

ABSTRACT

Title of Document: Rewiring Quorum Sensing Circuitry for
 Recombinant Protein Production in *E. coli*

 Chen-Yu Tsao, Doctor of Philosophy, 2007

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 Engineering

The global objective of this research is to rewire the circuitry of bacterial quorum sensing to facilitate recombinant protein production in bacteria. Previous research has shown that the activity of AI-2, the putative “universal” bacterial autoinducer, decreased in culture fluids when several proteins were overexpressed in *E. coli* W3110, suggesting bacteria communicate or possibly potentiate the “metabolic burden” associated with protein overexpression. Additionally, conditioned medium obtained from LuxS⁺ and LuxS⁻ strains was added to these cultures, resulting in a 2-4 fold increase in specific yield for both chloramphenicol acetyltransferase (CAT) and organophosphorus hydrolase (OPH). These simple observations set the stage for examining the role of quorum sensing in recombinant protein expression systems and also suggested that “rewiring” the quorum sensing circuitry would lead to significant improvement of yield.

In this dissertation, we have inserted *luxS* into expression vectors (IPTG inducible) which can co-synthesize target recombinant proteins (arabinose inducible) to accomplish the modulation of the metabolic landscape for protein synthesis via altered AI-2 signaling. Our results show significant enhancement in both protein yield and activity, and reveal a strong linkage between bacterial cell communication and cellular processes involved in synthesis and folding of recombinant proteins in *E. coli*. Second, we have attempted to rewire the native quorum sensing signaling circuitry and couple it to the widely-used T7 expression system for constructing an autoinducible recombinant protein expression platform. We demonstrate, for the first time, true autoinduction of recombinant proteins in *E. coli* or, in fact, any expression system. That is, our results showed that GFPuv, CAT, and LacZ were all expressed using this innovative system without cell growth monitoring or inducer addition.

REWIRING QUORUM SENSING CIRCUITRY FOR
RECOMBINANT PROTEIN PRODUCTION IN *E. COLI*

By

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Dedication

I dedicate the accomplishments of my learning to my parents, Chih-Fang Tsao and Hsiu-Mei Tsao Chang, and my wife, Yi-Chen Ko.

Acknowledgements

I would like to gratefully acknowledge my advisor Dr. William E. Bentley, for his dedication of my research and the guidance of this intellectual journey. From him, I have broadened the view of academia and learned the attitude of optimism, diligence, and humility. I would also like to thank my committee: Dr. Peter Kofinas, Dr. Nam Sun Wang, Dr. Vikram Vakharia, and Dr. William A. Weigand for their invaluable time, discussion, and encouragement.

I would also like to give my thanks to all the members of Dr. Bentley's Lab and friends in the University of Maryland. From them, I have not only earned the friendship, but also obtained their generous help, sincere suggestion, and inspirational discussion.

Finally, I would like to especially acknowledge my parents who give me the endless support, confidence, and warmth. Also, I would like to thank my wife and my elder sister for their consideration, assistance, and spiritual support. Without my families, I would have been like a boat without a sail and might have been lost in the journey of life.

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Chapter 1 Introduction

Recombinant protein technology has been extensively applied in manufacturing therapeutic, agricultural and numerous biological products in recent years. Therefore, the need to produce larger quantities of recombinant proteins of higher quality is in great demand. Understanding the underlying mechanisms, optimizing the procedures, improving the existing expression systems, and developing novel approaches are the main objectives for biochemical engineers to benefit this rising and flourishing industry.

The production of recombinant proteins is carried out by introducing various deoxyribonucleic acid (DNA) sequences encoding for the foreign proteins into various host cells as different expression systems. The gene encoding the desired foreign protein is typically downstream of an inducible promoter, so that its transcription level can be controlled by inducer addition. Transcription of the foreign gene produces a messenger ribonucleic acid (mRNA) transcript which is then translated into the target protein product via ribosomal complexes (Lewin 2000; Shuler and Kargi 1992).

Coordination of the knowledge of recombinant protein technology and bioprocess engineering has admitted large scale production of recombinant proteins by different genetically modified microorganisms. The gram-negative prokaryotic bacterium, *Escherichia coli* (*E. coli*), is one of the most often used microorganisms for industrial production of recombinant proteins if the posttranslational modifications are not necessary. Compared to other developed expression systems,

the advantages of *E. coli* are: (1) high growth rate; (2) inexpensive media; (3) availability of high-cell-density fermentation; (4) relative ease of process scale-up; (5) broad knowledge of the host; and (6) abundant selection of genetic control elements and the generally well-known methods for genetic manipulation (Baneyx and Mujacic 2004; Shuler and Kargi 1992; Sodayer 2004). However, there are still drawbacks using *E. coli* as a host: (1) misfolded recombinant proteins result in the accumulation of insoluble and biologically inactive inclusion bodies; (2) *E. coli* lack systems for post-translational modifications; and (3) there are poor secretors (Baneyx and Mujacic 2004; Lee 1996; Shuler and Kargi 1992). Furthermore, overexpression of recombinant proteins usurps the limited resources from the host and triggers physiological responses such as a decreased cell growth rate (Bentley et al. 1990; DeLisa et al. 2001b; Georgiou 1988; Glick 1995), coincidence with a rapid stress response which can lead to the upregulation of stress genes and their products (Andersson et al. 1996; Gill et al. 2001; Harcum and Bentley 1993b); and finally, plasmid instability (Andersson et al. 1996; Bentley et al. 1990). These factors can all result in the reduction of recombinant protein yield.

Therefore, in order to pursue improvements in productivity, several cell-based molecular strategies have been developed: (1) the regulation of heat shock regulator, σ^{32} , to depress the stress response by antisense RNA (Srivastava et al. 2000); (2) the utilization of protease minus mutants as hosts (Georgiou 1988); and (3) the co-expression recombinant “functional” proteins (e.g. chaperones) coincident with the target protein products (Baneyx and Mujacic 2004; Lee and Olins 1992; Thomas and

Baneyx 1996b). This study presents one other target for manipulating the host cells to increase yield.

To date, there is some preliminary evidence suggesting a linkage between recombinant protein overproduction in *E. coli* and bacterial quorum sensing or cell-to-cell communication (DeLisa et al. 2001b). Indeed, bacterial signaling circuits have been coupled to innovative biotechnological applications which have attracted more and more attention, suggesting a wealth of potentiality for advancing biotechnology (March and Bentley 2004).

1.1 Research Background

1.1.1 Quorum Sensing: How Bacteria Communicate with Each Other?

Quorum sensing is a process that bacteria communicate cell-to-cell via the production and detection of small secreted signal molecules termed autoinducers (AIs). It enables population-density-based regulation of gene expression, whereby a single cell senses and communicates a minimal population unit (or quorum) needed for intra- or inter-species orchestrating population behavior (Fuqua and Greenberg 1998a; Fuqua and Greenberg 1998b; Hastings and Greenberg 1999; Salmond et al. 1995). Therefore, while a threshold of an autoinducer level is achieved, a signal transduction cascade is initiated that results in diverse changes in bacterial behavior including: bioluminescence (Lilley and Bassler 2000; Miller and Bassler 2001), motility (Ren et al. 2004; Sperandio et al. 2001), competence (Perego and Hoch 1996), antibiotic synthesis (Bainton et al. 1992), virulence (Miller et al. 2002; Sperandio et al. 1999), and biofilm maturation (Davies et al. 1998; Prouty et al.

2002). In general, there are two paradigmatic quorum-sensing systems: (1) different oligopeptides used as autoinducers in Gram-positive bacteria (Figure 1-1A), and (2) various small molecules such as acylated homoserine lactones (AHLs) used as autoinducers in Gram-negative bacteria (Figure 1-1B) (Waters and Bassler 2005; Xavier and Bassler 2003).

Gram-positive Bacteria Communicate with Oligopeptides.

The oligopeptide signals (Figure 1-1A) are first synthesized as precursor peptides. After the modification processes, the quorum signals are exported using ATP binding cassette (ABC) transporters. When the cell densities increase, the secreted signals accumulate to a certain level, which can be detected by two-component phospho-relay circuits. Through phosphorylation/dephosphorylation processes, the signals are transduced from the sensors to transcriptional regulators and then the phosphorylated regulatory proteins stimulate the expression of target genes.

Gram-negative Bacteria Speak with Various AHLs.

The AHL autoinducers are derived from *S*-adenosyl methionine (SAM), and share a common homoserine lactone moiety with different lengths of acyl side-chain and the substitutions on the side chain (Fuqua et al. 1996; Schauder and Bassler 2001). Bacteria use similar LuxI/LuxR type protein pairs to regulate different cellular activities. The LuxI-like proteins produce specific AHL autoinducers, which are able to freely diffuse through the bacterial membrane and accumulate in concentration along with the cell growth. The LuxR-like proteins are responsible for binding with

the partner AHL autoinducers to specific DNA promoter regions for the regulation of target genes (Figure 1-1B) (Fuqua et al. 1994; Schauder and Bassler 2001).

Parallel Quorum Sensing Circuits in *Vibrio harveyi*

Quorum sensing processes in different microorganisms are variable. An interesting example is the bioluminescence of *Vibrio harveyi*, which is governed via a multi-component signal transduction cascade built upon three parallel sensory pathways channeled to a shared response regulator protein, LuxO (Figure 1-1C) (Bassler et al. 1994; Waters and Bassler 2005). The first pathway responds to an AHL autoinducer, *N*-(3-oxohexanoyl)-L-homoserine lactone (autoinducer-1 or AI-1) (Bassler et al. 1993; Cao and Meighen 1989), which is similar to other Gram-negative bacteria. However, the mechanism by which *V. harveyi* responds to AI-1 is mediated by a Gram-positive-like two-component phospho-relay system. The second pathway is mediated by autoinducer-2 (AI-2), dependent on the *luxS* gene product and identified as furanosyl borate diester (Bassler et al. 1994; Chen et al. 2002; Miller et al. 2004). The third pathway, responding to *cholerae* autoinducer 1 (CAI-1), which is produced by the CqsA enzyme, was recognized recently (Henke and Bassler 2004). Sensory signals from the three pathways are transduced by phosphorylation/dephosphorylation to the shared signal integrator protein, LuxU, which, in turn, signals to the common response regulator, LuxO. At high cell density, a condition in which the autoinducers exist in high level, the phosphate leaves LuxO, and dephosphorylated LuxO activates the expression of the transcriptional activator LuxR. In this way, the *lux* operon is transcribed and the

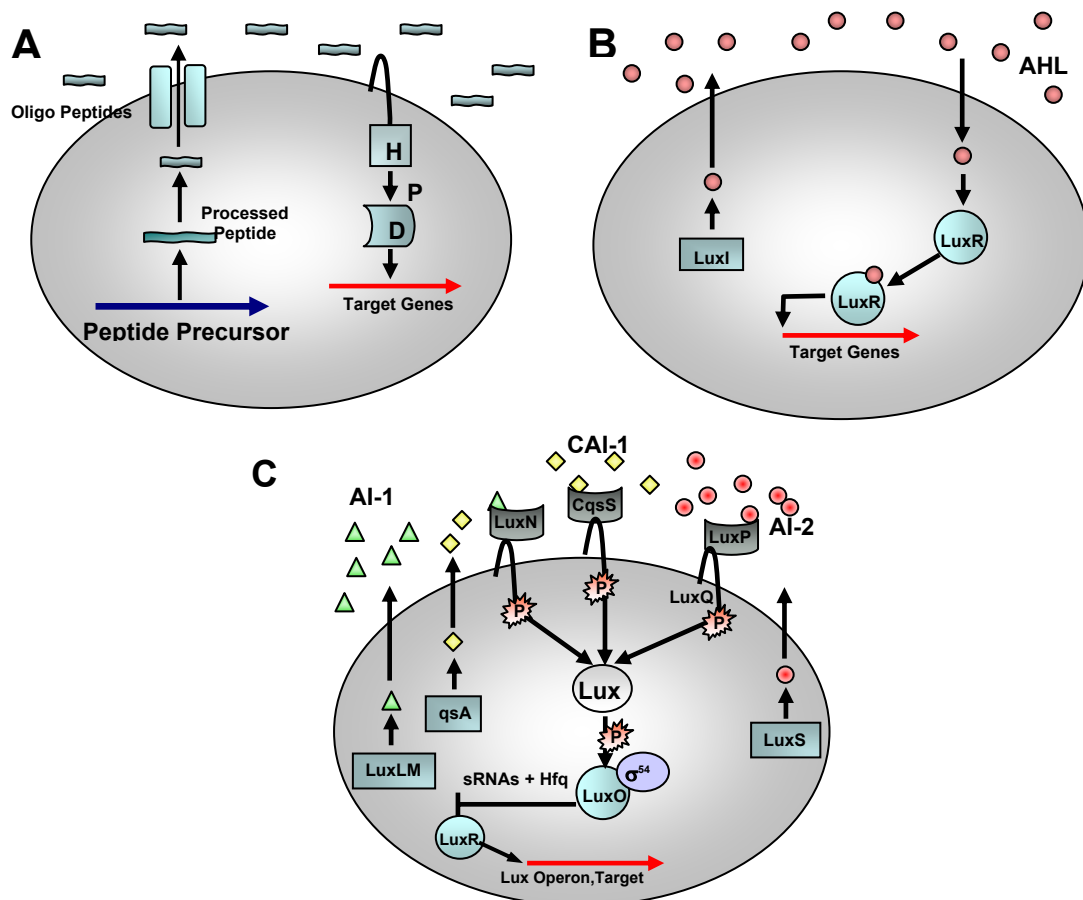


Figure 1-1 Different types of quorum-sensing circuits

(A) Quorum sensing in Gram-positive bacteria are mediated via oligopeptides. Two-component phosphorylation cascades are used for signal transduction. (B) In most Gram-negative bacteria, quorum circuits are mediated by species-specific AHLs, which are produced by the LuxI-type enzyme and detected by LuxR-type transcription regulator. (C) 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 σ^{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 the *lux* operon is eliminated. Differing from system 1 and 2, the CAI-1-CqsS system responds to CAI-1 at much lower cell densities (Waters and Bassler 2005; Xavier and Bassler 2003).

bacteria produce luminescence (Miller and Bassler 2001; Waters and Bassler 2005). Though the first pathway is species specific, signaling via AI-2 appears to be more widespread, occurring in numerous Gram-positive and Gram-negative bacteria and suggesting that the *V. harveyi* AI-2 quorum architecture may constitute an interspecies communication mechanism among bacteria. By comparing to the first two pathways, it was recognized that the third pathway responds to CAI-1 at a much lower cell density (Henke and Bassler 2004). These examples demonstrate the complexity and multiplicity of quorum sensing in *Vibrio harveyi*.

LuxS Quorum Sensing and Autoinducer-2

The presence of different cell-density-sensing systems to regulate bioluminescence in *V. harveyi* seems at first excessively redundant. To enable differentiation of the different systems, Bassler *et al.* developed *V. harveyi* mutant strains as reporters that are able to respond to the different autoinducers. In their study in 1997, cell-free culture fluids from various bacterial species were tested by various reporter strains: BB120 (sensor 1⁺ sensor 2⁺), the wild type strain, responds to both AI-1 and AI-2; BB886 (sensor 1⁺ sensor 2⁻) only responds to AI-1; BB170 (sensor 1⁻ sensor 2⁺) which only responds to AI-2 (Bassler *et al.* 1997). Interestingly, cell-free culture fluids from a number of different bacterial species induced bioluminescence in *V. harveyi*, and most the substances produced by these species mimicked the response of AI-2 but not AI-1. This result demonstrated that *V. harveyi* system 1 is specific, while *V. harveyi* system 2 is less specific. The gene encoding AI-2 synthase, *luxS*, exists in numbers of sequenced bacterial genomes and AI-2

production has been confirmed in many of these species. Therefore, because of its conserved nature, AI-2 is suggested to be a “universal” signal which is responsible to interspecies cell-to-cell communication (Bassler et al. 1997; Surette et al. 1999; Xavier and Bassler 2003).

The complete AI-2 biosynthesis pathway has demonstrated that AI-2 is derived from *S*-adenosylmethionine (SAM) (Figure 1-2) (Schauder et al. 2001; Xavier and Bassler 2003). SAM acts as a methyl donor dependent on methyltransferases in the biosynthesis or modification of DNA, RNA and cell proteins, and produces the toxic intermediate *S*-adenosylhomomocysteine (SAH). SAH is hydrolyzed to *S*-ribosylhomocysteine (SRH) and adenine by the nucleosidase, Pfs, and thereafter LuxS, the AI-2 synthase, cleaves homocysteine from SRH to 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is highly reactive and readily undergoes nucleophilic attack. After spontaneous cyclization and further rearrangement processes, it results in distinct but related derivatives (Figure 1-2) suggesting that distinct signal molecules can be distinguished as AI-2 by different bacterial species (Miller et al. 2004; Schauder et al. 2001; Waters and Bassler 2005; Xavier and Bassler 2003).

There have been two distinct DPD derivatives identified in *Salmonella typhimurium* (*S. typhimurium*) and *V. harveyi* by trapping the signal molecules in their individual receptors (LsrB in *S. typhimurium* and LuxP in *V. harveyi*) and resolving their structures by crystallography. The AI-2 in *V. harveyi* is (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate) and the AI-2 in *S. typhimurium* is (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF)

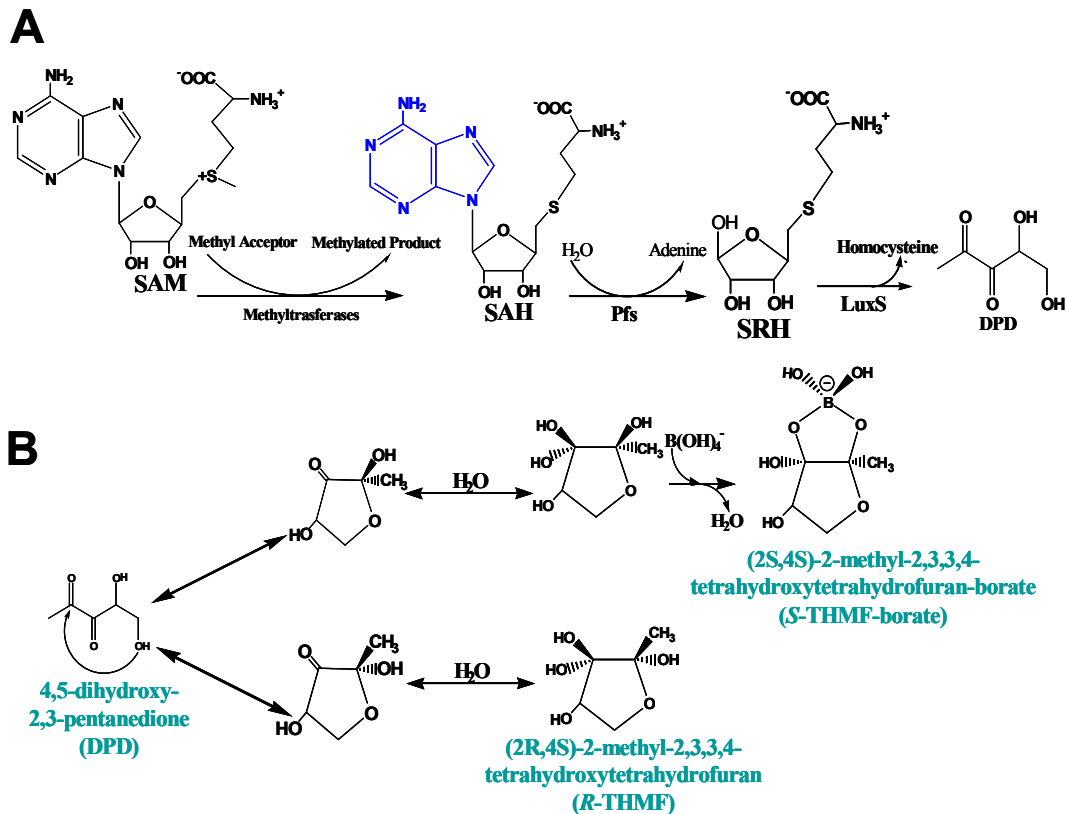


Figure 1-2 Biosynthesis and formation of AI-2 signaling molecules

(A) 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 (Xavier and Bassler 2003). (B) Formation of the AI-2 signaling molecules (Miller et al. 2004). The DPD molecules undergo self-cyclization and arrangement to form S-DHMF and R-DHMF, which are hydrated 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.

(Figure 1-2)(Chen et al. 2002; Miller et al. 2004; Waters and Bassler 2005). Though AI-2 structures are distinct, *V. harveyi* can report activity of *S. typhimurium* AI-2 under the presence of boric acid and *S. typhimurium* AI-2 bioassay can report the activity of *V. harveyi* AI-2(Miller et al. 2004). Additionally, the result of the co-culture of *V. harveyi* and *E. coli* has demonstrated the bacteria can manipulate AI-2 quorum sensing and interfere with other species' cell-to-cell communication (Xavier and Bassler 2005b). Therefore, the distinct AI-2 structures are presumably able to interconvert, which allows bacteria to sense not only their own AI-2 but also AI-2 produced by different species. These results further demonstrate that AI-2 is potentially a “universal” language between different bacterial species (Meijler et al. 2004; Miller et al. 2004; Xavier and Bassler 2005b).

1.1.2 Quorum Sensing in *E. coli*

There have been over 55 Gram-positive or Gram-negative bacterial species found to produce and secrete AI-2(Vendeville et al. 2005). The discovery that *E. coli* and *S. typhimurium* are also able to release AI-2 may have the most remarkable impact towards bioengineering applications. Surette and Bassler observed that cell-free culture media (conditioned media) from *E. coli* and *S. typhimurium* contain extracellular factors that can induce luminescence in *V. harveyi* through the non-selective autoinducer sensor, Autoinducer-2 sensor (Figure 1-3) (Surette and Bassler 1998). The authors' previous results also indicated that quorum sensing in *E. coli* was affected by several environmental factors. This suggested that the signal might have communicated the growth phase and metabolic activity of the cells (Surette and

Bassler 1998; Surette et al. 1999). After this discovery, more and more researchers attempted to characterize the potential roles that quorum sensing has in *E. coli*.

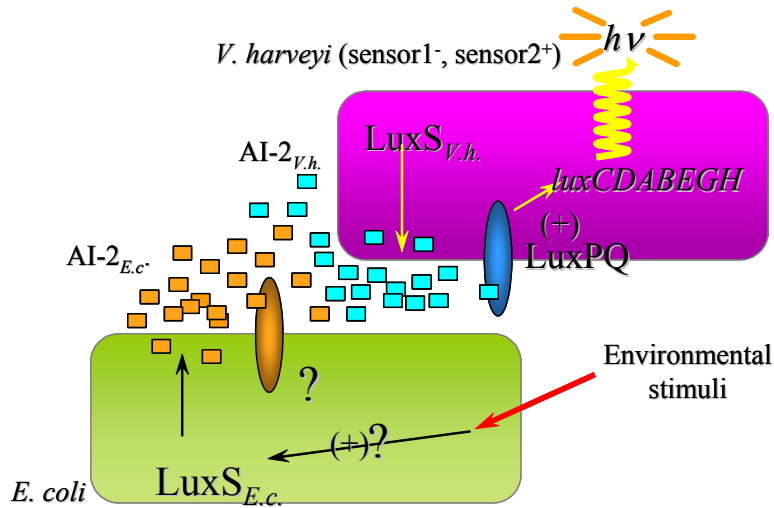


Figure 1-3 Listening in on the conversation of bacteria

Vibrio harveyi BB170 cross-species AI-2 activity assay for quantitative detection of autoinducer-2 (AI-2) in cell-free medium from *E. coli* W3110 cultures (DeLisa et al. 2001b).

Previously, our lab applied various environmental stimuli to chemostat cultures of *E. coli* K12 to investigate the response levels of AI-2 (DeLisa et al. 2001a). AI-2 was found to increase with the steady state-growth rates (*i.e.* dilution rate), but decreased during the overproduction of recombinant protein (DeLisa et al. 2001a). Further results demonstrated that there was a significant reduction of AI-2 in response to the overexpression of a variety of recombinant proteins in batch cultures of *E. coli*. This response can be attributed to a “metabolic burden” (Bentley et al. 1990), which is widely believe to be associated with the redistribution of metabolites

and nutrient resources (DeLisa et al. 2001b). These works suggested that recombinant *E. coli* communicate the burden of overexpressing heterologous genes via the AI-2 quorum sensing circuit, and elicited more curiosity towards determining the physiological roles of quorum sensing in *E. coli*.

The evidence that *S. typhimurium* can also detect AI-2 signaling is another milestone towards understanding quorum circuitry of *E. coli* and *S. typhimurium*. Although the quorum sensing role of AI-2 in the genus *Vibrio* has been determined, it was once disputed as to whether or not the molecule was just a metabolic byproduct or a nutrient (Ahmer 2004; Winzer et al. 2002b). Previous research indicated that quorum sensing in *E. coli* and *S. typhimurium* might be critical for regulating behavior prior to the stationary phase of growth. The maximal extracellular AI-2 activity was observed during mid- to late-exponential phases in the presence of glucose. AI-2 activity was observed to decrease, based on degradation, when glucose was depleted or during the onset of the stationary phase (Surette and Bassler 1998). Because the phenomenon is in contrast to general quorum sensing schemes, which are cell-density related, the roles that AI-2 played was a topic of debate until the *lsr* (*luxS* regulated) operon was identified in *S. typhimurium*, a homologous operon of *lsr* present in *E. coli* (Taga et al. 2003). Consequently, the discovery of the *lsr* operon is an important step towards understanding AI-2 quorum sensing in *E. coli* and *S. typhimurium* (Ahmer 2004; Taga et al. 2003; Taga et al. 2001).

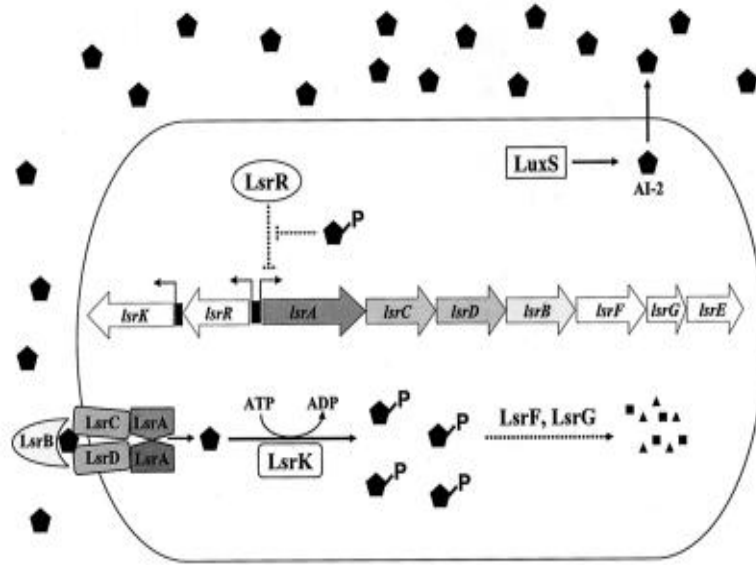
In order to identify and characterize AI-2 regulated genes of *S. typhimurium*, Taga *et al.* screened 11,000 isogenic wild type and *luxS* null strains harboring random MudJ (*lacZ*) reporter insertions in the chromosome. The *lsrACDBFGE* operon was

identified. The *lsr* operon encodes an ATP binding cassette (ABC)-type transporter system which is similar to the ribose transporter system of *E. coli* and *S. typhimurium* (Taga et al. 2001). Therefore, the Lsr transporter system is suggested to be responsible for AI-2 uptake and modification (Figure 1-4A). The *lsrACDB* genes encode the transport apparatus which seemingly bind to AI-2 specifically and function to import AI-2. The gene, *lsrR*, located adjacent to the upstream sequences of the *lsr* operon encodes the primary regulatory protein of the operon (Taga et al. 2001). Following the entry into the cells, AI-2 is phosphorylated by a kinase encoded by *lsrK*. The phospho-AI-2 is suggested to be the inducer responsible to inactivate the LsrR repressor, which results in the de-repression of *lsr* expression (Taga et al. 2003). In *E. coli*, *b1513* operon is homologous to *S. typhimurium* *lsr* operon. Also, *ydeV* and *ydeW* encode the proteins homologous to *S. typhimurium* LsrK and LsrR respectively (Figure 1-4B) (Taga et al. 2003; Wang et al. 2005a; Xavier and Bassler 2005b). Unsurprisingly, it is also shown that *b1513* operon of *E. coli* encodes AI-2 transport system which function to import AI-2 from the culture fluid (Xavier and Bassler 2005b). Additionally, the work from our group determined that AI-2 synthesis and uptake in *E. coli* are regulated by the catabolite repression through the cyclic AMP (cAMP) and cAMP receptor protein (CRP) complex, which directly stimulates transcription of the *lsr* operon and indirectly represses *luxS* expression (Wang et al. 2005a). The model describing the production, accumulation, and transduction of AI-2 in *E. coli* was proposed to interpret AI-2 regulation (Figure 1-4C) (Wang et al. 2005a).

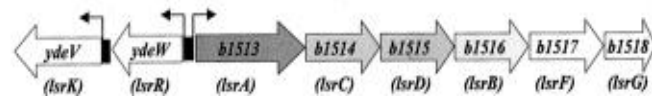
Figure 1-4 Conceptual models of AI-2 synthesis and internalization in *S. typhimurium* and *E. coli*

(A) AI-2 (pentagons) is produced by LuxS and accumulates extracellularly. Thereafter, AI-2 is internalized by the Lsr ABC-type transporter and following phosphorylation via the LsrK kinase. Phospho-AI-2 is an inducer of transcription of the *lsr* operon, and is proposed to bind to the *lsr* operon repressor LsrR and inactivate LsrR (Xavier and Bassler 2005b). **(B)** *E. coli* b1513 operon is homologous to *S. typhimurium* *lsr* operon. The proteins encoded by *ydeV* and *ydew* are homologous the AI-2 kinase LsrK and the *lsr* repressor LsrR respectively (Xavier and Bassler 2005b). **(C)** In the presence of glucose, low level of cAMP and CRP repress the *lsr* operon. Also, indirect upregulation and likely relatively abundant precursor flux result in the increases AI-2 synthesis. Both factors enable the rapid accumulation of AI-2 extracellularly. While the inducer of *lsr*, phospho-AI-2, is absent, LsrR represses the expression of *lsr* operon. After glucose is depleted, cAMP-CRP stimulates the expression of *lsr*. As AI-2 accumulates, *lsr* transcription is de-repressed and more AI-2 is internalized. Plus and minus indicate positive and negative regulations respectively (Wang et al. 2005a).

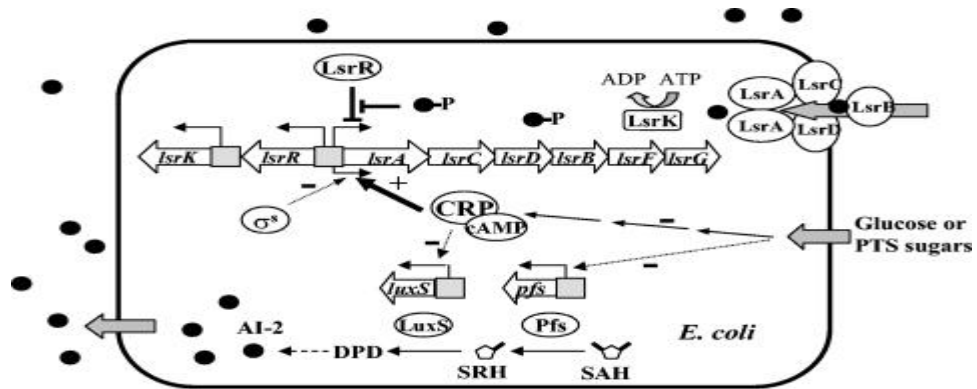
A



B



C



1.1.3 Biotechnological Applications of Quorum Sensing

Biotechnology researchers look for novel platforms that assist in the enhancement of efficiency, searching for wider applications, and maintaining or intensifying of process specificity. There have been several research reports directed at harnessing quorum sensing in areas such as novel antimicrobial therapies to the management of pathogen/pest (For reviews, see(March and Bentley 2004; Weber et al. 2005)). One of the most innovative application of quorum sensing research has been in metabolic engineering (March and Bentley 2004). Specifically, Neddermann *et al.* (Neddermann et al. 2003) developed a novel expression system in mammalian cells by transferring the bacterial quorum sensor of *Agrobacterium tumefaciens* into mammalian hosts under a eukaryotic transcriptional regulation. Also, Weber *et al.* (Weber et al. 2003) implanted *Streptomyces* bacterial quorum sensing system into a eukaryotic transactivation domain to engineer a tunable heterogeneous gene expression system in mammalian cell culture and mice. Finally, Bulter *et al.* (Bulter et al. 2004) created an artificial quorum circuit expression system via acetate for cell to cell signaling in *E. coli*.

1.2 Outline of the Dissertation

In this dissertation, Chapter 2 presents the work on the “rewiring” of *E. coli* quorum sensing by altering the induction level of AI-2 synthase LuxS. This work has linked a dramatic improvement in yield by LuxS manipulation to the level of *E. coli* chaperone, GroEL. Chapter 3 presents an entire new “autoinduction” expression system. The whole work is summarized in Chapter 4.

Chapter 2 Rewiring Native AI-2 Quorum Sensing Circuit Increases Yield of Recombinant Proteins in *E. coli* through Post-transcriptional Control of GroEL

2.1 Introduction

Quorum sensing (QS) enables population-density-based regulation of gene expression, whereby a single cell senses and communicates with a minimal population unit (or quorum) needed for orchestrating population behavior (Fuqua and Greenberg 1998a; Fuqua and Greenberg 1998b; Hastings and Greenberg 1999; Salmond et al. 1995). While there is intense interest in understanding the mechanisms of QS-signal transduction, there have been few technological or commercial applications that have directly resulted from adapting or rewiring this signaling process. One of the most striking targets is in the field of metabolic engineering, where signaling modules can be constructed to alter phenotype and aid in the synthesis of recombinant gene products (March and Bentley 2004; Tjalsma et al. 2004). For example, Bulter *et al.* (Bulter et al. 2004) created an artificial genetic switch using acetate for modulating cell to cell signaling in *Escherichia coli* (*E. coli*). Neddermann *et al.* (Neddermann et al. 2003) developed a hybrid expression system by incorporating the quorum circuitry of *Agrobacterium tumefaciens* into a eukaryotic transcriptional controller for HeLa cells. Weber *et al.* (Weber et al. 2003) implanted the *Streptomyces* bacterial QS system for tuning heterologous protein expression in mammalian cell culture and mice (human primary and mouse embryonic stem cells).

The molecular basis by which QS works varies among different species, both in the synthesis of the signaling compounds and in the modes of their perception. This

diversity affords a vast array of potential application – the types of rewired systems for achieving specific outcomes can vary greatly and are limited by our imagination and ability to abstract regulatory features and reconstruct them into modular, transplantable, controllers. For instance, bioluminescence of *Vibrio harveyi* is governed via a multi-component signal transduction cascade built upon three parallel sensory pathways channeled to a single response regulator protein, LuxO (Bassler et al. 1994; Waters and Bassler 2005). The first pathway responds to an acylated homoserine lactone (AHL), *N*-(3-oxohexanoyl)-L-homoserine lactone (autoinducer-1 or AI-1) (Bassler et al. 1993; Cao and Meighen 1989), while the second is mediated by autoinducer-2 (AI-2), (Chen et al. 2002; Miller et al. 2004), and the third pathway responds to CAI-1, which, in turn, is produced by CqsA (Henke and Bassler 2004). Though the first pathway is species specific, signaling via AI-2 appears to be widespread, as its terminal synthase, LuxS, is present in numerous Gram-positive and Gram-negative bacteria (Chen et al. 2002; Miller et al. 2004; Xavier and Bassler 2005a). Indeed, the mode by which an AI-2 signal is transduced also varies and can therefore be exploited by using these modalities in a variety of metabolic engineering applications. For these reasons, we have systematically studied both the synthesis and perception of AI-2 in *E. coli*, so that we might create novel modular QS-based approaches for enhancing bacterial performance in commercial processes.

The ability of bacteria such as *E. coli* to produce the AI-2 quorum signal has been attributed to the LuxS protein, a homodimeric zinc metalloenzyme originally identified in *V. harveyi* (Lewis et al. 2001; Surette et al. 1999). AI-2 signal generation results from LuxS-catalyzed cleavage of S-ribosylhomocysteine (SRH) yielding

homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) which is cyclized into AI-2 (Schauder et al. 2001; Zhu et al. 2004). The specific genes, proteins, pathways, and functions attributed to AI-2 signaling in *E. coli*, while described to be widespread (DeLisa et al. 2001a; DeLisa et al. 2001c), are not fully understood and are continually emerging (Ahmer 2004; Vendeville et al. 2005). Notably, important phenotypes have been attributed to AI-2 signaling (e.g. virulence, biofilm formation, etc.) (Barrios et al. 2006; Domka et al. 2006). We have demonstrated that AI-2 also communicates the “metabolic potential” of *E. coli*, particularly when they are expressing recombinant proteins (DeLisa et al. 2001a; DeLisa et al. 2001b). The signal level in the extracellular milieu decreased precipitously upon the overexpression of recombinant proteins at a rate proportional to their rate of synthesis. This observation was independent of the protein, whether of viral, bacterial, or eukaryotic cell origin (DeLisa et al. 2001a; DeLisa et al. 2001b). We subsequently hypothesized that the global protein synthesis landscape (e.g., chaperone, protease, polymerase activities) could be altered by shifting the window of quorum-dependent gene regulation through exogenous addition of AI-2 or modulation of AI-2 production via the regulation of *luxS*.

While metabolic engineering studies often target, via complementation or mutation, the proteins or enzymes directly involved in a particular pathway of interest, such as TraR above (Neddermann et al. 2003), an approach described here “tweaks” the native signal transduction pathway to alter the global landscape necessary for the desired objective. That is, we describe for the first time the intentional manipulation of native QS communication for improving the synthesis of

recombinant proteins. We have confirmed that the approach is general by testing several proteins of interest. Moreover, we attribute this to increased level of active GroEL, which, in turn, is shown for the first time to be post-transcriptionally modulated by AI-2. Such QS-mediated post-transcriptional modulation of protein level has never been reported in *E. coli*.

2.2 Materials and Methods

Bacterial strains and plasmid construction: Strains and plasmids used in this study are listed in Table 2-1. Chloramphenicol acetyltransferase (CAT)(Bentley 1991) and organophosphorus hydrolase (OPH)(Wu et al. 2000) were expressed using pTrcHisB (Invitrogen). In *luxS* co-expression experiments, plasmid pBO was constructed by digestion of the *opd* gene encoding organophosphorous hydrolase with *NcoI* and *HindIII* from pTO(Srivastava et al. 2000), and insertion into pBADHisA (Invitrogen). The *luxS* gene was amplified by PCR from genomic DNA of W3110 using primers LuxSF and LuxSR (Table 2-2) containing *EcoRI* restriction sequences and inserted into pKK223-3 (Amersham Pharmacia) yielding pKK-*luxS*. Plasmid pBOL was constructed by PCR amplification of the *tac* promoter-*luxS* fusion from pKK-*luxS* using primers pkk223LuxSF and pkk223LuxSR (Table 2-2), followed by ligation into *NdeI* digested pBO. Plasmid pBOL-LacI^q was built by PCR amplification of *lacI*^q encoding and overproducing Lac repressor from the vector pTrcHisB (Invitrogen) using primers LacI^qF and LacI^qR (Table 2-2). The PCR product was blunt cloned into *BstZ17I* digested pBOL. Two additional sets of plasmids were derived from pBO, pBOL, and pBOL-LacI^q to express two other recombinant proteins, CAT and the UV

variant green fluorescent protein (GFPuv). Plasmids pBC, pBCL, and pBCL-LacI^q, the PCR amplified *cat* gene from pTrcHisCAT (Invitrogen) using similar methods and primers FCAT and RCAT (Table 2-2). Likewise, pBG, pBGL, and pBGL-LacI^q were constructed to express GFPuv using pTrcHisGFPuv(Cha et al. 2000) and primers FCAT and RGFPuv (Table 2-2). All plasmids were transformed to TOP10 competent cells (Invitrogen) for sequencing (DNA sequencing facility, UMBI) and later transformed into W3110 or MDAI2. Recombinant model proteins were under the control of the arabinose-inducible *araBAD* promoter, and the *luxS* was controlled by the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *ptac* promoter. *In vitro* synthesized AI-2 was made using plasmids pTrcHis-luxS and pTrcHis-pfs(Barrios et al. 2006), which overproduce His₆-LuxS and His₆-Pfs in the host *E. coli* BL21 (Novagen) (Barrios et al. 2006). *Vibrio harveyi* BB170 (*luxN*::Tn5, sensor 1⁻, sensor 2⁺) and BB152 (*luxL*::Tn5, autoinducer 1⁻, autoinducer 2⁺) (Surette and Bassler 1998), were used for AI-2 activity assays (kindly provided by Dr. B. Bassler). Transformations, cloning procedures and DNA isolation were performed using standard protocols (Sambrook 2000).

Growth media: Luria Bertani (LB) medium contained 5 g L⁻¹ yeast extract (Sigma), 10 g L⁻¹ bacto tryptone (Difco), and 10 g L⁻¹ NaCl. *E. coli* defined growth medium was prepared according to Riesenber (Riesenber et al. 1991) and supplemented with 0.8% glucose (Sigma). Autoinducer bioassay (AB) medium was made according to Greenberg et al (Greenberg et al. 1979).

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	K12 strain, wild type, λ^- , F^- , IN(rrnD-rrnE)1, rph-1s	GSC, Yale University, New Haven, CT
MDAI2	W3110 <i>luxS</i> ::Tc ^r W3110-derived <i>luxS</i> mutant strain	(DeLisa et al. 2001a)
BL21	$F^- ompT [dcm] [lon] hsdS(r_B^- M_B^-) gal$	Novagen
<i>V. harveyi</i>		
BB152	BB120 <i>luxL</i> ::Tn5 (AI-1 ⁻ , AI-2 ⁺), Km ^r	(Surette and Bassler 1998)
BB170	BB120 <i>luxN</i> ::Tn5 (sensor 1 ⁻ , sensor 2 ⁺), Km ^r	(Bassler et al. 1993)
Plasmids		
pKK223-3	Cloning vector, Ap ^r	Pharmacia Biotech
pTrcHisA,B,C	Cloning vector, Ap ^r	Invitrogen
pBADHisA	Cloning vector, Ap ^r	Invitrogen
pTrcHisCAT	pTrcHis derivative, Ap ^r	Invitrogen
pKKluxS	pKK223-3 derivative, <i>luxS</i> ⁺ Ap ^r	This study
pTO	pTrcHisA derivative, containing <i>opd</i> , Ap ^r	(Srivastava et al. 2000)
pBO	pBADHisA derivative, containing <i>opd</i> , Ap ^r	This study
pBOL	pBO derivative, containing <i>luxS</i> from W3110, Ap ^r	This study
pBOL-LacI ^q	pBO derivative, containing <i>luxS</i> from W3110 and <i>lacI</i> ^q , Ap ^r	This study
pBC	pBO derivative, containing <i>cat</i> , Ap ^r	This study
pBCL	pBC derivative, containing <i>luxS</i> from W3110, Ap ^r	This study
pBCL-LacI ^q	pBC derivative, containing <i>luxS</i> from W3110 and <i>lacI</i> ^q , Ap ^r	This study
TrcHisGFPuv	pTrcHisB derivative, containing <i>gfpuv</i> , Ap ^r	(Cha et al. 2000)
pBG	pBO derivative, containing <i>gfpuv</i> , Ap ^r	This study
pBGL	pBG derivative, containing <i>luxS</i> from W3110, Ap ^r	This study
pBGL-LacI ^q	pBG derivative, w/ <i>luxS</i> from W3110 and <i>lacI</i> ^q , Ap ^r	This study
pTrcHis-LuxS	pTrcHisC derivative, containing <i>luxS</i> from W3110, Ap ^r	(Barrios et al. 2006)
pTrcHis-Pfs	pTrcHisC derivative, containing <i>pfs</i> from W3110, Ap ^r	(Barrios et al. 2006)

Table 2-2 Oligonucleotide primers used in this study

Name	Sequence	Relevant description
LuxSF	CCTTGAATTCAGGATGCCGTTGTTA GATAGC	Upstream primer for cloning <i>luxS</i> from W3110
LuxSR	AACTGAATTCGGCTAGATGTGCA GTT	Downstream primer for cloning <i>luxS</i> from W3110
pkk223LuxSF	ACGCATATGTCCTACTCAGGAGAG CGTTCA	Upstream primer for cloning <i>tac</i> promoter- <i>luxS</i> fusion from pKK- <i>luxS</i>
pkk223LuxSR	AGCCATATGTCGCTCAAGGCGCACT CCCG	Downstream primer for cloning <i>tac</i> promoter- <i>luxS</i> fusion from pKK- <i>luxS</i>
LacI ^q F	GGAGCTGCATGTGTCAGAGGTT	Upstream primer for cloning <i>lacI^q</i> from pTrcHisB
LacI ^q R	CAAAAAACATTATCCAGAACGGGA G	Downstream primer for cloning <i>lacI^q</i> from pTrcHisB
FCAT	TAAAAGACATGTGGGGTTCTCATCA TCATC	Upstream primer for cloning <i>cat</i> and <i>gfpuv</i> from pTrcHisCAT and pTrcHisGFPuv respectively.
RCAT2	TTAATGTTTAGCGGCCGCTTAAAAA AATTACGC	Downstream primer for cloning <i>cat</i> from pTrcHisCAT
RGFPuv	TTAATGTTTAGCGGCCAGCTTT CATTATTT	Downstream primer for cloning <i>gfpuv</i> from pTrcHisGFPuv

Culture conditions: Primary *E. coli* inoculums consisting of LB medium, glucose (0.8%), ampicillin (100 $\mu\text{g mL}^{-1}$, Sigma) and frozen *E. coli*, were grown for 4 hr at 37°C with 250rpm shaking, then 1% (v/v) inoculated into overnight cultures in defined medium (~16 h at 30°C and 250 rpm) (DeLisa et al. 2001b). To initiate experimental cell growths, overnight cultures were inoculated into 40 mL defined medium and volumes were adjusted to achieve similar initial cell densities ($\text{OD}_{600} = 0.10$). For conditioning experiments (Figure 2-1), mid-log phase ($\text{OD}_{600} \sim 0.25$) cells were spun down gently (2500 x g for 5 min, 4°C) and resuspended in either fresh defined medium, defined medium + 10% (v/v) CM or defined medium + 50% (v/v) CM. For co-expression experiments, arabinose (Sigma) or arabinose and IPTG (Sigma) were added to mid-log phase cultures ($\text{OD}_{600} \sim 0.40$).

Preparation of cell-free culture fluids and conditioned medium: Cell-free culture fluids were prepared by centrifugation of 1-mL *E. coli* whole broth samples for 10 min (10,000 x g at 4°C). Cleared supernatants were passed through 0.22 μ m Sterile Millex filters (Millipore) and stored at -20°C. *V. harveyi* BB152 cell-free culture fluids were prepared likewise to obtain positive control samples as reported previously. CM was prepared by growing W3110 or MDAI2 in LB + 50 mM glucose or defined medium +50 mM glucose to an OD₆₀₀ = 3.0 (~6-8 hr) followed by centrifugation (10 min, 10,000 x g at 4°C) and filtering of cleared supernatants by vacuum driven filter (Corning). The detailed preparation of cell-free culture fluids for AI-2 activity assays and for conditioning experiments were also described previously (DeLisa et al. 2001b; Wang et al. 2005a).

Analytical measurements of AI-2 activity The AI-2 activity assay was based on the reports of Surette and Bassler (Surette and Bassler 1998; Surette et al. 1999). Luminescence was measured hourly as a function of *V. harveyi* cell density by quantitating light production with a luminometer (EG&G Berthold). Data reported as fold activation were obtained by dividing the light produced by the reporter cells after addition of *E. coli* cell-free culture fluids by the light output from the reporter cells while growth medium alone was added.

Western blotting and protein activity assays: Culture volumes equivalent to 2 ml at OD₆₀₀=1.0 were withdrawn from experiments and centrifuged at 10,000xg for 10 min. The cell pellets were resuspended and lysed in 300 μ l BugBuster protein

extraction reagent (Novagen) under room temperature for 30 minutes, and then centrifuged again at $10,000\times g$ for 10 min to separate soluble and insoluble cell extracts. We found this lysis method was complete, systematic and reproducible. Protein concentration of soluble cell extracts was determined by the protein assay kit (Bio-Rad Lab). Insoluble cell debris was resuspended with 0.1 ml resuspension buffer (0.06M Tris-HCl (pH6.8)). The soluble cell extracts or insoluble debris were 1:1 (v/v) mixed with SDS sample buffer (12.5% 0.5M Tris-HCl (pH=6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β -mercaptoethanol, 0.0025% bromophenol blue), heated at 100°C for 5 min, and centrifuged for 1 min. Samples were loaded onto 12.5% SDS polyacrylamide gels for electrophoresis and blotted onto nitrocellulose membranes (BioRad) using a mini-trans blot cell (BioRad) and Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 29 mM glycine, 20% methanol) for 30 min at 20V. Monoclonal anti-polyhistidine (Sigma), polyclonal anti-OPH (kindly provided by Dr. J. Grimsley), monoclonal anti-GroEL, and monoclonal anti-DnaK (Stressgen) were diluted 1:4000 in antibody buffer (0.5% Tween-20 (v/v), Tris-buffered saline with 1% (w/v) non-fat dry milk) to probe recombinant proteins. The membranes were then transferred to a 1:4000 diluted goat-anti-mouse or goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). Membranes were developed with 1:50 diluted nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate (NBT/BCIP) solution (Roche Molecular Chemicals). Lastly, the membranes were scanned and the images were analyzed using ImageJ software (<http://rsb.info.nih.gov/ij/>). Activity of soluble CAT within crude cell extracts was measured according to Rodriguez and Tait(Surette and Bassler 1998), OPH activity was measured as per Wu et al.(Wu et al.

2000), and GFP activity of 1-mL whole cell samples was measured using a Perkin-Elmer LS-3B fluorescence spectrometer at excitation and emission wavelengths of 395 and 509 nm, respectively. Finally, specific CAT and OPH activity were reported as activity divided by total protein concentration (Rodriguez 1983; Wu et al. 2000).

Synthesis and fractionation of *in vitro* AI-2 His₆-Pfs and His₆-LuxS were overexpressed (Barrios et al. 2006; Schauder et al. 2001) under 1 mM IPTG induction of BL21(pTrcHis-pfs) and BL21(pTrcHis-luxS) as cell densities were grown to OD₆₀₀=0.4~0.6 at 37°C. The cells were harvested after 4 hr induction by centrifugation at 14000xg under 4°C for 20 min. After lysis using BugBuster solution (Novagen) at room temperature for 40 min, the soluble cell extracts were mixed with Co²⁺ affinity resin (BD TALONTM, BD Biosciences), and the bound His₆-Pfs and His₆-LuxS was washed three times using phosphate buffer (pH=7.4) (Sigma) to remove non-specifically bound proteins. The purified enzymes were eluted (125 mM imidazole in phosphate buffer, pH=7.4) and used to synthesize AI-2 from 1 mM S-adenosylhomocysteine (SAH) in 50 mM Tris-HCl (pH=7.8) under 37°C for 4hr (Barrios et al. 2006). The enzymatic reaction product was twice extracted by chloroform and recovered from the aqueous phase. To remove unreacted substrate, SAH, and byproducts, adenine and homocysteine, *in vitro* AI-2 reaction product was fractionated by HPLC with a preparative silica reverse-phase column (25×10cm), using 90% water : 10% acetonitrile eluent at flow rate of 3ml/min by Dynamax SD-200 pumps (Varian Inc., Walnut Creek CA). Absorbance at 210nm and 260nm was recorded using a UV-D II dual wavelength UV-vis detector. After fractionation,

acetonitrile was evaporated from each aliquot for 1.5 hr by CentriVap concentrator (Labconco) and analyzed for AI-2. Fractionated *in vitro* AI-2 was further confirmed by mass spectrometry using a JEOL AccuTOF electrical mass spectrometer (dual ESI+ ionization; mass ranges from 100 to 1000m/z were monitored).

2.3 Results

“AI-2-conditioned” cultures exhibit increased chloramphenicol acetyltransferase (CAT), and organophosphorus hydrolase (OPH). In earlier studies, we observed a significant drop in AI-2 level after the induction of recombinant proteins (DeLisa et al. 2001b); we asked a simple question: what if we were to restore AI-2 into the growth medium when we induce recombinant proteins? Would enhanced cross-talk result and would protein yield increase? We performed a series of simple experiments wherein conditioned medium (CM) with or without AI-2 was added to cultures at the same time as the inducer of recombinant proteins (IPTG). Thus, W3110/pTrcHis-X cells (where X = CAT and OPH) were cultured to mid-log phase and resuspended in varying concentrations of conditioned media (CM) (10%, 50%), which were from AI-2 producing (+AI-2) or *luxS* mutant (-AI-2) cells, then immediately induced with 1 mM IPTG. Accordingly, for the CAT producing cultures, AI-2 was initially highest in the 10% and 50% CM (+AI-2) cases, and thereafter approached similar levels as the remaining control cultures (Figure 2-1A). Similar AI-2 results were obtained for *E. coli* cultures producing OPH (not included here). W3110 produced AI-2 via the normal metabolic pathways and MDAI2 is an isogenic *luxS* knockout, so our results

reflect behavior due to an imposed large differential in AI-2 activity with presumably little other differences in the CM (DeLisa et al. 2001c).

Remarkably, the expression levels of CAT (25 kDa) and OPH (36 kDa) both increased 2 to 4 fold relative to control cells identically resuspended in CM from MDAI2 cells (-AI-2; Figure 2-1B & D). In both cases, the yield in terms of specific activity increased concomitantly, with activities in +AI-2 CM cultures reaching 4-fold higher than controls (Figure 2-1C & E). We note the enhancements observed were typically greatest during the periods where the AI-2 levels were most disparate (first three hours).

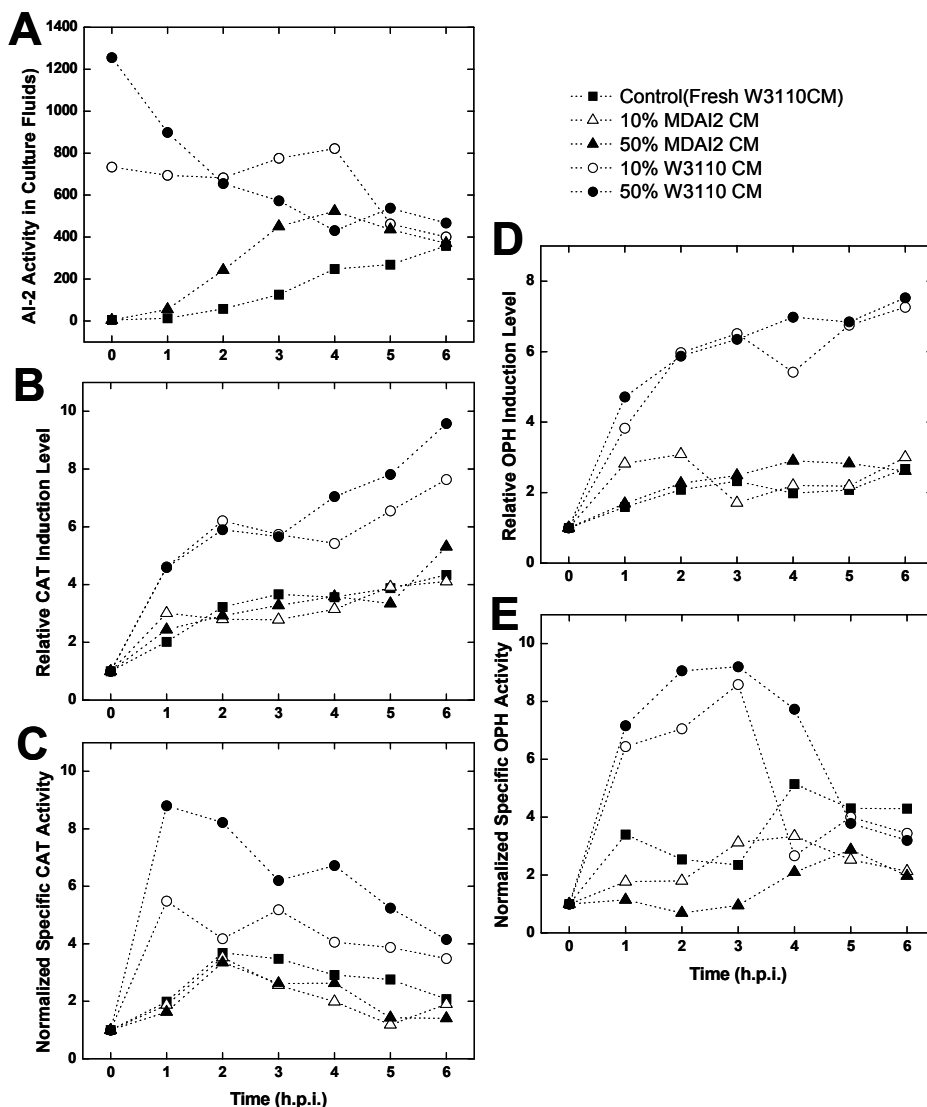


Figure 2-1 exogenous AI-2 from culture fluids enhances recombinant protein production

AI-2 level was modulated in W3110/pTrcHis-X (X=CAT or OPH) cell cultures by resuspending cells in CM containing AI-2 activity (+AI-2, circles, generated from W3110 wild type) or lacking AI-2 activity (-AI-2, triangle, generated from MDAI2 *luxS* cells). Recombinant protein expression was induced at t = 0 h (1 mM IPTG). **(A)** AI-2 activity in W3110/pTrcHis-CAT culture fluids. **(B)** Relative CAT induction level and **(C)** normalized specific CAT activity. Results demonstrate exogenously-added AI-2 enhances CAT production. **(D)** and **(E)** show similar results for W3110/pTrcHis-OPH. Induction level and normalized specific activity are reported as Western band intensity and specific activity of each sample, respectively, relative to the pre-induced (t = 0 h) value. Reported blot intensities and activity levels are the average of duplicate experiments and agreed to within 15%.

Construction of controllable LuxS co-expression system While excited by the results of condition medium addition experiments, we recognize that runtime addition of media and/or *in vitro*-produced signaling molecules is both inefficient and expensive for commercial systems. More importantly, directly ascribing the differences in yield to the presence or absence of AI-2 is premature based on these experiments. Notably, some reports have pointed to the dual function of LuxS as both a signal molecule synthase and a key metabolic enzyme (Sperandio et al. 2001; Winzer et al. 2002a; Winzer et al. 2003). While the experiments shown in Figure 2-1 suggest alternatives in AI-2 signaling are responsible for enhanced yield, they are not sufficient to validate this hypothesis.

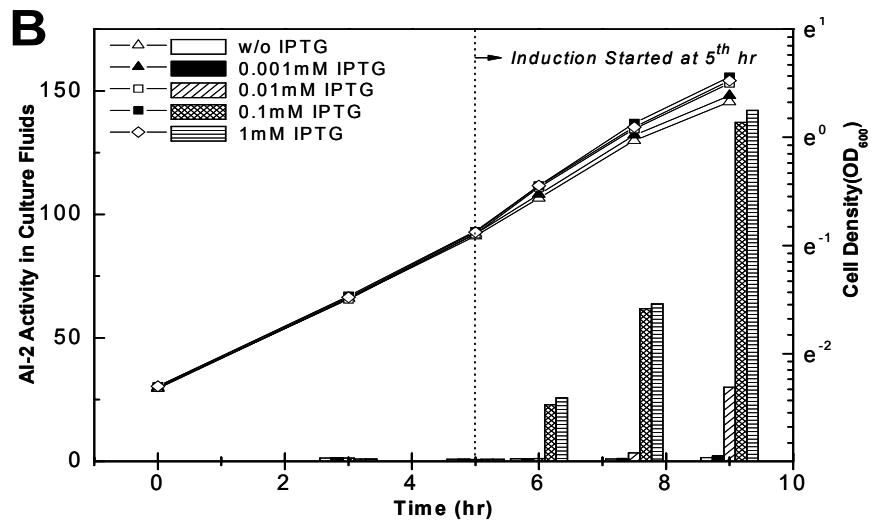
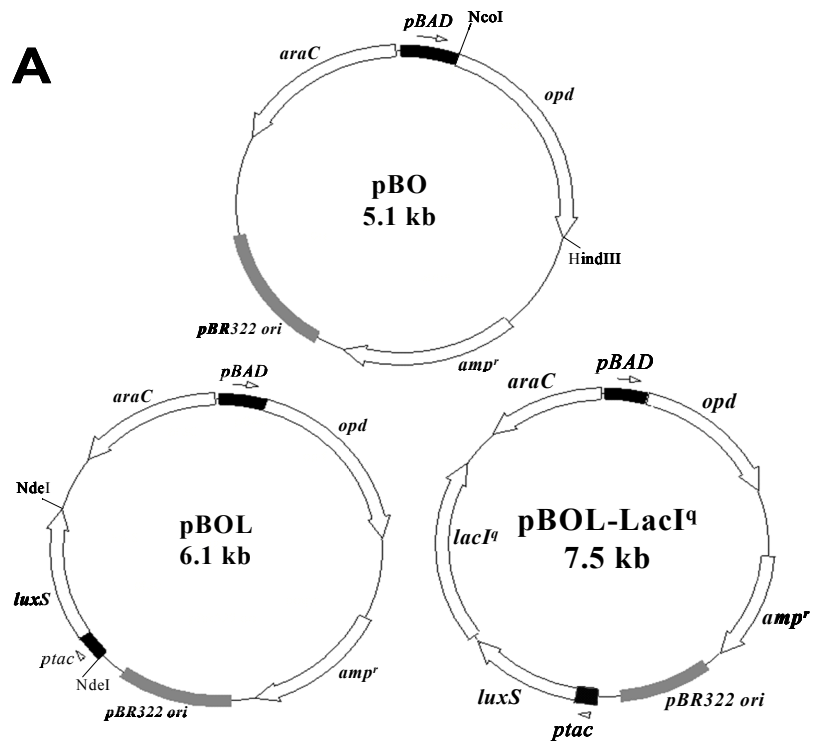
To generate a more commercializable scheme and to more systematically investigate how QS affects recombinant protein productivity, we constructed LuxS co-expression vectors for *in vivo* generation of AI-2 as well as the production of recombinant proteins – wherein LuxS and the product proteins were independently controlled under different controllable promoters. In this way, we also intentionally and systematically manipulate quorum sensing pathways for testing effects on recombinant protein productivity. MDAI2, a *luxS* null mutant host, was used as the background host to enable a full range of AI-2 “tuning” [from near zero (mutant and LacI^q-repressed culture) to high levels (LuxS overexpression)]. Organophosphorus hydrolase (OPH) was selected as the first model product because its expression in *E. coli* has been proven difficult (Wu et al. 2000). In order to co-express *luxS*, *ptac-luxS* (IPTG-inducible sequence) was inserted into pBO, which produces OPH under the control of *araBAD* (arabinose-inducible) promoter (Figure 2-2A). Further, to

minimize background *luxS* transcription, *lacI^q* was inserted into pBOL yielding pBOL-LacI^q (Figure 2-2A). These vectors enable independent control of *luxS* and *opd*.

To determine if *luxS* expression could modulate AI-2 level, MDAI2 (pBOL-LacI^q) were grown to mid-log phase ($OD_{600} \approx 0.4$) in defined minimal medium supplemented with 0.8% glucose which, in turn, ensures high AI-2 activity (Surette and Bassler 1998; Wang et al. 2005a). IPTG was added at varying levels (0 to 1mM) after 5 hr, when cells would otherwise be induced for recombinant protein expression. We found AI-2 levels in the extracellular media spanned a 150-fold range after an additional 4 hours. The more immediate AI-2 activity differences (< 1 hr) were substantially less, but a 25-fold difference was still observed between the 0 and 1mM IPTG cases. Interestingly, SDS-PAGE gels were run and no significant LuxS bands were revealed (LuxS MW=19.4kD), suggesting that even 1mM did not result in dramatic LuxS overexpression (not shown). These results demonstrate that LuxS expression in a *luxS* null mutant can significantly alter the AI-2 level found in extracellular fluids.

Figure 2-2 LuxS and recombinant protein co-expression vectors

(A) pBO expresses *opd* under arabinose-inducible promoter *araBAD* control. An expression cassette of the IPTG-inducible promoter *ptac* and the *luxS* gene was inserted into pBO, yielding pBOL. To more effectively regulate *luxS* expression, LacI^q was inserted into pBOL, yielding pBOL-LacI^q. (B) Modulation of AI-2 via varied *luxS* expression was carried out by addition of different IPTG levels to MDAI2 (pBOL-LacI^q) cultures. At different time points during cell growth, aliquots were collected for measurement of cell density (lines) and AI-2 activity (bars). The AI-2 values shown here are representative of three independent experiments. Replicate assays agree within 15%.



Co-expression of LuxS significantly improves recombinant OPH protein

production in MDAI2. Wild-type *E. coli* W3110 and *luxS* isogenic knockout MDAI2 were transformed with plasmids pBO, pBOL, and pBOL-LacI^q, and grown to mid-log phase (OD₆₀₀ ≈ 0.4) in defined minimal medium containing 0.8% glucose. Arabinose (0.2%) was added to each culture to induce *opd*. Additionally, IPTG was added at varied levels (0 and 0.01mM) to the cultures containing pBOL-LacI^q to induce *luxS*. The results from the 0.01mM case are depicted in Figure 2-3. We found that 0.01 mM IPTG was sufficient to generate, but not rapidly accumulate AI-2 (Figure 2-2). The growth rates of MDAI2 (pBOL- LacI^q) with or without *luxS* induction were both slower than MDAI2 (pBO) and MDAI2 (pBOL) (Figure 2-3A).

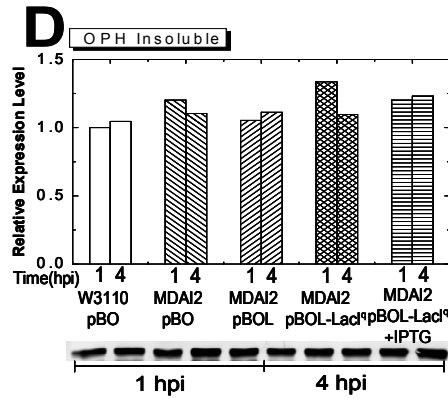
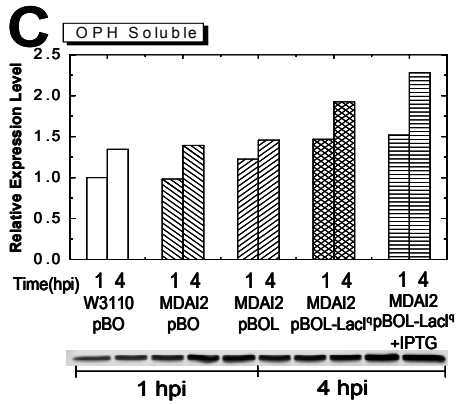
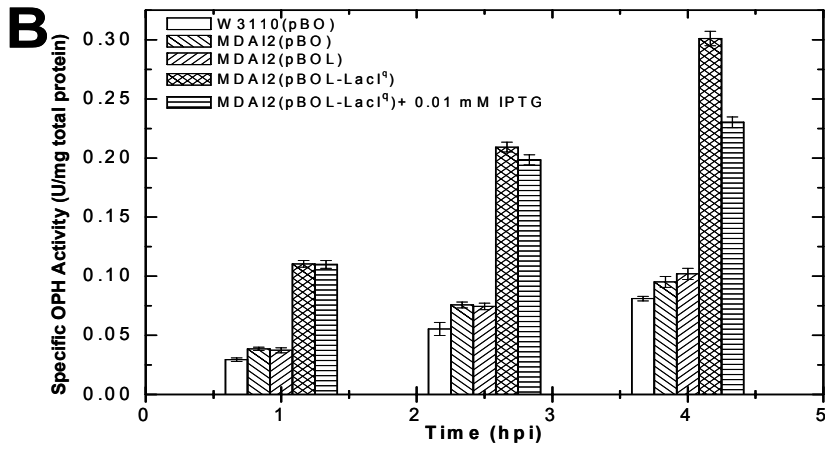
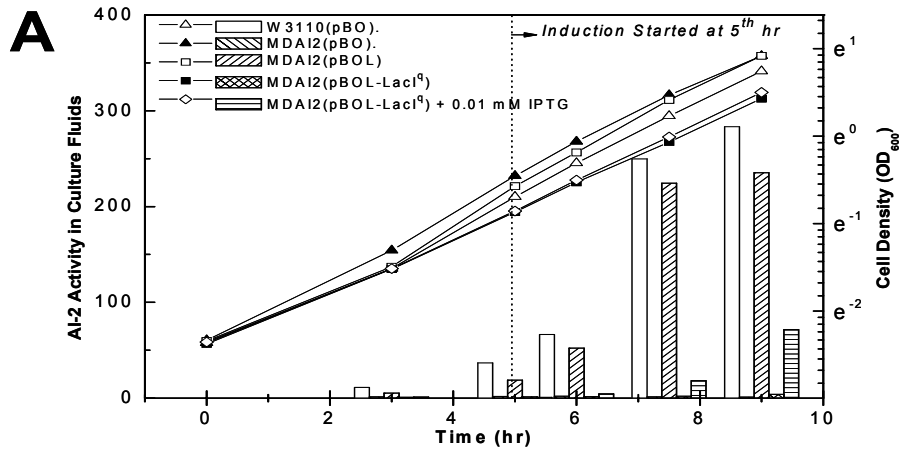
Remarkably, in both MDAI2 (pBOL- LacI^q) cases, a 3 to 4-fold increase in specific OPH activity was observed (Figure 2-3B). We also examined the relative expression levels of OPH via Western blot and found an appreciable increase (~1.5-fold) in OPH in the soluble fraction of cell extracts (Figure 2-3C). The level of OPH found in the insoluble fractions was constant among all cultures (Figure 2-3D). The nearly 1.5-fold increase in soluble OPH at 4 hpi, however, was insufficient to account for the increased specific activity (4-fold) (Figure 2-3B). Thus, the OPH was of higher activity due to mechanisms beyond simple expression rate and is attributed to the presence of LuxS.

The AI-2 activity in wild-type W3110 was highest among all cultures, reaching ~300 fold by 9 hrs. MDAI2 cells containing unrepressed pBOL, reached similar levels (~250 fold AI-2 activity). As expected, MDAI2 (pBO) produced no AI-2 and MDAI2 (pBOL- LacI^q) without IPTG also resulted in insignificant AI-2

accumulation. The addition of 0.01 mM IPTG to this culture, however, resulted in similar AI-2 levels as previous experiments without arabinose (~50 fold AI-2 activity at 4 hpi, Figure 2-2). These results demonstrate no proportional correlation between AI-2 level and OPH yield, but instead suggest that manipulation of AI-2 synthesis capacity and/or homocysteine synthesis via modulation of *luxS* gene expression can have a significant impact both on protein activity (quality) and yield (quantity). Alternatives in amino acid metabolism have previously proven beneficial (Ramirez and Bentley 1993). These results, therefore, provided further motivation to pinpoint the features of AI-2-mediated quorum circuitry which contributed to the apparent increase in yield. First, however, we checked to see whether improved yield was general by examining two additional recombinant proteins expressed in *E. coli*.

Figure 2-3 OPH accumulation and activity are both enhanced significantly by modulating LuxS expression in co-expression system.

(A) OPH was expressed in *E. coli* W3110 (wild type) and MDAI2 (*luxS*⁻) by 0.2% arabinose induction and altered AI-2 signaling. That is, MDAI2 (pBOL- LacI^q), with and without 0.01 mM IPTG, were compared with those in W3110 (pBO), MDAI2 (pBO), and MDAI2 (pBOL) when identical levels of arabinose (0.2%) were added. Throughout, the cell density (lines) and AI-2 activity (bars) were observed. **(B)** After induction, samples were collected and lysed. OPH activity in each sample was measured and divided by the total protein concentration to derive specific OPH activity. The data shown here are representative of two independent experiments. The errors shown are standard errors from triplicate OPH activity and total protein assays. **(C)** and **(D)** OPH accumulation levels in the soluble and insoluble fractions of cell extracts were examined 1 hpi and 4 hpi by Western blots. The results shown here are not pooled, but instead are representative of triplicate experiments (which agree to within 20%).



CAT and GFPuv as model proteins co-expressed with LuxS. In previous work, the recombinant proteins' amino acid composition proved to be important in observing an enhanced yield (Ramirez and Bentley 1993). We replaced the *opd* gene in the plasmids noted above (pBO, pBOL, and pBOL-LacI^q) with *cat* or *gfp_{uv}* for the overexpression of CAT and GFPuv. CAT, GFPuv and OPH all have dissimilar amino acid compositions. All recombinant proteins are under control of arabinose-inducible *araBAD* promoter, and LuxS remains independently regulated by the IPTG-inducible *ptac* promoter. Again, W3110 and MDAI2 were transformed with these two sets of plasmids and the LuxS co-expression experiments were executed under the same conditions as OPH and LuxS co-expression experiments depicted above. In all cases, co-expression of LuxS increased specific activities of recombinant model proteins, CAT (~1.5 fold) and GFPuv (4~6 fold) (Figure 2-4). Protein expression levels were also investigated via Western blot. In general, both CAT and GFPuv were found to increase in both soluble and insoluble fractions (not shown). These data support the notion that LuxS co-expression increased yield and activity of recombinant proteins, irrespective of the protein's native origin and composition (GFPuv, CAT, and OPH).

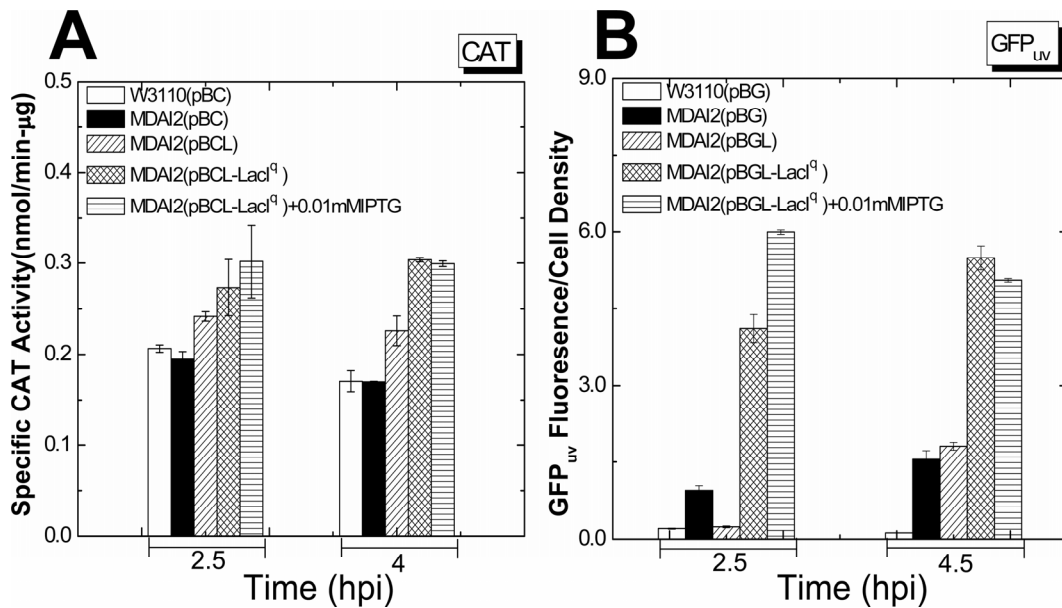


Figure 2-4 Specific activities of CAT and GFP_{uv} are enhanced in the LuxS co-expression system.

(A) and (B) CAT and GFP_{uv} were expressed in *E. coli* W3110 and MDAI2 by 0.2% arabinose induction and at different AI-2 levels (by varied IPTG). CAT activities were divided by total protein level of each cell extract to generate the specific CAT activities. However, in order to derive specific GFP_{uv} activities, fluorescence results of GFP_{uv} were divided by cell density (OD600) directly instead of total protein concentration of each sample. Both CAT and GFP_{uv} co-expression experiment were duplicated to confirm reproducibility; data shown here are representative and the errors are from triplicate assays.

Chaperone protein, GroEL, is affected by *luxS* co-expression. It is broadly recognized that chaperone proteins are accessory factors that play key roles in the assembly and folding of heterologous proteins synthesized in *E. coli* (Gragerov et al. 1992; Thomas and Baneyx 1996a). It is also recognized that the abundance of heat shock proteins (hsps, including chaperones and proteases) is influenced by heterologous protein overexpression and, in turn, can impact the protein yield (Bentley et al. 1990; Harcum and Bentley 1993a; Kanemori et al. 1994; Ramirez and Bentley 1995; Thomas and Baneyx 1996a). We have previously demonstrated that avoiding (Ramirez and Bentley 1995) or intentionally downregulating (Srivastava et al. 2000) the heat shock response coincident with protein overexpression can facilitate increased yield and activity of CAT and OPH (Srivastava et al. 2000). To ascertain whether *luxS* co-expression leads to increased yield through the pleiotropic regulation of hsps, we measured the levels of two important heat shock proteins, GroEL and DnaK, as well as transcription of several other proteins in the presence and absence of varied LuxS expression.

The amounts of GroEL and DnaK in MDAI2 cultures induced with arabinose to synthesize OPH were examined both 1 and 4 hpi (Figure 2-5). In all cases where *luxS* was present or intentionally induced, the GroEL level in the soluble fractions was higher (up to 3~4-fold) than in controls (W3110 (pBO) and MDAI2 (pBO), Figure 2-5A). The GroEL level in the insoluble fractions of all cultures was similar in all cases (Figure 2-5B). The level of DnaK in the soluble fractions was also typically unchanged, although there was a 60% increase in the cases where LuxS was regulated by LacI^q (Figure 2-5C). There was no detectable DnaK in any of the insoluble

fractions (not shown). Importantly, in the cases where soluble GroEL increased the most (MDAI2, *lacI^q*, +/- IPTG), we found the highest and most active levels of OPH (Figure 2-3B&C). In separate experiments (data not shown), the independent presence of *LacI^q* was tested; results demonstrated that *LacI^q* had no effect on protein yield or GroEL/DnaK.

While the overexpression of non-native proteins has previously been shown to increase the levels of GroEL and DnaK in *E. coli* (Kanemori et al. 1994; Srivastava et al. 2000), we attempted to explore whether LuxS had an independent effect on these important chaperones, irrespective of the recombinant product. Hence, MDAI2 (pBOL- *LacI^q*) cultures were supplemented with different levels of IPTG to vary LuxS expression and the two chaperones were examined by Western blot (Figure 2-5D-F). These experiments were also described in Figure 2-2 and depict altered levels of *luxS* induction with no background *opd* expression (as confirmed by activity measurements, data not shown). Interestingly, GroEL was notably upregulated in the soluble fractions in cultures with IPTG at or above 0.01 mM IPTG (Figure 2-5D) and was moderately downregulated in the insoluble fractions of the same cultures (Figure 2-5E). There was no significant difference in DnaK found in the soluble fractions (Figure 2-5F), and there was no observable DnaK in the insoluble fractions (not shown). These results demonstrate that LuxS expression in a *luxS⁻* host can modulate levels of GroEL in both soluble and insoluble fractions, and suggest that an appropriate LuxS expression level could be found that is coincident with an appropriate GroEL level that facilitates the folding of target proteins in *E. coli*.

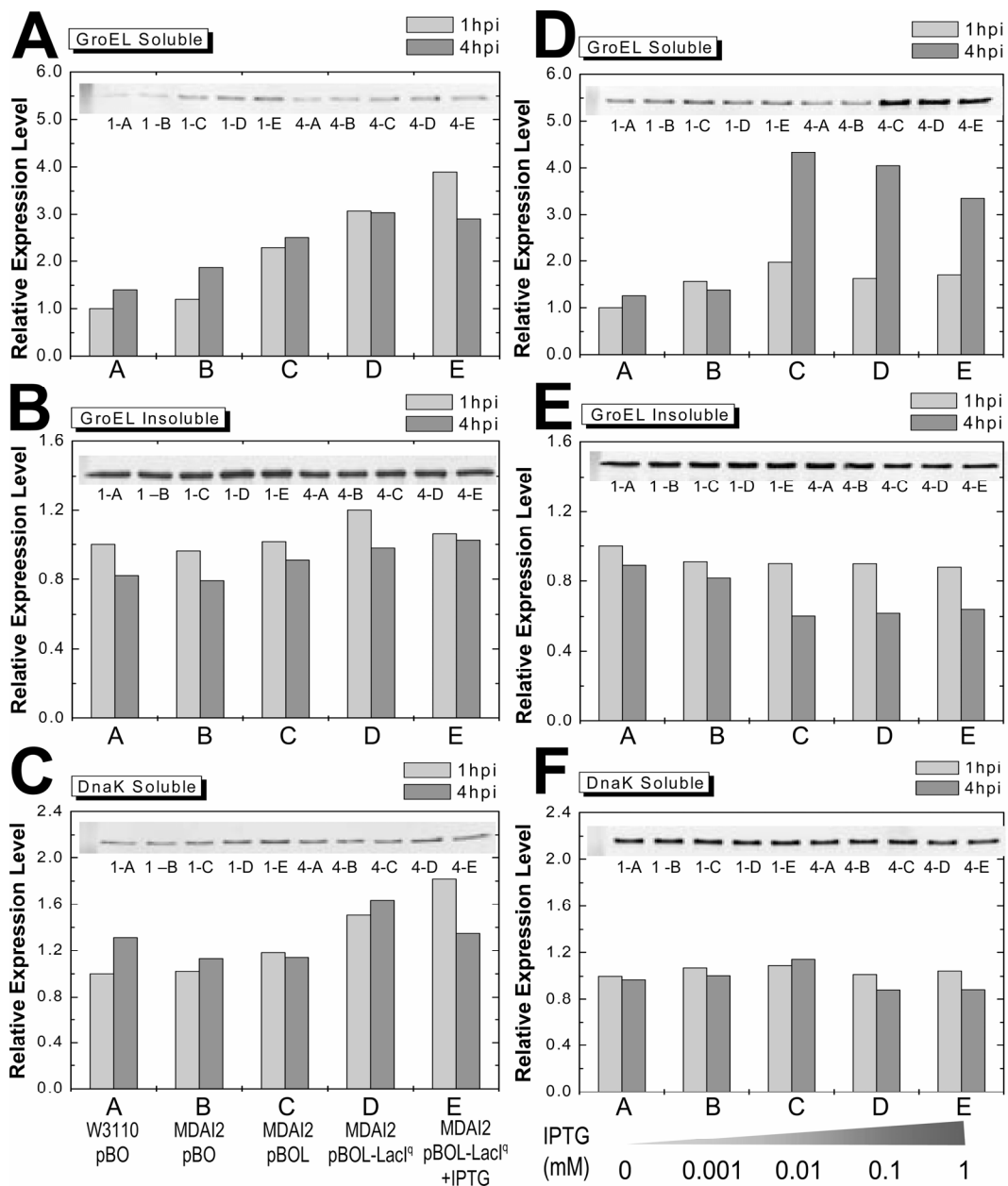


Figure 2-5 the expression level of chaperone protein GroEL in soluble cell extracts is significantly higher than controls in the *luxS* modulated system. (A), (B), and (C) the amounts of GroEL and DnaK in cultures induced with arabinose to synthesize OPH at both 1 and 4 hpi were examined by Western blot. MDAI2 (pBOL- LacI^q), with and without 0.01 mM IPTG, were compared with W3110 (pBO), MDAI2 (pBO), and MDAI2 (pBOL) when identical levels of arabinose (0.2%) were added. (D), (E), and (F) MDAI2 (pBOL- LacI^q) cultures were supplemented with different levels of IPTG to vary LuxS expression in the absence of recombinant protein synthesis. GroEL and DnaK were examined by Western blot.

Does AI-2 communicate with GroEL? To this point, we have manipulated QS by tuning LuxS expression, achieved higher yield of several proteins, and found that chaperone GroEL was upregulated. However, an open question remained. That was, whether the expression of LuxS led to increased GroEL (as a stress response) or whether AI-2 signaling played the key role in altering the expression landscape. In order to clarify this, we added *in vitro* synthesized AI-2 to MDAI2 cells and asked whether or not GroEL was increased in the soluble fraction. In Figure 2-6A., we synthesized AI-2 *in vitro* (Barrios et al. 2006; Schauder et al. 2001) and fractionated the reaction mixture using high performance liquid chromatography (HPLC) to remove the byproducts and any unreacted SAH (Figure 2-7 and Figure 2-8). AI-2 levels in treated MDAI2 cultures decreased steadily, and growth rates were unaffected (data not shown). The two chaperones, GroEL and DnaK, were observed by Western blot (Figure 2-6B-D). GroEL increased ~1.5 to 2-fold in the soluble fractions for the first hour when diluted AI-2 was added (20X-100X). There was no apparent difference in the level of GroEL in the insoluble fraction (Figure 2-6C). There was no observable trend in soluble DnaK (Figure 2-6D), and no insoluble DnaK was detected under any of the conditions (data not shown). Additionally, results after 2 hpi showed no conclusive changes in GroEL or DnaK levels in response to AI-2. We might attribute these phenomena to the rapid decrease in AI-2 activity that occurred when exogenous AI-2 was added to the mutant cultures. These results demonstrate that the QS signal molecule, AI-2, affects the level of GroEL in the soluble fraction of *E. coli*, and suggests further that the expression of LuxS in *luxS*

mutants, regulates the chaperone GroEL and that this contributes to the enhancement of the recombinant protein productivity in *E. coli*.

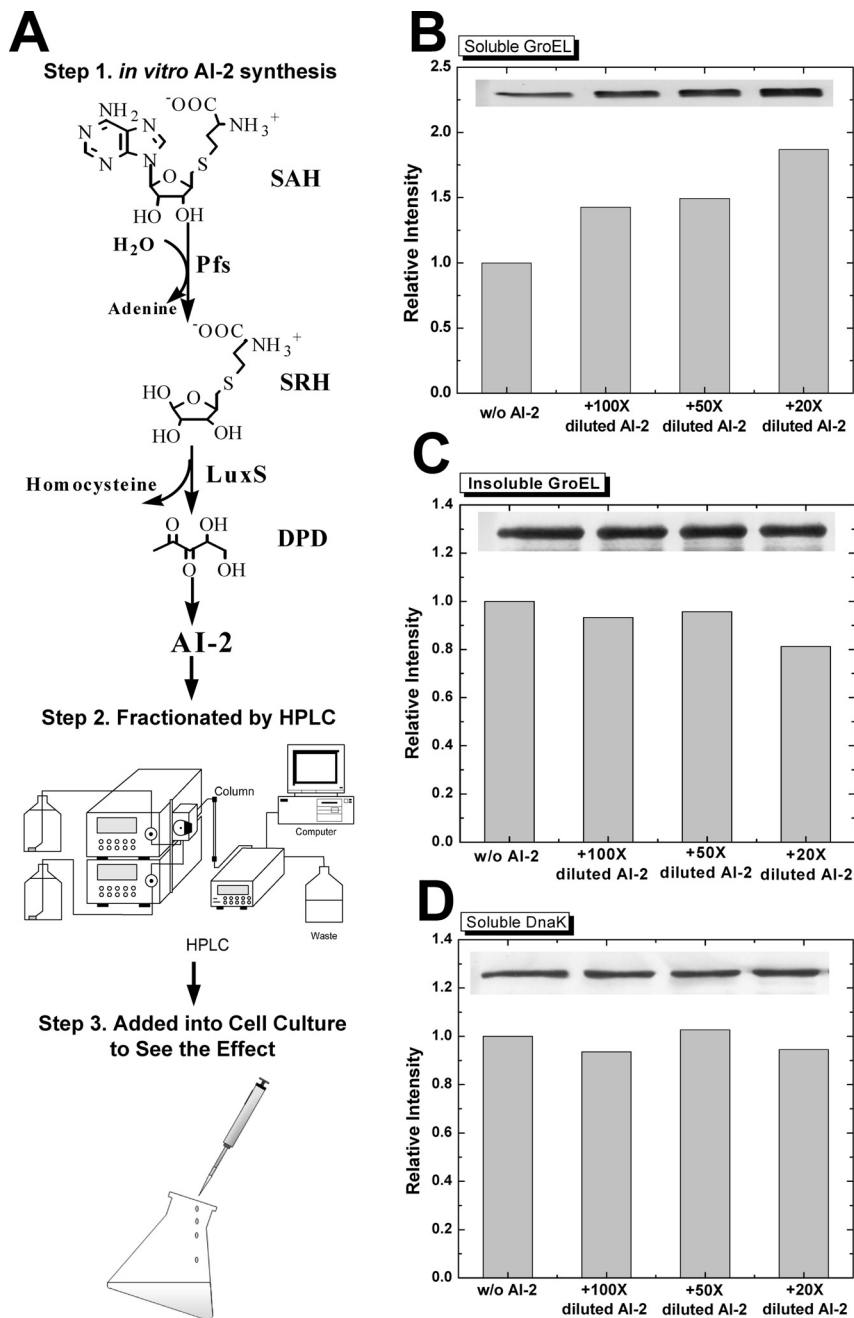


Figure 2-6 *in vitro* synthesized AI-2 increases soluble GroEL level.

(A) The scheme depicts synthesis, fractionation, and addition of AI-2 to cell cultures. First, AI-2 is synthesized *in vitro* from substrate SAH. Second, any un-reacted SAH and byproducts, homocysteine and adenine, are removed by HPLC. After mobile phase solvent removal via vacuum pump, the fractionated AI-2 was added to MDAI2 cell cultures. (B) Chaperones, GroEL and DnaK, were analyzed by Western blot. The results shown here are representative from duplicate experiments and triplicate assays. Results are within +/- 20%.

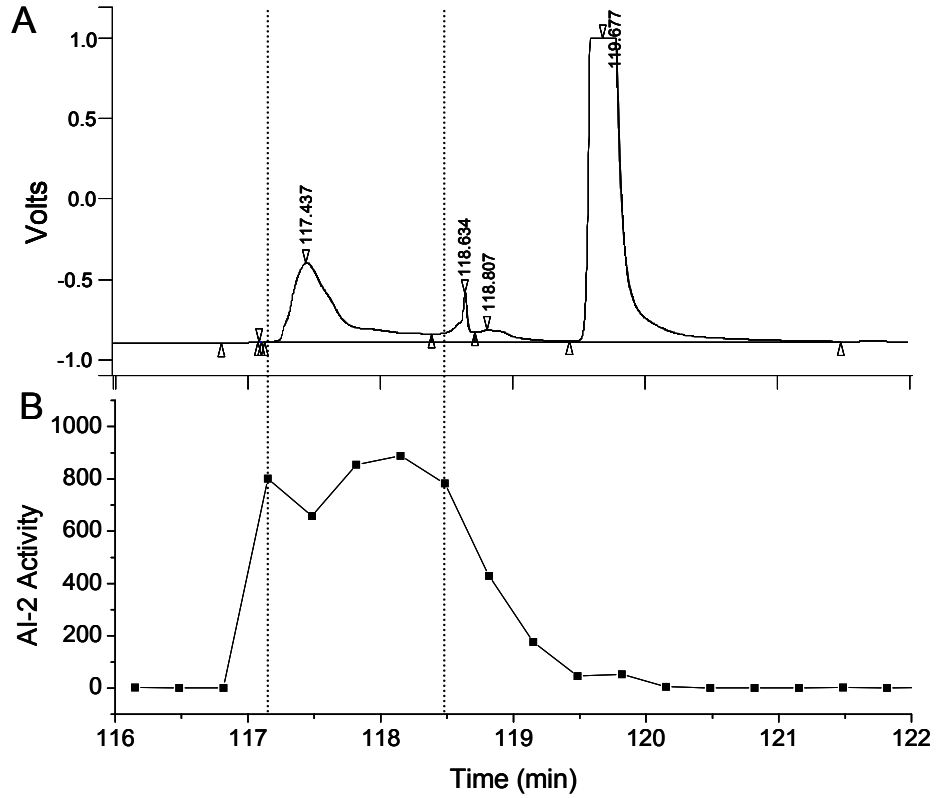
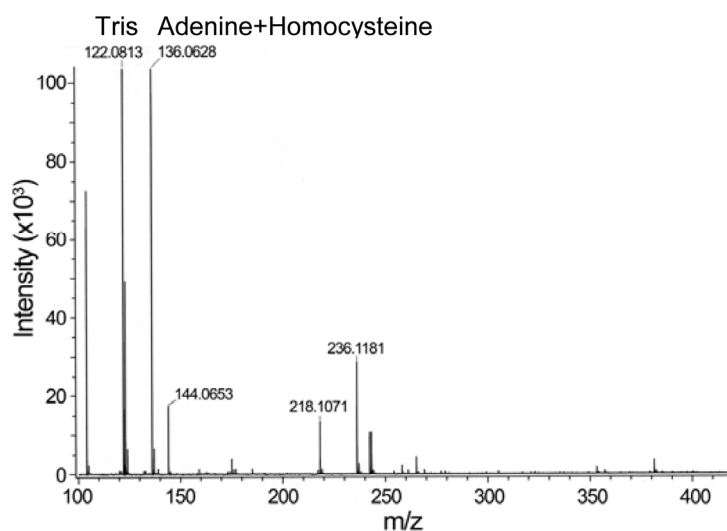


Figure 2-7 Collection of the HPLC fractionated *in vitro* AI-2 with activity
 (A) HPLC chromatogram of the *in vitro* synthesized AI-2 fractionation and (B) AI-2 activity of fractionated samples over different collection periods. We observed that the AI-2 activity was only in the void fraction (window between the dotted lines). The fractionated samples with AI-2 activity were then mixed for mass spectrometry and further experiments

A *In vitro* AI-2



B HPLC Void Fraction

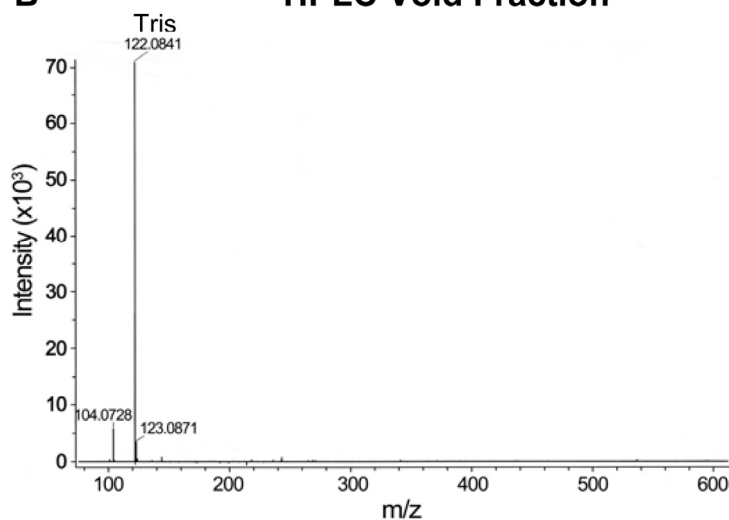


Figure 2-8 Mass spectrometry of *in vitro* synthesized AI-2 with and without HPLC purification

(A) MS analysis of *in vitro* synthesized AI-2 without HPLC fractionation. Adenine and homocysteine are found in superimposed peaks. There was no SAH or SRH detected after the 4hr reaction. (B) After HPLC separation of the *in vitro* synthesis product and AI-2 bioassay, we found the AI-2 activity was only in the void fraction. There was no SAH, SRH, adenine, or homocysteine in void fractions (mass spec result).

2.4 Discussion

Studies of AI-2 mediated QS suggest quorum signaling may communicate the prevailing metabolic condition (DeLisa et al. 2001a; DeLisa et al. 2001b) and that a “tuned” signaling process may potentially enable improved recombinant protein production. There have been no reports on the intentional manipulation of the AI-2 or any other QS system for improving recombinant protein synthesis in bacteria. In Figure 2-1, we demonstrate for the first time that exogenous addition of AI-2-containing CM enhances CAT and OPH production both in quantity (protein yield) and quality (protein activities). Recognizing the potential that many metabolites may have altered concentration in CM from *luxS*⁻ vs. *luxS*⁺ strains (DeLisa and Bentley 2002; Lee and Shuler 2000), we developed a carefully controlled study to investigate and “tune” *luxS*/AI-2 QS during recombinant protein overexpression.

We constructed a *luxS* coexpression system wherein *luxS* and target recombinant proteins (i.e. *opd*, *cat*, and *gfp_{uv}* genes) were expressed under the control of two independent promoters (Figure 2-2). In all cases, both the expression level and activity of the recombinant product was increased when cells were complemented with *luxS* under *lacI^q* control, with or without induction of *luxS* at the same time as the target recombinant protein. Unexpectedly, the best results were found in the *luxS* host, MDAI2. We also demonstrated that for all cases of dramatically improved recombinant protein production, GroEL levels were increased in the soluble fractions. Upregulation of hsps, including GroEL and DnaK, is commonly observed to accompany recombinant protein overexpression, owing to an upregulated heat shock response (Bentley et al. 1990; DeLisa et al. 2001a; Harcum and Bentley 1993a;

Kanemori et al. 1994; Ramirez and Bentley 1995; Thomas and Baneyx 1996a). That is, increases in both *groEL* and *dnaK* transcription (DeLisa et al. 2001a) and GroEL and DnaK protein levels (Bentley et al. 1990; Harcum and Bentley 1993a; Kanemori et al. 1994; Ramirez and Bentley 1995) are typically observed. Notably, in our *luxS* co-expression system, DnaK levels exhibited no systematic trends. Moreover, *dnaK* and *groEL* transcription are unaltered by *luxS* mutation (Wang et al. 2005b), and are seemingly uncorrelated with QS. Hence, the apparent decoupling of (1) GroEL level from its transcription and (2) GroEL from DnaK, suggested that the enhanced level of GroEL was due to other mechanisms than the classic heat-shock-like response (Gill et al. 2000; Gragerov et al. 1992; Kanemori et al. 1994; Schweder et al. 2002). Since *groEL* transcription is apparently unaffected by AI-2, the apparent linkage between the GroEL level and *luxS* co-expression is likely at the post-transcriptional level. We are aware of only one report in which an AI-2 mediated process affects the level of a protein in a manner other than transcriptional regulation. In that report, the AI-2 phospho-relay system of *V. harveyi* is shown to affect endogenous *lux* enzyme activity by modulating translation through the recruitment of small RNAs and RNA chaperone, Hfq (Lenz et al. 2004). There have been no reports of post-transcriptional regulation in *E. coli* that are attributed to QS.

In our experiments in which we added purified AI-2 to cultures of MDAI2, we found that the level of GroEL in the soluble fraction increased significantly within the first hour. Also, we found in several cases that the increase was accompanied by a decrease in the insoluble fraction supporting the absence of a linkage between AI-2 and *groEL* transcription. Rather, this was the first demonstration that AI-2 could alter

soluble GroEL level in *E. coli*. Our experiments with modulated LuxS level point to altered GroEL but do not confirm causality between endogenously synthesized AI-2 and increased GroEL. It is noteworthy that GroEL co-expression (or increased GroEL level) is used to increase the yield of recombinant proteins in *E. coli* (Gamez et al. 2000; Gragerov et al. 1992; Nishihara et al. 1998; Thomas and Baneyx 1996a; Thomas and Baneyx 1996b). Our results clearly demonstrate that altered LuxS expression also altered yield and activity. They also coincidentally demonstrate increased GroEL level with minimal perturbation on the cell growth. Hence, the results suggest a linkage between QS signaling, increased soluble GroEL, and increased yield. Such metabolic “tuning” suggests future efforts directed at varying AI-2, *luxS*, and protein synthesis via altered IPTG and arabinose addition in this system are warranted.

Chapter 3 Rewiring Native AI-2 Quorum Sensing Circuit for True Autoinduction of Recombinant Proteins in *E. coli*

3.1 Introduction

Bacteria communicates cell-to-cell using signal molecules, termed autoinducers (AIs), and this process which enables bacteria to sense and communicate with each other in a population-dependent manner has been termed quorum sensing (QS). This cell-to-cell communication process is mediated by the bacteria's production, secretion, and response to signal molecules (Fuqua and Greenberg 1998a; Surette and Bassler 1998; Waters and Bassler 2005). Many Gram-positive bacteria use peptides as autoinducers (Kleerebezem et al. 1997; March et al. 2003; Waters and Bassler 2005), while many Gram-negative bacteria use small chemical molecules as autoinducers (Fuqua and Greenberg 1998a; Surette and Bassler 1998; Waters and Bassler 2005). The QS signaling relying on autoinducer-2 (AI-2), has evoked researchers' intense interest, because its synthase, LuxS, is found in over 55 bacteria and is hypothesized AI-2 as an inter-species signal molecule (Meijler et al. 2004; Miller et al. 2004; Xavier and Bassler 2005b). QS systems, including the AI-2 system of *E. coli*, are characterized by signal accumulation in the exponential phase, a peak as the cell begin to enter the stationary phase, and rapid decrease (which is glucose dependent) shortly thereafter (Wang et al. 2005a). The rapid decrease is attributed to the uptake process (Wang et al. 2005a; Xavier and Bassler 2005b), and the ATP-binding cassette (ABC) transporter, denoted Lsr. As such, the extracellular activity of

the signal molecule represents an indication of a metabolic switch. In this work we intend to exploit this switch for recombinant protein production.

AI-2 is phosphorylated by LsrK, a cytoplasmic kinase, becoming phospho-AI-2. LsrR, the repressor of the *lsrACDBFGE* operon, interacts with the *lsr* promoter, and is de-repressed by phospho-AI-2 resulting in the induction of *lsr* genes. The conceptual model has been depicted in different reports (Li et al. 2007; Wang et al. 2005a; Xavier and Bassler 2005b). Our laboratory has been interested in understanding and modulating microbial behavior for enhancing recombinant protein production and we considered AI-2 quorum circuitry is a potential target. Several conceptual approaches for exploiting QS signal transduction have been proposed. For example, Bulter *et al.* (Bulter et al. 2004) created an artificial genetic switch using acetate for modulating cell to cell signaling in *Escherichia coli* (*E. coli*). Neddermann *et al.* (Neddermann et al. 2003) developed a hybrid expression system by incorporating the quorum circuitry of *Agrobacterium tumefaciens* into a eukaryotic transcriptional controller for HeLa cells. Weber *et al.* (Weber et al. 2003) implanted the *Streptomyces* bacterial QS system for tuning heterologous protein expression in mammalian cell culture and mice (human primary and mouse embryonic stem cells). Furthermore, recent “synthetic biology” studies were reported wherein synthetic genetic circuits were constructed for various purposes, including protein synthesis and understanding the native systems (Feng et al. 2004; Hooshangi et al. 2005; Yokobayashi et al. 2002). Few “autoinducible” expression models in bacteria have been reported to date (Carbonell et al. 2002; Studier 2005). These have made use of the auto-inducible promoters which are induced based on changing the culture

medium throughout the culture; one system is now commercially available (Novagen) (Studier 2005). In contrast, we have attempted to develop an innovative autoinducible expression platform by harnessing “native” QS signaling in *E. coli* for recombinant protein synthesis. Our belief is that the metabolic activity of the cell is minimally perturbed when the “native” QS circuit is exploited.

E. coli QS signaling circuitry consists of an inducible and repressible promoter, the *lsr* promoter, and its cognate repressor, LsrR, which in turn are the most critical units for any expression system (Georgiou 1988). In this study, to achieve high expression and compatibility with established expression systems, we have attempted to rewire the *E. coli* native QS circuitry and couple the popular expression system, pET-series vectors, to develop a genetic switching network. Our results have demonstrated that three model proteins (GFP_{uv}, CAT, and LacZ) were successfully expressed in the novel expression system, and the expression system can be also induced by adding *in vitro* synthesized AI-2 exogenously.

3.2 Materials and Methods

Bacterial strains and growth conditions: The strains used in this study are listed in Table 3-1. *E. coli* K-12 strain W3110 (Genetic Stock Center, Yale University, New Haven, CT), and W3110-derived *luxS* mutant strain, MDAI2 (W3110 *luxS*::Tc^r)(DeLisa et al. 2001a), were used as hosts. The *E. coli* B strain derivative, BL21 Star (DE3) [*F*⁻ *ompT hsdS_B(r_B⁻ m_B⁻) gal dcm rne131λ*(DE3)] (Invitrogen), was used as the host of the plasmids, pET200/D/X (X=*gfp_{uv}*, *cat*, and *lacZ*), to confirm plasmids’ expression abilities and also for comparative expression experiments.

Vibrio harveyi BB170 (*luxN::Tn5*, sensor 1⁻, sensor 2⁺) and BB152 (*luxL::Tn5*, autoinducer 1⁻, autoinducer 2⁺) (Surette and Bassler 1998), were used for AI-2 activity assays (kindly provided by Dr. B. Bassler). Luria Bertani (LB) medium contained 5 g L⁻¹ yeast extract (Sigma), 10 g L⁻¹ bacto tryptone (Difco), and 10 g L⁻¹ NaCl. The autoinducer bioassay (AB) media has been described in detail elsewhere (Bassler et al. 1994; Greenberg et al. 1979). Cultures of *E. coli* which had grown overnight in LB under 30°C were inoculated in 40mL fresh LB to achieve a similar initial cell densities (OD₆₀₀ = 0.10) in 250mL flasks. The cultures were then incubated at 30°C with shaking at 250 rpm. For the experiments with induction, the inducers [arabinose (Sigma), IPTG (Sigma) or *in vitro* synthesized AI-2], were added into cultures at mid-log growth phase (OD₆₀₀ ~ 0.40 to 0.60).

Construction of Plasmids for Autoinducible Expression Platform: The plasmids used in this study are listed in Table 3-1 and were constructed using standard procedures (Sambrook 2000) and have been described in previous reports (Tsao et al. 2007; Wang et al. 2005a). Oligonucleotides [Integrated DNA Technologies (Coralville, IA)] are listed in Table 3-2. DNA sequencing was performed at the DNA Core Facility of the Center of Biosystems Research (University of Maryland Biotechnology Institute). Plasmid pFZY1, a mini-F derivative (average copy number = 1~2/ cell) (Koop et al. 1987; Wang et al. 2005a), was used to construct plasmids pCT1, pCT2, pCT5 and pCT6. The promoter region of the *lsrACDBFG* operon (-248 to -1 relative to the start codon of *lsrA*) (abbreviated as P_{lsr}) was amplified by PCR using primers Flsrp and Rlsrp (Table 3-2). The P_{lsr} including the *lsrR* operon

Table 3-1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and property	Source or reference
Strains		
<i>E. coli</i>		
W3110	K12 strain, wild type, λ^- , F ⁻ , IN(<i>rrnD-rrnE</i>)1, <i>rph-1s</i>	Genetic Stock Center Yale University, New Haven, CT (DeLisa et al. 2001a)
MDAI2	W3110 <i>luxS::Tc^r</i> W3110-derived <i>luxS</i> mutant strain	Novagen
BL21	B strain, F ⁻ <i>ompT</i> [<i>dcm</i>]/[<i>lon</i>] <i>hsdS</i> (r _B ⁻ M _B ⁻) <i>gal</i>	Invitrogen
BL21Star (DE3)	B strain, F ⁻ <i>ompT</i> <i>hsdS</i> _B (r _B ⁻ m _B ⁻) <i>gal dcm rne131λ</i> (DE3)	Invitrogen
<i>V. harveyi</i>		
BB152	BB120 <i>luxL::Tn5</i> (AI-1 ⁻ , AI-2 ⁺), Km ^r	(Surette and Bassler 1998)
BB170	BB120 <i>luxN::Tn5</i> (sensor 1 ⁻ , sensor 2 ⁺), Km ^r	(Bassler et al. 1993)
Plasmids		
pFZY1	<i>galK'-lacZYA</i> transcriptional fusion vector, Ap ^r	(Koop et al. 1987; Wang et al. 2005a)
pET200/D-TOPO	Cloning vector, containing <i>T7</i> promoter, Km ^r	Invitrogen
pTOPO-lsrP	pCR-Blunt II(Invitrogen)derivative, containing <i>lsr</i> promoter, Km ^r	This study
pTOPO-lsrP&R	pCR-Blunt II(Invitrogen)derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region, Km ^r	This study
pTOPO-T7RP	pCR-Blunt II(Invitrogen)derivative, containing the gene encoding <i>T7</i> RNA polymerase, Km ^r	This study
pCT1	pFZY1 derivative, containing <i>lsr</i> promoter, Ap ^r	This study
pCT2	pFZY1 derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region, Ap ^r	This study
pCT5	pFZY1 derivative, containing <i>lsr</i> promoter fused with <i>T7RPol</i> , Ap ^r	This study
pCT6	pFZY1 derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region fused with <i>T7RPol</i> , Ap ^r	This study
pTF7-3	containing <i>T7RPol</i> , Ap ^r	(Fuerst et al. 1986)
pTrcHisCAT	pTrcHis derivative, Ap ^r	Invitrogen
pTrcHisGFP	pTrcHisB derivative, containing <i>gfpuv</i> , Ap ^r	(Cha et al. 2000)
pET200/GFP	pET200 derivative, containing <i>gfpuv</i> , Km ^r	This study
pET200/CAT	pET200 derivative, containing <i>cat</i> from pTrcHisCAT, Km ^r	This study
pET200/LacZ	pET200 derivative, containing <i>lacZ</i> , Km ^r	Invitrogen
pTrcHis-LuxS	pTrcHisC derivative, containing <i>luxS</i> from W3110, Ap ^r	(Barrios et al. 2006)
pTrcHis-Pfs	pTrcHisC derivative, containing <i>pfs</i> from W3110, Ap ^r	(Barrios et al. 2006)

Table 3-2 Oligonucleotide primers used in this study

Name	Sequence	Relevant description
Flsrp	CGAATGGATCCTCAATTCATTCTT CACTTTGAAC	Upstream primer for cloning <i>lsr</i> promoter
Rlsrp	GCTTGAAGCTTATAATTTCCCCCG TTCAGTTTTG	Downstream primer for cloning <i>lsr</i> promoter
FlsrP&R	CGCGAATGGATCCTCTTAACTACG TAAAATCG	Upstream primer for cloning <i>lsr</i> promoter region with <i>lsrR</i>
FT7RP	CCGCAAACCCAAGCTTGCATGAAC ACGATTAACATC	Upstream primer for cloning T7 RNA polymerase gene from pTF7-3
RT7RP	GTAAACGTACGGATCCTTGTTACG CGAACGCGAAG	Upstream primer for cloning T7 RNA polymerase gene from pTF7-3
GFPuvF	CACCATGAGTAAAGGAGAAGAAC TTTTCACTG	Upstream primer for cloning <i>gfp_{uv}</i> from pTrcHisGFPuv
GFPuvR	GGAATTCAGAGCTCATCCATGCCA TG	Downstream primer for cloning <i>gfp_{uv}</i> from pTrcHisGFPuv
FpETCAT	CACCATGGAGAAAAAATCACTG G	Upstream primer for cloning <i>cat</i> from pTrcHisCAT
RpETCAT	CTTAAAAAAATTACGCCCCGCC	Downstream primer for cloning <i>cat</i> from pTrcHisCAT

promoter region (abbreviated as P_{lsrR}) and *lsrR* gene (-1202 to -1 relative to the start codon of *lsrA*) (Wang et al. 2005a) was amplified by PCR using FlsrP&R and Rlsrp primers. These PCR products were respectively cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and resulted in pTOPO-lsrP and pTOPO-lsrP&R. To create pCT1 and pCT2, pTOPO-lsrP and pTOPO-lsrP&R were both digested with BamHI and HindIII. The digested fragment containing the promoter region of *lsr* operon or the fragment containing both the promoter region of *lsr* operon and *lsrR* structural gene was inserted into BamHI-HindIII digested FZY1. The resulting plasmids pCT1 and pCT2 were both transformed into Top10 chemical competent cells (Invitrogen), and the colonies were selected using blue/white screen. The gene encoding T7 RNA polymerase (simplified as *T7RPol*) was amplified by PCR from pTF7-3 (Fuerst et al. 1986) (kindly provided by Dr. V. N. Vakharia) using primers FT7RP and RT7RP (Table 3-2) and the PCR product was inserted into pCR-Blunt II-

TOPO vector (Invitrogen) resulting in plasmid pTOPO-T7RP. The plasmid pTOPO-T7RP was digested with HindIII; the fragment containing *T7RPol* was respectively inserted into the HindIII site of pCT1 and pCT2, resulting plasmids pCT5 and pCT6. The vector pET200/D-TOPO was used to construct plasmids pET200/GFP and pET200/CAT. Plasmid pET200/GFP was generated by PCR amplification of *gfp_{uv}* from pTrcHisGFP_{uv} (Cha et al. 2000) using primers GFP_{uv}F and GFP_{uv}R followed by TOPO directionally cloning into pET200/D-TOPO. Similarly, plasmid pET200/CAT was generated by PCR amplification of *cat* from pTrcHisCAT (Invitrogen) using primers FpETCAT and RpETCAT followed by TOPO directionally cloning into pET200/D-TOPO. In order to create the AI-2-inducible expression strains, the plasmid pCT5 or pCT6 was chemically transformed into hosts W3110 and MDAI2 and two resulting strains were transformed with pET200/X (X=GFP, CAT, and LacZ) respectively. The constructed plasmids pCT6 and pET200/GFP and the interactions between them are shown in Figure 3-1.

RT-PCR: To determine relative transcription levels of *T7RPol*, cell pellets were lysed and RNA extracted using an RNAqueous kit (Ambion) according to the manufacturer's instructions. Total RNA concentration was determined by the measurement of the absorbance of a diluted sample at the 260 nm wavelength using UV spectrophotometer (Beckman). To synthesize cDNA, 300ng total RNA was subject to reverse transcription using gene specific primer RT7RP. The cDNA template was followed by PCR with gene-specific primers (FT7RP and RT7RP).

PCR products were run on a 1% agarose gel to compare band intensities using ImageJ software (<http://rsb.info.nih.gov/ij/>).

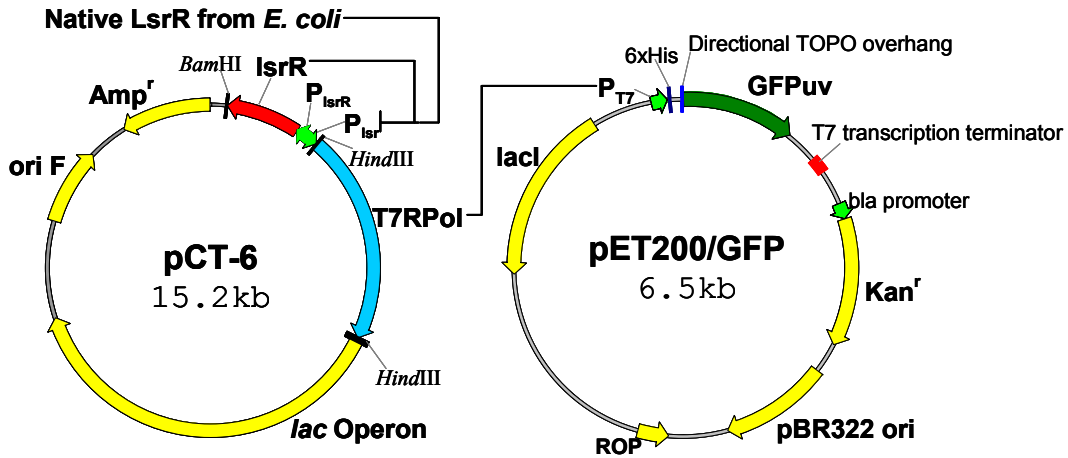


Figure 3-1 the plasmids of two genetic switching network

There are two units in the genetic switching network. The plasmid pCT6 contains *lsr* promoter fused with *T7RPol*. The plasmid pET200/GFP contains T7 promoter along with the protein of interest, GFPuv. In this design, *lsr* promoter can be induced when the repressor LsrR is de-repressed by phospho-AI2, resulting in the transcription and translation of T7RPol. T7RPol can induce the T7 promoter, and the target protein GFPuv can be expressed thereafter. In order to strengthen the regulation of the *lsr* promoter and achieve a tighter control, *lsrR* was also included into pCT6.

Protein Activity Assays: Fluorescence of GFPuv in 1-mL cell culture samples was measured using a Perkin-Elmer LS-3B fluorescence spectrometer which was at an excitation wavelength 395nm and emission wavelengths 509 nm. Specific GFPuv fluorescence was reported as fluorescence divided by cell density (OD₆₀₀). In order to

measure the activity of soluble CAT, the crude cell extracts were prepared as described (Tsao et al. 2007) and the CAT activity was measured as per Rodriguez and Tait (Rodriguez 1983). Specific CAT activity was reported as activity divided by total protein concentration which was measured using Bio-Rad protein assay kit. The Miller assay was performed to measure β -galactosidase activity (Miller 1972).

Analytical measurements: Preparation of cell-free culture fluids for AI-2 activity assays and for conditioning experiments were described previously (DeLisa et al. 2001b; Tsao et al. 2007; Wang et al. 2005a). The AI-2 activity assays were based on Surette and Bassler's previous reports (Surette and Bassler 1998; Surette et al. 1999) and have been described (Tsao et al. 2007; Wang et al. 2005a). In order to observe the expression levels of the proteins of interest, Western blotting was performed with the Anti-His₆ primary antibody (Invitrogen) according to the earlier standard protocols (Sambrook 2000). Finally, the membranes of the blots were scanned and the images were also analyzed using ImageJ.

Synthesis of *in vitro* AI-2: In order to observe the effects of *in vitro* synthesized AI-2 supplemented to the autoinducible expression system, AI-2 was synthesized according previous reports (Barrios et al. 2006; Schauder et al. 2001; Tsao et al. 2007). Briefly, His₆-Pfs and His₆-LuxS were overexpressed with 1 mM IPTG induction of BL21(pTrcHis-pfs) and BL21(pTrcHis-luxS) cells as cell densities were grown to OD₆₀₀=0.4~0.6 at 37°C. After 4 to 6 hours induction, the cells were lysed using BugBuster solution (Novagen), and His₆-Pfs and His₆-LuxS were purified with

Co²⁺ affinity resin (BD TALONTM, BD Biosciences). The purified enzymes were eluted with 125 mM imidazole in phosphate buffer (pH=7.4) and used to synthesize AI-2 from 1 mM *S*-adenosylhomocysteine (SAH) in 50 mM Tris-HCl (pH=7.8) under 37°C for 4hr (Barrios et al. 2006; Tsao et al. 2007). Lastly, the enzymatic reaction product was twice extracted by chloroform and recovered from the aqueous phase.

3.3 Results

Rewiring AI-2 QS network for recombinant protein expression: In *E. coli*, the *lsr* operon has been shown to directly respond to AI-2, and directly regulates its secretion and uptake (Wang et al. 2005a; Xavier and Bassler 2005b). In order to harness *E. coli* AI-2 QS to develop a true autoinducible recombinant protein expression platform, a two-step genetic switching network was transformed into *E. coli* augmenting its native AI-2 QS circuit (Figure 3-2). The schematic plot of the autoinducible system are shown in Figure 3-2A. There are two units (plasmids) involved in the network: the first unit (pCT5 or pCT6) is responsible for expression T7 RNA polymerase (T7RPol) under the control of the *lsr* promoter; the second unit (plasmid pET200/X) expresses a protein of interest X (X=GFP, CAT, or LacZ) under the control of T7 promoter. The T7 promoter can be induced by the product of the first unit, T7RPol. The polymerase then amplifies the initial AI-2 signal for the synthesis and overproduction of the product protein.

This design is an attempt to exploiting the native signaling and decrease the artificially imposed “metabolic burden” on hosts (DeLisa et al. 2001b). The system also incorporates with the well-known pET-system. Thus, the expression system

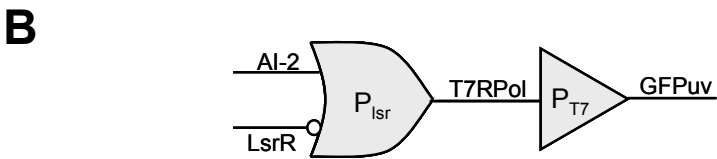
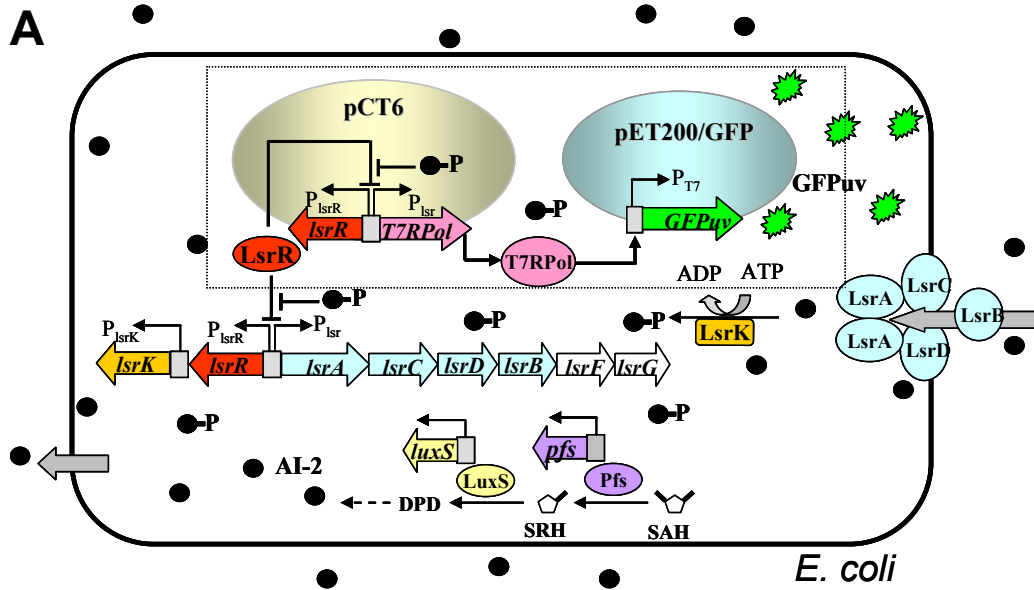
exploiting the *lsr* promoter enables true auto-induction of a protein of interest.

Further, a second parallel system was constructed (plasmid pCT6) that exploits the fine tuning of the native *lsr* regulon (Taga et al. 2003; Wang et al. 2005a; Xavier and Bassler 2005b). The circuit includes the *lsrR* repressor enabling tighter control for the expression of potentially toxic or harmful proteins to the hosts.

Figure 3-2B shows the logic circuit of the genetic network and a truth table of the logic function for different input factors. LsrR is a transcriptional regulator of the native *E. coli lsr* operon (Li et al. 2007; Wang et al. 2005a; Xavier and Bassler 2005b). In the system with pCT6, *lsrR* is included in the circuit in order to strengthen the regulation of P_{lsr} and decrease its basal transcription. The *E. coli* QS signal molecule, AI-2, is phosphorylated by LsrK and becomes phospho-AI-2 which inactivate the LsrR repressor and induce the *lsr* promoter. This results in the transcription and translation of T7RPol (plasmid pCT5 or pCT6), which, in turn, is the inducer of the T7 promoter on the plasmid pET200/X (X=GFP, CAT, or LacZ). After the T7 promoter is induced by T7RPol, the target genes (i.e. *gfp_{uv}*, *cat*, or *lacZ*) can be expressed. Therefore, the final output of this genetic network, or the expression level of the proteins of interest (i.e. GFP_{uv}, CAT, or LacZ), is putatively correlated to the strength of the input, AI-2 level. The first two columns of the truth table (Figure 3-2B) illustrate different inputs; the third column shows the T7RPol output, which represents the transcriptional activity of the *lsr* promoter. As shown, the T7RPol can be expressed when LsrR does not exist in the circuit or the *lsr* promoter is de-repressed by the signal molecule, AI-2. In this study, because LsrR always exists in the hosts, only the last two input cases are met. Lastly, because

T7RPol is the inducer of the promoter of the target genes, the expression levels of the final products are directly related to T7RPol expression level.

In order to investigate the performance of the expression system with or without AI-2, the two units of the genetic network were respectively transformed into wild-type *E. coli* W3110 and the W3110-derived AI-2 synthase, *luxS* knockout MDA12.



AI-2	LsrR	T7RPol	GFP
0	0	1	1
1	0	1	1
0	1	0	0
1	1	1	1

$$\text{Logic function} = \text{AI-2} + \overline{\text{LsrR}}$$

Figure 3-2 (A) Schematic plot of the rewired QS signaling circuitry for autoinducible expression system. AI-2 is produced by LuxS and accumulated extracellularly. Thereafter, AI-2 is internalized by the LsrABC-type transporter and following phosphorylation via the LsrK kinase. Phospho-AI-2 is an inducer of the *l sr* promoter and can de-repress the *l sr* repressor LsrR. After the insertion of the genetic network, the *l sr* promoter on pCT6 can also be induced by de-repression of LsrR and initiate the cascade network, resulting in the expression of the target protein. (B) The logic circuit of the genetic switching network and a truth table of the logic function for different factors. When the *l sr* promoter is with the inputs being the repressor LsrR and inducer AI-2, the output is T7RPol. T7RPol is the input of the inverter, T7 promoter, and induces the synthesis of the target protein GFP. In the truth table, “1” and “0” represent the high and low level of the circuit components respectively.

Expression of the genes of interest using the autoinducible expression system

GFPuv Expression: At first, GFPuv was studied as a model protein investigation due to its stability and simple detection (March et al. 2003). Sequential transformation of plasmid pCT5 or pCT6 followed by pET200/GFP was used for hosts W3110 and MDAI2. Cells were grown in LB medium shaking at 30°C without inducers or additives. Figure 3-3A shows a time course of cell growth, demonstrating no apparent difference between the strains.

AI-2 activities in the extracellular mediums are also shown in Figure 3-3B. In W3110, AI-2 activity increased to a maximum at 4 hr and then sharply decreased to zero shortly thereafter. In W3110 (pCT5+pET200/GFP), which is the expression cascade network without the exogenous insertion of *lsrR*, the trend of AI-2 activity was similar to W3110 except at the maximum level was ~30% lower. In W3110(pCT6+pET200/GFP), which is the expression switching network including the exogenous insertion of *lsrR*, the AI-2 activity also accumulated to the maximum at 4 hr, but decreased more slowly than both W3110 and W3110(pCT5+pET200/GFP). As expected, no AI-2 activity was observed throughout the time course in the cultures of MDAI2(pCT5+pET200/GFP) and MDAI2(pCT6+pET200/GFP). The expression levels of the final product GFPuv in different strains are represented by the specific fluorescence and are depicted in Figure 3-3C. In both the W3110 (pCT5+pET200/GFP) and the W3110(pCT6+pET200/GFP) cultures, the specific expression levels of GFPuv increased along the time course. However, GFPuv expression levels in W3110(pCT6+pET200/GFP) were slightly lower than W3110(pCT5+pET200/GFP).

There was also no apparent increase of specific GFPuv expression observed in MDAI2(pCT5+pET200/GFP) and MDAI2(pCT6+pET200/GFP). Nevertheless, the specific GFP expression in the MDAI2(pCT6+pET200/GFP) culture was even lower than in the MDAI2(pCT5+pET200/GFP) culture. The expression levels of GFPuv were also confirmed by Western blotting (data not shown).

RT-PCR for the samples from the 4 hr and 8 hr time points was performed to further check the transcriptional levels of the gene *T7RPol*, which is under the control of the *lsr* promoter and encodes the inducer of the second unit in the genetic cascade network. The results are shown in Figure 3-3D and support our hypothesis showing in wild type host W3110 (1 and 2 in Figure 3-3D), the *T7RPol* transcriptional levels were higher than in MDAI2 host (3 and 4 in Figure 3-3D) at the 4 hr and 8 hr time points. Additionally, the *T7RPol* transcriptional levels were lower in the case with the exogenous insertion of *lsrR* into the genetic network (column 1 vs. column 2; column 3 vs. column 4) in both the 4 hr and 8 hr time points. Comparing the results from the same cultures at these two time points reveals that the *T7RPol* transcriptional levels at 8 hr were higher than at 4 hr. The trends of *T7RPol* transcriptional levels in different cultures compared closely to the corresponding trends in specific GFPuv in the same cultures (Figure 3C vs. Figure 3D), showing that the specific expression levels of GFPuv were directly related to the transcriptional levels of *T7RPol*. These results demonstrate realization of our switching design.

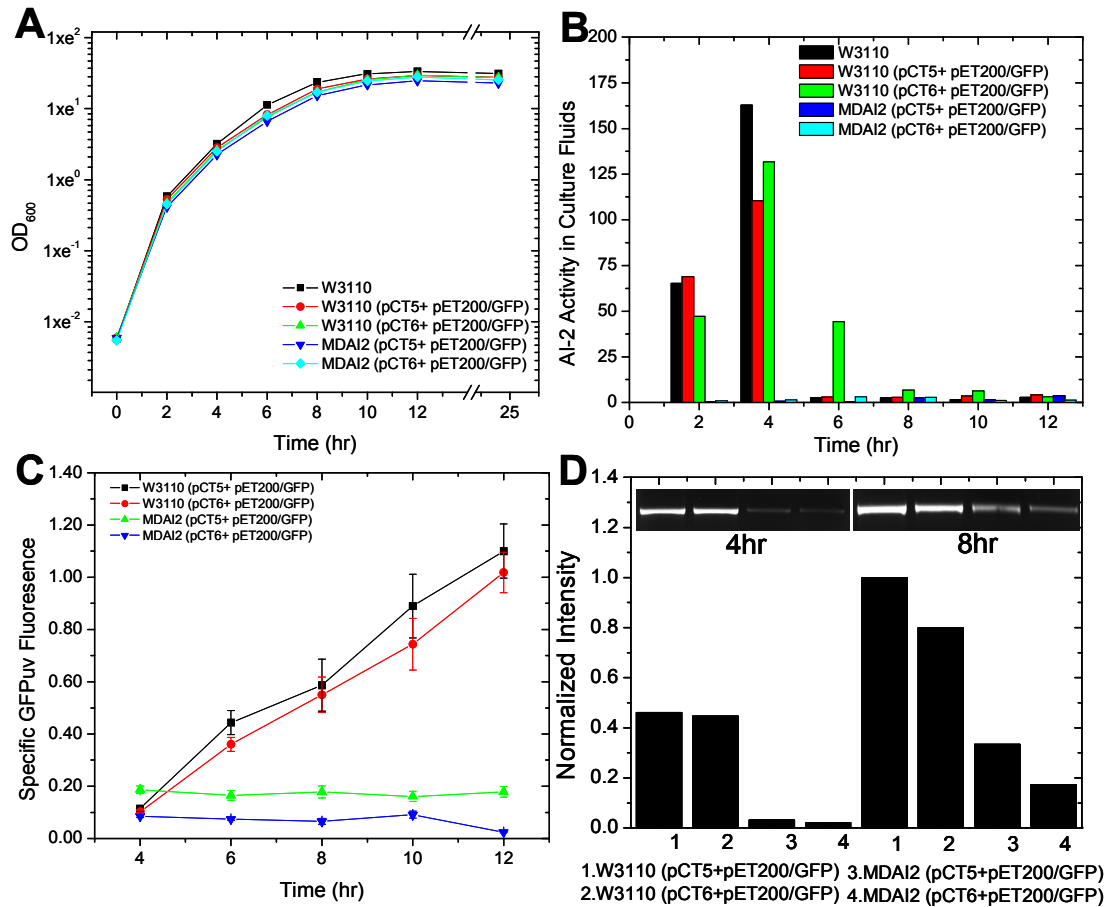


Figure 3-3 GFPuv expression using the autoinducible expression system in W3110 and MDAI2.

Cells were grown in LB medium shaking at 30°C. The fluorescence detection is described in Materials and Methods. **(A)** Time course of cell growth; **(B)** extracellular AI-2 activity; **(C)** specific GFPuv expression results have been normalized by the expression level in the W3110(pCT6+pET200/GFP) culture at 12 hr; **(D)** The transcriptional analysis of T7RPol. The agarose gel was run to show DNA fragment obtained from RT-PCR of total RNA extracted at 4hr and 8hr.

CAT and LacZ Expression: To investigate whether the designed expression platform could be used with different recombinant target proteins, CAT and LacZ constructs were introduced. Plasmid pET200/GFP was therefore replaced by the plasmids pET200/CAT or pET200/LacZ. The conditions of the cell cultures for the expression experiments were the same as GFPuv expression experiments. The growth curves and the AI-2 activities are similar to the results of GFPuv expression experiment depicted in Figure 3-3 (data not shown).

The expression levels of CAT in different strains are represented by the specific activity and the results are depicted in Figure 3-4A. Similar to the GFPuv expression results, the specific CAT activity increased in time in both the W3110(pCT5+pET200/CAT) and the W3110(pCT6+pET200/CAT) cultures. However, the increase of the CAT expression level in W3110(pCT5+pET200/CAT) culture was more steady and reached a higher level as compared to the W3110(pCT6+pET200/CAT) culture. In the MDAI2(pCT5+pET200/CAT) culture, there was a basal CAT activity observed but without any obvious and accumulation along the time course. In the MDAI2(pCT6+pET200/CAT) culture, there was almost no observable CAT activity. These results show that the exogenous insertion of *lsrR* into pCT6 resulted in tighter regulation for the cascaded genetic network. The expression levels of CAT were also confirmed by Western blotting.

In Figure 3-4B, the LacZ expression results in different strains are represented by β -galactosidase activity. The negative control, W3110, exhibited almost no β -galactosidase activity. Expression results from the cultures of W3110(pCT5+pET200/LacZ) and W3110(pCT6+pET200/LacZ) show similar

patterns to GFPuv and CAT expression results. Expression results from the cultures of MDAI2(pCT5+pET200/LacZ) and MDAI2(pCT6+pET200/LacZ) are also similar to the GFPuv and CAT MDAI2 expression results.

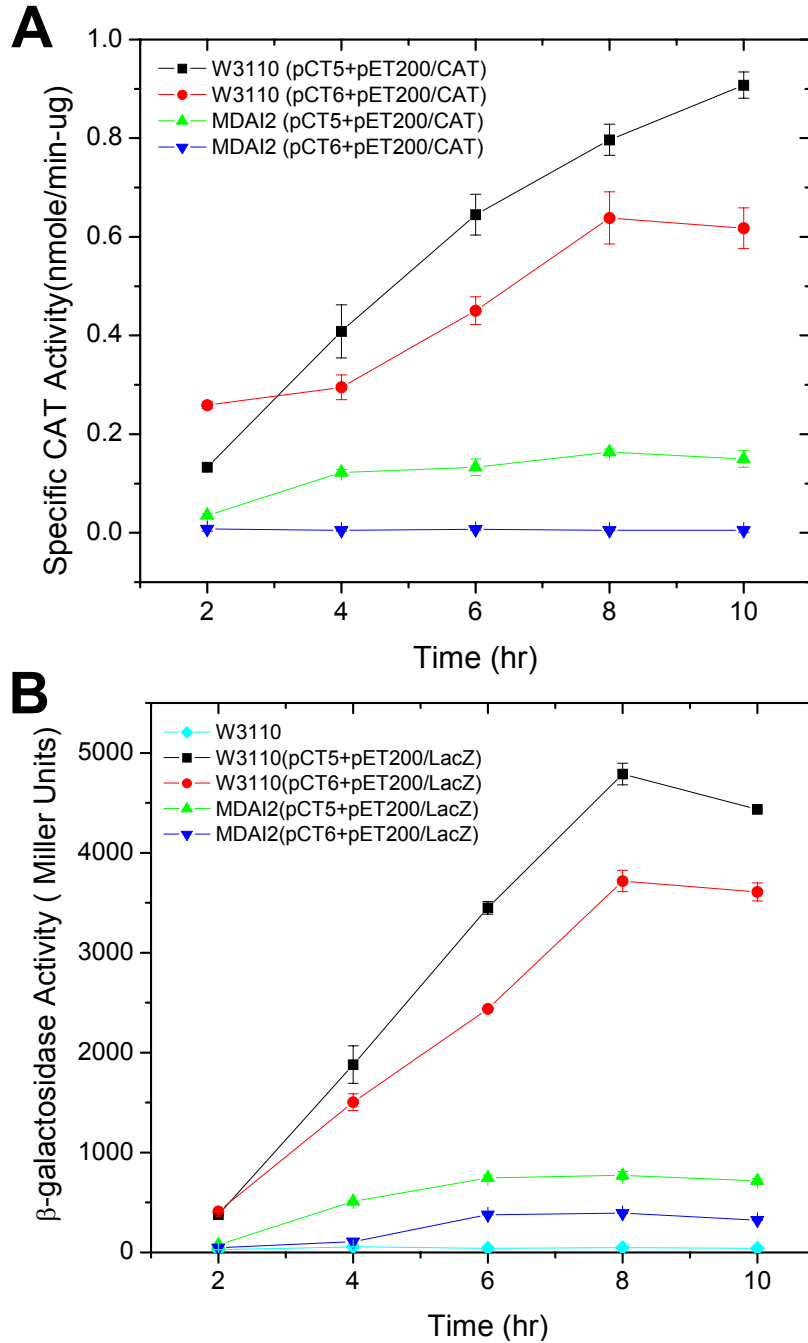


Figure 3-4 (A) CAT expression using the autoinducible expression system in W3110 and MDAI2.

Cells were grown in LB medium shaking at 30°C. The CAT activity was measured by CAT assay. **(B) LacZ expression using the autoinducible expression system in W3110 and MDAI2.** Cells were grown in LB medium shaking at 30°C. The LacZ expression level is represented by β -galactosidase activity and β -galactosidase activity is measured by Miller assay.

***In vitro* synthesized AI-2 for switching on the genetic cascade network:** There have been several reports demonstrated that the *lsr* promoter can be activated via phospho-AI-2 inactivating the repressor LsrR (Taga et al. 2003; Wang et al. 2005a; Xavier and Bassler 2005b). In order to determine whether the expression platform can also be induced artificially, AI-2 was synthesized *in vitro* and exogenously added into MDAI2(pCT6+pET200/GFP) cultures. The W3110(pCT6+pET200/GFP) culture was used as the positive control. When the cells were grown to mid-log phase ($OD_{600} \approx 0.4\text{--}0.6$) at 2 hr, *in vitro* AI-2 was added into MDAI2(pCT6+pET200/GFP) cell cultures at two supplemental levels [4% (v/v) and 1% (v/v)]. Figure 3-5A shows the results of AI-2 activities. In the W3110(pCT6+pET200/GFP) culture (black bars), the trend of AI-2 activity is consistent with the result shown in Figure 3-3B. In the MDAI2(pCT6+pET200/GFP) culture, without the addition of *in vitro* AI-2, there was no observable AI-2 activity. In the MDAI2(pCT6+pET200/GFP) culture with 4% (v/v) *in vitro* AI-2 addition (green bars), AI-2 activity was highest (~450 folded units) immediately after the *in vitro* AI-2 was added and the AI-2 activity in the medium monotonically decreased thereafter. However, in the MDAI2(pCT6+pET200/GFP) culture with 1% (v/v) *in vitro* AI-2 addition (blue bars), the AI-2 activity maintained at a similar level (~300 folded units) until 6 hr and followed by a sharp decline. The GFPuv expression level results for this artificial *in vitro* AI-2 induction system are shown in Figure 3-5B. Consistent with the trend in Figure 3-3C, the GFPuv expression levels in the W3110(pCT6+pET200/GFP) culture (black bars) increased in time, and only a basal expression level was observed in the MDAI2(pCT6+pET200/GFP) cultures without the addition of *in vitro* AI-2 (red bars).

Confirming our hypothesis, the addition of *in vitro* AI-2 did lead to the induction of the genetic network to produce GFPuv. However, the GFPuv expression levels in the MDAI2(pCT6+pET200/GFP) culture with 4% (v/v) *in vitro* AI-2 addition (green bars) were similar to expression levels in the W3110(pCT6+pET200/GFP) culture and apparently higher than in the MDAI2(pCT6+pET200/GFP) culture with 1% (v/v) *in vitro* AI-2 addition (blue bars). Although initially the expression levels in the MDAI2(pCT6+pET200/GFP) culture with 1% (v/v) *in vitro* AI-2 addition were close to the expression levels in the MDAI2(pCT6+pET200/GFP) culture with 4% (v/v) *in vitro* AI-2 addition at 4 hr, the GFPuv expression levels in the MDAI2(pCT6+pET200/GFP) culture adding 1% (v/v) *in vitro* AI-2 were only slightly higher than in the MDAI2(pCT6+pET200/GFP) culture without *in vitro* AI-2.

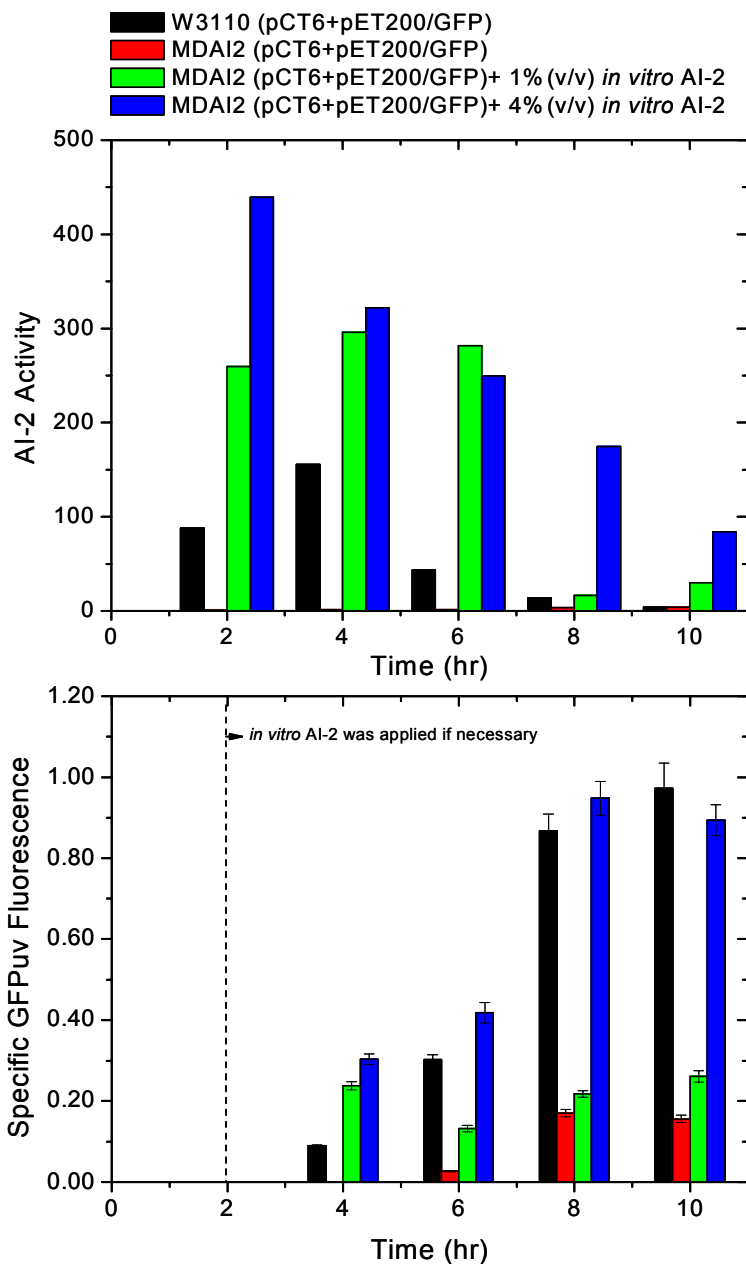


Figure 3-5 Induced the expression system by *in vitro* synthesized AI-2 addition. Cells were grown in LB medium shaking at 30°C and the *in vitro* AI-2 was applied at 2hr. W3110(pCT6+pET200/GFP) was conducted for the autoinducible expression which was the positive control. **(A)** Extracellular AI-2 activity. In the case with 4%(v/v) *in vitro* AI-2 addition, AI-2 activity decrease steadily; in the case with 1%(v/v) *in vitro* AI-2 addition, AI-2 activity maintained at a similar level until 6 hr. **(B)** Specific GFPuv expression results have been normalized by the expression level in the W3110(pCT6+pET200/GFP) culture at 10 hr.

Comparison of the autoinducible expression system with other protein

expression platforms: In order to further understand the performance of the autoinducible recombinant protein expression platform, GFPuv expression using the QS autoinducible genetic network was compared to the expression using two other popular commercially available expression platforms, the *trc* expression system [BL21(pTrcHis-GFPuv)] and the T7 expression system [BL21(DE3) (pET200/GFP)]. BL21(pTrcHis-GFPuv) and BL21(DE3) (pET200/GFP) cells were grown to mid-log phase ($OD_{600} \approx 0.4-0.6$) at 37°C and induced by 1mM IPTG.

MDAI2(pCT5+pET200/GFP) cells were induced with 4% (v/v) *in vitro* AI-2, and compared to the GFPuv auto-expression in W3110 (pCT5+pET200/GFP) grown similarly. Figure 3-6A shows the growth curves of all cell cultures. The growth curve for W3110(pCT5+pET200/GFP) and MDAI2(pCT5+pET200/GFP) were very similar to W3110. However, the growth rates of BL21(DE3) (pET200/GFP) and BL21(pTrcHis-GFPuv) decreased significantly after IPTG induction and were slower than W3110, W3110(pCT5+pET200/GFP), and MDAI2(pCT5+pET200/GFP).

The GFPuv expression performance in the different systems were compared on a volumetric basis and the results are shown in Figure 3-6B. The volumetric expression in BL21(DE3) (pET200/GFP) culture reached the highest final level, though the culture grew much slower as compared to the other strains after induction. The volumetric expression in BL21(pTrcHis-GFPuv) reached the second highest level and was ~20% less than the final volumetric expression level of BL21(DE3) (pET200/GFP). The volumetric expression in the auto-inducible system, W3110 (pCT5+pET200/GFP), was only ~33% of that in BL21(DE3) (pET200/GFP) and was

~50% of that in BL21(pTrcHis-GFPuv). The volumetric expression levels in MDAI2(pCT5+pET200/GFP) induced by 4% (v/v) *in vitro* AI-2 were only ~33% of the level in auto-inducible culture.

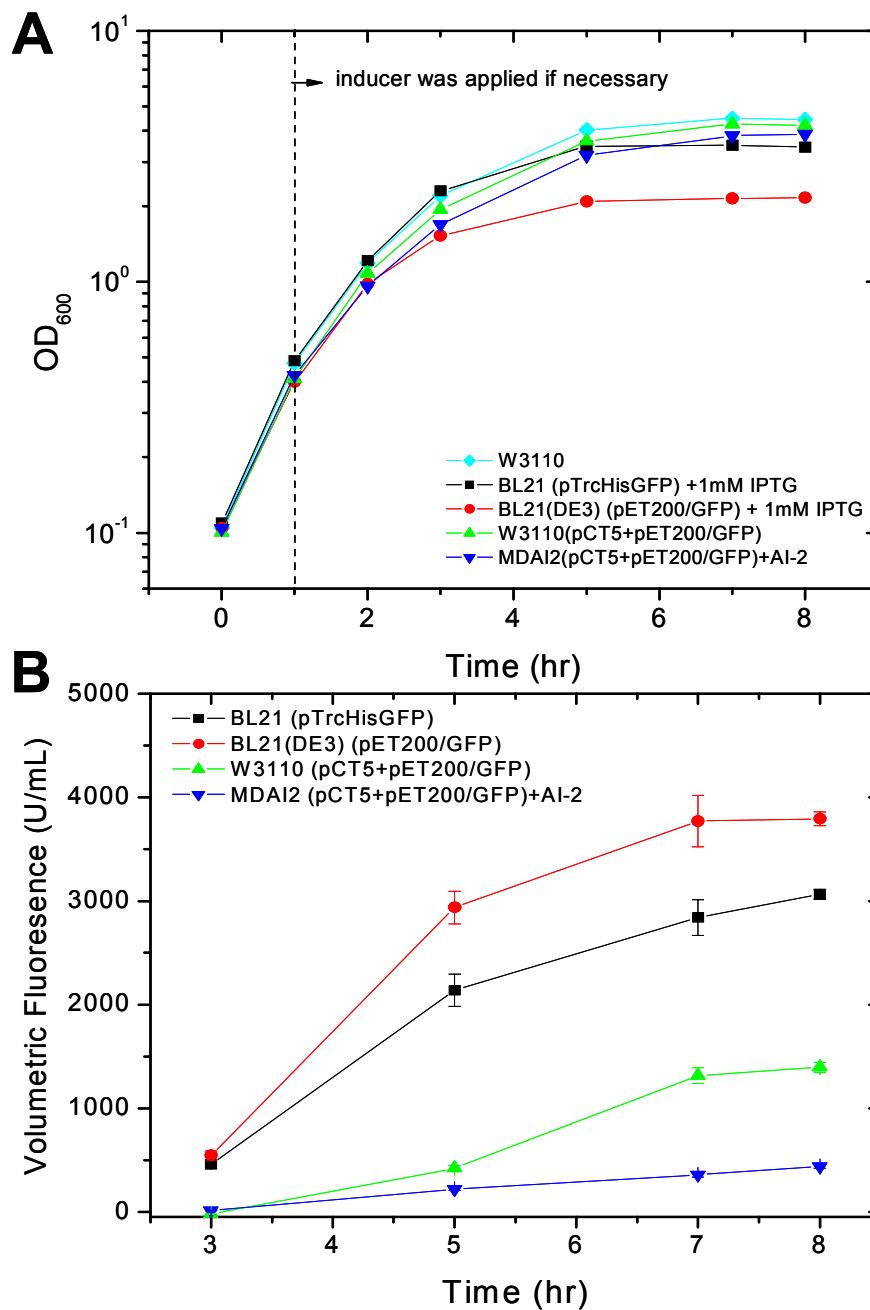


Figure 3-6 Expressed GFPuv using different expression systems.

The cells were grown in LB medium and shaking at 37°C to compare the expression performance of different expression systems. (A) Time course of cell growth shows that BL21(DE3)(pET200/GFP) culture grew slowest after induced by 1mM IPTG; BL21(pTrcHisGFP) culture grew the second slowest after induced by 1mM IPTG. In the MDAI2(pCT5+pET200/GFP) culture, 4% (v/v) *in vitro* AI-2 was added at 2 hr. (B) The expression performance was represented by volumetric fluorescence.

3.4 Discussion

Quorum sensing (QS) systems are characterized by signal molecule production, transduction, and regulation of genes in a growth-dependent manner. An auto-inducible recombinant protein expression platform was constructed in this study that exploits the native QS signaling process in *E. coli*. Specifically, *E. coli* AI-2 signaling transduction pathway is regulated by the *lsr* and *lsrRK* operons (Li et al. 2007; Wang et al. 2005a; Xavier and Bassler 2005b). The principles of our protein expression system design include high levels of expression, simplicity, and compatibility with commercially available expression vectors. At first glance, QS signaling may provide simplicity but not compatibility or high expression levels. In order to amplify the *E. coli* QS auto-inducible effect to achieve a higher expression level, we have attempted to rewire the widely used T7 expression system by inserting QS signaling as a switch, thereby generating a new genetic circuit. Our expression results for the three model proteins (GFPuv, CAT, and LacZ) demonstrated the system could be auto-induced and target proteins could be expressed in high levels (Figure 3-3 and Figure 3-4). Moreover, our results demonstrate coupling to the established and popular T7 expression system, pET-series vectors which include the T7 promoter (Studier and Moffatt 1986; Studier et al. 1990). That is, our second unit is transformed directly to pET host vector system, and QS signaling system is created.

Notably, when the plasmid pCT6, including the gene *lsrR* with its promoter, was introduced as the first unit, the expression of the target proteins in the wild type host W3110 showed only slightly lower levels compared to the pCT5 system in W3110 (Figure 3-3C and Figure 3-4). As expected, the AI-2 activity (Figure 3-3B) in

the W3110(pCT6+pET200/GFP) culture decreased more slowly than both W3110 and W3110(pCT5+pET200/GFP) cultures. Because the native *lsrACDBFG* operon encodes the AI-2 transduction apparatus proteins in *E. coli* (Li et al. 2007; Wang et al. 2005a; Xavier and Bassler 2005b), these results not only demonstrate that the insertion of *lsrR* reduces the leaky expression of the target proteins in the host MDAI2, but also suggest that the stronger LsrR expression gives stronger repression to the native *lsrACDBFG* operon in the host W3110, resulting in a slower AI-2 uptake rate from the medium. Correspondingly, the expression in the pCT6 system is delayed relative to the pCT5 system. This was found to be the case for all model proteins (Figure 3-4B). The minor differences in the protein accumulation patterns might be caused from different processing properties of different target proteins (Georgiou 1988; March et al. 2003; Tsien 1998) and a slight decrease in T7RPol expression.

Since there is no AI-2 production in MDAI2(DeLisa et al. 2001a), the AI-2 synthase *luxS* knockout, we attempted to investigate whether the target protein in MDAI2(pCT6+pET200/GFP) culture could be induced by *in vitro* synthesized AI-2 added exogenously. The data shown in Figure 3-5B demonstrate that at a 4% (v/v) supplemental level of *in vitro* AI-2, the specific GFP_{uv} expressed was comparable to the W3110(pCT6+pET200/GFP) auto-expression results. However, at the 1% (v/v) supplemental level there was only slightly higher expression than the MDAI2(pCT6+pET200/GFP) culture without induction. The AI-2 uptake in the 4% case was uniform throughout, while in the 1 % case the AI-2 level stayed steady upon addition and then decreased when the culture moved into the stationary phase. This

suggests that the increment of AI-2 (4% vs. 1%) was needed to stimulate AI-2 uptake in this system. Correspondingly, this increment is observed to induce protein synthesis. Because ours is the first true AI-2 responding reporter system in *E. coli*, such QS signaling phenomenon has never been observed with this level of precision. These results demonstrate that the inserted *lsr* promoter on the plasmid and native *lsr* promoter regulating *lsr* operon in *E. coli* can be both induced by sufficient level of *in vitro* synthesized AI-2.

Importantly, though the AI-2 activity in W3110 (pCT6+pET200/GFP) cultures were lower than MDAI2(pCT6+pET200/GFP) culture with 1% (v/v) of *in vitro* AI-2 induction, the GFPuv expression levels were much higher. In addition, though the AI-2 activity levels in W3110 (pCT6+pET200/GFP) was apparently lower than MDAI2(pCT6+pET200/GFP) culture with 4% (v/v) of *in vitro* AI-2 induction after 6 hr, the expression levels of GFPuv were almost identical. These results suggest that endogenous AI-2 of W3110, without the secretion and uptake process, can still be phosphorylated by LsrK, or otherwise used to stimulate *lsr* expression directly. In our previous studies, the investigation of the *lsr* promoter transcriptional level in *ΔlsrK* and *ΔlsrACDBFG* mutants was conducted, and the results suggested that both the exogenous and endogenous AI-2 can be phosphorylated by LsrK (Li et al. 2007; Wang et al. 2005a). Therefore, the results of the *in vitro* AI-2 induction experiment in this study provide new evidence supporting our previous inference.

The original purpose of this study was to develop a new recombinant protein expression system. Therefore, there is always a critical question that will be asked: “How good does it perform?” In order to determine the performance of the novel

expression system, we expressed GFPuv in the autoinducible strain, W3110(pCT5+pET200/GFP), and compared the volumetric yield with the expression of GFPuv by two well established and popular expression systems, *trc* expression system and traditional T7 expression system [pET-X express in BL21(DE3) host]. All these experiments conducted were at 37°C, a common temperature for recombinant protein production in *E. coli*. Our results demonstrate the autoinducible expression system performs only modestly compared to BL21(pTrcHis-GFP) (c.a. 2 fold higher) and BL21(DE3)(pET200/GFP) (c.a. 3 fold higher). Furthermore, the volumetric yield of the MDAI2(pCT5+pET200/GFP) with 4% (v/v) of *in vitro* AI-2 induction was obviously lower than the volumetric yield of W3110(pCT5+pET200/GFP). This result is not consistent to the results of the induction experiment conducted at 30°C, suggesting that the *in vitro* AI-2 may degrade or its signaling process be attenuated and lose activity faster at 37°C. Our initial comparison results suggest that the new autoinducible system might not be as powerful as the two popular expression systems at the conditions used. Note, however, BL21 and BL21(DE3) were constructed specifically to achieve high yields and used in concert with the pTrcHis and pET-series vectors. It would be interesting to transform our autoinducible system into BL21 host to conduct a further comparison. Also, we might transfer our system into different hosts which possess AI-2 QS circuitry or screen different culture conditions to optimize the novel expression system in the future. One important characteristic of the autoinducible expression system is: there is no apparent metabolic burden shown based on the growth course, but there were different levels of metabolic burden was shown in BL21(pTrcHis-GFP) and BL21(DE3)(pET200/GFP) (Figure 3-6B).

3.5 Conclusion

In this study, we have developed a novel autoinducible recombinant protein expression platform via rewiring *E. coli* native AI-2 QS signaling circuit. We also demonstrate that the genetic cascade network can be induced by exogenously adding *in vitro* AI-2. Therefore, this expression system provides not only the convenience of operation and significance in yield for recombinant protein production, but also a new tool to investigate the cell-to-cell communication in bacteria.

Chapter 4 General Conclusions

4.1 Exploiting quorum circuitry for enhancement for recombinant protein production.

We inserted *luxS* (AI-2 synthase) into a vector which co-synthesizes proteins of interest CAT, OPH, or GFPuv and found dramatically increased yield in all cases. Moreover, we have attributed this yield to the upregulation of the chaperone, GroEL, which is shown for the first time to be positively regulated at the post-transcriptional level by AI-2. This research is the first to tuning the quorum sensing signaling to improve recombinant protein synthesis.

4.2 Harness bacterial cell-to-cell communication for an innovative autoinducible recombinant protein expression system.

We rewired the QS signaling to develop a true autoinducible expression platform by transforming a genetic switching network into *E. coli*. Three model protein, GFPuv, CAT, and LacZ were all successfully expressed at significant yields. The genetic switching network can also be induced by *in vitro* synthesized AI-2. The novel autoinducible system facilitates protein synthesis without culture monitoring or inducer addition.

4.3 Future Directions

Though our results suggest that AI-2 can upregulate the chaperone GroEL, further investigation must be conducted to understand the mechanisms. Also,

extracellular AI-2 activity does not faithfully indicate the effect of AI-2 signaling. That is, AI-2 level signals a dynamic process but does not itself indicate the amplitude of a “switch”. We can measure *luxS* transcript levels using RT-PCR to correlate the transcriptional level of *luxS* with the chaperon GroEL in transcriptional or translation level. Further, we also can apply the antisense RNA to modulate the endogenous *luxS* and AI-2 activity to further confirm the phenomena we observed in this study.

For the novel autoinducible expression system, we only use three reporter proteins as models. Some problematic recombinant proteins may need to be examined. The *lsr* promoter can be stimulated by cyclic AMP, which is related to the absence of glucose or other phosphotransferase system sugars(Wang et al. 2005a; Xavier and Bassler 2005b). Therefore, we may screen different medium and different culture conditions to get optimal expression results. Also, we can transform the expression system into different *E. coli* hosts to investigate the optimal host. Furthermore, the novel autoinducible expression system can be used as new cell-to-cell communication reporter, which provides us a new tool to understand how bacteria communicate between different species. QS signaling in *E. coli* also relates to the formation of biofilm which is correlated to the pathogenesis(Davies et al. 1998; Li et al. 2007; Prouty et al. 2002), the autoinducible system may also provide us a new drug discovery platform.

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