogenic bacteria to survive the oxidative environment created by the host (178, 192). Wang *et al.* reported that the *mdaB* mutant of *Helicobacter pylori* is much more sensitive to oxidative stress and less successful in colonization of the host stomach (192). Actually, Wen *et al.* observed that the *luxS* mutant of the pathogenic bacterium, *Streptococcus mutans*, has an increased survival rate in the presence of 58.8 mM hydrogen peroxide, although it showed the increased acid sensitivity and reduced

biofilm formation (200). The induced *mdaB* expression in the  $\Delta luxS$  mutant may suggest a defensive response of cells to a more oxidative intracellular condition or a preparation for living in a more oxidative environment.

### **Carbohydrate utilization.**

Deletion of the *luxS* gene results in induction of several genes involved in utilization of various carbohydrates. The *rhaBAD* operon, which encodes enzymes responsible for utilization of L-rhamnose, and its regulatory gene *rhaR*, all have increased expression in the *luxS* mutant (1.44, 1.96, 1.65, and 2.47-fold respectively). Expression of *glvCBG* operon, which encodes putative proteins involved in utilization of arbutin, is also induced by 2.2, 1.68, and 1.78-fold respectively. In addition, the *luxS* mutant has higher expression of the *dcuC* and the *xapB*, which encode proteins involved in transport of dicarboxylates and xanthosine (2.09 and 1.93-fold respectively). It is not clear why the *luxS* deletion increases expression of these carbohydrate utilization genes. The *luxS* mutant appears to be more in a carbon limitation compared to the wild type under the investigated conditions.

### **Genes controlled by *luxS* in the presence of glucose at OD 1.0.**

Table 3-3 lists the specific genes that were affected by the deletion of *luxS* when the cells were grown to OD<sub>600</sub> of 1.0 in LB plus 0.8% glucose. The functions for most of them are not clear. As shown in the condition of OD 2.4 in LB, expression of *metE* is again decreased in the  $\Delta luxS$  mutant, but with much less repression compared to the wild type at lower OD (1.8-fold) (Figure 3-3). This difference is probably due to more increased production of the homocysteine, which is caused by higher levels of

metabolic flux in the presence of glucose (194). Interestingly, expression of *metT* and *metU*, which encode methionine tRNA<sup>Met</sup>, was elevated by *luxS* (1.76 and 1.53-fold respectively), while expression of *metZ*, *metW*, and *metV*, which encode initiator methionine tRNA<sup>Met</sup>, does not change between the wild type and the *luxS* mutant. In addition, the expression of b0667 and b0771, which encode two putative RNAs (not proteins) and are close to *metT* and *metU* in chromosomal location, is also increased (1.93 and 1.84-fold respectively) by *luxS*. It is not clear how the production of methionine is correlated to the expression of methionine tRNA<sup>Met</sup> and certain putative RNAs.

Several iron transport genes were shown to be affected by deletion of *luxS*. Namely, the expression of *ybiL* and *fhuE*, which encode outer membrane receptors for ferric iron uptake, and *fhuF*, which encodes a ferric hydroxamate transport protein, is induced by *luxS* (1.97, 1.52, and 1.82-fold respectively). The iron receptors are usually the gated pores involved in ferrisiderophore uptake, which are induced by iron limitation. When the ferrisiderophore complexes are transported into the cells, the iron is reduced by the NADH (or NADPH) oxidoreductase and released into the cytoplasm (46). Our results show that the wild type cells have increased expression of *ydiT* and *hcr*, which encode a putative ferredoxin and a NADH oxidoreductase for HCP (hybrid cluster protein). The induction of the iron transport genes by *luxS* suggests a potential signaling role for AI-2 in control of the iron acquisition. Consistent with this role, the AI-2 containing conditioned medium prepared from *E. coli* was shown to stimulate expression of the *afuA* (encoding a periplasmic iron transport protein) in this *Actinobacillus actinomycetemcomitans* (54). The AI-2/*luxS* mediated modulation of



Table 3-3 Genes regulated by *luxS* at OD 1.0 in LB plus glucose

Functional group	B number	Gene	Gene product/function	Fold change <sup>a</sup> (WT / $\Delta luxS$ )
Amino acids	b3829	<i>metE</i>	tetrahydropteroyltri-glutamate methyltransferase	1.81
Energy metabolism	b1700	<i>ydiT</i>	putative ferredoxin	2.58
	b3580	<i>lyxK</i>	L-xylulose kinase, cryptic	2.32
Transport and binding proteins	b0805	<i>ybiL</i>	putative outer membrane receptor for iron transport	1.97
	b2715	<i>ascF</i>	PTS system enzyme II ABC ( <i>asc</i> ), cryptic, transports specific beta-glucosides	-2.02
	b3110	<i>yhaO</i>	putative transport system permease protein	-1.98
Hypothetical, unclassified, unknown	b0667		putative RNA	1.93
	b0671		putative RNA	1.84
	b0872	<i>hcr</i>	putative enzyme, NADH oxidoreductase for HCP	1.8
	b1461	<i>ydcE</i>	orf, hypothetical protein	-1.92
	b1482	<i>osmC</i>	osmotically inducible protein	-1.91
	b1561	<i>rem</i>	orf, hypothetical protein	2.67
	b1567	<i>ydfW</i>	orf, hypothetical protein	2.25
	b1834	<i>yebT</i>	orf, hypothetical protein, putative membrane protein	1.92
	b2597	<i>yfiA</i>	putative YhbH sigma 54 modulator	-1.83
	b2687	<i>luxS</i>	autoinducer 2 synthase	57.53
	b3004		orf, hypothetical protein	2.19
	b3108	<i>yhaM</i>	orf, hypothetical protein	-2.14
	b3109	<i>yhaN</i>	orf, hypothetical protein	-2.87
	b3267	<i>yhdV</i>	orf, hypothetical protein	-1.92
	b3711	<i>yidZ</i>	putative transcriptional regulator LYSR-type	2.42
	b4002	<i>zraP</i>	orf, hypothetical protein, Zn-binding periplasmic protein	2.04
	b4186	<i>yjfC</i>	putative synthetase/amidase	2.51
	b4367	<i>fhuF</i>	orf, hypothetical protein, ferric hydroxamate transport protein	1.82

<sup>a</sup> only the genes with not less than +/-1.8 fold changes are shown. Genes with multiple functions may appear in different groups.

iron acquisition was also reported in *Porphyromonas gingivalis* (31), and *V. harveyi* (96). Although confirmed in several bacterial species, the exact mechanism for the control of iron uptake by AI-2 needs further investigation.

### Conclusion

Using DNA microarrays, we have investigated the growth dependent gene regulation by AI-2/*luxS* and have identified *luxS* controlled genes in *E. coli* K12 W3110 strain. Some of these genes, such as *metE* and the *lsrACDBFG* operon, were identified previously in *S. e. typhimurium* as *luxS*-regulated using a different method (186). The identified *luxS* controlled genes in W3110 are different from those identified in the EHEC strain (173), and there are significantly fewer genes regulated by *luxS* in W3110. In addition, our results have demonstrated that more genes are significantly affected by *luxS* deletion when cells were obtained at higher cell density in the absence of glucose than when they were cultivated with glucose and sampled at a low cell density. These observations suggest that *luxS* -mediated gene regulation and resulting phenotypic changes may be observed only in specific conditions or genetic background. It is noteworthy that a 2-D gel analysis of proteins from the wild type and the  $\Delta luxS$  mutant of *Helicobacter pylori* did not reveal apparent difference in protein expression (82). This has been attributed to the modest changes in protein expression and sensitivity limitation of 2-D gel analysis (82). Another possibility may be that the selected growth conditions did not cause obvious differences between the *luxS* mutant and its parent strain.

It was shown in this study that deletion of *luxS* affects genes associated with diverse cell activities in *E. coli* K12, such as AI-2 transport, biosynthesis of methionine, transfer of methyl group, iron uptake, resistance to oxidative stress, utilization of different carbon sources, and virulence. Many of the differentially expressed proteins have unknown functions. Although the microarray experiments were duplicated and analyzed by the four-comparison survival method (28), additional confirmation (eg. RT-PCR, Northern blot, or *lacZ* fusion) may be needed. Expression of the *lsr* operon and *lsrR* revealed by microarray experiments, has been confirmed by the *lacZ* fusions of the *lsr* and *lsrR* promoter regions, respectively (chapter 2 and the work by J. Li and L. Wang (unpublished data)). We are undertaking further investigation to study genes and cell activities likely regulated by AI-2. Currently, except the *lsrACDBFG* operon, we can not tell what other genes are actually responsive to the AI-2/phospho-AI-2. The *lsrK*, *lsrR*, *tam*, and *yneE* genes are good candidates based on their high induction by *luxS* and close location to the *lsr* operon in chromosome. Induction of LsrR expression by *luxS* suggests more LsrR-mediated cellular functions may exist. There may be a regulon controlled by the LsrR/phospho-AI-2. We are testing this possibility right now.

Some of the genes identified by this work may respond directly to AI-2, while regulation of the others may reflect indirect response to the metabolic effects of the *luxS* deletion, such as accumulation of the *S*-ribosylhomocysteine and reduced level of homocysteine. As a matter of fact, most of the highly induced genes (such as *lsr* operon, *lsrR*, *lsrK*, *tam*, and *metK*) are related to AI-2 production and transport, while the genes involved in other activities are induced to a less degree. This data are consistent with the function of AI-2 as an autoinducer, but do not support its role as a signaling molecule as

used by *V. harveyi* in stimulation of luminescence. In chapter 2, we have shown that the presence of glucose highly inhibits expression of the *lsr* operon through control of cAMP-CRP. That *lsr* operon is induced only in the absence of glucose suggests AI-2 is used as a carbon source. Although Taga *et al* reported that *S. e. typhimurium* could not grow on AI-2 as the sole carbon source (186), additional conditions may be needed for AI-2 utilization as a carbon source, as suggested by Winzer *et al.* (202). It is possible that AI-2 is used as a true signal only in specific bacterial species or in specific conditions.

## Chapter 4 Genomic Expression Profiling of *Escherichia coli* Grown on Glucose

### Abstract

Glucose has long been known to cause “catabolite repression” in *Escherichia coli*. In this study, we investigated genomic expression profiling of *Escherichia coli* in response to glucose using a DNA microarray. It is shown that, of the total 4346 genes in *E. coli* genome, 464 (10.7%) and 237 (5.5%) genes are up and down regulated at least 2 fold respectively by the presence of glucose, indicating the global effects of glucose on *E. coli* gene expression. In the overall pattern, the cells increase expression of genes involved in biosynthesis of amino acids, nucleotides, cofactors, and autoinducer-2 (AI-2), Pi assimilation, and glycolysis, but reduce expression of genes involved in TCA cycle and transport of the alternative carbon source. With this strategy, the cells appears to prepare well for depletion of the glucose with ‘storage’ of a large pool of building block for production of protein, DNA and RNA, and are more adaptive to the quickly varied environment.

### Introduction

Bacterial cells are capable of adjusting their physiological activities to survive frequently changed environmental conditions. Regulation of carbon utilization has been intensely studied in *Escherichia coli* (161). It has long been known that the presence of glucose in the growth medium can inhibit induction of many genes and operons controlling enzymes involved in carbohydrate catabolism. This phenomenon is referred to as “catabolite repression” or the glucose effect. The principle pathway for glucose

input involves the internalized phosphorylation of sugars. The phosphotransferase system (PTS) is believed to be the principal mechanism accounting for the glucose effect in *E. coli* (145, 160, 161). The proposed model is: glucose uptake by the PTS results in deactivation of adenylate cyclase and inhibition of many permeases and catabolic enzymes. This, in turn, causes reduction of both cytoplasmic cAMP and inducer levels, which coordinately control catabolic gene expression through CRP and several other regulatory proteins. In addition to the well known cAMP/CRP complex, there exist additional regulatory mechanisms that contribute to the glucose effect: (1) FruR, the repressor of the fructose regulon, is involved in controlling expression of many key enzymes of the central carbohydrate metabolic pathways (161); (2) Sensor kinase-response regulator system (CreC-CreB system) modulates expression of a set of *cre* regulon genes which are involved in carbon utilization (6, 161); (3) Alternative sigma factors,  $\sigma^s$  and  $\sigma^{32}$ , are involved in the activation of many carbon starvation responsive genes (161).

Despite the enhanced understanding of carbon utilization and the mechanisms associated with the glucose effect in *E. coli*, there is no transcript profile of the bacterial response to the limitation of glucose as a principal carbon source. Hence, it is difficult to examine the interactions between various physiological activities, when glucose is present or absent or during oscillation between the two extremes, as is prevalent in bioprocess industries that rely exclusively on bioreactor cultivated *E. coli*. The technology of DNA microarray provides us with a powerful tool to study genome-wide transcriptional changes in parallel. In this work, we investigated the global transcriptional response of *E. coli* cells to the presence of glucose using the Affymetrix

*E. coli* antisense genome arrays, and we have attempted to unravel new connections between the different cell activities.

### Materials and Methods

#### **Bacterial strains and growth conditions.**

*E. coli* K-12 strain W3110 (F<sup>-</sup>, λ<sup>-</sup>, in (*rrnD-rrnE*)) was obtained from Genetic Stock Center (New Haven, Connecticut). Luria-Bertani broth (LB) contains 5 g L<sup>-1</sup> yeast extract (Difco), 10 g L<sup>-1</sup> bacto tryptone (Difco), and 10 g L<sup>-1</sup> NaCl. Cultures of *E. coli* that had been grown overnight in LB or LB plus 0.8% glucose, were diluted to OD<sub>600</sub> about 0.02 in LB or LB plus 0.8% glucose. The cultures were then incubated at 30°C with shaking at 250 rpm in 50 ml flasks. When the cultures reached the OD<sub>600</sub> of 1.0, the cells were harvested for RNA extraction.

#### **Analysis of glucose in growth media.**

Glucose concentrations in fresh growth media or conditioned media (cell-free culture fluids) prepared from *E. coli* were determined by using a Trinder assay (Diagnostic Chemicals, Oxford, CT) according to manufacturer's instructions.

#### **RNA isolation.**

Total RNA was isolated from the cultures using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The procedure for RNA isolation combines the selective RNA binding properties of a silica-gel-based membrane with the speed of microspin technology. RNeasy Protect Bacteria Reagent

(containing tetradecyltrimethylammonium bromide) (Qiagen, Inc., Valencia, CA) was added to the cultures to stabilize RNA before isolation. The RNase-Free DNase Set (Qiagen, Inc., Valencia, CA) was used for on-column DNase digestion to remove residue DNA during RNA purification. Finally, RNA was eluted with nuclease-free water.

### **cDNA synthesis and labeling.**

cDNA was synthesized and labeled according to the manufacturer's suggestions for the Affymetrix *E. coli* Antisense Genome Array (Affymetrix, Inc., Santa Clara, CA). Briefly, in 60  $\mu$ l of reaction mixture, 10  $\mu$ g of total RNA was used for cDNA synthesis by random primers (12.5 ng/ $\mu$ l) and SuperScript II reverse transcriptase (25 U/ $\mu$ l) (both from Invitrogen Corp., Carlsbad, CA). RNA was removed by addition of 20  $\mu$ l of 1N NaOH and incubation at 65°C for 30 minutes. cDNA was purified with Qiaquick PCR Purification Kit (Qiagen, Inc., Valencia, CA), then fragmented using DNase I (0.6 U/ $\mu$ g of DNA) (Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C for 10 minutes. The Enzo BioArray Terminal Labeling Kit with Biotin-ddUTP (Affymetrix, Inc., Santa Clara, CA) was used to label the 3' termini of the fragmented cDNA by terminal deoxynucleotide transferase. A gel-shift assay with NeutrAvidin (Pierce Biothehnology, Inc. Rockford, IL) was performed to estimate the labeling efficiency based on the instructions from Affymetrix.



**Microarray hybridization, washing and scanning.**

Hybridization solution mix was made with the labeled cDNA according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA), and the mixture was hybridized to the *E. coli* Antisense Genome Arrays at 45°C for 16 hours. GeneChip Fluidics Station (Affymetrix, Inc., Santa Clara, CA) was used to automate the washing and staining of the arrays. Sequentially, the arrays were stained with ImmunoPure streptavidin (Pierce Biotechnology, Inc., Rockford, IL), Anti-streptavidin goat antibody (Vector Laboratories, Inc., Burlingame, CA), and R-Phycoerythrin streptavidin (Molecular probes, Inc., Eugene, OR). Finally, the probe arrays were scanned using the Affymetrix GeneArray scanner.

**Data analysis.**

Microarray Data was analyzed with the Affymetrix Microarray Suite Software 5.1 (Affymetrix, Inc., Santa Clara, CA) and the four-comparison survival method (28). The fluorescence of each array was normalized by scaling total chip fluorescence intensities to a common value of 500. Two independent experimental cell cultures (LB plus 0.8% glucose) were compared with two independent control groups (LB) and four comparisons were made. The fold change for each gene was calculated as division of signal intensity for the experiment group by the signal intensity for the control. The reported value for the fold change is the average of the four comparisons. Genes with consistent increase or decrease in all comparisons were determined and used for the analysis. However, the induced genes with absent calls of the array signal in the experimental groups, and the repressed genes with absent calls of the array signal in the control groups are eliminated for the analysis. The determination of gene functional

categories was based on the *E. coli* K12-MG1655 role category database from TIGR ([http://www.tigr.org/tigr-scripts/CMR2/gene\\_table.spl?db=ntec01](http://www.tigr.org/tigr-scripts/CMR2/gene_table.spl?db=ntec01)).

### Results and Discussion

#### **Transcriptional analysis of *E. coli* whole genome.**

The goal of this study was to investigate the global effects of glucose on gene transcription of *E. coli* cells. We also attempted to reveal the regulatory mechanism for AI-2 biosynthesis and uptake. The growth medium was Luria-Bertani broth (LB), which is widely used to study a variety of physiological activities of this micro-organism. This rich medium provides cells with a variety of building blocks such as amino acids, nucleosides, vitamins, and carbon sources, lipids, minerals, etc. to support substantial cell growth in simple laboratory shake flasks. The level of glucose in fresh LB medium (originally from yeast extract) is very low (about 0.26 mM), and it is consumed rapidly during the early growth of the cells. In this study, we compared genomic expression profiles of *E. coli* grown in LB with or without addition of 0.8% (or 44.4 mM) glucose by using Affymetrix DNA microarrays. This addition maintains glucose at levels substantially higher than the Monod constant (166) for several hours of cultivation, so that the growth rate is unaffected by the glucose levels. In Figure 4-1, *E. coli* cells were shown to grow similarly during exponential phase with or without glucose, but presence of glucose caused inhibited cell growth upon entry into the stationary phase probably due to accumulation of acetate. Total RNA was purified from cells grown to the cell density of 1.0 OD<sub>600</sub>. A four-comparison survival method by Chen *et al* (28) was used to identify genes differentially expressed in transcription. Although the previous studies

**Figure 4-1 Effects of glucose on growth of the *E. coli* cells**

Overnight cultures of *E. coli* W3110 were diluted in LB (square) or LB plus 0.8% glucose (triangle) to OD<sub>600</sub> below 0.03. At different time points during cell growth, aliquots were collected for measurement of OD<sub>600</sub> and glucose concentration. The glucose levels were only shown for the culture grown in the presence of glucose (diamond). The arrow indicates the sampling time for mRNA preparation used for the microarray experiments.

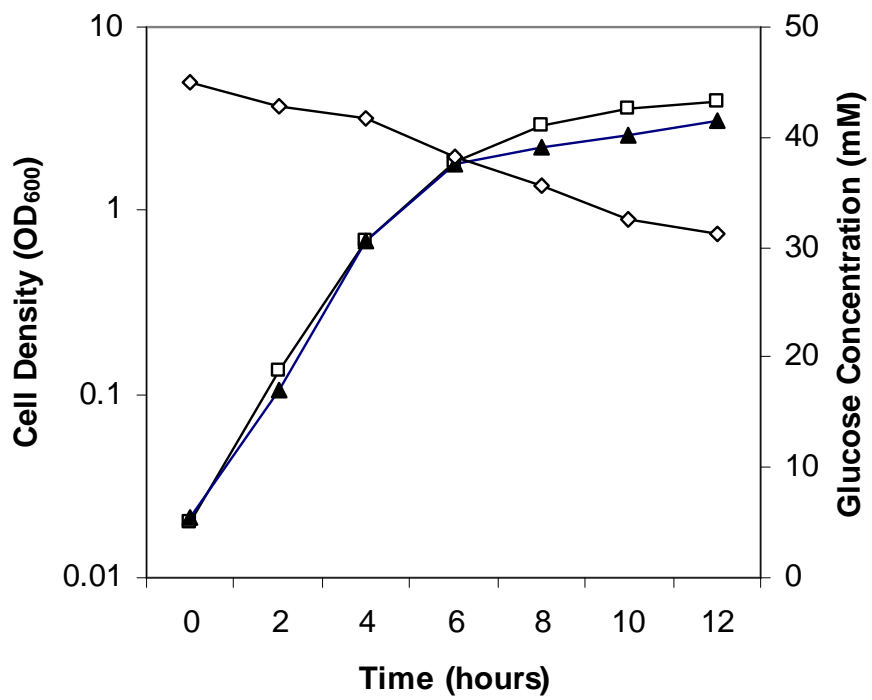


Table 4-1 Functional categories of the differentially expressed genes

Functional categories	Total	Higher on LB +Glc		Higher on LB	
		Number <sup>a</sup>	%	Number <sup>a</sup>	%
Total	4346	464	10.7	237	5.5
Amino acid biosynthesis	115	31	27.0	4	3.5
Biosynthesis of cofactors, prosthetic groups and carriers	120	5	4.2	0	0.0
Cell envelop	176	14	8.0	4	2.3
Cellular processes	189	20	10.6	4	2.1
Central intermediary metabolism	73	10	13.7	4	5.5
DNA metabolism	107	4	3.7	2	1.9
Energy metabolism	399	48	12.0	37	9.3
Fatty acid and phospholipid metabolism	70	5	7.1	7	10.0
Mobile and extrachromosomal element	39	4	10.3	0	0.0
Protein fate	117	6	5.1	5	4.3
Protein synthesis	123	1	0.8	4	3.3
Purines, pyrimidines, nucleosides, nucleotides	82	9	11.0	4	4.9
Regulatory functions	177	20	11.3	7	4.0
Transcription	45	0	0.0	1	2.2
Transport and binding proteins	322	28	8.7	37	11.5
Hypothetical, unclassified, unknown	2244	273	12.2	123	5.5

<sup>a</sup> only the genes with not less than +/-2 fold changes are calculated. Some genes with multiple functions appear in different groups, but are counted once in total item.

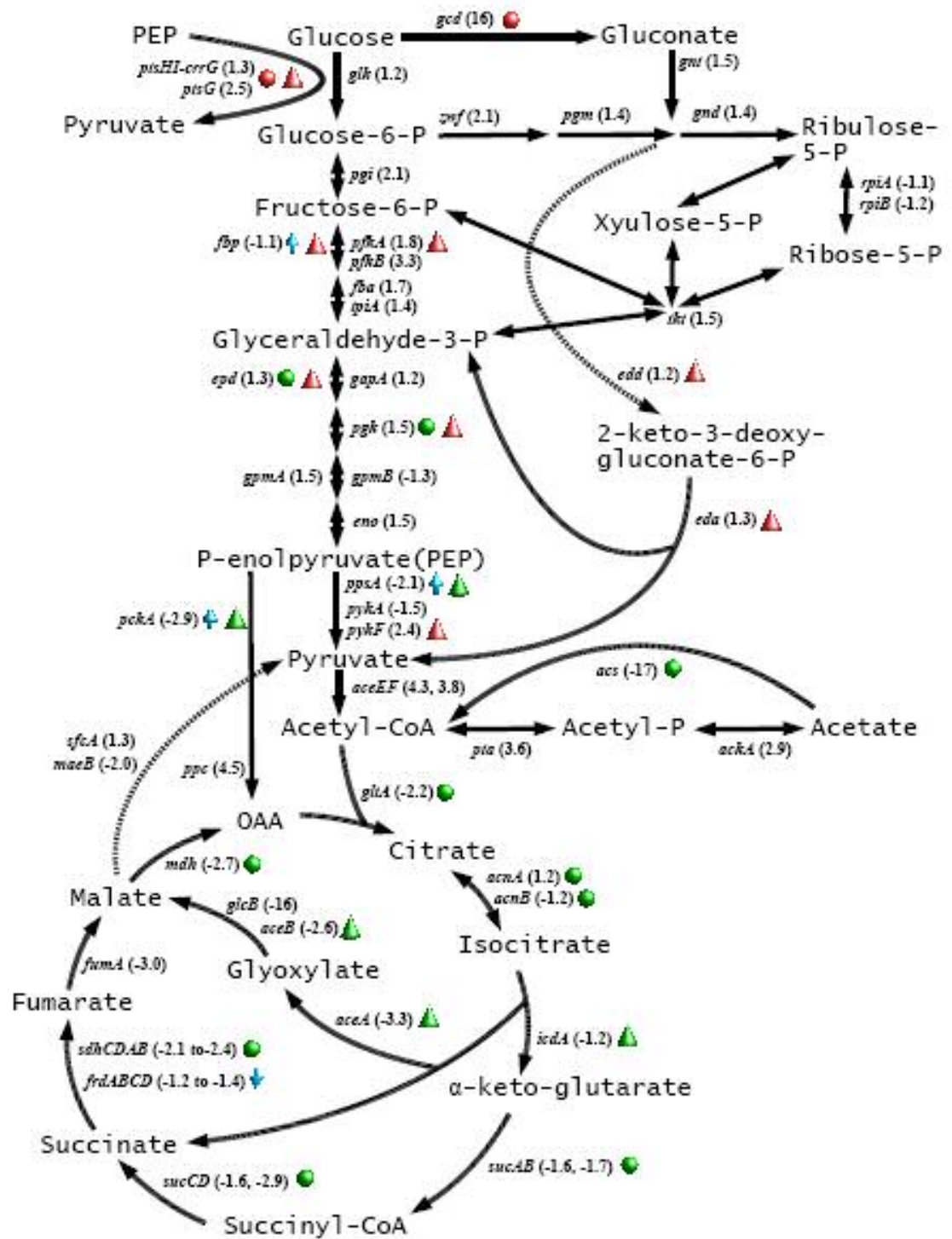
have indicated that a 1.5-change in gene expression can be biologically significant (20, 76, 168), we have chosen 2.0 as cutoff for the fold change to identify genes significantly affected by the glucose addition. Table 4-1 shows, of the total 4346 genes, 464 and 237 genes are up and down regulated at least 2-fold, respectively, by the presence of glucose. These account for 10.7% and 5.5% of the whole genome, respectively, indicating that addition of glucose causes widespread and significant changes in the gene transcription profile of *E. coli*. In most of the functional categories, the presence of glucose results in more genes being upregulated than downregulated. For example, in the category corresponding to amino acid biosynthesis and cellular processes, the induced genes exceed repressed genes by 31 to 4, and 20 to 4 respectively. However, in the category of transport and binding proteins, 28 and 37 genes are up- and down-regulated, respectively. The differential response in gene expression of these cells in due to glucose addition was interpreted in detail as follows.

#### **Central metabolic pathway.**

Due to the role of glucose as a preferred energy and carbon source, it is not surprising that the genes involved in central metabolic pathway are tightly and coordinately controlled by the presence of glucose. Our results indicated that, in the category denoted energy metabolism, 48 and 37 genes were up- and down-regulated, respectively. The expression of 10 genes was increased whereas expression of 4 genes was reduced in the category denoted central intermediary metabolism. The influence of glucose on many of these genes is carried out through two main global regulators, the cAMP-CRP complex and FruR protein (161). Figure 4-2 presents the primary glycolytic,

**Figure 4-2 The expression of central metabolic pathway components in response to the presence of glucose**

For each gene, the fold level of induction (positive numbers) or repression (negative numbers) is indicated as the number in parenthesis. For multiple genes controlling one step of the pathway, the range of expression is given. Gene products that drive a reaction in a direction opposite that of the black arrows are indicated by a blue arrow. Genes that are regulated by cAMP-CRP are denoted as follows: up-regulated (green ball) or down-regulated (red ball). Genes that are regulated by FruR are denoted as follows: up-regulated (green cone) or down-regulated (red cone)





pentose-phosphate, pyruvate dissimilation, and TCA-related genes and their transcription fold-changes in response to glucose. Overlaid onto this figure are the genes regulated by cAMP-CRP and FruR based on previous literature studies and predictions. That our data correlate well with previous studies serves to indirectly validate our findings.

Most of the genes involved in glycolysis and pyruvate dissimilation are up-regulated, while genes involved in TCA cycle are down-regulated. This is reasonable because the cells do not need full oxidation of acetyl-CoA to obtain energy when grown on high levels of glucose. Expression of the *aceEF* genes, which encode enzymes responsible for pyruvate dissimilation to acetyl-CoA, are highly induced (4.3 and 3.8-fold respectively) by glucose. Expression of *pflA* encoding pyruvate formate lyase, which is involved in second mechanism for pyruvate dissimilation to acetyl-CoA by the formation of formate, is not affected by glucose under this condition. Transcription of *pta* and *ackA*, which encode phosphotransacetylase and acetate kinase involved in formation of acetate from acetyl-CoA, is also elevated significantly (3.6 and 2.9-fold respectively) in the presence of glucose. The expression of these two genes is controlled by the CreC-CreB system, which is responsive to the presence of glucose (6, 161) (discussed more below).

We compared the expression levels of the glycolytic and TCA genes to those reported by Oh and others in their investigation of the transcriptional differences between cells grown on acetate and on glucose (132). Most of the genes involved in glycolysis have similar regulatory patterns, however the expression of TCA and glyoxylate shunt genes in the presence of acetate was, in some cases, higher than it was

in our data. Key differences between the two studies are summarized in Table 4-2, and these genes are probably those more influenced by acetate. In addition, the *acs* gene, which is suggested as an acetate induced gene in the previous report (132), is highly repressed by the presence of glucose (-17-fold), consistent with its regulation by cAMP-CRP.

### **Biosynthesis of amino acids.**

Although addition of glucose to the LB medium did not result in faster proliferation of the cells as per constant growth rate using OD<sub>600</sub> data, the production of amino acids appeared strongly enhanced by the presence of glucose (Table 4-1 & 4-3). There were 31 genes of this group that were induced at least 2 fold. It is known that glutamate and glutamine, the main products of ammonia assimilation, provide nitrogen for almost all nitrogen-containing compounds when bacteria are grown in minimal medium containing ammonium salt (151). In this study, expression of the enzymes involved in ammonia assimilation, glutamine synthase (GS, encoded by *glnA*), glutamate synthase (GOGAT, encoded by *gltBD*), and glutamate dehydrogenase (GDH, encoded by *gdhA*), are all increased by the glucose addition (1.9, 6, 4.5, and 2.2-fold, respectively). These are consistent with previous observations and predictions that these genes have CRP binding sites in their promoter regions (134, 152, 154). These results suggest that, under the investigated condition, the *E. coli* cells enhance ammonia incorporation by increasing expression of all enzymes involved in its assimilation. Our results also

Table 4-2 Effects of carbon sources on expression of several genes

Gene	M9+Glc / M9+Acetate <sup>a,b</sup>	LB+Glc / LB
<i>acnB</i>	-6.9	-1.2
<i>aceAK</i>	-(15-39)	-(2.4-3.3)
<i>pckA</i>	-8.3	-2.9
<i>sfcA</i>	-1.7	1.3
<i>ppsA</i>	-13	-2.1
<i>fbp</i>	-3.3	-1.1
<i>maeB</i>	-5.1	-1.5

<sup>a</sup>Cultures of *E. coli* were grown in either M9+0.5% glucose, M9+0.25% acetate, LB, or LB+0.8% glucose. The M9 media cultures were compared in, and the results reported in the first column. The LB media cultures are from this work and the results are reported in the third column.

<sup>b</sup>Data in this column taken from.

Table 4-3 Differentially expressed genes involved in nitrogen metabolism and biosynthesis

	B	Gene	Gene Product	Fold change (+Glc/-Glc)
	number			
Amino acid biosynthesis	b0002	<i>thrA</i>	aspartokinase I, homoserine dehydrogenase I	2.21
	b0003	<i>thrB</i>	homoserine kinase	2.04
	b0033	<i>carB</i>	carbamoyl-phosphate synthase large subunit	6.99
	b0071	<i>leuD</i>	isopropylmalate isomerase subunit	2.23
	b0073	<i>leuB</i>	3-isopropylmalate dehydrogenase	3.32
	b0074	<i>leuA</i>	2-isopropylmalate synthase	2.13
	b0077	<i>ilvI</i>	acetolactate synthase III, valine sensitive, large subunit	3.25
	b0078	<i>ilvH</i>	acetolactate synthase III, valine sensitive, small subunit	2.36
	b0268	<i>yagE</i>	putative lyase/synthase 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	-4.3
	b0754	<i>aroG</i>		2.64
	b0907	<i>serC</i>	3-phosphoserine aminotransferase	2.7
	b0908	<i>aroA</i>	5-enolpyruvylshikimate-3-phosphate synthetase	3.03
	b1261	<i>trpB</i>	tryptophan synthase, beta protein	2.09
	b1693	<i>aroD</i>	3-dehydroquininate dehydratase	2.06
	b1748	<i>cstC</i>	acetylornithine delta-aminotransferase	-6.13
	b1761	<i>gdhA</i>	NADP-specific glutamate dehydrogenase	2.18
	b2020	<i>hisD</i>	L-histidinol:NAD <sup>+</sup> oxidoreductase; L-histidinol:NAD <sup>+</sup> oxidoreductase	3.23
	b2021	<i>hisC</i>	histidinol-phosphate aminotransferase	2.74
	b2023	<i>hisH</i>	glutamine amidotransferase subunit of heterodimer with HisF	3.1
	b2025	<i>his</i>	imidazole glycerol phosphate synthase subunit in heterodimer with HisH	2.34
	b2026	<i>hisI</i>	phosphoribosyl-amp cyclohydrolase; phosphoribosyl-ATP pyrophosphatase	2.13
	b2290	<i>yfbQ</i>	putative aminotransferase	2.05
	b2838	<i>lysA</i>	diaminopimelate decarboxylase	3.68
	b2913	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	5.16
	b3073	<i>yjG</i>	probable ornithine aminotransferase	3.38
	b3172	<i>argG</i>	argininosuccinate synthetase	2.09
	b3212	<i>gltB</i>	glutamate synthase, large subunit	5.98
	b3213	<i>gltD</i>	glutamate synthase, small subunit	4.5
	b3433	<i>asd</i>	aspartate-semialdehyde dehydrogenase acetolactate synthase I, valine sensitive, small subunit	2.19
	b3670	<i>ilvN</i>	acetolactate synthase I, valine-sensitive, large subunit	-2.71
	b3671	<i>ilvB</i>		-2.57
	b3774	<i>ilvC</i>	keto acid reductoisomerase	2.76
	b3958	<i>argC</i>	N-acetyl-gamma-glutamylphosphate reductase	2.78
b3960	<i>argH</i>	argininosuccinate lyase	2.74	
b4054	<i>tyrB</i>	tyrosine aminotransferase, tyrosine repressible	2.01	
Cofactors, prosthetic groups and carriers	b0628	<i>lipA</i>	lipoate synthesis, sulfur insertion	2.24
	b1740	<i>nadE</i>	NAD synthetase, prefers NH <sub>3</sub> over glutamine	2.43
	b1991	<i>cobT</i>	nicotinate-nucleotide dimethylbenzimidazole-P phosphoribosyl transferase	2.05

	b3551	<i>bisC</i>	biotin sulfoxide reductase	2.37
	b3856	<i>mobB</i>	molybdopterin-guanine dinucleotide biosynthesis protein B	2.01
Purines, Pyrimidines, nucleosides, nucleotides	b0032	<i>carA</i>	carbamoyl-phosphate synthetase, glutamine (small) subunit	18.11
	b0033	<i>carB</i>	carbamoyl-phosphate synthase large subunit	6.99
	b0160	<i>dgt</i>	deoxyguanosine triphosphate triphosphohydrolase	2.14
	b0758	<i>galT</i>	galactose-1-phosphate uridylyltransferase	-2.09
	b0945	<i>pyrD</i>	dihydro-orotate dehydrogenase	2.27
	b2143	<i>cdd</i>	cytidine/deoxycytidine deaminase	2.81
	b2518	<i>ndk</i>	nucleoside diphosphate kinase	-5.36
	b3642	<i>pyrE</i>	orotate phosphoribosyltransferase	2.22
	b3994	<i>thiC</i>	thiamin biosynthesis, pyrimidine moiety anaerobic ribonucleotide reductase activating protein	2.48
	b4237	<i>nrdG</i>	protein	-10.38
	b4238	<i>nrdD</i>	anaerobic ribonucleoside-triphosphate reductase	-12.73
	b4244	<i>pyrI</i>	aspartate carbamoyltransferase, regulatory subunit	6.98
	b4245	<i>pyrB</i>	aspartate carbamoyltransferase, catalytic subunit	8.03

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showed that all of the genes responsible for arginine biosynthesis were expressed at elevated levels in the presence of glucose (Table 4-3) (the genes with fold changes lower than 2 were not shown in the table.). The *carAB* genes, which encode small and large subunits of carbamoylphosphate synthase, were the most significantly regulated genes in the arginine regulon (with fold changes of 18 and 7, respectively), suggesting an important role of this enzyme in arginine production under this growth condition.

Most of the genes encoding enzymes responsible for biosynthesis of the branch-chain amino acids (isoleucine, valine, and leucine) are shown to be transcribed at higher levels in response to the addition of glucose, except the *ilvBN* operon which is downregulated about 2.6-fold. Since the *ilvBN* operon, *ilvIH* operon, and *ilvGM* genes (in *ilvGNEDA* operon) encode enzymes catalyzing formation of the acetohydroxy (188), the increase in expression of *ilvIH* (2.4-3.4 fold) and *ilvGM* (1.3 fold) probably are enough for the biosynthesis of isoleucine and valine, suggesting differential expression pattern of the encoded enzymes under the investigated condition. The *aroG* gene, which encodes the first enzyme in the common pathway of aromatic acid biosynthesis, and the other two (*aroD* and *aroA*), are expressed at significantly higher levels in the presence of glucose. Histidine biosynthesis was highly enhanced, suggested by increased expression of the genes encoding most of the enzymes involved in the pathway. In summary, presence of glucose in LB medium resulted in overall increased expression of the genes encoding the enzymes of amino acid biosynthesis.

## **Nucleotide Biosynthesis.**

Pyrimidine biosynthesis is enhanced by increased induction of *carAB* (7-18-fold) and *pyrBDEI* (2.3-8-fold) by the addition of glucose (Table 4-3). As mentioned earlier, the CarAB is involved in arginine production. Therefore, induction of this operon probably assures necessary supply of the enzyme required for the synthesis of both pyrimidine and arginine. Regulation of *carAB* is necessarily complicated due to its role as a precursor to arginine and pyrimidine residues. Control is primarily achieved at the transcriptional level through integration host factor (IHF), aminopeptidase A (PepA), pur regulon repressor (PurR), and the arg regulon repressor (ArgR), as well as purine and pyrimidine. Interestingly, we do not find any apparent increase in purine synthesis genes. This probably results from availability of high levels of intracellular ATP and potential interconversion between purines.

The *nrdDG* genes, which encode anaerobic ribonucleoside-triphosphate reductase and anaerobic ribonucleotide reductase activating protein, are found to be strongly repressed by the presence of glucose (-13 and -10, respectively). The NrdD reduces ribonucleoside triphosphates in the absence of oxygen. Other ribonucleotide reductases (encoded by *nrdA* and *nrdB*) are active in the presence of oxygen (128). It is widely accepted that the *nrdDG* operon is activated under microaerophilic to anaerobic conditions. The strong repression of *nrdD* and *nrdG* in the presence of glucose could indicate transition from either aerobic to anaerobic growth in the culture grown without glucose (63). However, expression of other genes that are expected to be transcribed in the presence of the anaerobic transcriptional regulator *fnr* (-1.6-fold change), such as *icd* (-1.2-fold change) and *dcuC* (no change) was not significantly changed when cells were

grown on glucose. Further investigation is needed to clarify the regulation of *nrdDG* and *ndk* transcription in the presence of glucose.

### **Transport and binding proteins.**

The genes encoding enzymes involved in transport of carbohydrates, amino acids, and peptides comprise the primary subgroups that are induced or decreased in this group (Table 4-4). Consistent with the role of glucose in catabolite repression, most of the genes responsible for the use of other carbohydrates were dramatically down-regulated in the presence of glucose. Some examples are: lactose transport system (*lacZYA* operon, 6-10-fold), maltose/maltodextrin transport system (*lamB*, *malK*, and *malEFG* operon, 4-7-fold), galactose transport system (*mglBAC* operon, 45, 48, 17-fold respectively), ribose transport system (*rbsDACB* operon, 2-6-fold), and the C4-dicarboxylic acid transport gene (*dctA*, 5-fold). The galactose transport system genes were among the most significantly repressed genes following glucose addition. In contrast to the overall repression pattern of the genes involved in carbohydrate use, the expression of some genes encoding enzymes responsible for amino acid transport are increased with glucose addition such as *artJ*, *cadB*, *prop*, *yifK*, and *yjdE*, while the others show reduced expression such as *argT*, *putP*, and *sdaC*. Interestingly, the expression of two peptide ABC transport systems, encoded by operon *dppABCDF* and *oppABCDF*, are reduced significantly in the presence of glucose, 2.3-4 and 1.8-3 fold respectively. In addition, several genes involved in cation transport are upregulated more than 2-fold, which include, *fecA*, *kch*, *nikC*, while expression of sulfate transport system genes (*cysP*, *cysU*, *cysW*) are reduced markedly (5-7-fold). The differential



Table 4-4 Differentially expressed transport genes

B number	Gene	Gene Product	Fold change (+Glc/-Glc)
b0019	<i>nhaA</i>	Na <sup>+</sup> /H antiporter, pH dependent	-2.3
b0040	<i>caiT</i>	probable carnitine transporter transcriptional regulator for nitrite reductase (cytochrome c552)	2.07
b0211	<i>dniR</i>		-3.21
b0241	<i>phoE</i>	outer membrane pore protein E (E,Ic,NmpAB)	2.06
b0343	<i>lacY</i>	galactoside permease (M protein)	-10.78
b0411	<i>tsx</i>	nucleoside channel; receptor of phage T6 and colicin K	-3.33
b0490	<i>ybbL</i>	putative ATP-binding component of a transport system	2.23
b0553	<i>nmpC</i>	outer membrane porin protein; locus of <i>qsr</i> prophage	-16.93
b0679	<i>nagE</i>	PTS system, N-acetylglucosamine-specific enzyme IIABC	-3.69
b0860	<i>artJ</i>	arginine 3rd transport system periplasmic binding protein	2.18
b1006	<i>ycdG</i>	putative transport protein	3.38
b1015	<i>putP</i>	major sodium/proline symporter	-5.8
b1101	<i>ptsG</i>	PTS system, glucose-specific IIBC component	2.45
b1198	<i>ycgC</i>	putative PTS system enzyme I	2.89
b1244	<i>oppB</i>	oligopeptide transport permease protein homolog of Salmonella oligopeptide transport permease protein	-2.82
b1245	<i>oppC</i>		-2.98
b1246	<i>oppD</i>	homolog of Salmonella ATP-binding protein of oligopeptide ABC transport system	-2.31
b1250	<i>kch</i>	putative potassium channel protein bifunctional: putative transport protein (N-terminal); putative kinase (C-terminal)	2.53
b1729	<i>ydiN</i>		-3.64
b1736	<i>celC</i>	PEP-dependent phosphotransferase enzyme III for cellobiose, arbutin, and salicin	-2.53
b1738	<i>celA</i>	PEP-dependent phosphotransferase enzyme IV for cellobiose, arbutin, and salicin galactose-binding transport protein; receptor for galactose taxis	-2.81
b2150	<i>mglB</i>		-45.14
b2167	<i>fruA</i>	PTS system, fructose-specific transport protein	-2.38
b2310	<i>argT</i>	lysine-, arginine-, ornithine-binding periplasmic protein	-2.41
b2344	<i>fadL</i>	transport of long-chain fatty acids; sensitivity to phage T2	-2.13
b2423	<i>cysW</i>	sulfate transport system permease W protein	-4.63
b2424	<i>cysU</i>	sulfate, thiosulfate transport system permease T protein	-6.95
b2425	<i>cysP</i>	thiosulfate binding protein	-7.2
b2497	<i>uraA</i>	uracil transport	6.73
b2547	<i>yphE</i>	putative ATP-binding component of a transport system	2.23
b2796	<i>sdaC</i>	probable serine transporter	-2.44
b2975	<i>yghK</i>	putative permease	-4.06
b3110	<i>yhaO</i>	putative transport system permease protein	3
b3224	<i>nanT</i>	sialic acid transporter	-15.96
b3270	<i>yhdY</i>	putative transport system permease protein	2.58
b3271	<i>yhdZ</i>	putative ATP-binding component of a transport system	2.45
b3364	<i>yhfC</i>	putative transport	4.53
b3478	<i>nikC</i>	transport of nickel, membrane protein	2.4
b3523	<i>yhjE</i>	putative transport protein	4.84
b3528	<i>dctA</i>	uptake of C4-dicarboxylic acids	-5.17
b3540	<i>dppF</i>	putative ATP-binding component of dipeptide transport	-2.7

		system	
		putative ATP -binding component of dipeptide transport system	
b3541	<i>dppD</i>	system	-4
b3542	<i>dppC</i>	dipeptide transport system permease protein 2	-3.89
b3543	<i>dppB</i>	dipeptide transport system permease protein 1	-3.11
b3544	<i>dppA</i>	dipeptide transport protein	-2.33
b3547	<i>yhjX</i>	putative resistance protein	30.28
b3720	<i>yieC</i>	putative receptor protein	2.74
		D-ribose high-affinity transport system; membrane-associated protein	
b3748	<i>rbsD</i>	protein	-2.97
b3750	<i>rbsC</i>	D-ribose high-affinity transport system	-6.36
b3751	<i>rbsB</i>	D-ribose periplasmic binding protein	-5.47
b3795	<i>yifK</i>	putative amino acid/amine transport protein	2.93
b3909	<i>kdgT</i>	2-keto-3-deoxy-D-gluconate transport system	2.13
b4032	<i>malG</i>	part of maltose permease, inner membrane	-5.3
b4033	<i>malF</i>	part of maltose permease, periplasmic	-4.95
b4034	<i>malE</i>	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis	-3.8
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	-7.41
b4086	<i>yjcV</i>	putative transport system permease protein	2.13
b4096	<i>phnL</i>	ATP-binding component of phosphonate transport	2.19
b4111	<i>proP</i>	low-affinity transport system; proline permease II	8.67
b4115	<i>yjdE</i>	putative amino acid/amine transport protein, cryptic	3.15
b4123	<i>dcuB</i>	anaerobic dicarboxylate transport	2.89
b4132	<i>cadB</i>	transport of lysine/cadaverine	2.21
b4229	<i>ytfS</i>	putative ATP-binding component of a transport system	2.76
b4240	<i>treB</i>	PTS system enzyme II, trehalose specific	-11.41
b4291	<i>fecA</i>	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	2.24

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expression of the genes responsible for the transport for amino acids, peptides, amines, and ions may reflect the various nutrient requirements by the cells under the investigated condition, which are affected by availability of the energy source.

### **Regulatory proteins.**

The expression of *cya*, encoding adenylate cyclase, is increased about 3.4-fold in the presence of glucose (Table 4-5), which is consistent with the previous reports (3, 19, 161). Although *cya* is expressed at higher level, its activity showed a 100-fold decrease in this condition, resulting in much lower level of cAMP (103). This has been interpreted as a preparation period for the transition from carbon excess to carbon depletion (161). Under the growth conditions of this study, there was no difference in expression of the *crp* gene, which encodes cAMP receptor protein (CRP).

Phosphorus assimilation is also affected by the presence of glucose. The PhoR-PhoB two component regulatory system is a master control for the phosphate (Pho) regulon (196). A Pi limitation is sensed by the PhoR sensor kinase and transferred to PhoB, the transcriptional regulator, resulting phosphorylation and activation of this protein. Our results show the expression of *phoR* is elevated about 2.4-fold by glucose addition, while the *phoB* expression is only slightly increased (1.3-fold). Many genes belonging Pho regulon were found to be up-regulated in the presence of glucose. For example, the expression of *phnC*, *phnK*, *phnL* and *phnN*, which encode proteins involved in phosphonate transport, were elevated (4.3, 2.4, 2.2, and 1.6-fold respectively). The *pstSCAB*, which encodes Pi transporter, and *phoU*, which encodes protein involved in Pho regulation and Pi metabolism, were expressed at higher levels

Table 4-5 Differentially expressed genes encoding regulatory proteins

B number	Gene	Gene Product	Fold change (+Glc/-Glc)
b0208	<i>yafC</i>	putative transcriptional regulator LYSR-type	2.23
b0241	<i>phoE</i>	outer membrane pore protein E (E,Ic,NmpAB)	2.06
b0346	<i>mhpR</i>	transcriptional regulator for mhp operon	-2.64
b0400	<i>phoR</i>	positive and negative sensor protein for pho regulon	2.36
b0598	<i>cstA</i>	carbon starvation protein	-10.8
b1303	<i>pspF</i>	<i>psp</i> operon transcriptional activator	2.02
b1422	<i>ydcl</i>	putative transcriptional regulator LYSR-type	-3.95
b1569	<i>dicC</i>	regulator of <i>dicB</i>	2.38
b2126	<i>yehU</i>	putative 2-component sensor protein	-2.56
b2151	<i>galS</i>	<i>mgl</i> repressor, galactose operon inducer	-3.09
b2248	<i>yfaX</i>	putative regulator	2.76
b2292	<i>yfbS</i>	putative transport protein	2.32
b2370	<i>evgS</i>	putative sensor for regulator EvgA	4.3
b2437	<i>yfeG</i>	putative ARAC-type regulatory protein	2.04
b2537	<i>hcaR</i>	transcriptional activator of hca cluster	-3.07
b2869	<i>ygeV</i>	putative transcriptional regulator	-2.54
b2921	<i>ygfl</i>	putative transcriptional regulator LYSR-type	2
b3026	<i>ygiY</i>	putative 2-component sensor protein	2.46
b3243	<i>yhcS</i>	putative transcriptional regulator LYSR-type	2.26
b3515	<i>yhiW</i>	putative ARAC-type regulatory protein	4.73
b3569	<i>xylR</i>	putative regulator of <i>xyl</i> operon	2.63
b3662	<i>yicM</i>	putative transport protein	5.7
b3680	<i>yidL</i>	putative ARAC -type regulatory protein	3.09
b3724	<i>phoU</i>	negative regulator for pho regulon and putative enzyme in phosphate metabolism	2.29
b3806	<i>cyaA</i>	adenylate cyclase	3.41
b4084	<i>yjcT</i>	putative NAGC-like transcriptional regulator	2.13
b4116	<i>adiY</i>	putative ARAC-type regulatory protein	4.24

(1.4-1.7, 2.3-fold respectively). The expression of PhoE, a polyanion porin, was also increased (2.1-fold). However, the expression of *ugpBAE* operon, which encodes proteins involved in G3P and glycerophosphoryl diester, was not affected significantly by the glucose addition, suggesting additional control mechanism.

In the PhoR null mutants, the CreC sensor kinase and acetyl phosphate can also phosphorylate and activate PhoB in the Pi-independent controls of the Pho regulon. However, in the PhoR wild type cells, it appears not necessary to have CreC and acetyl phosphate to control the Pho regulon since the PhoB is activated completely by the PhoR under Pi limitation (196). The CreB-CreC system has long been known to be responsive to carbon source, but its function in carbon utilization was unclear until recent identification of the cre regulon (6). Interestingly, the *pta* and *ackA*, which encode enzymes involved in the production of acetyl phosphate and further formation of the acetate and ATP, belong to the cre regulon (6). In this study, it is shown that the expression of *creB*, *creC*, *pta*, and *ackA* increased 1.8, 2.1, 3.6, and 2.9 fold, respectively, by growth on glucose.

### **Effects of glucose on biosynthesis and transport of the quorum signal, AI-2.**

AI-2 is suggested to be a signal molecule involved in various cell activities (206). Glucose and the other PTS sugars are well known to influence extracellular AI-2 activities of *E. coli* and *S. e. typhimurium* (179). We recently showed that the cAMP-CRP complex is involved in the control of both biosynthesis and uptake of AI-2 in *E. coli* (193). This complex directly stimulates transcription of AI-2 uptake genes, the *lsrACDBFG* operon, and indirectly represses expression of AI-2 synthase, encoded by

the *luxS* (*ygaG*) gene (193). The DNA microarray provides us a powerful tool to study the global expression of those genes involved in AI-2 biosynthesis and uptake.

Biosynthesis of AI-2 from SAM involves Pfs, LuxS, and several methyltransferases (163). The SAM is derived directly from methionine, and supplies methyl group in methyl transferring reactions and propylimino group in spermidine biosynthesis (68). Homoserine is a common source for the biosynthesis of threonine and methionine (68, 139). In this study, we found, ThrA, LsyC, and Asd, the enzymes for homoserine biosynthesis, were expressed at higher levels in the presence of glucose (2.2, 1.8, and 2.2 fold, respectively). In addition, the expression of *metC*, *metE*, and *metH*, which encode enzymes specifically responsible for biosynthesis of methionine, are also elevated (1.6, 1.6, and 1.7-fold, respectively). In the specific pathway for AI-2 biosynthesis, the expression of the *luxS* was increased about 1.6-fold, which is consistent with our previous data with a *luxS-lacZ* fusion plasmid (193). There was no significant difference in *pfs* expression in the conditions with or without glucose, which is also consistent with our previous results obtained using *lacZ* fusion, before the stationary phase (during late stationary phase, the presence of glucose results lower *pfs* expression) (193). CheR, an enzyme involved in SAM methyl transfer, showed no change in its expression. In the SAM decarboxylation pathway, in which AI-2 levels may be affected by the alternative consumption of the SAM source, the expression of *speED* is reduced slightly (1.2-fold) by addition of glucose, suggesting small influence of AI-2 biosynthesis by spermidine production under the investigated conditions. In addition, we did not observe significant changes in transcription of the *lsrACDBFG* operon, which encodes AI-2 uptake system. This data is also consistent with our

previous results with the *lsr-lacZ* fusion plasmid, which showed that the expression of this operon is growth phase dependent and is increased during stationary phase (193). The expression of *lsrR*, which encodes a repressor of the *lsr* operon, shows no difference as well. Therefore, an increase in metabolic flux resulting from methionine biosynthesis, together with the increase of *luxS* expression, are among the most significant reasons for AI-2 accumulation observed during the exponential phase (193). Glucose was shown to play a role in influencing AI-2 production and likely regulating quorum signalling.

#### **Other transcriptional changes in response to growth on glucose.**

The presence of glucose in growth medium also affects the expression of genes involved in other cell activities. In the fimbrial operon *fimBEACDFGH*, the *fimBEACDFGH* genes, which encode structural components of the fimbrial had lower expression (-1.3 to -3.1-fold) in the presence of glucose, while expression of the regulatory gene, *fimB*, which is required for inversion-dependent phase variation (Table 4-6) (99), increased about 4.1-fold. The expression of *fimE* was not affected significantly. The reduced expression of fimbrial structural genes in presence of glucose is consistent with previous observance that poor growth conditions (low glucose and amino acid levels) activate fimbrial expression (99).

The presence of glucose induced expression of the *emrAB* and *emrK*, which encode proteins involved in multidrug resistance, but highly repressed expression of two genes (*cspD* and *cspF*) encoding cold shock proteins. The actual mechanisms for these are not known.

Table 4-6 Differentially expressed genes involved in cell envelope and cellular processes

	B number	Gene	Gene product	Fold change (+Glc/-Glc)	
Cell envelope	b4053	<i>alr</i>	alanine racemase 1	5.46	
	b3433	<i>asd</i>	aspartate-semialdehyde dehydrogenase	2.19	
	b2060	<i>wzc</i>	orf, hypothetical protein, tyrosine-protein kinase	2.31	
	b2253	<i>yfbE</i>	putative aminotransferase	2.76	
	b2505	<i>yfgH</i>	putative outer membrane lipoprotein	2.18	
			curlin major subunit, coiled surface structures;		
	b1042	<i>csgA</i>	cryptic	3.9	
			transport of long-chain fatty acids; sensitivity to		
	b2344	<i>fadL</i>	phage T2	-2.13	
			periplasmic chaperone, required for type 1		
	b4316	<i>fimC</i>	fimbriae	-2.92	
	b4315	<i>fimI</i>	fimbrial protein	-3.14	
	b3661	<i>nlpA</i>	lipoprotein-28	2.27	
	b0814	<i>ompX</i>	outer membrane protein X	2.46	
	b0700	<i>rhcC</i>	rhcC protein in rhs element	3.28	
			outer membrane protein induced after carbon		
	b3506	<i>slp</i>	starvation	3.28	
	b2175	<i>spr</i>	putative lipoprotein	-2.03	
	Cellular processes	b2045	<i>wcaK</i>	putative ga	2.22
		b0938	<i>ycbQ</i>	putative fimbrial-like protein	2.51
b4333		<i>yjiK</i>	orf, hypothetical protein	2.4	
b3014		<i>yqhH</i>	orf, hypothetical protein	2.55	
b2470		<i>acrD</i>	sensitivity to acriflavine, integral membrane protein, possible efflux pump	2.33	
b1415		<i>aldA</i>	aldehyde dehydrogenase, NAD-linked	-8.49	
b1978		<i>yeeJ</i>	putative factor	2.43	
b0880		<i>cspD</i>	cold shock protein	-3.6	
b1558		<i>cspF</i>	cold shock protein	-5.55	
			membrane protein required for colicin V		
b2313		<i>cvpA</i>	production	-2.07	
b2685		<i>emrA</i>	multidrug resistance secretion protein	2.05	
			multidrug resistance; probably membrane		
b2686		<i>emrB</i>	translocase	3.18	
b2368		<i>emrK</i>	multidrug resistance protein K	2.54	
b2370		<i>evgS</i>	putative sensor for regulator EvgA	4.3	
b0229		<i>fhiA</i>	flagellar biosynthesis	3.32	
b1896		<i>otsA</i>	trehalose-6-phosphate synthase	2.01	
b3285		<i>smf</i>	orf, fragment 2	3.03	
b0483		<i>ybaQ</i>	orf, hypothetical protein	2.36	
b0877	<i>ybjX</i>	putative enzyme	2.22		
b1220	<i>yehP</i>	putative factor	2.61		
b1797	<i>yeaR</i>	orf, hypothetical protein	5.16		
b1895	<i>yecG</i>	putative regulator	5.04		
b3515	<i>yhiW</i>	putative ARAC-type regulatory protein	4.73		
b3586	<i>yiaV</i>	putative membrane protein	2.5		
b3765	<i>yifB</i>	putative 2-component regulator	2.46		
b3923	<i>yiiT</i>	putative regulator	4.4		



b4082	<i>yjcR</i>	putative membrane protein	4.54
b2847	<i>yqeI</i>	putative sensory transducer	2.09

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There is relatively small number of genes influenced by glucose on the group of DNA metabolism, protein fate, protein synthesis, and transcription, suggesting importance of the stability of the necessary cell machinery and functions to the bacterial cells.

### Conclusion

Concentrations of glucose as outlined in our investigation provide the *E. coli* cells the “preferred” energy and carbon source. As a consequence, they down-regulate the expression of most of the genes involved in the energy-producing TCA cycle, while the expression of many glycolytic genes is elevated, resulting in sequestration and accumulation of the carbon source and its metabolic intermediates. Consistent with the accumulation of intermediates and down-regulation of TCA cycle, the expression of the genes involved in production of the amino acids, nucleotides and cofactors, and Pi assimilation, which normally requires expenditure of significant quantities of energy, is increased significantly. The AI-2 production is also elevated by an increase in metabolic flux together with the moderate increase of *luxS* expression. In addition, the cells reduce expression of genes involved in transport and utilization of alternative energy sources, as expected. However, it seems the bacteria do “prepare” for the depletion of the glucose. Our samples were at OD 1.0 which is less than one doubling away from stationary phase. This suggests some quorum or nutrient level regulation was occurring although we found only minor changes in the *luxS*-regulated genes found in chapter 3. Our proposed model is: once the glucose is consumed, the “prepared” cells have retained ‘stockpiles’ of a large pool of building blocks for production of protein, DNA

and RNA, which in the end makes them more quickly adaptable to the conditions with limited carbon sources.

## Chapter 5 General Conclusions and Future Directions

Quorum sensing was first discovered in two species of luminous marine bacteria, *V. fischeri* and *V. harveyi* over twenty eight years ago (126). In the past several years, AI-2 mediated quorum sensing has been among the most energized topics in the field of bacterial cell-to-cell communication, because the gene for its synthase, *luxS*, is present in over 55 species of diverse bacteria. The work by Schauder *et al.* (163) suggested that pathways for AI-2 biosynthesis are similar. Many bacteria possessing *luxS* were shown to produce AI-2 activity that can be measured by the *V. harveyi*BB170 reporter strain (181, 206). Because of the prevalence of *luxS*, it was believed that AI-2 may be used as a common language between different bacterial species (118, 206). Thus, the most important questions in this field are what genes and cell activities are regulated by AI-2/*luxS*, and how the AI-2 mediates cell-to-cell communication. To answer these questions, *luxS* mutants have been made in various bacterial species. *luxS* was shown to regulate different cell activities, such as biofilm production in *Streptococcus mutans*, *S. e. typhimurium*, and *V. cholerae* (69, 115, 147); motility in *Campylobacter jejuni* and *E. coli* EHEC and EPEC(50, 64, 173) ; iron acquisition in *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *V. harveyi*(31, 54, 96) ; expression of virulence factors in *A. actinomycetemcomitans*, *E. coli* EHEC, *P. gingivalis*, *V. cholerae*, and *Clostridium perfringenes* (31, 54, 133, 172, 208). These studies have contributed to our understanding of the AI-2 /*luxS* mediated regulation of gene expression and cell activities, but questions remained as few genes appeared to be directly influenced by the AI-2 (206).

Some researchers have attempted to reveal the global effects of *luxS* deletion on cells by using DNA microarrays or protein 2-D gel analysis (82, 173). However, such investigations have been muddled by the faster growth rate of the  $\Delta luxS$  mutant, or have not been successful in identifying genes controlled by *luxS*. Until our work, there has remained a cloudy view of the effects of *luxS* deletion on gene expression in a genomic scale, irrespective of bacterial species.

AI-2/*luxS* controlled genes have now been identified for the first time in *E. coli* K12 W3110 through our work using DNA microarrays. Deletion of *luxS* in W3110 influenced expression of genes involved in diverse physiological activities such as methionine biosynthesis, methyl transfer, iron uptake, AI-2 uptake, resistance to oxidative stress, carbon source utilization, and virulence. However, growth rate, motility, biofilm formation were not affected for the  $\Delta luxS$  mutant in our investigated conditions. Importantly, we have shown that the growth conditions are a crucial factor in the AI-2/*luxS* mediated gene regulation. In our microarray results at mid exponential phase, there had been few genes identified that were influenced by *luxS*. Then, at later times (higher OD), the genes presumably responding to AI-2, were revealed (including the *lsr* operon). Note, induction of the *luxS* regulated genes identified by this work may have resulted from a direct response to AI-2, or from the disturbance of the intracellular metabolic environment caused by a blockage in the AI-2 biosynthesis pathway. We have found that most of the highly induced genes (such as *lsr* operon, *lsrR*, *lsrK*, *tam*, and *metK*) are related to AI-2 production and transport, while the genes involved in other activities are induced to a less degree. This data are consistent with the function of AI-2 as an autoinducer, but do not support its role as a receptor-mediated signaling

molecule as used by *V. harveyi* in luminescence stimulation. In chapter 2, we have shown that the presence of glucose highly inhibits expression of the *lsr* operon through control of cAMP-CRP. That *lsr* operon is induced only in the absence of glucose suggests AI-2 is used as a carbon source. Although Taga *et al* reported that *S. e. typhimurium* could not grow on AI-2 as the sole carbon source (186), additional conditions may be needed for AI-2 utilization as a carbon source, as suggested by Winzer *et al.* (202). It is possible that AI-2 may be used as a true signal molecule only in specific bacterial species or in specific conditions.

To determine the AI-2 regulated gene, in vitro synthesized AI-2 could be added to the  $\Delta luxS$  mutant to test whether the expression of the genes identified above is affected by AI-2 (through RT-PCR or Northern blot). In addition, since the transcriptional regulator LsrR is involved in AI-2 mediated modulation, the wild type and *lsrR* null mutant could be compared to see whether *lsrR* deletion affects transcription of genes identified by the *luxS* microarray experiments. This work could be done through another set of DNA microarrays, RT-PCR, Northern blot, or *lacZ* fusion. The LsrR regulated genes could be further tested by their response to the in vitro synthesized AI-2.

In addition to completing the first *luxS* null mutant DNA microarray study in *E. coli* K12, this work has further elucidated the regulation of AI-2 biosynthesis and transport in this organism. This is, in our view, one of the most important aspects that has to be addressed in study of the AI-2 mediated quorum sensing. There have been many reports showing that, in various bacterial species, extracellular AI-2 activity peaks in late exponential phase, then drops during the stationary phase. Addition of glucose

greatly increases AI-2 level. Surprisingly, the mechanism for this glucose effect has remained a mystery for several years in this field. Through the glucose microarray experiments, we showed that glucose likely affects AI-2 levels by increasing the metabolic flux in biosynthesis of methionine and AI-2 through upregulation of the genes involved. We further showed that the cAMP-CRP complex indirectly represses *luxS* expression and directly stimulates transcription of the *lsrACDBFG* operon. The cAMP-CRP is shown to bind to a CRP binding site located in the upstream region of the *lsr* promoter and works with LsrR repressor to regulate AI-2 uptake. We found that the function of the *lsr* operon and its genetic regulation are very similar between *E. coli* and *S. e. typhimurium* (185, 186). Moreover, it is likely that the *lsr* operon and AI-2 uptake are similarly controlled in the other *lsr*-possessing bacteria, such as *A. actinomycetemcomitans* and *Yersinia pestis*.

Although likely, it is not clear whether and where the LsrR binds to the promoter of the *lsr* operon. Future work could be directed toward producing and purifying the LsrR protein, through a His-tag, to test its binding activities to the *lsr* promoter region (by gel mobility shift assay). In addition, transcription of *lsrR* and *lsrK* could be monitored by constructing *lacZ* fusion plasmids and studying the effects of different host background mutations on their transcription. This is a natural extension of this work since microarray results suggest these two genes probably belong to the same AI-2 controlled regulon as the *lsr* operon. Other interesting genes such as *tam*, *yneE*, *arpA*, and *mdaB* etc. could be investigated as well. For example, *tam* and *yneE*, which are located immediate downstream of the *lsr* operon and are induced by *luxS*, may be important in regulation of AI-2 production and modification. This hypothesis could be

simply tested by measuring extracellular AI-2 activity of the mutants of *tam* and *yneE*. Careful elucidation of the regulation of AI-2 production and removal is very important for the study of AI-2 mediated quorum sensing and allows us to correlate the level of quorum signal with its controlled behaviors. This may provide a way for us to modulate the signal production and corresponding cell activities (eg. virulence). In particular, these may be a most promising avenue for study of microbial virulences among such pathogens as *P. aereofaciens* and EHEC etc. by blocking cell signalling.

With information obtained from this work, one of applications may be construction of a protein expression system controlled by the *lsr* promoter. This will allow autoinduced production of proteins in *E. coli*, a technology currently unavailable. Current expression systems rely heavily on addition of expensive inducer molecules such as IPTG. An autoinduction based system would have built-in control and require minimal cost (cell endogenously produce inducer).

In summary, this dissertation has led to a better understanding of AI-2/*luxS* mediated gene regulation and the control of the AI-2 synthesis and transport in *Escherichia coli*. It has opened a window for us to view quorum sensing controlled cell activities. With fast growing interest in the study of AI-2 mediated quorum sensing, scientists and engineers from different fields are all focusing on this fascinating phenomenon, bringing an invigorating and productive multidisciplinary approach to elucidate these behaviors. In this way, we are likely to develop a better understanding of the language of bacteria and may more easily control bacterial behavior by manipulating quorum signalling.



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