

ABSTRACT

Title: VIEWING QUORUM SENSING IN A GLOBAL NETWORK: STUDIES ON SDIA-DEPENDENT SYSTEM 1 AND AUTOINDUCER-2 MEDIATED SYSTEM-2 OF NON-PATHOGENIC ESCHERICHIA COLI K-12

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Quorum sensing is a process by which bacteria send out and perceive signal molecules that enumerate their population density. When a threshold signal level is achieved, these bacteria coordinate their behavior and exhibit multicellularity. This can be viewed as a sort of sociological behavior, wherein bacteria communicate with each other resulting in altered societal structure. Studies on the quorum sensing of common laboratory strains of *E. coli* K-12 have been independently directed on two mainly recognized systems, each associated with an autoinducer family (AI-1 and AI-2). This study has specifically addressed a question of the functionality of a sensor associated with system 1 (a LuxR type sensor, SdiA). Interestingly, this sensor, which is also a transcriptional regulator, was found to positively regulate the uptake of the quorum system 2 signaling molecule (AI-2). An *sdiA* mutant showed altered mRNA levels for motility genes, purine de novo synthesis and rRNAs, which are likely to link to phenotypic changes such as decreased motility and increased aggregation and stress resistance. This study also describes the

regulatory effects of major molecular chaperones, DnaJKE and GroESL, on AI-2 level found in conditioned medium, suggesting a linkage between the well-characterized heat shock stress response and quorum sensing. The finding that there is cross-regulation between *E. coli* quorum system 1 and 2, and the interplay with global regulatory networks such as the heat shock response, will contribute to the development of “engineered” quorum systems in *E. coli* that serve industrial purposes and also to the potential control of pathogenic bacteria.

VIEWING QUORUM SENSING IN A GLOBAL NETWORK:
STUDIES ON SDIA-DEPENDENT SYSTEM 1 AND AUTOINDUCER-2
MEDIATED SYSTEM-2 OF NON-PATHOGENIC ESCHERICHIA COLI K-12.

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Dedication

To my loved ones, to whom I will always be indebted: Hyun Kyung Kim, my mother-in-law, Kyung Ja Kim and Jang Ok Kim, my mother and my father.

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I feel like I have arrived at one stop after a long journey. I appreciate the support and direction of my advisors Dr. Bentley and Dr. Joseph. Their enthusiasm toward the research and their compassion and humor are so precious to me. I thank Dr. Culver, Dr. Liu, and Dr. Sukharev for the valuable advices and guidance they provided during the course of my study. I also extend my sincerest thanks to the members of the Bentley Lab, who share the memory and friendship during the voyage, especially to John.

During the last 6 years, I have been so blessed with the births of my two sons, Jaeyoon and Jaeyoung. Most of all, I give my best thanks to my loved husband, Hojeong Park, whose trust never wavered encouraging me even in my restless moments, and who has provided the shoulder to lean on.

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Chapter 1: Introduction

Overview

Unicellular microorganisms have evolved to dynamically adjust their physiological characteristics to changing environments for their own survival and propagation. Accordingly, bacterial sensory machineries have been developed that enable cells to perceive signals emanating from both abiotic and biotic sources. The idea that bacteria may sense their own population density and that of other bacterial species' and then transform this knowledge into a coordinated phenotype dates from studies in the '70's with a variety of microorganisms including Cyanobacteria, Firmicutes, Actinobacteria, and Proteobacteria. The first discovery of bacterial mass-action was centered on the luminescence of *Vibrio fischeri*, a symbiotic marine microorganism (Eberhard, 1972; Nealson *et al.*, 1970). This concept was coined "Quorum Sensing" in the early 1990s (Fuqua *et al.*, 1994).

Now, quorum sensing has become a widespread subject of research, as bacterial interactions with eukaryotic systems, i.e. plant and animal kingdoms, have been shown to be quorum-sensing associated. Topics including pathogenesis, symbiotic associations, and even bacterial consortia in biofilm communities are linked to quorum sensing.

Advantages of gauging cell density within certain physical ranges were inferred by researchers using ecological or teleological arguments, such as in competitions among microbes faced with limited nutrients, or from the necessity to avoid impending anti-bacterial immune system for human pathogens. The advantages in quorum sensing may have placed some part of selective pressure on bacterial speciation (McFall-Ngai, 2002).

It is not surprising that quorum regulation on non-virulent genes has thus far received only minimal attention unlike that of virulent ones. The original intent of my research was to test the possibility of engineering bacterial metabolism by disturbing or stimulating the quorum sensing mechanism for the purpose of increasing the yield of heterologous protein overexpression. Measurement of the *Escherichia coli* produced quorum signal molecule, AI-2, was an important element in early experiments. It became clear early on, that very little was known about the AI-2 synthesis and uptake mechanisms in *E. coli*, and importantly, what genes were regulated by this autoinducer. Besides the architecture of *E. coli* quorum system was incompletely composed of two systems (or more) and several conflicting experimental results were reported on the functionality and interaction between quorum systems. Drawing linkages between quorum sensing and other signal transduction mechanisms turned out to be more difficult than we had imagined, so my study was refocused on determining the functionality of a quorum sensing transcriptional regulator. In short, opposing the ideas that non-pathogenic *E. coli* strains do not have potential for behaving group-wise, this study was motivated to examine *E. coli* quorum sensing.

We expect that this basic questioning on the quorum regulation can be applied through engineering and/or interfering cell-cell communication in the future; controlling bacterial virulence and recombinant protein expression. Unlike antibiotics-based treatment of problematic bacteria (from human-interest standpoint) dauntingly challenged by occurrence of resistant ones, this new approach is envisioned not to place unwanted selective pressure on the bacteria. Also the idea associated with the signal-mediated gene transcription can be engineered develop tightly controlled bacterial signal mediated

transcription system, to control recombinant gene expression [Reviewed in (March and Bentley, 2004)].

In the rest of this Chapter, the diverse features of Gram negative bacterial quorum sensing are highlighted by the required components for successful quorum sensing autoregulation. Further, I introduce fundamental questions we have on quorum sensing in *E. coli*. In Chapter 2, I present evidence of AI-2 signaling that links global signal networks through molecular chaperones. In particular, heat shock experiments are used to probe the role of molecular chaperones on AI-2 synthesis. Chapter 3 mainly describes results from transcriptome analyses of *sdiA* deletion and attempts to provide an association with phenotype. Finally, conclusions drawn out from my research are presented along with the remaining questions suggested for future research.

Literature review

1-1. Discovery of quorum sensing

Euprymna scolopes and *Vibrio fischeri* interaction

During nocturnal feeding periods, Hawaiian squid *Euprymna scolopes* emits luminescent light matching the intensity of moonlight, downward from their bodies. The luminescence is made possible by a symbiotic marine bacterium, *Vibrio fischeri*. Living in the center of the squid body cavity, *V. fischeri* exerts about 20 % of its metabolic potential for light emission when found at high cell density (e.g., $\sim 10^{10-11}$ cells/ml) (Makemson, 1986). What they gain by spending this much energy has intrigued many scientists.

Importantly, the luminescence from *E. scolopes* exhibits periodicity that is controlled by the internalized *V. fischeri* cell density. Each morning, more than 95 % of the symbionts are expelled from the animal host into the surrounding seawater thereby dissipating the luminescence. The squids stay in the sand, hiding during daytime hours. Meanwhile unexpelled 5 % bacterial cells grow and repopulate the light organ as nightfall approaches (Lee and Ruby, 1994; McFall-Ngai, 1990). In this way, a periodic cycle of luminescence can be synchronized with that of bacterial mass population.

Initiated from the discovery by ecologists, the squid-bacterial symbiosis became the prototypic model of quorum sensing and has intrigued many other research groups in the field of development and evolution who noted the importance of its dynamicity. For instance, reciprocal interactions and competition between host and bacteria, or genes between bacterial species, have been studied (Lee and Ruby, 1994). Also, mechanisms

governing the essential bacterial communication including its biochemical features have been actively studied at the molecular level (Graf and Ruby, 1998; Lupp *et al.*, 2003; Miyamoto *et al.*, 2003). Since the discovery of quorum sensing in *V. fischeri*, a diverse array of bacterial organisms have been studied for possible density dependent phenotypic changes and have often been reported as quorum sensing organisms. From these pools of proposed quorum-sensing bacteria, discerning real quorum-dependent bacteria has become an issue.

1-2. Molecular biology of quorum sensing: qualifying features for quorum sensing

We define a quorum sensing bacterium as one that detects a small molecule that is either self-secreted or is otherwise present in its immediate growth environment, and through the detection of this signal molecule, it coordinates its own behavior as a function of its own cell density. Traditionally, qualified quorum sensing bacteria are equipped with at least three components: signal molecules produced by autoinducer synthases, their cognate receptors or transcriptional regulators, and genes that are placed under the control of the signals. Note that non-self secreted signals from other bacteria can function as effectors, which are specifically referred as alloinduction. Hence, the genetic requirement that a signal synthase encoding component be present is not as critical.

In general, researchers pursue fundamental insight on quorum sensing systems in both directions; by starting with phenotypes that appear to be cell density dependent and unraveling their molecular componentry or by starting with known molecular components and searching for phenotypic differences. Searching for genes involved with bacterial luminescence is one example of the first case, while screening genes affected by exogenous autoinducers or by mutation in regulators (receptors) is exemplary of the second case. I have chosen the latter and have focused on SdiA, an *E. coli* homologue to *V. fischeri* quorum sensing transcriptional regulator, LuxR.

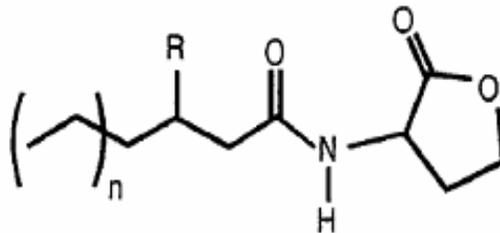
1-2-1. Signals: diversity versus universality Signal-molecules are usually produced as byproducts of pivotal enzymatic reactions. S-adenosylmethionine (SAM) is the common precursor for both autoinducer families, AI-1 and AI-2. In plants, SAM has also been known as the precursor of the plant signal, ethylene, a gaseous plant hormone that induces the ripening of fruits (White *et al.*, 1994). The apparent uniqueness of SAM in its utility to serve as a precursor to signals among various biological systems is reviewed in (Fontecave *et al.*, 2004). Using SAM as precursors, either species-specific signals by diversifying signal molecules and/or use species-non specific signals by using seemingly uniform molecules are produced by many Gram negative bacteria.

Diversity

Diverse forms of Gram-negative bacteria commonly in the form of Acylated Homoserine Lactone signals, whose differences are crucial in the signal specificity (AHL or hereafter AI-1). Over ten different AI-1s have been identified from different bacterial species, often through the use of a combination of high performance liquid chromatography (HPLC) purification, nuclear magnetic resonance (NMR) spectroscopy, and mass spectroscopy (Schaefer *et al.*, 2000). AI-1s have a common five-membered homoserine lactone ring and can be diversified by conjugated acyl side chains differing in length, backbone saturation, or the side chain substitution (Table 1). The acyl chains identified thus far vary from 4 to 14 carbons (all even-numbered) in length. The length of the acyl chain proportionately increases the hydrophobicity of the signals and determines each signal's membrane permeability. Fundamentally, AI-1 is able to freely move across cell membranes. For example, titrated *V. fischeri* 3-oxo-C6-HSL was used to characterize free

diffusion across cell membranes (Kaplan and Greenberg, 1985). However, when the acyl chain is too long, it is hard to rely on the simple diffusion thus active secretion is needed. The length of the acyl chain also affects signal's turnover rate (see below).

In the synthesis of AI-1 molecules, amide bond formation between the acyl- group and the amino group of S-adenosyl methionine (SAM) is critical (Hanzelka and Greenberg, 1996). Three types of AHL synthases, LuxI, AinS (LuxM), and HdtS types, have been identified (Laue *et al.*, 2000; Swift *et al.*, 2001). Amongst them, LuxI family members are evolutionarily conserved. In vitro catalysis of AI-1 synthesis was reported with *V. fischeri* LuxI (Schaefer *et al.*, 1996), *A. tumefaciens* TraI protein (More *et al.*, 1996) and *P. aeruginosa* RhlI protein (Parsek *et al.*, 1999). The second type of AI-1 synthase (AinS family) is non-homologous to the typical LuxI proteins. In this group, products of *luxM* (*V. harveyi*, 25-kDa) and *ainS* (*V. fischeri*, 46 -kDa) genes are included. LuxM has 34 % identity with the C-terminal 218 residues of AinS (Fuqua *et al.*, 2001). The putative third group of AI-1 synthase, HdtS (homolog of PlsC from *E. coli*), was identified from *P. fluorescens* (Laue *et al.*, 2000). It is closely related to lysophosphatidic acid acyltransferase, which is needed for cell membrane biosynthesis.



AI-1

Bacteria	Regulators	Chain length	β R-group ^a	Target function
<i>Vibrio fischeri</i>	LuxR-LuxI	6	=O	Bioluminescence
	AinR-AinS ^b	8	-H	Bioluminescence
<i>Vibrio harveyi</i>	LuxM/LuxN-LuxO-LuxRvh ^c	4	-OH	Luminescence, proteases, polyhydroxybutyrate synthesis, and others
<i>Vibrio anguillarum</i>	VanR-VanI	10	=O	Proteases
<i>Agrobacterium tumefaciens</i>	TraR-TraI	8	=O	Conjugal transfer *
		6	=O	
<i>Pantoea stewartii</i>	EsaR-EsaI	6	=O	Exopolysaccharide
<i>Rhodobacter sphaeroides</i>	CerR-CerI	14 ^d	-H	Aggregation
<i>Yersenia enterocolitica</i>	YenR-YenI	6	-H, =O	Target functions unknown
<i>Pseudomonas aeruginosa</i>	LasR-LasI	12	=O	Host interaction
	RhlR-RhII	4	-H	Rhamnolipids
	QscR ^c	?	?	Host interaction
<i>Erwinia stewartii</i>	EsaR-EsaI	6	=O	virulence, exopolysaccharide
<i>Escherichia coli</i>	SdiA-?	?	?	Cell division,?

Table sources are from reviews of (Fuqua *et al.*, 1996; Fuqua *et al.*, 2001) and * from (Zhang *et al.*, 1993)

- Moiety at β position (R in diagram; fully reduced, H; hydroxyl, OH; carbonyl, O)
- AinR is homologous to two component sensor kinases; AinS is not homologous to LuxI
- QscR is a LuxR homologue that inhibits the activity of LasR
- Has an unsaturated bond between position 7-8 on the acyl chain
- LuxM/LuxN-LuxO-LuxR^c Phosphorelay, LuxRvh is not a LuxR family protein

Table 1. The examples of acylated homoserine lactones and corresponding sensors (denoted as AI-1 in this dissertation)

Although the three family proteins apparently differ in structure, they catalyze a common reaction recognizing SAM as an amino acid substrate, not as a methyl-donor, unlike most other SAM-utilizing enzymes. Acyl-acyl carrier protein (acyl-ACP) is known to be most efficient as an acyl donor for LuxI proteins, while acyl-ACP and acyl-CoA conjugates provide acyl side chains for AinS family proteins (Hanzelka and Greenberg, 1996; Hanzelka *et al.*, 1999). Acyl-ACP conjugates are intermediates in fatty acid synthesis, while acyl-CoA compounds are typically intermediates of fatty acid β -oxidation. Sequential reactions initiated by SAM binding followed by butyryl-ACP have been suggested from a study using purified soluble *P. aeruginosa* RhII (Parsek *et al.*, 1999). Studies on HdtS are incomplete, but based on the activity of *E. coli* PlsC, both acyl-coA and acyl-ACP could be used as substrates for autoinducer synthesis (Laue *et al.*, 2000).

Several reports show that single AI-1 synthases can produce multiple types of AI-1 molecules (Zhu *et al.*, 1998), implying a range of Acyl-substrate specificity for the enzymes. Also many bacteria are reported to have multiple AI-1 synthases. Production of a range of AI-1s with different length of acyl side chains may provide bacteria with greater versatility in adaptation to various ecosystems (Yates *et al.*, 2002). How they differentiate self- or non-self-derived signals is a key in the signal specificity.

In natural environments, AI-1 is not accumulated to high concentrations. This is partly explained by natural lactone ring hydrolysis by hydroxide ions in alkaline conditions. Such lactonolysis increases with increased temperature and decreases as the acyl side chains become more elongated (C12 and beyond). Opened lactone rings can be closed reversibly by lowering the pH. Enzymatic lactonolysis also occurs in the natural environments. Bacterial species, such as *Variovorax paradoxus* and *Bacillus* subspecies., can degrade AI-

1s as a source of organic compounds, energy, and nitrogen (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000).

Quorum signals are often detected with bioassays using luminescence (*lux*) or β -galactosidase (*lacZ*) genes or by the production of purple compound, violacein, in response to the presence of signal molecules. Currently used AI-1 monitoring systems are CviR-based *Chromobacterium violaceum* CV026 (Atkinson *et al.*, 1999), LuxR based *E. coli* biosensor strains *E. coli* (pSB536) for C4-HSL and *E. coli* (pSB1705) for 3-oxo-12-HSL (Winson *et al.*, 1998), and TraR-based *A. tumefaciens* NT1 (pZLR4). Using multiple biosensors in combination enables one to detect the entire range of known AI-1s (Ravn *et al.*, 2001).

Universality

AI-2 is the most recently identified signal molecule and it is considered to be “universal” since about half of the fully sequenced bacterial genomes, both Gram-positives and Gram-negatives, have genetic potential to produce this molecule, as they have a *V. harveyi luxS* homologue in their genome (Waters and Bassler, 2005).

The AI-2 biosynthetic pathway is now elucidated in which SAM is demethylated to S-adenosylhomocysteine (SAH), and then sequentially degraded to S-ribosylhomocysteine (SRH), and (4S)-4,5-dihydroxy-2,3-pentanedion (DPD) as depicted (Fig. 1). Most prokaryotes use this two step conversion of SAH to homocysteine, by enzymes Pfs (5'-Methylthioadenosine/S-adenosylhomocystein (MTA/AdoHcy) nucleosidase) and LuxS. While LuxS is essential for AI-2 synthesis, Pfs appears not as essential and works also in the SAM decarboxylation pathway producing polyamines (Lee *et al.*, 2003). In contrast,

Archea, Eukarya, and Alphaproteobacteria prefer the one-step conversion of SAH to adenosine and homocysteine (Sun et al. 2004). The resulting homocysteine can be degraded further to α -ketobutyrate and then to succinyl CoA or can be recycled back to methionine and SAM (Berg et al.2001).

The final structure of *V. harveyi* AI-2 was first reported to be a borated furanosyl diester as co-crystallized with LuxP protein, which specifically binds AI-2 in the periplasmic space. However, AI-2 from *S. typhimurium* is not borated (Miller *et al.*, 2004). Considering borate is abundant in the ocean (~0.4 mM) but not in the terrestrial environment (Waters and Bassler, 2005), another level of species-dependent variation must exist even in AI-2 reflecting the condition of natural habitats (Miller *et al.*, 2004). However, AI-2 is still considered to be a universal quorum signal molecule.

While *Vibrio* species use the periplasmic protein, LuxP, for AI-2 binding and then subsequently initiate signaling via a two-component phosphotransfer from the inner-membrane, both *E. coli* and *Salmonella* uptake AI-2 through an ATP-dependent cassette transporter into the cytoplasm (Taga *et al.*, 2003; Wang *et al.*, 2005). Although there remains speculation as to the reasons for ATP consumption in AI-2 uptake, none have been experimentally verified. Especially noteworthy is that in the gut, high concentrations of AI-2 are expected since AI-2 synthesis is associated with many enteric bacteria. Indeed, from the human stool specimens and from in vitro artificial intestine model, AI-2 activities were detected (Sperandio *et al.*, 2003). Presence of AI-2 was also found from stored foods (Cloak *et al.*, 2002).

So far, the most commonly used AI-2 biosensor strain is *V. harveyi* derivative BB170, which was developed by null-mutation of *V. harveyi* quorum receptor of AI-1, but keeping

the sensory of AI-2 intact (Bassler *et al.*, 1997). Recently, a third sensory of *V. harveyi* was identified (Henke and Bassler, 2004). Discovery of the third parallel sensory has not raised concerns on BB170 reporter activity (Fig. 4.A).

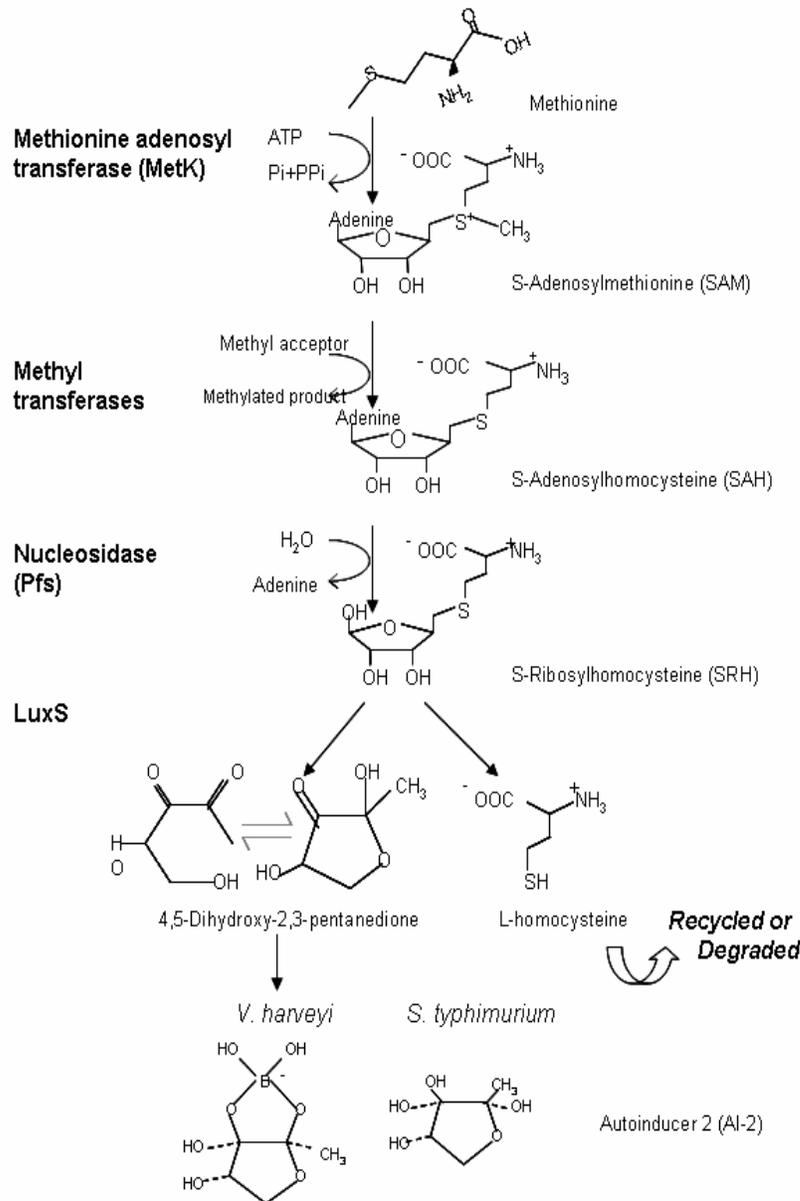


Figure 1. The conversion of S-adenosyl methionine (SAM) to AI-2.

Modified from (Miller *et al.*, 2004; Zhao *et al.*, 2003). The activated methyl group of SAM is used for methylating RNA, DNA, certain metabolites and some proteins leading to the formation of the toxic metabolite S-adenoylhomocysteine (SAH). Bacteria commonly remove the SAH using two enzymatic steps using Pfs and LuxS, which simultaneously generates 4,5-dihydroxy-2,3-pentandione (DPD). DPD is an extremely unstable molecule and cyclized as a furanone. AI-2 molecule co-crystallized as a ligand for LsrB of *S. typhimurium* is (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuranone (R-THMF), while AI-2 molecule as a ligand of *V. harveyi* is reported as a borated S-THMF. LuxS reaction also produces homocysteine, which can be either recycled to methionine synthesis (see Fig. 23) or be degraded.

1-2-2. Signal dependent transcriptional regulator

Structure: signal binding and DNA binding

Most well known regulatory elements for the autoinducer AI-1 quorum circuit, are an evolutionarily conserved group of proteins known as the LuxR family. Working as transcriptional regulators, this group of proteins has two functional domains, an N-terminal (about two-thirds) domain for binding autoinducer and a C-terminal (one third) domain for DNA binding and transcriptional activation. They also interact with RNAP α -subunit C-terminal domain (α CTD) to recruit RNA polymerase to target promoters (e.g., *V. fischeri* LuxR system (Stevens *et al.*, 1999)). Sequence similarities in C-terminus helix-turn-helix-containing regions define the group [Reviewed in (Choi and Greenberg, 1991)], while sequence variations in N-terminus signal binding domains with a hydrophobic pouch indicates signal-specificity driven protein evolution (Fig. 2). Coevolution between the LuxI and LuxR may have enabled bacterial cells to differentiate subtle structural features of signals from the diverse pool of quorum signals present in the environments.

Structural features of the regulatory proteins are as well understood as those of signal molecules, partly due to difficulties in protein purification and the time involved with structural determination. Generally LuxR-type proteins aggregate and become insoluble upon overexpression in *E. coli*. This problem has at times been solved by the addition of signal molecules at the same time as overexpression, which implies a critical requirement of AI-1 for effective translation and folding of the LuxR polypeptide. This was noted in studies with TraR (co-crystallized as a complex with its cognate signal, 3OC8-HSL) and *V. fischeri* LuxR, where cognate AI-1 addition increased the protein-yield by stabilizing LuxR conformation and increasing its DNA binding affinity after multimerization (Stevens and

Greenberg, 1999)(Urbanowski *et al.*, 2004). In this way, full length of *V. fischeri* LuxR was purified for the first time. Sometimes, an autoinducer-independent truncated form of LuxR has been used to study its transcriptional activity (Sitnikov *et al.*, 1996). Additional LuxR family proteins that have been successfully crystallized are: *Pantoea stewartii* ssp. *stewartii* EsaR (Minogue *et al.*, 2002), *Erwinia chrysanthemi* ExpR (Nasser *et al.*, 1998), *Erwinia* CarR (Welch *et al.*, 2000), and *A. tumefaciens* TraR (Zhu and Winans, 1999). These proteins are sometimes cocrystallized with their cognate signal or DNA showing the strength of the interaction.

Conserved DNA sequences for *V. fischeri* LuxR protein binding have been reported as a “lux box”: a 20-bp inverted repeat (ACCTGTAGGATCGTACAGGT). This sequence is centered at position -42.5 b away from the transcriptional start site. The lux box and its location with respect to other promoter elements is critical for LuxR-dependent activation of the lux genes (Egland and Greenberg, 2000)). A “Las box” has also been defined by the presence of a conserved sequence identified from all the genes known to be activated by the LasR/RhlR/QscR regulators. The las box, NNCT (N)₁₂ AGAA, is centered at -40 region (Egland and Greenberg, 2000; Whiteley and Greenberg, 2001). Suggested *A. tumefaciens* “Tra boxes” are ATGTGCAGATCTGCACAT, ATGTGCAGATCTGCACGT, and ATGTGCAAATCTGCACTT. Each of these is positioned differently relative to the transcription start site (Fuqua and Winans, 1996). The consensus sequences with dyad structures observed from the three LuxR proteins support bimolecular binding of LuxR on the promoter and indicate varied target motifs in LuxR family proteins among species.

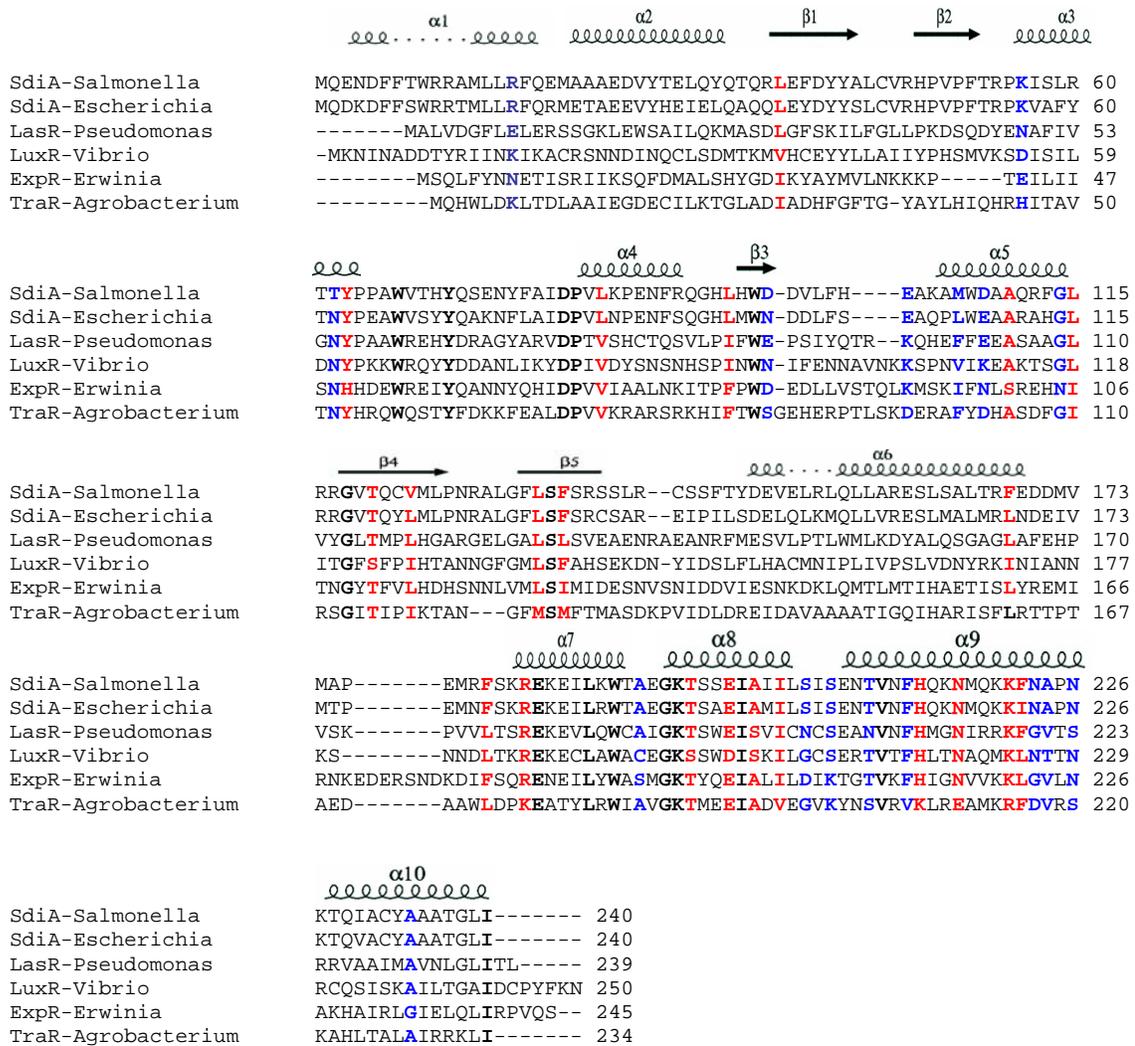


Figure 2. The multiple sequence alignment of six members of the LuxR family. *E. coli* SdiA, *S. typhimurium* SdiA, *P. aeruginosa* LasR, *V. fischeri* LuxR, *E. carotovora* ExpR, and *A. tumefaciens* TraR protein sequences were aligned by ClustalW algorithm (EMBL-EBI). Identical residues are distinguished by black bold letters and conserved residues are done by blue (less conserved) or red (more conserved) ones. Secondary structures were adopted from the TraR structure suggested from (Vannini *et al.*, 2004).

Transcription activation or repression

Members of the LuxR family proteins have different mechanisms for transcriptional activation. *P. aeruginosa* LasR, *A. tumefaciens* TraR, *V. fischeri* LuxR, and *Erwinia carotovora* CarR, the proteins bind to the DNA target sequences only when complexed with their corresponding AI-1. On the other hand, *E. chrysanthemi* ExpR can bind to its DNA target sequence even in the absence of its corresponding AI-1 (Reverchon *et al.*, 1998). ExpR can also function as a transcriptional repressor of its own synthesis. For this bimodular regulation, it represses DNA transcription in the absence of AI-1, while the presence of AI causes a conformational change making it dissociate from the target DNA. Similar examples can be found from *Pantotea stewartii* EsaR and *Serratia marcescens* SpnR. Most of the studied LuxR family proteins are not able to bind to the target sequences when complexed with AI-1 and when not complexed with AI-1 (Medina *et al.*, 2003). Another exception is RhIR, which can bind on *rhlAB* promoter in the presence and absence of AI-1.

1-2-3. Target genes (function)

By far, the most dominant targets for quorum sensing regulated genes are in association with pathogenesis (Ahmer, 2004; Newton and Fray, 2004; Suga and Smith, 2003). In particular, those that are reported to influence or control virulence are the most widely reported and studied, although there is an exception in that of luciferase encoding genes from *Vibrio*.

AI-1 controlling functions

Genes regulated by AI-1/LuxR family can be grouped into (exo) enzymes, antibiotics and siderophore production, and surface motility for gaining nutritional and spatial advantages in natural niches, capsular polysaccharide for virulence and aggregation, and producing gene transfer and conjugation agent for propagation. Certain AI-1s possess pharmacological and immunomodulatory activity such that their direct role as virulence determinants was implied (Yates *et al.*, 2002).

AI-2 controlling functions

The *luxS* and AI-2 are reported to be involved in global regulation of physiologic functions and virulence. However, due to the fact that AI-2 synthesis is associated with activated methyl cycle, the actual mechanisms of signal transduction and gene regulation by the autoinducer are difficult to determine. Still, an increasing number of reports show phenotypic changes by in conjunction with the *luxS* gene (e.g., a *Streptococcus pyogenes* mutant showed enhanced haemolytic activity and a reduction in secreted proteolytic activity (*sagA* and *speB*)(Lyon *et al.*, 2001). LuxS has also been shown to induce a hemin

acquisition protein and arginine-specific protein (RgpA), but repressed a hemin-regulated protein and an exonuclease (Chung *et al.*, 2001). LuxS was shown to be associated with acid susceptibility (Wen and Burne, 2004) and biofilm development in mixed cultures of *Streptococcus gordonii* and *Porphyromonas gingivalis* cells (Davies *et al.*, 1998; Li *et al.*, 2002; Merritt *et al.*, 2003). Also, in the gram positive *Clostridium perfringens*, the importance of LuxS and AI-2 was reported in the production of the three toxins, *alpha*-, *kappa*-, and *theta*- toxins (Ohtani *et al.*, 2002).

Independent microarray-based transcriptome analyses were done to show the consequence of *luxS* mutation in EHEC 0157::H7 (Sperandio *et al.*, 2001) and the effects of conditioned media with or without AI-2 on W3110 (DeLisa *et al.*, 2001). Both studies showed global effects of AI-2/LuxS, in varied functions including DNA processing, carbohydrate metabolism, surface- and outer membrane-associated function, and virulence.

To date, the only confirmed AI-2 dependent gene transcription identified in *E. coli* was also shown in *Salmonella*, is limited to AI-2 uptake machinery, *lsr* (*luxS* regulated) operon and the methionine synthase gene, *metE*.

1-2-4. Methodology for identifying quorum signaling components

Identification of quorum sensing components has been accomplished by various molecular biological techniques. Commonly, *E. coli* is used as a surrogate host, as its relatively well-annotated genome and ease of genetic manipulation for harboring reporters or regulatory genes on multicopy plasmids, make it amenable to a variety of techniques. Interestingly, so far ignored *E. coli* quorum signaling (as a host) affects the activity of heterogeneous quorum system (Lindsay and Ahmer, 2005).

Advanced from the non-quantitative Northern analysis techniques, recently-developed microarray technologies have been used as systemic tools for identifying the architecture and dynamics of gene networks (Tegner *et al.*, 2003). In vivo expression technology using promoterless reporter genes, are also used for positive selection of promoters that are specifically activated under certain conditions. Although microarrays and promoter-fusions are carried out at similarly large scales, they differ in that one represents the real quantity of RNA levels while the other is inevitably associated with protein expression. Screens of putative quorum components require further confirmation with in vitro assays, which provide information on the mechanisms of gene expression regulation. Importantly, with the increasing number of fully sequenced bacterial genomes, homologous sequence searches have proven quite effective and efficient in identifying conserved quorum components.

1-3. Quorum signal activation

1-3-1. Well-known model organisms, *Vibrio* species

Direct signal activation

Perhaps the simplest quorum sensing mechanism is based on the direct binding of AI-1·LuxR to activate transcription, as shown in *Vibrio fischeri* (Fig. 3). For this, AI-1 diffuses out and then comes back into the cell as the cell density increases. A second, more complex, type of signal transfer was also identified from bacteria of the genus, *Vibrio* (see below).

Phosphorelay

V. harveyi quorum sensing uses two component sensors initiating phosphorelay cascade for density dependent activation of the *lux* operon. In this model (Lenz *et al.*, 2004), at low cell density when the autoinducers are present at low concentrations, phosphate flows toward LuxO, which belongs to a large and highly conserved family of σ^{54} -dependent transcriptional regulators. LuxO has three conserved domains: the response regulator domain, the σ^{54} activation domain, and a HTH motif for direct DNA binding (Lilley and Bassler, 2000). Together with σ^{54} , phospho-LuxO can activate the expression of small regulatory RNAs (sRNAs). The complexes of the sRNAs and sRNA chaperone protein Hfq destabilize the mRNA of the quorum-sensing master regulator LuxR_{VH}, resulting in the indirect repression of *lux* operon transcription. LuxR_{VH} is not homologous to *V. fischeri* LuxR.

At high cell density, the binding of autoinducers to membrane receptors makes the phosphate flow away from LuxO. In this condition, LuxO is dephosphorelyated and the regulatory sRNAs are not expressed. As a consequence, LuxR translation and its expression are not free from the repression of destabilizing sRNA-Hfq complex. The result is the transcriptional activation of the *lux* operon.

In the suggested *V. harveyi* models, autoinducers do not need to reenter the cells and can initiate the phosphorelay from the innermembrane bound sensory proteins, LuxN and LuxPQ (Freeman *et al.*, 2000). Multiple components for quorum signaling were identified from *V. harveyi*, but LuxI-LuxR_{VF} homologues have not been found to date.

Bacterial phosphorelay is close to eukaryotic phosphotransfer featured with a conversion of response regulator from kinase to phosphatase. However it is distinguished from eukaryotes in that aspartate and histidine residues are used for phosphotransfer rather than threonine and serine.

Interestingly, a second quorum component pair (synthase/regulator) was identified in *V. fischeri*. AinS, and its corresponding regulator, LitR, are suggested to be operated at low cell density in laboratory cultures. Along with LuxS, the AinS/LitR pair, is homologous to quorum components of *V. harveyi* and is therefore, considered to operate in an analogous manner to the LuxO system of *V. harveyi*. It is not known whether sRNAs are also involved in bioluminescence control in *V. fischeri* (Lupp and Ruby, 2005).

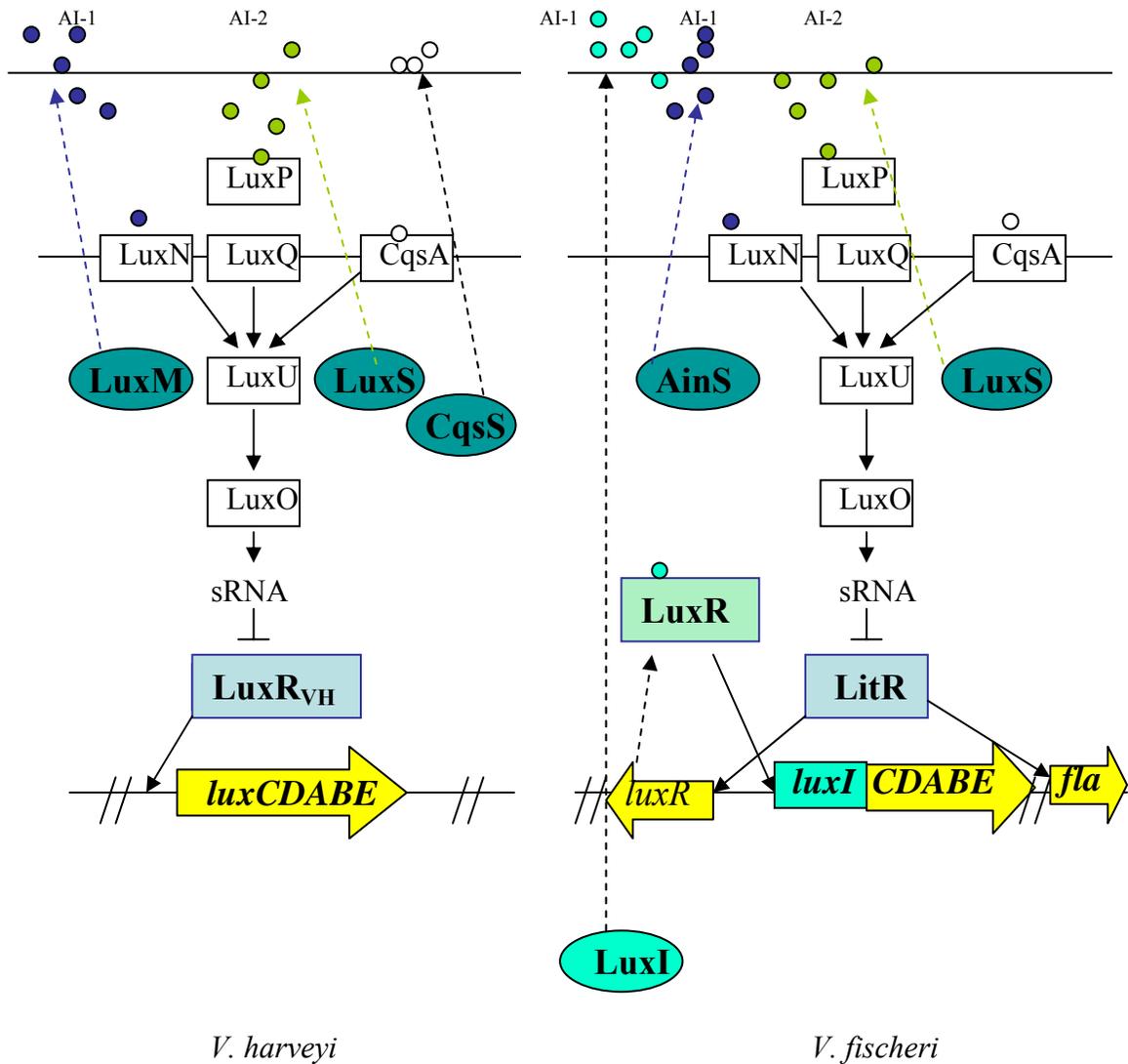


Figure 3. Currently suggested *Vibrio harveyi* and *V. fischeri* quorum signal pathways. Currently identified three parallel quorum signaling pathways of *V. harveyi* and *V. fischeri* are depicted with signal synthase (ovals), transcriptional regulators (colored rectangle), and the target genes (yellow arrows). *V. fischeri* LitR is homologous to *V. harveyi* LuxR_{VH}, while *V. fischeri* LuxR does not share homology to it. Note that LuxI-originated signal molecule binds (light blue circles) the *V. fischeri* LuxR to activated the *lux* operon while other quorum signal molecules initiate the signaling from the inner membrane (Visick, 2005).

1-3-2. Quantitative analysis signal activation (dose-responsive)

The physiologically relevant AI-1 concentrations for target gene activation have been calculated from dose-response activation curves (Blosser and Gray, 2000; Michael *et al.*, 2001; Zhu *et al.*, 1998). For this purpose, *in vitro* synthesized signal molecules are preferred over conditioned media (CM) due to their directed action and the potential for directly assigning activity to the presence of the specific molecule (as opposed to an array of molecules present in CM).

A study with *C. violaceum* reporter strains tested with a synthetic AI-1(C6) showed the maximum production of pigment at a concentration of 20 nM, while a minimum detectable response occurred with 1 nM of the signal (Blosser and Gray, 2000). TraR activity on TraI promoter by additional cognate AI-1 (3-oxo-C8-HSL) showed that *c.a.* 1 nM of AI-1 can induce the half maximal response, while *c.a.* 5 nM can saturate the activity (Zhu *et al.*, 1998). When transcriptional activity of *S. typhimurium* SdiA on the *rck* promoter was tested with 10 AI-1 type analogues, it showed a half-maximum response concentration of 1-5 nM, using the most potent signal (oxoC10). *rck* is the gene which was found from the promoterless *lacZ* genetic screen searching for *sdiA*-dependent gene expression (Michael *et al.*, 2001). From the three studies presented here, approximately 5 nM of AI-1 seems to be sufficient to activate target gene transcription at levels up to half the maxima.

At present, direct quantification of AI-2 is not available due to its extreme polarity. So the concentration is measured rather indirectly from *in vitro* synthesized AI-2 (Ellman's test for free sulfhydryl groups, calculated assuming a 1:1 ratio between homocysteine and AI-2)(Schauder *et al.*, 2001) or by measuring luminescence of BB170 cultures after adding

serially diluted conditioned media. About 100 nM of in vitro-synthesized AI-2 accomplished the half-maximal level of light production from BB170 reporter (Fig. 4.B) (Schauder *et al.*, 2001).

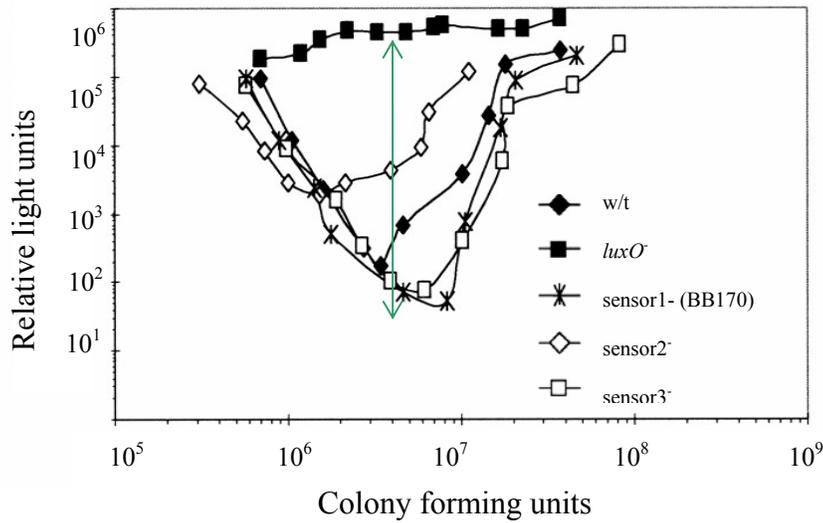
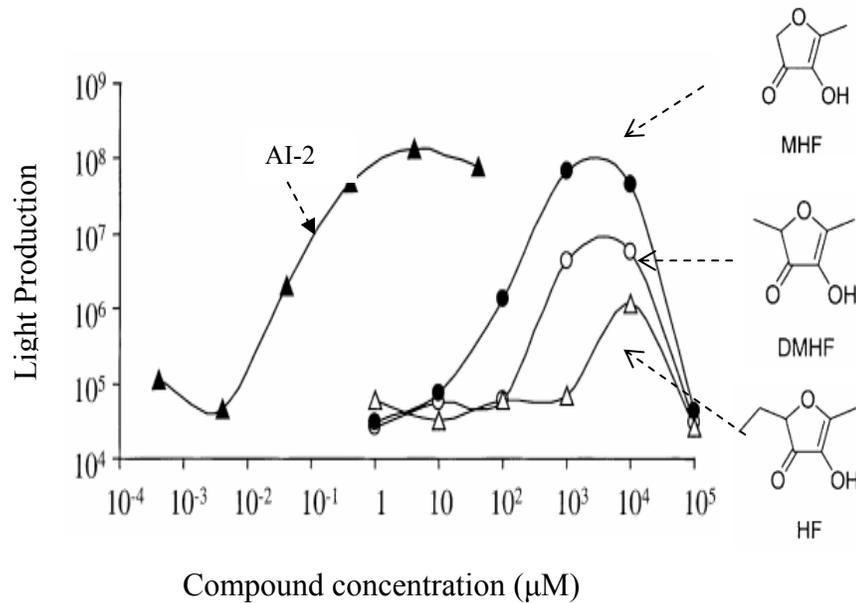
A.**B**

Figure 4. The activity of quorum signals on *Vibrio harveyi* luminescence.

(A) Density dependent luminescence of *V. harveyi* single, double, and triple autoinducer sensor. Light units are reported as counts $\text{min}^{-1} \text{ml}^{-1} \times 1,000/\text{CFU ml}^{-1}$. Arrow is to show the increase luminescence by exogenous quorum signal. Figure was adopted and modified from (Henke and Bassler, 2004). **(B)** Activities of in vitro synthesized AI-2 and its structure analogues (MHF, DMHF, and HF) were measured using *V. harveyi* reporter BB170. Figure was adopted from (Schauder *et al.*, 2001).

1- 3-3. Signal interception and eavesdropping in environments

The diversity or universality of signals imply intra- or inter bacterial species communication as well as cross kingdom gene regulation. Situations, such as the presence of quorum sensing-dependent target genes in the absence of the endogenous signal synthases, have intrigued many research groups. In the environment, bacteria such as *Pseudomonas aeruginosa* may modulate their behavior by monitoring environmental conditions and probably by eavesdropping on the other bacterial AI-2 and/or other signals. *P. aeruginosa* does not have the *luxS* genes, but has genes responding to the AI-2.

Examples of inter-kingdom signal events can be found from studies using halogenated furanones from marine algae *Delisea pulchra* inhibiting infection by plant pathogen *Serratia liquefaciens*. Structurally similar to the AI-1, halogenated furanones can competitively inhibit the bacterium's quorum sensing system (Rasmussen *et al.*, 2000). Also mammalian hormone, such as epinephrine, is reported to be analogue of autoinducers of enterohemorrhagic *E. coli* autoinducer (Sperandio *et al.*, 2003).

1-4. Quorum sensing in the global regulatory circuits

1-4-1. Catabolite repression and autoinduction

There are more factors controlling the luminescence of *V. fischeri* besides the commonly reported autoinduction system. It has long been appreciated that autoinduction of bioluminescence in many marine bacteria is also subject to catabolite repression of AI-1 synthesis. That is, the presence of glucose was reported to delay bioluminescence of *Vibrio* (Ruby and Nealson, 1976). In contrast, the addition of glucose to the enteric bacteria, *E. coli* and *Salmonella* cultures increases and prolongs the duration of AI-2 activity (Surette *et al.*, 1999), implying a different physiological role of glucose in the metabolism-linked Quorum signal regulation.

1-4-2. Global regulators

Quorum sensing, as a global gene regulation system, was first shown in *P. aeruginosa* (ChaponHerve *et al.*, 1997). In this study, the roles of stationary phase sigma factor, *rpoS*, and genes coding for components of the general secretory pathway (*xcp*) suggested that cells may anticipate stationary phase by sensing the crowdedness. Induction of RhlR by RpoS was reported, but this is controversial, since opposite observation of induction of RpoS by RhlR and C4-HSL mediated quorum sensing was also reported from *P. aeruginosa* (Latifi *et al.*, 1996; Whiteley *et al.*, 2000). It is not conclusive which one is hierarchically higher, or take precedence, but it is highly likely that RpoS and quorum regulators are closely linked and reciprocally influencing the other.

1-4-3. Molecular chaperones and proteases

Molecular chaperones and proteases control the quality of proteins newly emerging from ribosomes (about 10 to 20 % of the total) by binding to exposed hydrophobic patches on proteins. Chaperones prevent partially unfolded from being aggregated to functionally inactive states. They also facilitate the destruction of structurally damaged and/or misfolded proteins occurring from various stresses, such as oxidative stress and heat shock stress. This process consumes a substantial amount of energy in the form of ATP and requires a balance between the levels of chaperones and proteases (Wickner *et al.*, 1999).

The first introduction of molecular chaperones as components within quorum sensing systems was demonstrated by the overexpression of a cloned *V. fischeri luxR* gene in *E. coli* (Dolan and Greenberg, 1992). For proper expression of LuxR, GroES·GroEL complex also was overexpressed. Another group has focused on the role of proteases in controlling the life-span of the transcriptional regulator (Zhu and Winans, 2001). An alarmed state resulting from “crowdedness” needs to be tuned so that when the cell density level decreases, the activated system should be down-regulated. Proteolysis of the transcriptional regulator has been suggested as one way to terminate the activated LuxR-AI system in *A. tumefaciens*.

1-4-4. Small RNA

Small regulatory RNA first appeared in quorum circuitry in the *V. harveyi* model, as demonstrated by (Lenz *et al.*, 2004). The discovery of regulatory sRNA among the prokaryotes is very recent (last 5 years). Prokaryotic sRNA activity can be found in the transition to stationary phase, iron homeostasis, and polarity in some operons. A major

class of sRNAs works by binding to RNA chaperone, Hfq, followed by base-pairing with target mRNAs. This base pairing affects the translation or mRNA decay either positively or negatively, depending on the prevailing conditions and targets (Gottesman, 2004). Although it is highly likely there are many sRNA lurking in the genome, their small size and the requirements of suitable conditions for their expression make it difficult to confirm them. Various approaches using microarrays, bioinformatics, and immunoprecipitation with Hfq have been employed to hunt down and characterize novel orphan sRNAs.

Sensitivity to environmental conditions endows small RNA-based regulators the advantage of a shortened adjustment period, which make them appropriate for sensing changes of population density.

1-4-5. Others

Some environmental conditions, such as iron and oxygen limitation, promote *V. fischeri* luminescence at low cell density in monocentric fish, while also limiting the growth of *V. fischeri* cells (Dunlap and Kuo, 1992). Also, the production of AI-2 in *E. coli* was regulated by environmental parameters, such as the availability of preferred carbon sources (DeLisa *et al.*, 2001; Surette *et al.*, 1999) and acid stresses (Kirkpatrick *et al.*, 2001; Stancik *et al.*, 2002) suggesting that the AI-2 mediated quorum sensing is linked to nutritional limitation and stress responses. Other control elements, such as *gacA*, *rsaL*, and *Vfr* were reported along with the specific quinolones produced by *P. aeruginosa* (Parsek and Greenberg, 2000)

1-5. Quorum sensing in enteric bacteria (problem recognition)

1-5-1. Gene phylogenies of the quorum sensing components

Despite having a highly annotated genome, understanding of *E. coli* quorum sensing is not as advanced as it is in less-well characterized species. Currently identified quorum sensing components are; a *luxR* homolog, *sdiA*, and the only signal molecule with a known synthetic pathway (AI-2, *luxS*). It is noteworthy that although hormone-like signal molecule AI-3 was reported from *E. coli* serotype O157:H7 (EHEC), its synthesis is still *luxS* dependent. There are no *luxI* homologs for AI-1 synthesis (Fig. 5).

Regarding its functionality (as mentioned above), we now know that AI-2 activates its own up-take machinery encoding genes, *lsr* operon. The functionality of *sdiA* is associated with cell-division. However, its expression and functionality are not fully understood and agreed on. Details regarding SdiA are described further in Chapter 3.

Three molecular phylogeny studies regarding the genetic components for quorum sensing were reported. From them, we could find information relating to *E. coli* quorum sensory components. From the two studies regarding the phylogenetic trees of LuxR homologues, *E. coli* SdiA is reported to be closer to that of *Pseudomonas* spp., which in turn, are not as closely related to other enterobacterial species as to *E. coli* in the SSU rRNA phylogenetic tree (Gray and Garey, 2001; Lerat and Moran, 2004; Sun *et al.*, 2004). While cognate *luxI* and *luxR* gene pairs seem to have become linked physically and/or functionally early in history, still, many bacterial species contain multiple LuxI and /or LuxR homologues and product or detect distinct signals.

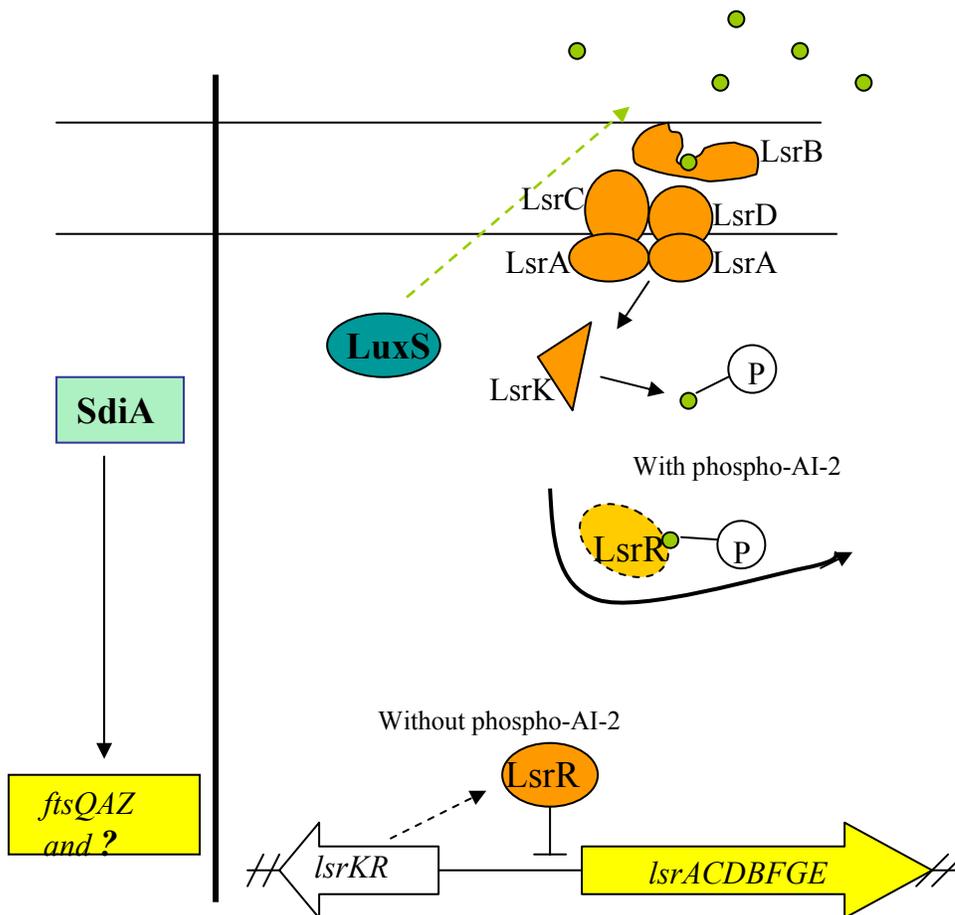


Figure 5. The genetic components of *E. coli* and *Salmonella* quorum sensing.

Currently identified quorum components are depicted. In the quorum system 1, LuxR type transcriptional regulator, SdiA, is identified with its target operon, *ftsQAZ*. In the quorum system 2, signal synthase LuxS produces AI-2 molecules, which are secreted outside the cells and reenter the cell through ATP binding cassette type-transporter, Lsr. Once entering the cells, AI-2 is phosphorelated by LsrK and binds the LsrR. While AI-2-free LsrR represses the *lsr* structural gene transcript, phospho-AI-2 bound LsrR cannot bind the *lsr* promoter anymore, derepressing the transcription of the *lsr* structural genes. The *lsrR* and *lsrK* are divergently transcribed from the *lsrACDBFGE*. Note that *E. coli* does not have the most distal gene, *lsrE*, which is present in *Salmonella* *lsr* operon. Model figure was adopted and modified from (Ahmer, 2004).

Multiple LuxR homologues can also permit the independent activation of different gene functions in response to a single autoinducer signal (Gray and Garey, 2001). The authors' view is that the *E. coli luxI* component was lost in evolution. As previously mentioned, the absence of signals could not be pivotal for their functionality, especially in mixed cell culture environments. Theoretically, without endogenous signal production, bacteria can still detect the presence of coexisting or competing bacterial species by sensing the environmental concentration of signals. Supporting this notion, *Pseudomonas aeruginosa* is reported to sense the AI-2 from the other bacterial species (Duan *et al.*, 2003). Also, Ahmer and colleagues have shown that AI-1 originated from other species can modulate *Salmonella* SdiA activity (Michael *et al.*, 2001; Smith and Ahmer, 2003). However genes are robustly changing their functions in the long run, it is also possible that SdiA may have gained new functions and can recognize new types of signal molecules as effectors present in pure cell culture environments.

Two independent phylogenetic trees for LuxS (Lerat and Moran, 2004; Sun *et al.*, 2004), agree with each other showing the ancestral presence of *luxS* in γ -Proteobacteria. Some cases of incongruence with 16S rRNA based microbial phylogeny can be found from *Bifidobacterium longum*, *Helicobacter pylori*, *Clostridium acetobutylicum* and *Borrelia burgdorferi*. The orthologs of the *S. typhimurium lsr* genes were found in most Enterobacteriales as well as in *Sinorhizobium meliloti* and some *Bacillus* species (Sun *et al.*, 2004).

Unlike the broad distribution of LuxS, AI-2 signal cascades components (Fig. 3.) are present in only *Vibrio* strains tested by a reciprocal best hit strategy (the periplasmic AI-2 binding protein LuxP, hybrid sensor kinase LuxQ, and the regulator protein LuxU, and the

terminal two component response regulator LuxO orthologs). However, the unidirectional best hit search expanded the LuxP homologues search to 28 species with D-ribose binding proteins and 104 organisms both with the hybrid sensor kinase LuxQ, and the two-component regulator LuxO. These studies show the uniqueness of *Vibrio* species quorum sensing different from that of *E. coli* and *Salmonella*.

1-5-2. Enteric environmental conditions

Gastrointestinal tracts of the stomach, small intestine, and large intestine are the site of food digestion and the major habitat of *E. coli*. Both commensal and pathogenic *E. coli* should pass the acid barriers of the stomach (pH 2) and enter the small intestine, in which pH becomes gradually more alkaline. In the intestinal cavity of the small intestine lower ileum, bacterial cell numbers are commonly about 10^5 - 10^7 per gram. In the large intestine, there commonly exist 10^7 facultative anaerobes and 10^{10} - 10^{11} obligate anaerobe cells per gram of intestinal content. Facultative *E. coli* are present in the intestines but at numbers smaller than obligate anaerobes, such as *Clostridium* and *Bacteroides*. The growth rate of bacteria in the lumen is reportedly one to two doublings per day. Intestinal flora has a profound influence on the animal, carrying out a wide variety of metabolic reactions. Of special note are the roles of the intestinal flora in modifying compounds of the bile. Bile acids are steroids produced in the liver and excreted into the intestine via the gall bladder. Their role is to promote emulsification of fats in the diet so they can be effectively digested. Intestinal microbes transform these bile acids (Brock and Madigan, 1991).

Purpose of study and approach

Quorum sensing is now a common terminology describing sociological group-wise bacterial adaptation to changes in its own or others' cell density. In this chapter, I contrasted quorum systems by categorizing them under the criteria of diversity and universality of the signal molecules. Uses of diverse or universal forms of signals or both may give cells different kinds of advantages, which are not yet understood and is needed to be investigated.

Challenges properly describing quorum dependent features from non-pathogenic *E. coli* were from the absence of “diversified” signal, AI-1 and its synthase, although they are genetically competent for receiving AI-1 type signals by possessing LuxR homologue encoding gene, *sdiA*. Does it mean that they are neither to reveal their presence to others nor to count themselves; but rather, only interested in counting the other species? This basic question on the functionality of System 1 was complicated or overwhelmed System 2, which actively produces and takes up its signal molecule, AI-2. System 2 is easily affected by environmental factors, but it is not quite clear “how”. Looking at this puzzling architecture of the *E. coli* quorum sensing, comprised of the seemingly incomplete system 1 and the environmentally sensitive system 2, has intrigued me and inspired me to investigate the functionality of system 1 and find, at the molecular level responsible regulators effecting the changes in AI-2 upon stress exposure.

Experimental approach to solve the above questions follows below.

1. The functionality of *E. coli* quorum system 1 was examined by deleting *sdiA* and performing microarray analysis. It was expected when combined with the microarray

results from the overexpression of *sdiA*, these data could help to identify *sdiA*-dependent genes, which may begin to explain SdiA function.

2. Different kinds of molecular chaperones and their wild type parental strains were examined for the AI-2 activity under different culturing conditions. By varying environmental stimuli, reproducible effects of chaperone activity on system 2 were expected.

Chapter 2: Quorum-sensing under stress-effects of heat shock on *Escherichia coli* AI-2 signaling

Abstract

Sensing cell-density and subsequent physiological adjustments are tuned to a global signaling network that receives various cues from the environment. In an effort to identify components linking quorum-sensing to the global sensory system, we examined whether defects in the molecular chaperones, DnaJKE and GroESL, influence AI-2 profiling. We tested the conditioned media from 20 different *E. coli* strains grown in LB media in order to characterize the kinetics of AI-2 accumulation and depletion, and to link these phenomena to specific molecular chaperones reportedly involved in the folding of quorum sensing transcriptional regulators. The examined *E. coli* strains include those with null and point mutations in the major chaperones, DnaJKE or GroESL and their isogenic parental strains. Imposing “high temperature” as an environmental stress to chaperone mutated *E. coli* cells growing in Luria-Bertani media with glucose supplementation showed uncontrolled AI-2 production beyond the stationary phase, while wildtype cells in the same conditions showed the commonly reported increase and decline exemplified by the growth of cells at 30°C. We present AI-2 profiles in various genetic backgrounds as evidence of active AI-2 decrease towards the stationary phase that is controlled by the molecular chaperone complexes, DnaJKE and GroESL.

Key words: AI-2 profile, molecular chaperones, heat shock, glucose

Introduction

The dynamic physico-chemical conditions of natural habitats place evolutionary pressures on bacteria for the development of sensory signal mediated stress-adjustment processes. Blocking or stimulating such signaling pathways, interwoven as a complex web of interacting genes and proteins, often leads to changes in other signaling pathways. Quorum sensing, signal mediated bacterial assessment of population size in confined environments and subsequently adjusted phenotypic changes in response, is not an exception and is open to cues of not only biotic conditions but also many abiotic conditions. Thus “cell density” is more likely to be a relative term reflecting the number of cells within a particular environment. A most recent review on quorum sensing can be found in (Waters and Bassler, 2005).

Physico-chemical conditions can influence the onset of quorum activation. Examples include the luminescence of the *Vibrio* species, which was noted to change with the chemical composition of the culture medium. The presence of glucose delayed luciferase gene expression (Dunlap and Greenberg, 1988), while iron restricted conditions caused the earlier induction of luminescence, as compared to fast-growing iron sufficient cultures (Dunlap, 1992). An artificially induced stringent response, a nutrient stress response in *Pseudomonas aeruginosa*, was shown to induce premature expression of secreted virulence factors such as elastase (Van Delden *et al.*, 2001).

One exemplary signal AI-2, produced both from Gram+ and Gram- bacteria, has been suggested as a universal quorum signal molecule and has distinct characteristics in response to the growth conditions. Not alone to the specificity of AI-2 as a quorum

signal, its production level and pattern to environmental conditions have been mainly paid attention to. Related with its culminating timing, interesting observations were noted wherein AI-2 was produced early from *Campylobacter jejuni*, *C. coli*, *Salmonella typhimurium*, and *E. coli* grown in brucella broth at 4 °C. This observation was media dependent, since the same strains in different food or broth conditions at the similar temperatures did not produce AI-2. The reason for the early appearance of AI-2 activity at 4 °C is not known (Cloak *et al.*, 2002).

However, most of growth condition responsive AI-2 has been reported in a quantitative manner. Surette and Bassler reported that the presence of glucose, high osmolarity (0.4 M), and low pH (pH5) activated AI-2 production in *Salmonella typhimurium* LT2. In contrast, low osmolarity (0.1%) and heat shock (43°C) affected AI-2 production negatively (Surette and Bassler, 1999). Low pH was also shown to induce AI-2 synthase, LuxS, expression in *E. coli* (Kirkpatrick *et al.*, 2001) and also in *Lactococcus lactis* (Frees *et al.*, 2003). Our laboratory, using chemostat cultures, has also reported changes of AI-2 activity by various environmental stimuli from *E. coli* K-12. Namely, AI-2 levels increased with pulsed addition of glucose, Fe(III), NaCl, and dithiothreitol, and decreased with aerobiosis, amino acid starvation, and overexpression of cloned human interleukin-2 (DeLisa *et al.*, 2001).

To explain the quorum sensing responding to varied environmental perturbations as mentioned above, many investigators have turned to experimentally examining cross regulation between quorum regulators and other global transcriptional regulators. For example, Cyclic AMP receptor protein (CRP) frequently activates the expression of

LuxR family proteins, i.e. *V. fischeri luxR* (Dunlap and Greenberg, 1988) and *P. aeruginosa lasR* (Albus *et al.*, 1997). The cAMP-CRP associated repression of quorum sensing genes was reported, less frequently. Repression of *expI* in *Erwinia chrysanthemi* by CRP was suggested as a reason for declined signal concentration in the stationary phase (Reverchon *et al.*, 1998). AI-2 profiling of *E. coli* and *Salmonella typhimurium* is such that its activity increases along with growth until reaching a maximum in the late exponential phase. When cells enter the stationary phase, the AI-2 levels drop. Now it is known that the disappearance of AI-2 is actively mediated by an ATP-binding cassette type transporter (Taga *et al.*, 2003; Taga *et al.*, 2001). The decrease of *E. coli* AI-2 upon stationary phase can be explained by cAMP-CRP; it not only downregulates the AI-2 synthesis (indirect) but also activates AI-2 uptake machinery expression (*lsr* operon) (Wang *et al.*, 2005). In *Pseudomonas putida*, cross-regulation of global response regulator GacA and stationary-phase sigma factor RpoS on the regulation of AHL system was shown (McDougald *et al.*, 2003). Related with this, Affymetrix-based transcriptome analysis of *P. aeruginosa* further testified that more than 40 % of previously suggested quorum-controlled genes were also RpoS-dependent (Schuster *et al.*, 2003) .

Here, we are especially interested in the possibility of molecular chaperones as another type of quorum regulator. Molecular chaperones constitute a group of proteins that stabilize otherwise unstable, non-native states through controlled binding and release thus preventing aggregation [reviewed in (Wickner *et al.*, 1999)]. This group of proteins has multi-functionality for facilitating the correct folding, oligomeric assembly and transport of proteins (and even RNAs) to a particular subcellular compartment, or to a

location for disposal by degradation (Aoki *et al.*, 2000; Hartl, 1997; Reid and Flynn, 1996).

Most folding of newly synthesized proteins in the cytosol is aided by two different sets of molecular chaperones; a 70-kDa chaperone (also called as heat shock proteins, hsp70s, DnaK) and a 60kDa chaperone family (hsp60s, GroEL). They fulfill distinct roles in protein folding so that Hsp70s and their partner proteins can stabilize translating and/or newly synthesized polypeptides until all segments of the chain necessary for folding are available (co-translational binding), whereas chaperonins (GroESL) promote protein folding in the sequestered environment of their central cavity (Hartl, 1997). The chaperonin, GroEL, along with its cofactor GroES, is the only known chaperone system in *E. coli* that is essential under all growth conditions (Fayet *et al.*, 1989; Horwich *et al.*, 1993). Various structurally unrelated proteins can be the targets of GroESL. Especially related with stress-induced changes, chaperones are thought to control both intercellular and extracytoplasmic stress activated σ factors, although there is an opposing observation that the control of GroESL was not important for *Bacillus subtilis*, σ^B , a general stress response transcription factor (Scott *et al.*, 1999). A central role of DnaJKE is to down regulate the σ^{32} -dependent heat shock response. σ^{32} is one of the principal σ factors regulating heat shock gene expression in *E. coli* [reviewed in (Bukau, 1993)]. While expression of DnaJKE itself is dependent on heat and σ^{32} , a negative feed-back loop exists such that the σ^{32} -dependent heat shock response is eliminated by DnaJKE. The function of the mechanism apparently is to maintain optimal levels of the heat shock proteins needed for normal abundance of unfolded proteins (Craig and Gross, 1991)

Molecular chaperones have received attention from researchers working in quorum sensing especially those who tried to overexpress LuxR proteins for *in vitro* experiments or who tried to construct heterogeneous quorum systems using *E. coli* as host cells. While the former group emphasizes the importance of protease activity of molecular chaperones in non-quorum conditions (Zhu and Winans, 1999), the latter group emphasizes the requirement of GroESL in the conformational activation of LuxR proteins available for quorum activation on the *lux* operon (Dolan and Greenberg, 1992).

In this study, hypothesis was made that molecular chaperones would be a hub interconnecting quorum sensing to other global signaling networks, as shown from its pleiotropic functionality. We examined AI-2 signaling profiles affected by mutations in molecular chaperones. A high temperature condition was imposed to various chaperone mutants and their otherwise isogenic parental strains. Cells were cultured in Luria-Bertani media (LB) or LB supplemented with non-limiting concentrations of glucose. By combining the three factors, chaperones, high temperature, and glucose, we aimed to clarify a general effect of heat shock on AI-2 activity and to see the functional manifestation of the major chaperone as a stress modulator or as a cell process maintainer. Finally, we wanted to see if the experimental method (heat shock or high temperature) could be used as a parameter for discerning the steps on which the major chaperones are exerting their regulatory influence: AI-2 synthesis or uptake/degradation.

Material and Methods

Strains *E. coli* chaperone mutants were a gift of Drs. Costa Georgopolous, Debbie Ang, Pierre Genevaux (Département de Biochimie Médicale, Centre Médical Universitaire, Université de Genève, Geneva, Switzerland). *Vibrio harveyi* reporter strain BB170 was provided by Dr. Bonnie Bassler (Department of Molecular Biology, Princeton University, Princeton, USA). The genotypes of each strain are shown in Table 2.

Media Cells were cultured in Luria-Bertani media (pH 7.4). Glucose supplemented LB media was prepared by adding sterile 20 % (w/v) glucose stock at a 1/25 fold dilution (final concentration of 0.8 % (v/v), 8.88 mM).

Culture condition and heat shock Overnight seed cultures were prepared in LB media at 30 °C. Then cells were freshly cultured either as tube cultures or flask cultures. Tube culture experiments were initiated by 1/50 fold dilution of overnight seeds in new LB media. Polytest tube: 17x100 mm polystyrene test tubes were used (Fisher Scientific C., Inc., Pittsburgh, PA). Each of 20 *E. coli* strains was dispensed to four 15 ml test tubes with the volume of 2 ml. Two replicate sets of cultures were used to obtain growth curves at either constant 30 °C or 42 °C. For flask culturing of *E. coli* cells, overnight cells were 100-fold diluted to 25 ml LB (250 ml flask culture). Cell culturing conditions were identical to those of tube cultures.

Especially for 1 hr heat shock treatment, three replicate sets of tube cultures were prepared (1/50 fold dilution of overnight seeds) and were initially cultured at 30 °C until temperature upshift at either 3 hours (HS-case study 2) or 7 hours (HS-case study 1) after

initial inoculation. The three replicate tube cultures were used for obtaining AI-2 activities of initial cultures without stress, the cultures grown the additional 1 hour at 30 °C or cultured for the 1 hour at 42 °C, respectively. Temperature upshift was done by transferring each set of *E. coli* cultures for heat shock into a 42 °C incubator (air chamber). Cells were placed without shaking during the period (1 hr) and culture tubes were placed on ice for 2 minute to stop the reaction.

In this report, three independent experiments were performed for constant temperature cell cultures (30 °C or 42 °C) to obtain the AI-2 levels, while 1 hr heat shock experiments were performed just once, but in duplicate.

Conditioned media Conditioned media were prepared by centrifugation of *E. coli* cultures at 16,000xg for 10 min 4 °C. Supernatants were filtered with 0.2 µm HT Tuffryn filters (Pall Corp., Ann Arbor, MI).

AI-2 assay and reported values The AI-2 activity in the *E. coli* conditioned media was measured following the method in (Bassler *et al.*, 1997). BB170 was grown for 16 h with shaking at 30°C in AB medium with 10 µg/ml of kanamycin, diluted 1:5000 in fresh AB medium and aliquoted to sterile 12x75mm tubes (Fisher Scientific C., Inc., Pittsburgh, PA). Cell-free culture fluids were added to a final concentration of 10 % (v/v) to these tubes. Negative controls contained 10 % (v/v) sterile LB or LB plus 0.8 % glucose. Tubes were shaken at 175 rpm and 30 °C in a shaker (New Brunswick Scientific). Luminescence was measured by quantifying light production as a function of *V. harveyi* cell density (EG&G Berthold, Gaithersburg, MD). Here, AI-2 levels are reported either as AI-2 fold-induction (normalized luminescence by negative control) or as relative AI-2 fold-induction (normalized luminescence by isogenic parent strains).

Especially for 1 hr heat shock experiments, AI-2 levels are reported after two-step normalization to discern the portion attributable to natural changes over time. Calculation was done as follows. When the initial AI-2 activity at time 0 is set as $AI2_0$, AI-2 activity after addition 1 hr incubation at 30 °C as $AI2_1$, and AI-2 activity after 1 hr incubation at 42 °C $AI2_1'$, changes by heat and time is $AI2_1' / AI2_0$, changes by time is $AI2_1 / AI2_0$. Net changes by heat shock (relative) is defined to be $(AI2_1' / AI2_0) / (AI2_1 / AI2_0) = AI2_1' / AI2_1$.

Strains	Relevant characteristics or Genotypes
<i>E. coli</i>	
C600	F ⁻ <i>thi1 thr1 leuB6 lacY1 tonA21 supE44λ⁻</i>
MF746	C600 <i>dnaK756</i>
MF634	C600 <i>dnaJ259</i>
CG938	C600 <i>dnaJ259</i> <i>mini</i> Kan ^R
W3110	F ⁻ λ ⁻ <i>IN(rrnD-rrnE) rph-1</i>
B178	W3110 <i>galE relA</i>
CG712	B178 <i>zjd::Tn10 groES30</i>
CG714	B178 <i>groEL140</i>
CG2245	B178 <i>groES⁺L⁺ Tn10</i>
CG2244	B178 <i>groES619</i> Tn10
CG2241	B178 <i>groEL44</i> Tn10
DA15	B178 <i>phoA::Tn10</i>
DA16	DA15 <i>grpE280</i>
MC4100	<i>(argF-lac)U169 araD139 rpsL150 deoC1 relA1 ptsF25 flbB5501 rbsR F</i>
CU247	MC4100 <i>cbpA::Kan^R dnaJ::Tn10-42(Tet^R)</i>
WKG15	MC4100 <i>djlA::Spc^R</i>
GP108	MC4100 <i>dnaJ::Tn10-42(Tet^R)</i>
GP109	MC4100 <i>cbpA::Kan^R</i>
GP110	MC4100 <i>djlA::Spc^R dnaJ::Tn10-42(Tet^R)</i>
GP111	MC4100 <i>djlA::Spc^R cbpA::Kan^R</i>
GP112	MC4100 <i>dnaJ::Tn10-42(Tet^R) cbpA::Kan^R</i>
GP113	MC4100 <i>djlA::Spc^R dnaJ::Tn10-42(Tet^R) cbpA::Kan^R</i>
<i>V. harveyi</i>	
BB170	BB120 <i>luxN::Tn5</i> sensor1 ⁻ , sensor 2 ⁺

Tet^R, tetracycline resistance gene; Kan^R, kanamycin resistance; Spc^R, spectinomycin
Mutated gene of interest is marked with bold letters
Mutations are designated by single letter amino acid code and residue number.

Table 2. Bacterial strains used for studying involvement of molecular chaperones.

Results and Discussion

Chaperones are likely to be involved in nearly every cellular function. We examined whether this involvement could be manifested in AI-2 signaling by examining the effects of mutations in major chaperone, DnaJKE and GroELS, on AI-2 levels. Strains surveyed in these experiments are listed in Table 2. *E. coli* chaperone mutants have either substituted amino acid residues in the GroESL or DnaJKE complexes or have null-mutations in one or more of the *hsp40* homologues, *dnaJ*, *cbpA*, and *djlA*.

Two different experimental scales were adopted, test tube cultures (holding capacity of 15 ml) and flask cultures (holding capacity of 250 ml). Conditioned media from the tube cultures were collected at 4 hr and 8 hr after inoculation, while conditioned media from the flask cultures were collected every 2 hr for a comprehensive time course study.

AI-2 activity of chaperone mutants w/out heat shock (LB, 30 °C)

Thermally permissive conditions were expected to minimize the role of heat-shock proteins DnaJKE and GroESL on AI-2 signaling. All mutants tested in our study did not show noticeable changes in growth rate when cultured at 30 °C (Fig. 6). It was noteworthy (and perhaps fortunate) that the mutations examined had minimal impact on cell growth (Fig. 6). This is particularly important as deciphering effects of these chaperone mutants on AI-2 mediated signaling could be difficult, as results might be due to secondary effects of delayed or otherwise altered cell density. When normalized by the AI-2 level of parental strain, both DnaJKE and GroESL mutants showed higher AI-2

levels (values >1) in both scales of experiments in the late exponential phase, which corresponded to 4 hrs of tube cultures or 6 hrs of flask cultures. In this permissive condition, mutations in *groESL* (Fig. 1.C) exhibited significantly increased AI-2 levels, especially at 4 and 6 hr of flask cultures. Similar results were obtained at 6 hr with the *dnaJKE* point mutants but to a slightly lesser extent (Fig. 6.B). Examining the different combinations of *hsp40* knockout mutants, it appeared as though there was no synergism between the effects of $\Delta dnaJ$, $\Delta dnaK$, and $\Delta grpE$, as double mutants and the triple mutant were not distinguished from the single mutants at any of the sampled time points. The lone exception to this was found with the triple mutant, $\Delta dnaJ\text{-}cbpA\text{-}djlA$, at the 4 hr sample of the tube experiments (Fig. 6.A).

It is therefore noteworthy that in almost all cases, deletion or impairment of the chaperones investigated resulted in increased AI-2 levels at the times (entrée to stationary phase) when AI-2 is typically highest. Exceptions to this increase were tube cultures of $\Delta dnaJ$ at 4 hr, *grpE280* at 4 and 8 hr, *dnaK756* at 8 hr, *groES30* at 8hr and the flask cultures of *grpE280* at 6hr. These showed slightly lower levels of AI-2 compared to the controls, however, but these observations did not appear to be systematically consistent. The apparent AI-2 decrease by *grpE280* from both tube and flask cultures remains intriguing.

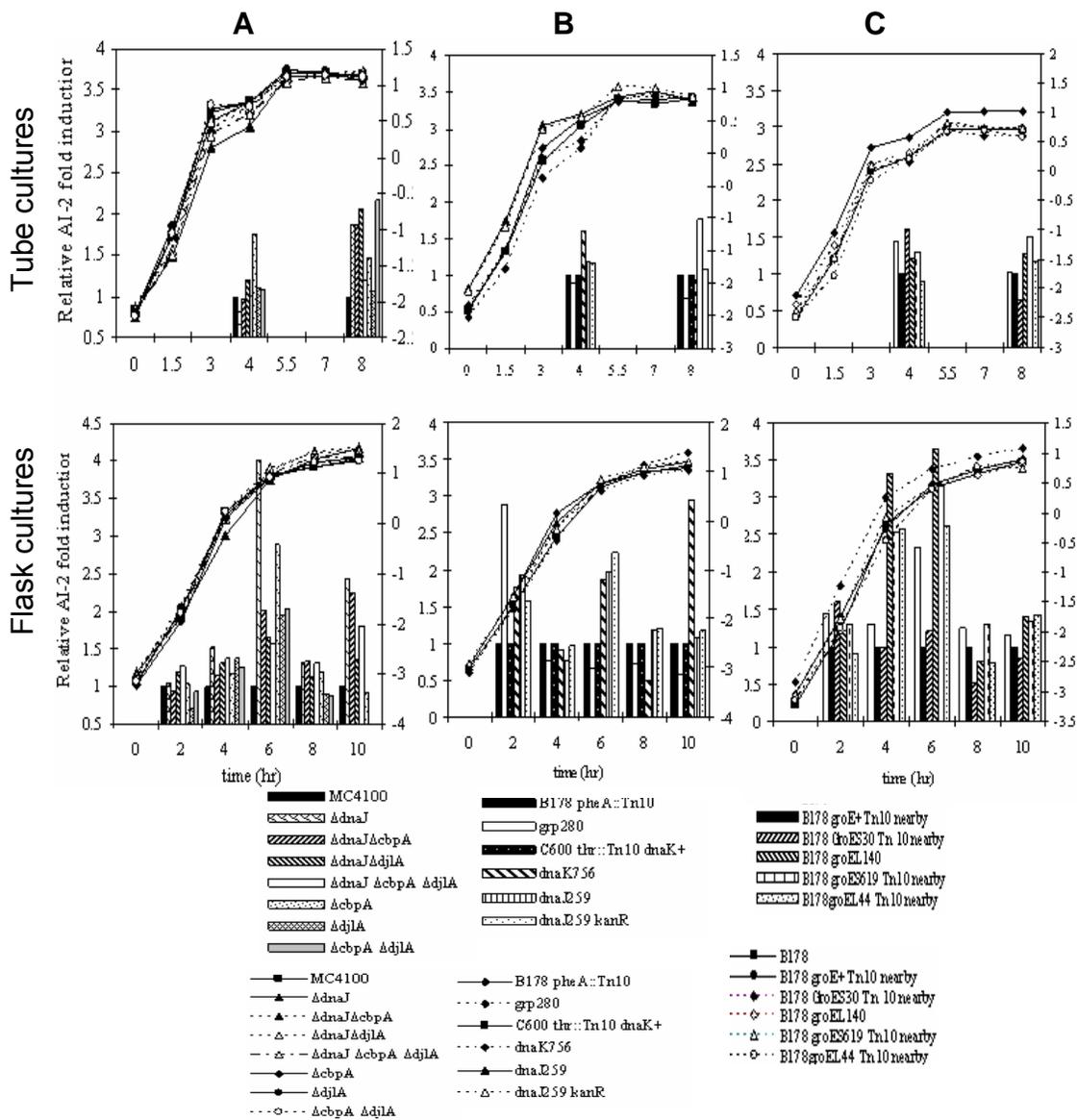


Figure 6. The comparison of tube culture and flask culture (30°C). The 20 strains were cultured either in culture tube (upper panels) or flasks (lower panels). Reported data are normalized AI-2 levels by parental control strains. The control strains are represented with black bars in graphs. (A) MC4100 and its 8 derivatives with deleted hsp40s, (B) point mutants of *dnaJ*, *dnaK* and *grpE*. Control strains for *dnaJ* and *dnaK* mutants are C600 derivative, while control of *grpE* is a B178 derivative. (C) *groESL* point mutants are B178 derivatives (*groE*+ Tn10 nearby). From the two independent experiments with tube culture and three independent experiments with flask cultures, representative experiment is presented. X-axis represents the time after initiating culturing. Left-y axis represents relative fold induction of AI-2, while right-y axis represents OD600nm. Errors associated with AI-2 measurement were less than 10% of the value.

A continuous high-temperature condition affected both growth rate and AI-2 profiling

Raising the temperature increases the cellular requirement of chaperone function. This is partly due to the increased rates of growth and certain types of protein occurring at higher temperature or due to the increase of temperature sensitive folding-intermediates of many proteins [reviews in (King *et al.*, 1996)].

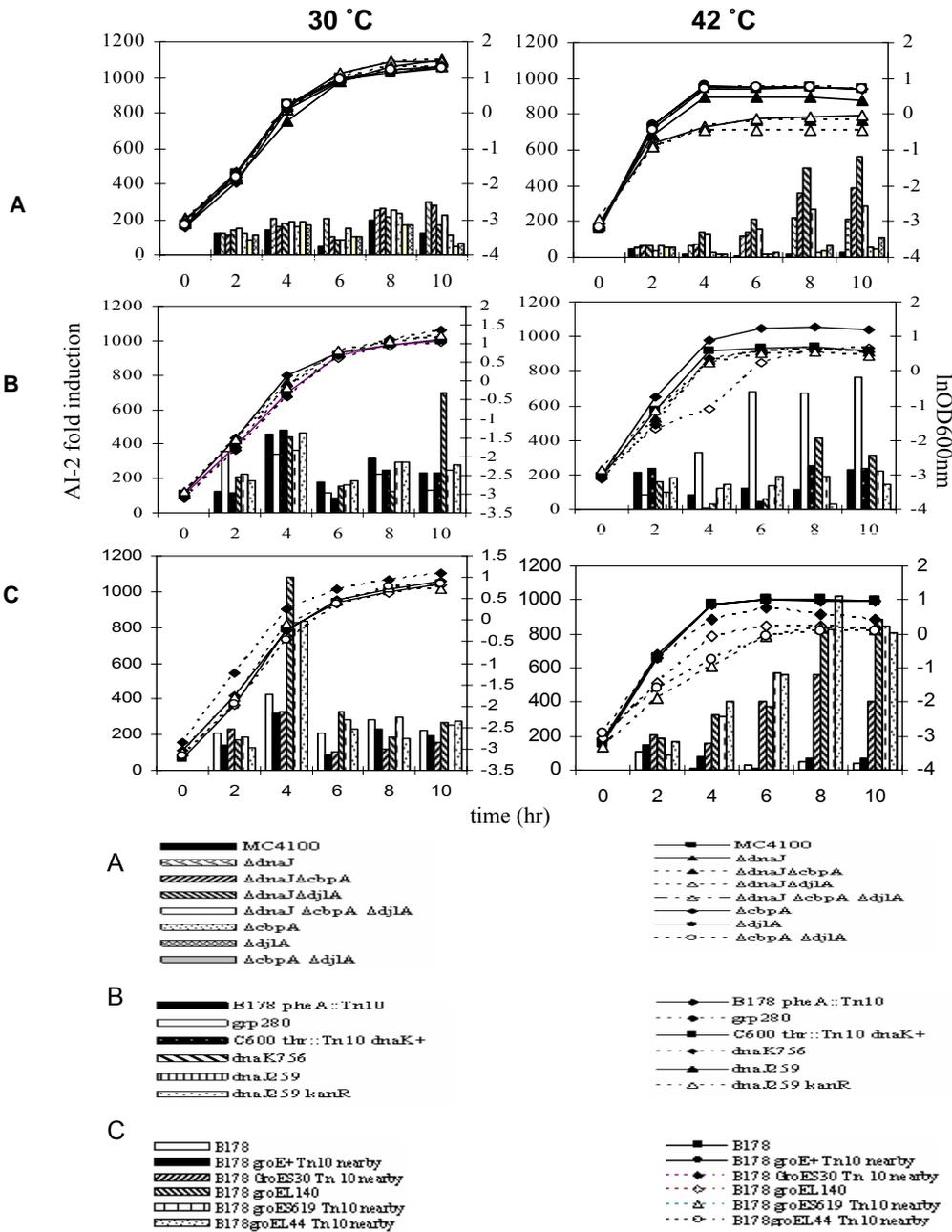
Constant or prolonged high temperature condition is considered different from traditional heat shock (typically short time period). In this condition, the majority of DnaJKE would be occupied by structure-damaged proteins rather exerting negative regulatory effects on σ^{32} regulon expected immediately following transient heat shock. Further, heat-inducible but mutated chaperone expression are assumed to lower the activity on structurally liable proteins, due to the decrease either in substrate-turnover rates or in the interaction with other chaperones or proteases.

When the 20 *E. coli* strains were cultured at higher temperature (42 °C), growth defects were found from most of the chaperone mutants, which can be ascribed to accumulated toxic protein aggregates in the cells. As shown in Fig. 7, deletion of *dnaJ* (Fig. 7.A), *grpE280* and point mutations in *groESL* (Fig. 7.C) showed significant growth defects. The triple *hsp40* mutant showed decreased turbidity all along the growth profile, particularly after 3 hrs. Increasingly affected growth could be found in the sequence of *groES30*, *groEL140*, *groEL44*, and *groES619*. Unlike the growth defects by *grpE280*, the *dnaK756* or *dnaJ259* mutants were resistant to 42 °C (Fig. 8.B). It is noteworthy that high temperature cultivation at 42 °C, of resistant cells increased their growth rates in the exponential phase so that they reached the stationary phase only 4 hr after inoculation

(and only 3 hr for the tube cultures, data not shown). This is about 1 hr earlier than the cultures at 30 °C or without heat shock conditions.

Decreases in the growth rate and yield of chaperone mutants were coincident with alterations in AI-2 production. As seen in Fig. 7.A, the deletion in *dnaJ* had a stronger impact on cell growth than deletion in *cbpA* or *djlA*. The lowest growth yield was found from the $\Delta dnaJ\text{-}cbpA\text{-}djlA$ strain indicating apparent synergistic effects on growth and yield (Fig. 8.A). Significantly increased AI-2 levels were found from $\Delta dnaJ\text{-}djlA$, and elevated AI-2 levels were also detected from *dnaJ* mutants ($\Delta dnaJ\text{-}djlA > \Delta dnaJ\text{-}cbpA > \Delta dnaJ\text{-}cbpA\text{-}djlA > \Delta dnaJ$ in series). The $\Delta cbpA$ or $\Delta djlA$ or the double mutants did not display altered growth and AI-2 production characteristics relative to their controls. Notable growth decreases were observed for *grpE280*, again yielding profound changes in AI-2 levels. This was in contrast to *dnaK756* and *dnaJ259*. The influence of growth rate was clear with *groESL* mutant strains, which increased AI-2 levels sequentially *groES30*, *groEL140*, *groES619*, and *groEL44*. Thus, the increase of AI-2 was apparently inversely-proportionate to the growth rate changes.

Growth defects observed from *grpE* and *groESL* mutants occurred during the exponential phase (2-4 hrs). Intriguingly, its influence on AI-2 signaling lasted longer and was progressive over the stationary phase (limited by our observation period of 10 hrs).



Application of heat shock to tube cultures (LB)

1 hr duration for heat shock was chosen as a relatively short but effective duration to enunciate shock effects. Surette and Bassler used 2 hr at 43 °C to cultures previously grown for 6 hr at 30 °C (Surette and Bassler, 1999). Delisa et al., (DeLisa *et al.*, 2001) upshifted the culture temperature to 42 °C within a steady-state culture ($\mu=0.75 \text{ h}^{-1}$) and continuously monitored AI-2 for the ensuing 4 hr. In both studies, lowered levels of AI-2 were attributed to the applied heat shock. Here, we report that a culture's growth state, during which the shock is applied, affects the subsequent AI-2 profiling. We present two case studies, performed at the exponential phase or at the stationary phase.

Case study of heat shock 1: heat shock in the stationary phase increases AI-2 activity (slowed decrease in AI-2 over time)

To distinguish changes due to heat shock from “normal” changes along typical batch growth, AI-2 activities of a strain at three conditions were measured and compared: base case culture without stress of cells cultivated for 3 hr (denoted AI2₀), the same culture grown an additional 1 hr at 30 °C (denoted AI2₁) or the same cells cultured for an additional 1 hr at 42 °C (denoted AI2₁').

Heat shock of the stationary phase cultures (at 7 hr post inoculation in tubes) resulted in cells maintaining a higher level of AI-2 activity than the cells kept at 30 °C for the same duration (expressed as AI2₁' /AI2₁). During this time, control cells take up AI-2 and the levels naturally decline over time (expressed as AI2₁/ AI2₀). We observed that heat shock increased the AI-2 levels both from the wild type and chaperone mutants. Changes in AI-2 among the parental strains are depicted in Fig. 8. The higher AI-2 levels observed after heat shock agreed with the general increase shown in cultures kept at 42

°C for the entire culture (shown previously in Fig. 6 and 7). Heat shock induced AI-2 increases were observed during the stationary phase from point mutated chaperones (B178 and C600 and their derivatives). MC4100 and its derivative hsp40 knockouts showed no differences due to the heat shock, which is surprising, considering previous observations of increased AI-2 with $\Delta dnaJ$ mutants (both at 42°C or 30 °C regardless of presence of glucose). There was no hierarchical dominance of *dnaJ* over *cbpA* and *djlA* in this decrease.

The relative insensitivity of *dnaJ* mutants from the control is intriguing. As an explanatory scenario, presuming that AI-2 synthesis is negatively targeted by chaperones, the chosen time point of temperature upshift (at 7 hr) may have been too late to see a “chaperone effect”. That is, if the chaperones have already worked on the AI-2 synthesis machinery, then a subsequent heat shock would be of no consequence to the AI-2 profile. This appears to have been the scenario, hence we believe that the involvement of the chaperones is on the synthesis pathway as opposed to the depletion pathway. We note, however, $\Delta dnaJ\text{-}cbpA\text{-}djlA$ was resistant to the decrease over the 1 hour (Table 3), which implies that when combined, Hsp40s have synergistically affected AI-2 uptake to a certain degree.

B178 *pheA::Tn10* · C600 *thr::Tn10* and their derivatives also showed decreases in AI-2 over time ($AI2_1 / AI2_0$), but had higher levels of AI-2 after the heat shock ($AI2_1' / AI2_1, AI2_1' / AI2_0$). From the C600 series, a decrease in AI-2 over time was not shown from the two *dnaJ259* mutants. Higher AI-2 activity after heat shock is clear from *dnaK756*, and *grpE280*. The *grpE280* increased more than 56 % than that of its parental cells. The *groESL* mutants showed increase of AI-2 from 6 to 60 %.

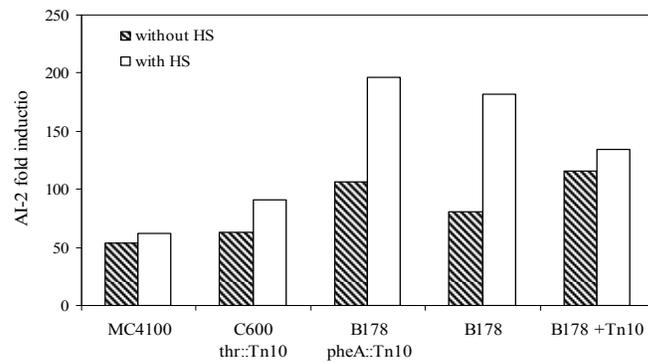


Figure 8. AI-2 activity before and after 1 hr heat shock to stationary phase cells. AI-2 activity of the stationary phase parental cells (precultured 7 hrs) was compared before and after the shock. Over the 1hr of temperature-shift, parental control cells showed increased AI-2 levels (single experiment based data).

Genotypes	Change by time (AI2 ₁ / AI2 ₀)	Change by HS over time (AI2 ₁ '/ AI2 ₀)	Net Change by HS (AI2 ₁ '/ AI2 ₁)
w/t (MC4110)	0.59	0.67	1.14
Δ dnaJ	0.73	0.83	1.15
Δ dnaJ Δ cbpA	0.63	0.58	0.92
Δ dnaJ Δ djlA	0.80	0.81	1.02
Δ dnaJ Δ cbpA Δ djlA	0.85	1.01	1.18
Δ cbpA	0.62	0.81	1.30
Δ djlA	0.59	0.61	1.03
Δ cbpA Δ djlA	0.68	0.70	1.03
w/t (B178 pheA::Tn10)	0.58	1.07	1.84
grpE280	0.69	1.97	2.88
w/t (C600 thr::Tn10)	0.53	0.77	1.45
dnaK756	0.53	1.13	2.11
dnaJ259	0.99	0.70	0.70
dnaJ259 mini-kanR nearby	1.05	1.23	1.16
w/t (B178)	0.68	1.53	2.26
w/t (B178 +Tn10)	0.98	1.14	1.16
B178 groES30 Tn10 nearby	0.59	1.13	1.91
GroES619	0.80	1.00	1.24
GroEL140	0.69	1.10	1.59
B178groEL44 Tn10 nearby	0.88	1.28	1.46

Table 3. Changes of AI-2 activity by 1 hr heat shock at stationary phase.

Relative AI-2 changes were reported as normalized values. Wild type parental strains are marked with boldic letters. Net change ratio by heat (AI2₁' /AI2₁) is marked with bold letters. AI2₀, initial AI-2 activity at 30 °C (at time 0), AI2₁, AI-2 activity after addition 1 hr incubation at 30 °C (at time 1hr), AI2₁': AI-2 activity after 1 hr 42 °C heat shock (at time 1hr) (single experiment based data).

Case studies of heat shock 2: heat shock in the exponential state

Heat shock to exponentially growing cells either decreased AI-2 or showed no effects (Table 4). The change patterns in AI-2 level of the parental strains are depicted in Fig. 9. Examples of chaperone mutants following the pattern of parental controls could be found in MC4110 and its *hsp40* knockouts and C600 and its *dnaJK* point mutants. In contrast, *grpE280* showed resistance to the decreases otherwise shown in its parental control. Looking closely at the AI-2 decreases of MC4100 and its derivative strain cells, the $\Delta dnaJ \cdot cbp \cdot djlA$ mutant did not decrease AI-2 levels (only 2 % decrease) as it didn't in the stationary HS experiment (Table 3). This again implies a synergistic effect on uptake or degradation machinery delaying the normally-hastened AI-2 uptake by heat shock at this growth state. Combined with $\Delta dnaJ$, deletion of *cbpA* ($\Delta dnaJ \cdot cbp$, 10 % decrease) seems to have contributed the higher AI-2 levels after the HS than *djlA* ($\Delta dnaJ \cdot djlA$ 30 % decrease), however, alone as Δcbp and $\Delta djlA$, they did not differ (c.a. 50 % decrease).

Point mutations in *groESL* showed clear decreases more by heat shock than by time, while the parental strain did not change its AI-2 level by either time or heat shock.

The AI-2 decrease by temperature upshift (exponential phase) described here was later found to be a general phenomenon of heat shock effects in LB glucose cultures (below).

Various patterns in AI-2 levels were observed among the chaperone mutants. No differences could be attributed to *dnaJK* point mutations, *groESL* point mutations, or to $\Delta dnaJ \cdot cbp \cdot djlA$. Of course, this set of experiments only tests a short time period and at one condition. Thus, differences here might not be uniquely ascribed to the mutations tested. That is, there are other conditions wherein the mutations might be more relevant. In this

context, the clearer differences seen in stationary phase HS appear to be associated with the required chaperone functionality. Stationary phase induced chaperon activities were reported (Dukan and Nystrom, 1998).

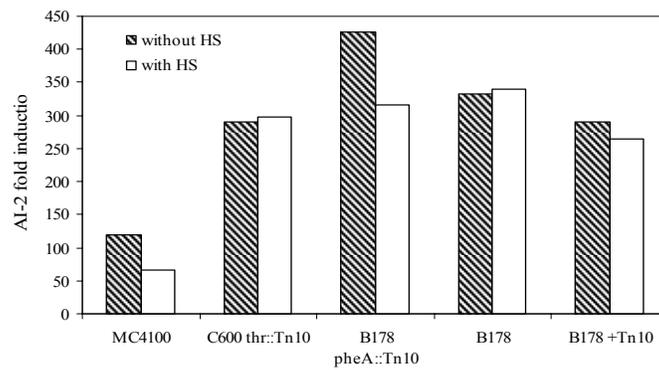


Figure 9. AI-2 activity before and after 1hr heat shock to exponential phase cells. AI-2 activity of the exponential phase parental cells (precultured 3 hrs) was compared before and after the shock. Over the 1hr of temperature-shift, parental control cells showed increased AI-2 levels (single experiment based data).

Genotypes	Change by time (AI2 ₁ / AI2 ₀)	Change by HS over time (AI2 ₁ ' / AI2 ₀)	Net Change by HS (AI2 ₁ ' / AI2 ₁)
w/t (MC4100)	1.02	0.57	0.56
ΔdnaJ	1.02	0.68	0.67
ΔdnaJΔcbpA	0.93	0.84	0.90
ΔdnaJΔdjIA	1.16	0.81	0.70
ΔdnaJ ΔcbpA ΔdjIA	0.97	0.95	0.98
ΔcbpA	1.34	0.70	0.52
ΔdjIA	1.03	0.52	0.51
ΔcbpA ΔdjIA	0.91	0.63	0.70
w/t (B178 pheA::Tn10)	0.89	0.66	0.74
grpE280	0.87	0.87	1.00
C600 thr::Tn10	0.82	0.84	1.03
dnaK756	1.23	1.12	0.91
dnaJ259	0.79	0.91	1.15
dnaJ259 mini-kanR nearby	0.88	0.93	1.05
w/t (B178)	0.94	0.96	1.02
w/t (B178 +Tn10)	0.78	0.71	0.91
B178 groES30 Tn10 nearby	0.98	0.95	0.97
GroES619	0.78	0.58	0.75
GroEL140	0.98	0.54	0.55
B178groEL44 Tn10 nearby	0.80	0.50	0.63

Table 4. Changes of AI-2 levels by 1 hr heat shock at exponential phase. Relative AI-2 change values were reported. Wild type parental strains are marked with bold letters. Net change ratio by heat (AI21' / AI21) is marked with bold letters. AI20: initial AI-2 activity at 30 °C (at time 0), AI21: AI-2 activity after addition 1 hr incubation at 30 °C (at time 1hr), and AI21': AI-2 activity after 1 hr 42 °C heat shock (at time 1hr) (single experiment based data).

Glucose effects: Presence of glucose at high temperature pronounced the chaperone activity

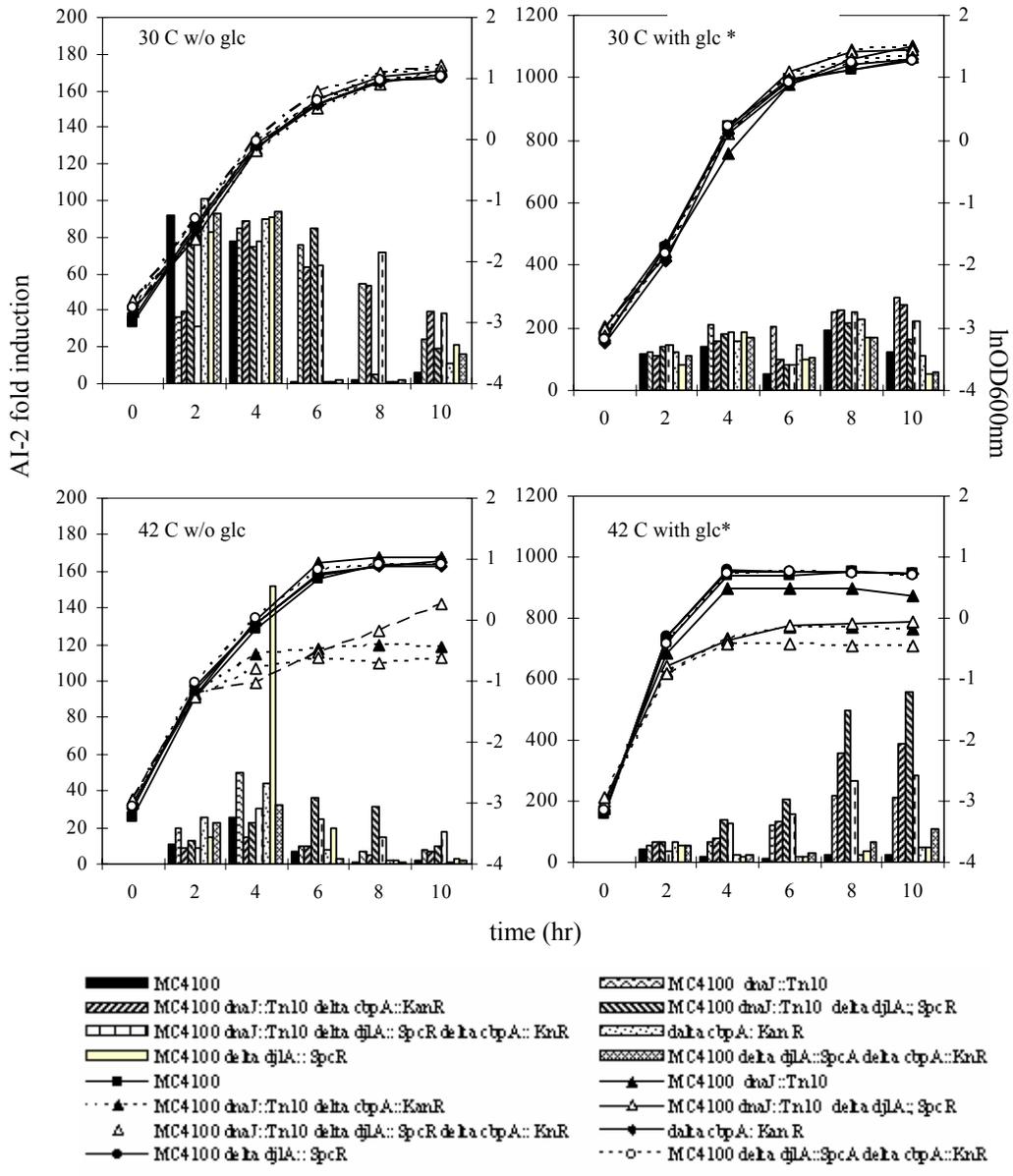
Glucose has been shown to increase AI-2 level and its period of sustained increase in conditioned media (Surette and Bassler, 1998). Our lab has also have noted the global impact of glucose in the transcriptome [microarray analysis (Wang et al. in preparation)], aspiring to reveal possible glucose-specific-effects. At the same time, we tried to discern glucose effects from AI-2 profile changes among chaperone mutations. For this, cells were grown in 4 different conditions: in the presence or absence of glucose combined with different culturing temperature of 37 °C or 42 °C. Conditioned media from the cultures were collected every 2 hours for AI-2 measurement (Fig. 10).

Of the 4 conditions, the strongest effects of chaperone mutants were found at 42 °C with glucose. Growth rate affected chaperone mutants (*dnaJ* knockouts, *grpE280* and *groESL* mutants) progressively increased AI-2 toward the stationary phase (Fig. 10. A.and C). The progressive increase in AI-2 is not clear at the other three conditions; when glucose is not supplemented or when the temperature is low or when those two conditions are combined.

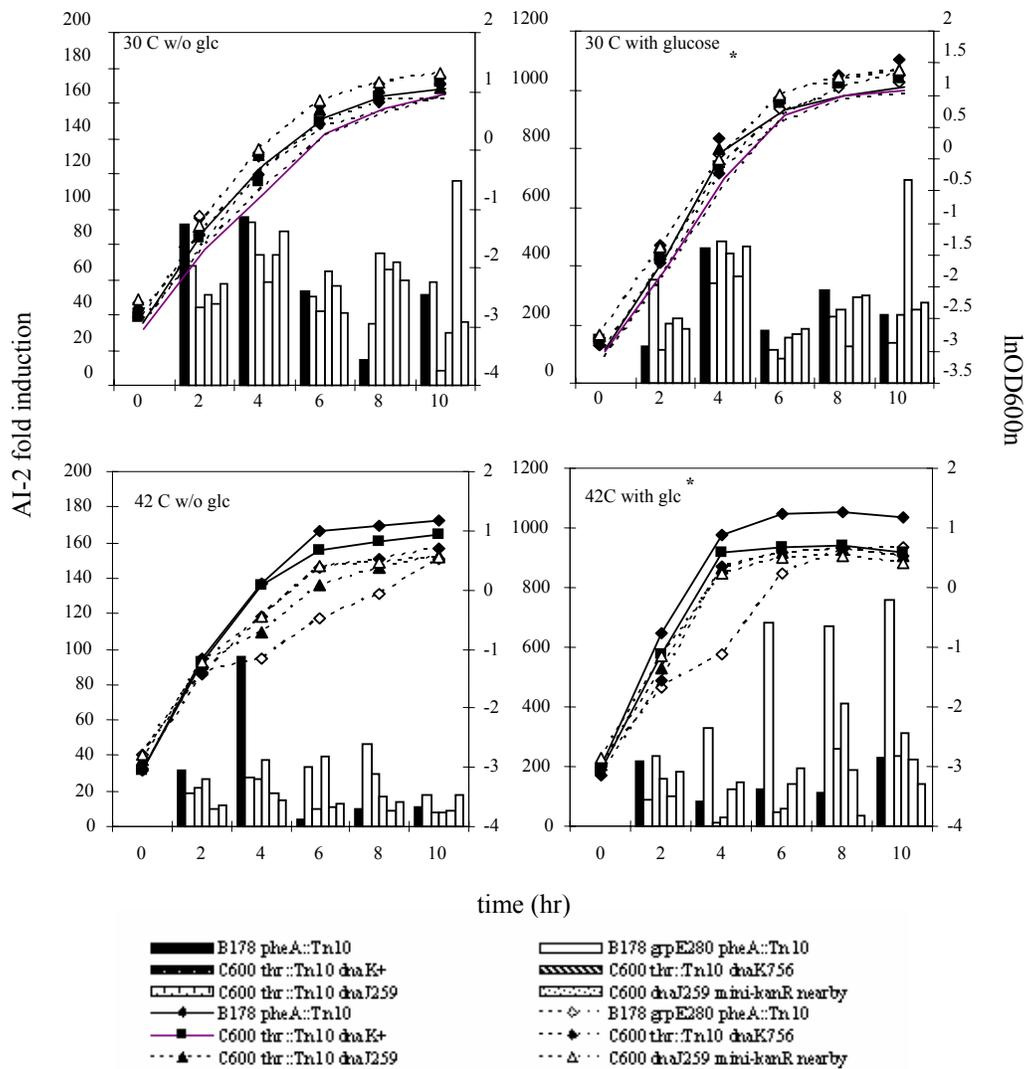
At 42°C, without glucose, complete depletion of AI-2 was observed within the time range studied from the wild type strains. Sustained AI-2 activity of *hsp40* mutants and *groESL* point mutants was clear (Fig. 10.C), which is not seen in the *dnaJKE* point mutants (Fig. 10.B). Despite the affected growth rate of *dnaJ259*, its AI-2 activity did not differ from that of the parental strain. We have marked this as an exceptional case of the general idea that the increase of AI-2 is inversely-proportional to the growth rate.

At 30°C, without glucose supplementation, no significant growth rate changes were found. Only *dnaJ* knockouts showed higher level of AI-2 and its slow disappearance,

while point mutants of *dnaJKE* did not differ much from the parental strains in the AI-2 profiles. The most noticeable difference was found at 10 hr and at this time, again, the *dnaJ259* mutant showed the biggest effects. The *groESL* mutants showed generally 1-3 fold higher AI-2 levels along growth, especially at 8 hr, where the biggest difference was seen (~10 to 20 fold).

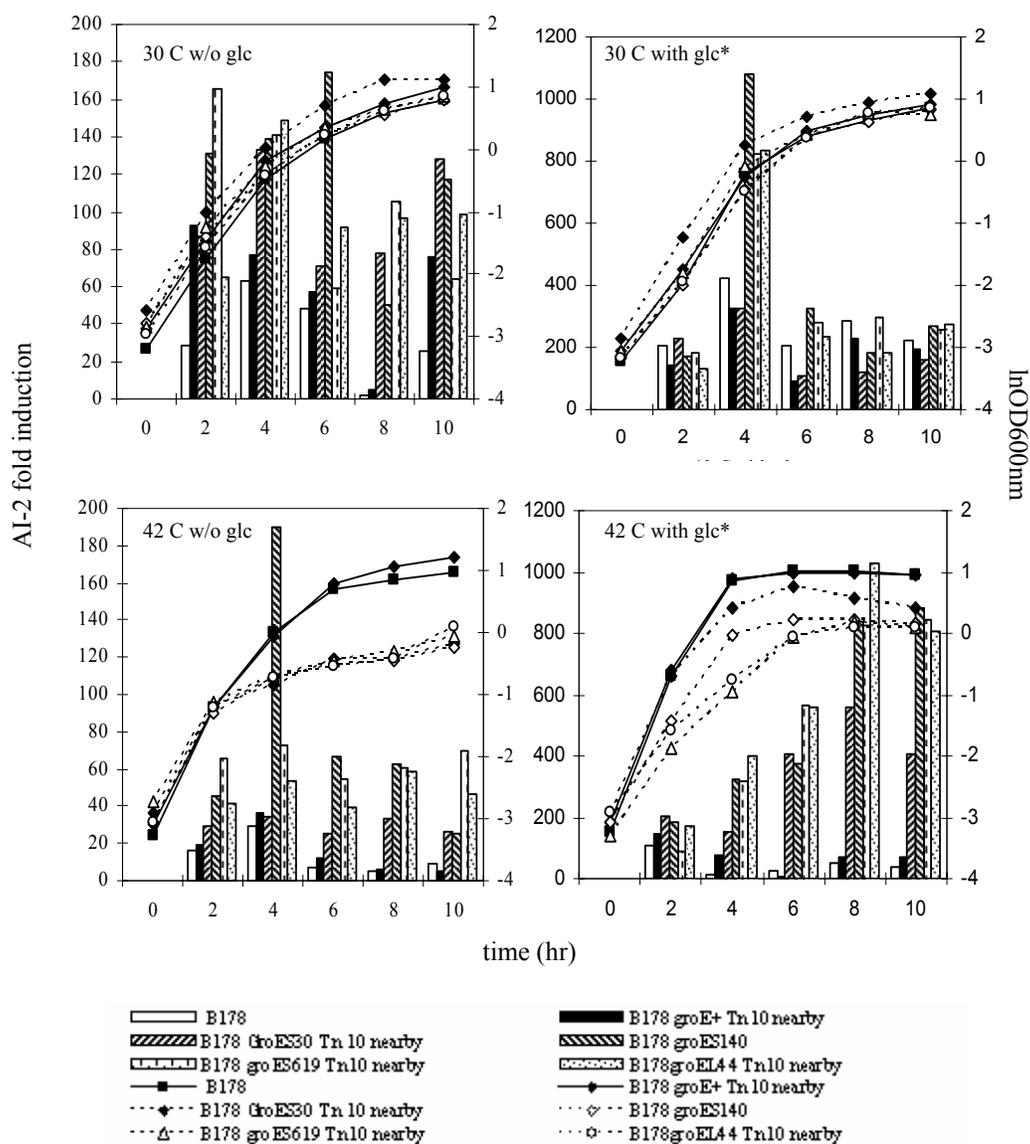


A.
Figure 10. The effects of glucose and temperature on AI-2. (A) MC4100 and its 8 derivatives with deletions in three *hsp40s*. (B) Point mutants of *dnaJ*, *dnaK* and *grpE*. Control strains for *dnaJ* and *dnaK* mutants are C600 derivative, while control of *grpE* is a B178 derivative. (C) The *groESL* point mutants are B178 derivatives (*groE*⁺ Tn10 nearby). Culture conditions are indicated. From three independent experiments with flask cultures, a representative experiment is presented. Errors associated with AI-2 measurement were than 10% of the value. * Figures are presented in the other section.



B.

Figure 10. The effects of glucose and temperature on AI-2 (contd.). **B)** Point mutants of *dnaJ*, *dnaK* and *grpE*. Control strains for *dnaJ* and *dnaK* mutants are C600 derivative, while control of *grpE* is a B178 derivative.



C.

Figure 10. The effects of glucose and temperature on AI-2 (contd.). C) The *groESL* point mutants are B178 derivatives (*groE*⁺ Tn10 nearby).

Presented data for the changed AI-2 profiles elucidate the importance of chaperone in the control of AI-2 toward the stationary phase. Especially when contrasted with parental cells with wildtype molecular chaperones in the high temperature glucose culture, chaperone mutants showed a surprising and preserved pattern of AI-2, progressively increasing AI-2, while wild type cells reduce AI-2 levels under the same condition

The results presented in this study provide clear evidence that the decrease of AI-2 shown in the stationary phase requires the activity of molecular chaperones, DnaJKE and GroESL complex (Fig. 10). Interestingly, we view this decrease in AI2 concentration (fold activity) as a function of altered synthesis rate. When the involved chaperones are mutated, the AI-2 level seems to increase unabated well into the late-exponential and stationary phases.

Glucose drives the metabolic flow towards AI-2 synthesis. We cannot pinpoint exact major target proteins under the regulatory effects of the chaperone from the methodology chosen here (screening AI-2 profiles). The observed phenomena could be attributed to one or a multiple number of proteins that are influenced by the chaperones. When we couple AI-2 profiles of wildtype cells and mutants grown under standard conditions to the same cultivated under heat shock, we can clearly conclude that the chaperones are negatively regulating the AI-2 synthesis so that AI-2 is not accumulated at stationary phase culture media. Since the concentration of glucose (0.8%) used in this study is high enough to totally inhibit the AI-2 uptake machinery (*lsr* operon), the chances of defects in AI-2 uptake machinery by the chaperone mutants are rare.

It is obvious from the results obtained here, that the level of AI-2 and the direction of AI-2 accumulation depend on chaperones and on the prevailing metabolic state of the cells. At stationary phase, heat shock increased AI-2 levels in the conditioned media and especially did from some of the chaperone mutants. During exponential phase, heat shock reduced AI-2 levels; however clear differences from the chaperone mutants were limited. Hence, the changes in AI-2 were different at the different states. This is entirely consistent with a recent microarray study by Wang et al, wherein they found discordant sets of genes that were affected by LuxS mutation when tested in the presence/absence of glucose at different ODs. Thus, perhaps it is now understandable, that the cells use quorum sensing signaling in a finely tuned and regulated manner, wherein the genes regulated and the signals perceived vary, but the prevailing environmental conditions, which themselves are ever changing.

Chapter 3: Transcriptome analysis of SdiA mutation; potential for AI-1·AI-2 crosstalk in *Escherichia coli*

Abstract

Enteric bacteria *Salmonella* and *Escherichia coli* has genetic potential to deploy a cell density dependent response with common tools, LuxI type transcriptional regulator, SdiA (system 1), and seemingly independent AI-2 signal synthase (system 2). While signal synthesis and uptake machinery of the system 2 has been widely studied, absence of identified signal and its synthase for the system 1 has suspended a consensus on its functionality. In this work, comparative analysis of transcriptome was performed between *E. coli* W3110 and its otherwise isotype *sdiA* mutant collected at late exponential phase in Luria-Bertani media. Using *E. coli* K-12 Affymetrix microarray, significant changes (cutoff of 2 fold) were found from small numbers of genes and IGs by the mutation, with notable reduction in rRNAs levels counterbalanced by induced purine *de novo* synthesis genes and a reduction in AI-2 uptake machinery transcripts (*lsr* operon). Additionally reporting increased resistance to general stresses and decrease in motility of the mutant, we also presented consensus sequences as possible binding motifs and predicted small RNA expression based on the information of sub-transcript level of changes in the intergenic regions (IGs).

Contrasting with the most of the previous studies focused on SdiA overexpression, this work investigated the effects of *sdiA* deletion systemically for the first time demonstrating the the possibility of its functionality in the pure culture condition especially on the quorum system 2.

Key words: SdiA, *lsr* operon, rRNA, purine *de novo* synthesis.

Introduction

Many prokaryotic species sense neighboring populations, adapt their physiological states to environmental cues, and trigger group-wide or coordinated physiological processes in a process termed “quorum sensing”. Quorum sensing involves an array of external signaling molecules (from peptides to furanones), several internal regulatory molecules (from proteins to small RNA), and an assortment of regulatory motifs (from direct signal binding of transcriptional regulators to hierarchical phosphorelay cascades). Among the most well known quorum regulator proteins are the LuxR-type proteins that have specificity toward acylated homoserine lactones (AHL), referred to as autoinducer-1 (AI-1). AI-1s consist of diverse acyl-side chains that are linked to S-Adenosylmethionine (SAM). There are currently three known protein families catalyzing AI-1 synthesis, including the well-studied LuxI-family, AinS, and HdtS. The signal synthase/regulator pair, LuxI-LuxR, are conserved among many bacterial species and are considered to have co-evolved (Lerat and Moran, 2004). Numerous examples of AI-1 synthase/regulator pairs can be found in organisms such as *Vibrio fischeri* (LuxI-LuxR, AinS-AinR), *Pseudomonas aeruginosa* (LasI-LasRI, RhII-RhIR), *Agrobacterium tumefaciens* (TraI-TraR), *Pantoea stewartii* (EsaI-EsaR), *Rhodobacter sphaeroides* (CerI-CerR), *Vibrio anguillarum* (VanI-VanR), *Yersinia enterocolitica* (YenI-YenR) among others [Review in (Fuqua *et al.*, 2001)].

The enteric bacterium *Escherichia coli* also has a LuxR homologue named *sdiA* (suppressor of *d*ivision *i*nhibition, 67 % aa similarity with 27 % identical aa compared with *V. fischeri* LuxR). SdiA was initially cloned and characterized as a factor restoring a cell division defect of *ftsZ84* (*fts*: *f*ilamentation *t*emperature *s*ensitive) upon

overexpression (Wang *et al.*, 1991). *Escherichia coli* and *Salmonella* appear to be distinct from the above group of bacteria, because they do not have a *luxI* homologue (Lerat and Moran, 2004). Experiments with several different sensor cells were indeed unsuccessful in detecting AI-1 derivatives from *E. coli*. Instead, *E. coli* has components associated with an alternative pathway proposed as a cross-species quorum sensing system. This signal molecule, autoinducer AI-2, is synthesized by three enzymatic reactions involving a set of methyltransferases, a nucleosidase (Pfs) and AI-2 synthase (LuxS). Consistent with many QS signal molecules, the AI-2 concentration in the media of *E. coli* cultures increases with growth until the late exponential phase. Surprisingly, *E. coli* and *Salmonella* actively uptake AI-2 and decrease its level in the culturing media. Conserved among *Enterobacteriales*, *lsr* operon (*lsr*: *luxS* regulated) encodes a ABC-type transporter and subsequent modification enzymes controlling the consumption of AI-2 (Chen *et al.*, 2002; Sun *et al.*, 2004; Taga *et al.*, 2003; Taga *et al.*, 2001; Wang *et al.*, 2005).

Despite the absence of AI-1 signal molecules in CM from *E. coli*, unknown signals to control SdiA activity have been speculated for over a decade. For example, unidentified signals present in stationary phase enterohaemorrhagic *E. coli* (EHEC) 0157:H7 DMEM (serum-free Dulbecco's modified Eagle medium) CM were shown to bind SdiA and negatively regulate the expression of virulence factors (Kanamaru *et al.*, 2000). Chemically synthesized AHLs were shown to activate SdiA mediated *ftsQ₂p* transcription (*ftsQAZ* cluster, *fts*: filamentation temperature sensitive) (Sitnikov *et al.*, 1996). Also, SdiA expression itself was affected by exogenously added quorum signals. Depending on the experimental conditions, increased *sdiA* expression was reported from

AI-2 synthesizing *E. coli* O157:H7 DMEM cultures (11.6 fold compared with AI-2 non-producers) (Sperandio *et al.*, 2001) and *E. coli* W3110 exposed to CM with an 800-fold differential in AI-2 concentration. (2 fold) (DeLisa *et al.*, 2001). Conflicting observation of decreased *sdiA* transcript by CM addition was reported from a study with stationary phase CM of *S. enterica* serovar. Typhimurium (30 %) (Volf *et al.*, 2002).

These studies have clearly failed to delineate the functional role of SdiA in quorum sensing systems and, while not intended, also did not find SdiA-regulated genes beyond *ftsQAZ*. Ahmer and colleagues (Ahmer *et al.*, 1998) adopted a genetic screen for SdiA dependent genes in *Salmonella typhimurium* (using MudJ transposon mutations) where they found three chromosomally located genes that were differentially controlled by plasmid-encoded SdiA (Michael *et al.*, 2001). None had sequence similarity to *E. coli* genes. Interestingly, none of these genes were differentially regulated by SdiA when *sdiA* was expressed from its natural position in the chromosome. The remaining of seven fusions were located within an operon on a virulence plasmid (Ahmer *et al.*, 1998; Michael *et al.*, 2001; Smith and Ahmer, 2003). Also, Wei *et al.* performed a transcriptome analysis using PCR-based spotted microarray slides to determine the consequence of overexpressed *sdiA* in exponentially growing cells ($OD_{600nm} \approx 0.45$) (Wei *et al.*, 2001). Their study was preceded by their discovery of increased resistance to DNA damaging mitomycin C (MCM) of *E. coli* by *sdiA* amplification (Wei *et al.*, 2001) and thus focused on changes in acridine efflux pumps that relate to MCM resistance.

Herewith, we have attempted to unveil the functionality of *sdiA* when expressed from the natural chromosomal position instead of using plasmid vectors. That is, since overexpressed SdiA is aggregate-prone (Kanamaru *et al.*, 2000; Yamamoto *et al.*, 2001),

our approach which employs gene knockout, was expected to reduce possible interference from the stress response associated with protein overexpression (Bentley *et al.*, 1990). Indeed, while culture conditions and assay formats were different from Wei *et al.*, distinctive changes in transcriptome profile were observed and reported here, with a notable evidence for the cross-talk between the AI-1 and AI-2 quorum sensing circuits in *E. coli*. Phenotypic changes associated with the *sdiA* deletion were presented ranging from increased tolerance toward general stresses to decreased motility. Finally, utilizing the antisense probe format spanning intergenic regions and partly trying to link the transcriptome and phenotypes, expression of novel small RNA or possible promoter region was predicted.

Material and Methods

Strains and growth conditions. The strains used in this study are derivatives of *E. coli* K-12, W3110 (Table 5). P1_{vir} transduction (Sternberg and Maurer) was done to transfer *sdiA::kan^R* from WX2 (Wang et al. 1991) to W3110 and ZK126 (W3110 *lac⁻*) chromosomes, and the new mutant strains were named W2X2 and ZKW2, respectively. Successful transduction was checked by antibiotic resistance (*sdiA::kan^R*) and the change in genomic DNA surrounding the *sdiA* using PCR and the absence of *sdiA* mRNA expression (RT-PCR).

Overnight cultures were prepared in Luria-Bertani media (pH adjusted to 7.4 with NaOH·HCl) with 10 µg/ml kanamycin and/or 100 µg/ml ampicillin according to Sambrook (Sambrook and Russell 2001). The overnight cultures were diluted 1/100 fold to initiate fresh cultures until OD_{600nm} reaches to be 1.8 at 37 °C at 250 rpm (250 ml flask, 50 ml vol of cell culture). Note that kanamycin was only used for maintaining W2X2 (in O/N culture), and was not used in experiments.

LB agar plates were used for *E. coli* colony counting in shock experiments and L-marine (LM) agar plates were used for *V. harveyi* BB170 colony counting in AI-2 assay.

Media composition:

RNA extraction and quantification of mRNA transcript 200 µl of cell cultures samples were immediately treated with RNA stabilizer (400 µl, Qiagen, Valencia CA) and stored at -80 °C before the RNA extraction. RNeasy kit was used with DNase treatment to eliminate chromosomal DNA contamination (Qiagen, Valencia CA). Isolated RNA was resuspended in diethylpyrocarbonated (DEPC)-treated water and the

RNA concentration was measured by UV absorption (260nm). RNA quality and the rRNA quantity of the total RNA were checked by electrophoresis in a 1.2 % (w/v) of agarose gel with formaldehyde (220 mM).

The initial quantification of mRNA of *sdiA* along the time course was done by using 0.05 µg of total RNA as a template for reverse transcription using 40 units of SuperScript III™ RT and 50 ng of random-primer. Samples for quantitative real-time PCR was prepared with qPCR SYBR®Green mix (Invitrogen, Calsbad CA). Fluorescence intensity was recorded in time using GeneAmp 5700 (Applied Biosystems, Foster City, CA). Time course data was normalized by making a ribosomal protein expression a constant (internal control, *rpsK*). The primers used for *sdiA* expressions are; 5'-GAATTTCTTTTCGCGCTTG-3' and 5'-TTTTTGTCCCTTCCCGTTG-3'.

Affymetrix microarray analysis and data mining Antisense GeneChip® arrays were used as specified by the manufacturer. Two independent experiments were performed for each strain, W3110 and W2X2. Total RNA (10µg) was used for synthesis of cDNA. Subsequent steps (cDNA conversion, RNaseI treatment, biotinylation, hybridization and scanning) were performed at the CBR Microarray facility. Image files were then analyzed using Gene Chip Operation System (GCOS version 1.12) (Affymetrix, Inc., Santa Clara, CA).

(<http://www.umbi.umd.edu/~cbr/macore/macorestart.htm>)

For detection call analysis, each of the two replicates needed to get present called. Detection calls in Affymetrix chips are assessed by probe pair saturation and calculation of a detection *p*-value. Default values of signal threshold (Tau 0.015) for a discrimination

score, $R = (PM-MM)/(PM+MM)$ and cut-offs of significance p -values (reflecting the confidence of the detection call, $\alpha_1=0.04$ and $\alpha_2=0.06$) were used in this study.

For differential call analysis, 4 pairwise batch analyses were performed for W3110 (baseline) and W2X2 (experiment). The results were averaged and reported as \log_2 IR (Induction Ratio). From the genes with differential calls, absolute \log_2 IR values of >1.0 was used for “significantly” changed genes and values between 0.5 and 1 for “moderate” transcript changes. Genes with absolute \log_2 IR < 0.5 were denoted to be with marginally altered. Description of probe set design:

Attempted to lower apparent signal loss by averaging over large regions, individual probe intensities of selected genes and intergenic region were interrogated. For each probe, corrected intensity (CI) was calculated by subtracting the value of mismatch (MM) probe from the value of the corresponding perfect match (PM) probe (PM-MM values). Bad probes are defined when the signal of MM is bigger than that of PM (CI <0) and were given a value of 0. Results from the biological duplicate experiments were averaged and then relative induction ratio (IR: CI_{W2X2}/CI_{W3110}) was reported in \log_2 scale (\log_2 IR). High resolution graphs of expression- and change patterns across ORFs and IGs were used to determine the expression of small RNA and promoter.

In silico consensus motif search To equally represent 5'UTR of each promoter region, each coding sequence of IG was chosen to cover 200 bp upstream of translation start sites (except *ftsQ_{2p}*) as inputs in an *in silico* study using BioProspector. Two block model was adopted with a spacer from 8 to 10 bp between the blocks.

Analytic assays β -galactosidase assays were performed following the method of Miller (Miller, 1972). Transcriptional fusion reporter for *lsrA* is a derivative of pFYZ1 (Wang *et al.*, 2005) (Table 1). Miller Units = $1000 \times [(OD_{420} - 1.75 \times OD_{550}) / (TxV \times OD_{600})]$. AI-2 levels were determined following Bassler *et al.* (Bassler *et al.*, 1993). Briefly, *E. coli* conditioned media were collected by removing cells with centrifugation (for 10 min of 16000xg at 4°C) and subsequent filtration (Miller pore, 0.2 μ m). 20 μ l of *E. coli* CM was added to 180 μ l of freshly diluted overnight cultured BB170 (D=1/5000, AI-2 reporter strain) to initiate culturing at 30 °C with aeration (175 rpm). Light production was measured with a luminometer (EG&G Berthold, Gaithersburg, MD) and the reported AI-2 levels were normalized by counts of *Vibrio* cells.

Phenotypic characterization

Shock resistance Late exponential state cells were exposed to different shock conditions, oxidative, acidic, and thermal shock. For oxidative and acidic shock, 10 μ l of cells were placed to 1ml of PBS with H₂O₂ (from 5mM to 40 mM) and LB (pH 3.0, adjusted with HCl), respectively. At each sample point, 100 μ l of shocked cells were withdrawn, immediately serially diluted by PBS (pH 7.5), and spread on LB agar plates. For heat shock, cells (40 μ l) were loaded into 1.5 ml Eppendorf tubes and placed into a 55 °C water bath. At indicated time points, aliquots were withdrawn and immediately mixed with room temperature PBS (pH 7.5) and then serially diluted before spreading onto LB agar plates. All the viable colony counting was done after 24 hr incubation of the plates at 37 °C.

Autoaggregation To see bacterial auto-aggregation, late-exponentially growing cells were transferred to 15 ml falcon tubes and stored statically at 4°C. Cells were stored for 12 hrs and 1 week before taking the image pictures.

Motility Swimming motility was examined by swimming zone on soft agar plates (0.3 %). Late exponential phase broth cultures were placed on top of the plates and then stored at 37 °C chamber for 12 hrs.

SEM (scanning electron microscopy) Images for late exponential phase broth culture cells were taken. For this, cells were collected and gently washed three times with PBS buffer (pH 7.4, centrifugation by 2000g for 10 min) and then fixed with 2 % glutaldehyde (1hr at RT and 9 hr at 4 °C). Cells were collected with 0.2 µl filters and the residual glutaldehyde was washed out with PBS buffer three times before the filters were dehydrated with ethanol (70 %, 95 % and 100 %). The filters were fully dehydrated with Denton Vacuum freezer (Denton DCP-1 critical point dryer) and coated with Ag-Pd (Denton DV 502/503 vacuum evaporator). Coated filters were examined under EM (Model). This process was performed in the Biological Ultrastructure Laboratory in UMCP.

Strains or plasmid	Characteristics	Source
<i>E. coli</i>		
	F ⁻ λ : <i>rph1</i>	
W3110		Lab stock
WX2	Δlac - <i>pro met pro zzz::Tn10 thy supD</i> r _K ⁻ m _K <i>sdiA::kanR</i>	Rothfield
W2X2	W3110 <i>sdiA::kanR</i>	
ZK126	W3110 $\Delta lacU169$ <i>tna-2</i>	This study
ZKWX2	ZK126 <i>sdiA::KanT</i>	This study
<i>V. harveyi</i>		
BB170	BB120 <i>luxN::Tn5</i> (sensor 1 ⁻ , sensor 2 ⁺)	B. Bassler
Plasmid		
	pLW11 (<i>lsrA</i> fusion, pFYZ1 derivative)	Lab stock

Table 5. Bacterial strains and a plasmid used for the transcriptome analysis.

Results and Discussion

Phenotypic changes from *sdiA* null mutation

Growth and colony morphology As previous researchers linked SdiA to a cell division defect, we examined W3110 *sdiA*⁻ for growth defects and other growth-related phenotypic changes. *E. coli* W3110 and W3110*sdiA*⁻(W2X2) cells were grown identically in LB medium (Fig. 11.A). When we examined the wild type *sdiA* expression level over the course of a typical culture using quantitative RT-PCR, an apparent mid-exponential peak was observed (OD₆₀₀1.8, Fig. 1.A); expression remained high thereafter. Based on this observation, we performed phenotypic characterization of the *sdiA* mutant at the same growth phase, late-exponential state. Colony shape and cell morphology under a light microscope did not differ between *E. coli* W3110 and W3110 *sdiA*⁻ (W2X2) cells (Fig. 11.B).

Swimming and SEM Swimming motility, however, was significantly attenuated in W2X2 cells inoculated onto soft agar plates (0.3 %) (Fig. 12.C) and in the LB broth (Fig. 12.B). Also, while cells showed no apparent sign of defects in division under the microscope, SEM images revealed clear differences between the two strains such that W3110 had significantly more flagellar or fimbrial appendages as well as a more developed extracellular matrix (ECM) (Fig. 12.D.E).

Autoaggregation We also noticed that LB cultured W2X2 cells immediately settled to the bottom of centrifuge tubes within an hour, while W3110 cells remained suspended in the broth. After long-term static storage, W3110 cells eventually settled down to the bottom along with a slimy layer compressed on top of cell pellet, which is not shown in W2X2 culture (Fig. 11.F, right panel). It is highly likely that the extracellular matrix

delayed direct cell-cell contact, which is mediated by short appendage protein such as Agn43. *E. coli* autoaggregation was reported to be only possible in the minimal media but not in LB media.

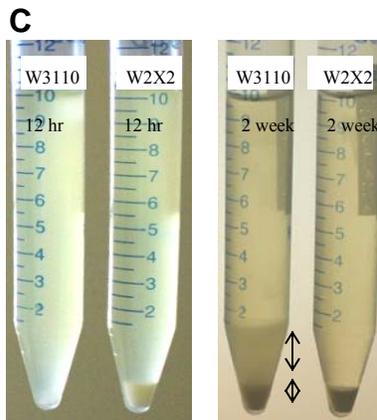
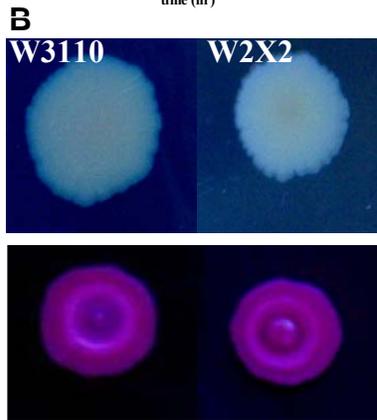
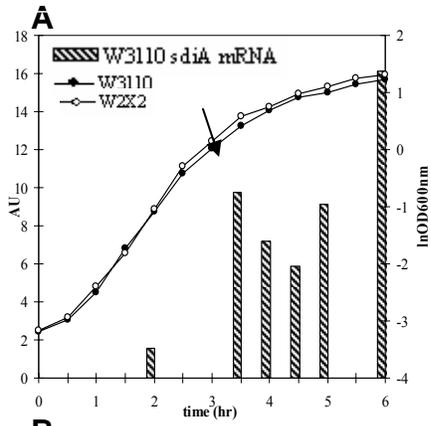


Figure 11. Macroscale phenotypic differences.

(A) Cell growth and expression of the *sdiA* gene. Growth curve for W3110 and W2X2 in LB media (semi-log graph: X-axis culture indicates sampling time and y-axis indicates logOD600nm). Quantitative Real time PCR was performed to see the expression pattern of *sdiA* along the time course. One of ribosomal protein-encoding gene (*rpsK*) was used as an internal control and used for normalizing the *sdiA* expression at different time points. Y-axis represents relative abundance (arbitrary units). (B) Colony morphology W3110 and W2X2 were cultured on LB (upper panel) and McConkey (lower panel) agar plates for 3 days at 37°C. (C) Autoaggregation of W2X2. Late exponential phase W3110 and W2X2 cultures were statically stored at 4 °C for 12 hrs (left panel). Same tubes after prolonged incubation (1week, right panel) showed the slimy top on the pellet. Autoaggregation of W2X2 is visible within an hour of static storage.

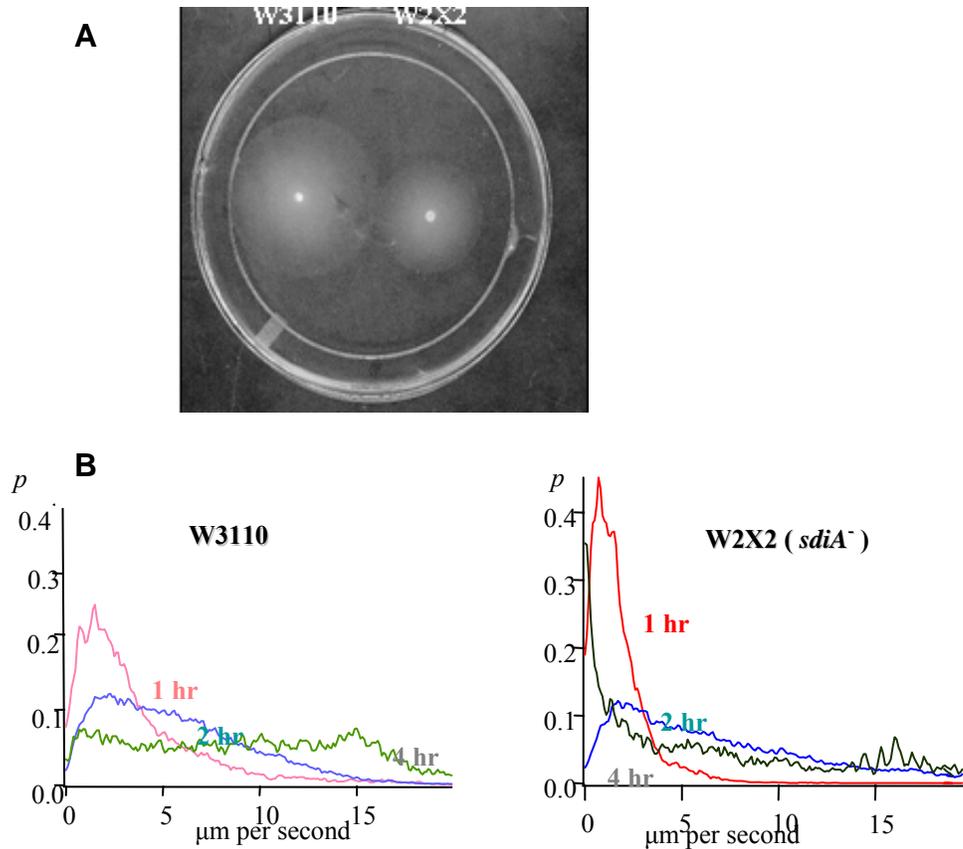
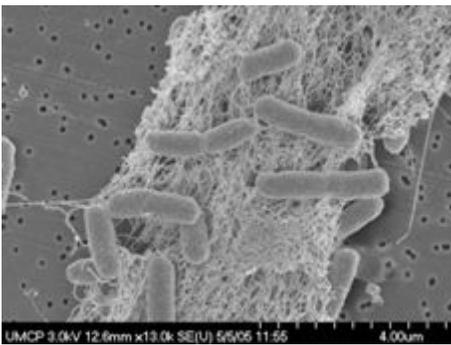
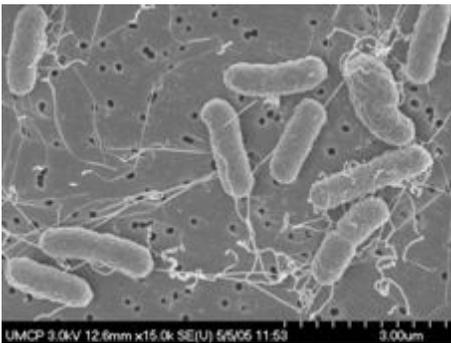
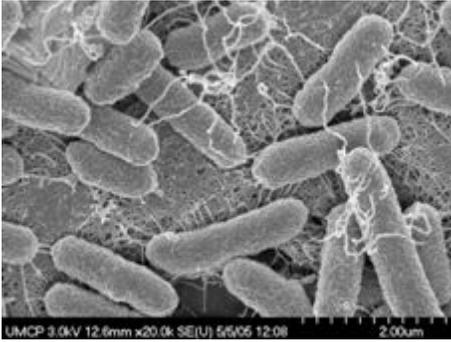


Figure 12. The reduced swimming motility of W2X2 (W3110 *sdiA*⁻). Swimming ability of *sdiA* deletion mutant, W2X2, was examined. **(A)** Overnight cultured W2X2 and W3110 in LB broth were placed on the LB agar containing 0.3 % Bacto agar (Difo) and incubated at regular 37 °C for 11 hr and then scanned. **(B)** Swimming speed of the wild type and mutant cells were compared by motility tracking (Dr. Losert, W. Dept of Physics, UMCP). 300 *E. coli* cells of each strain were individually tracked and distributed by probability. 3 time points of broth cultures were compared (1 hr, 2 hr and 4 hr after initiation of culture, each representing an early-, or mid-, or late exponential state).

C. W3110



D. W2X2

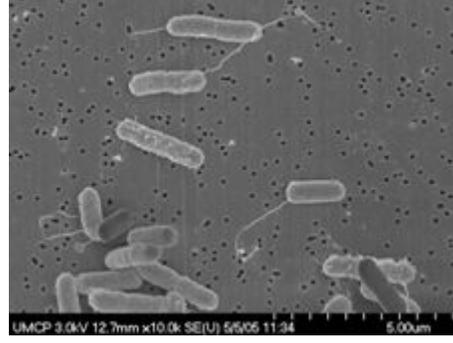


Figure 12. (contd)

(C) and (D) are scanning electron microscopy images of W3110 and W2X2 at late exponential stage.

Shock resistance assay When examined on the resistance toward severe insults, W2X2 survived better than wild type W3110 in general (heat shock at 55°C, acid shock at pH 3, and oxidative stress with 5 and 15 mM H₂O₂). For heat shock tests, cells were aliquoted in 1.5 ml Eppendorf tubes (40 µl) and set in a hot water bath (55°C). Every 1 min, each of the tubes was withdrawn and immediately serially diluted in PBS buffer (RT) and was plated on nutrient agar plates for counting the viable colonies (Fig. 13.A). In both cases, there was an apparent lag in cell death, but the lag was sustained in the case of W2X2. Ultimately the first order specific cell death rate was indistinguishable between the two, suggesting the death mechanism was consistent. Increased physical aggregation of W2X2 is speculated to have provided chances of the deep-core-centered cells from being slowly heated up about 1 min in our experiment set-up. For acid shock tests (Fig. 13.B), cells were centrifuged, resuspended in pH 3.0 LB for the indicated times, and rapidly serially diluted in PBS (pH 7.4). Both wild type and *sdhA* null mutant W2X2 were sensitive to acid shock, however, the specific cell death rate for W2X2 was significantly lower than that in wild type W3110. Hydrogen peroxide tests were performed identically, with cell resuspension in PBS (pH 7.4) containing 15mM H₂O₂. Again, W2X2 cells were more tolerate to the applied stress (Fig. 13.C).

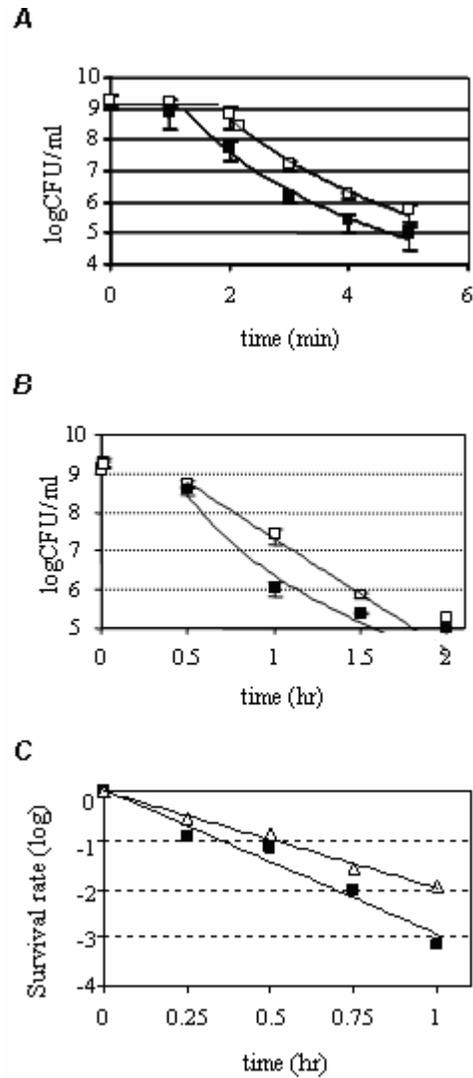


Figure 13. Lethal shock assays on the late exponential phase cells. W2X2 showed stronger resistance to lethal condition than wild type W3110. **(A)** 55 °C thermal shock. Lines along the data points are predicted as follows. W3110: $y=5 \times 10^9 X^{-7.0}$, $b=-7.0 \pm 0.63$; W2X2: $y=10^{11} X^{-7.8}$, $b=-7.8 \pm 0.67$. **(B)** pH3 acid shock responses are reported as the number of colony forming units of cells (CFU/ml) after the stress. **(C)** 15 mM H₂O₂ oxidative stress response is reported as the ratio of the survived cells after the stress. Error bars of A and B are based on 3 independent experiments. Closed squares stand for W3110 and open squares stand for W2X2.

Comparative analysis using DNA microarrays

Having established significant phenotypic variations between W3110 and W2X2 cultivated in LB media and/or during late exponential phase (OD_{600} 1.8), a transcriptome analysis was performed using Affymetrix *E. coli* antisense microarrays. Biological replicates were prepared on separate occasions and total RNA mixtures were extracted for the repeated chips. The RNA mixtures were converted to cDNA for further analysis using the chips (see Material and Methods). The antisense arrays are made of DNA oligo probes designed to hybridize all cDNAs from all open reading frames (ORFs) and intergenic regions (IGs). It is noteworthy that IG probes are designed to reflect the orientation of neighboring ORFs.

Initially limiting our analysis to detectable ORFs and IGs by signal intensity and reliability, our microarray data were initially grouped based on the present-absent calls, resulting in ~70 % of W3110 and 64 % of W2X2 of the total 4347 ORFs and 60 % of W3110 and 56 % of W2X2 of the total 2886 IGs assessed as present (Fig. 14). Beyond differential responses that are discussed in detail below, spots only expressed in W3110 were presumably attributed to a positive role of *sdiA*. Vice versa a putative negative role of SdiA can be found from spots expressed only in W2X2. SdiA is more likely to function as an activator than a repressor because the expression of 165 more ORFs and 92 more IGs were reduced with *sdiA* deletion. This is further supported by the fact that correlation with the overexpression study was limited to genes induced with the increase of *sdiA* transcripts (discussed below). A complete list of \log_2 IR for the ORFs and IGs is available on our group website (http://www.umbi.umd.edu/~cbr/lab_web/home.htm).

Next, ORFs and IGs both absent called from W3110 and W2X2 (absent in both from Fig. 3, black pies) were omitted and a total of 100 ORF (2.2 % of the total ORFs)

and 33 IGs were identified outside a 2-fold induction ratio ($\log_2 2=1$) cutoff (Table 6 and 9). Judging from the signal intensity in the microarray, the expression level of *sdiA* in wild type W3110 was quite low compared to the other global regulators such as RpoS. The low differential value of $\log_2 \text{IR}_{\text{W2X2/W3110}}$ (-2.4) is considered due to the low expression or short life-span of *sdiA* transcript. Whether this caused the generally mild differential IR between the strains is not known.

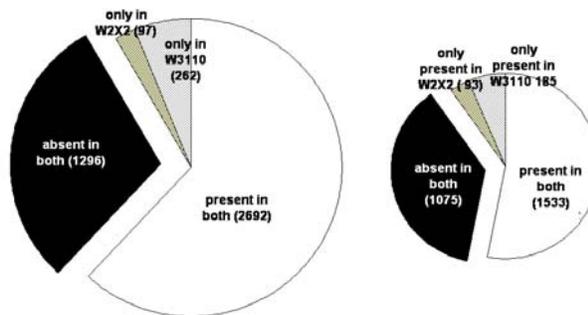


Figure 14. Differential transcriptome expression.

ORFs and IGs are grouped by detection calls. Absent called ORFs and IGs both from W3110 and W2X2 are colored black and were not further included in Table 6 and 9. Remaining data were further discussed in the text which include; the spots present only in W3110 or W2X2 (dotted or lined in the pie graph) and spots both present in W3110 and W2X2 (the white portion).

ORF

The 102 ORFs were grouped functionally based on the Affymetrix database; metabolism/nucleotide synthesis/motility related (42), regulatory proteins (13), transport /binding proteins (6), other functions (3) and uncharacterized or hypothesized functions (38). Interestingly, many of regulon- or operon-wide changes were noted.

Metabolism and cell processes The most highly affected ORF by *sdiA* deletion was *hdhA*, which encodes a bile acid modifying 7- α -hydroxysteroid dehydrogenase (*hdhA*, -2.4) (Yoshimoto *et al.*, 1991). Produced from liver, bile acids are secreted to digestive systems to emulsify dietary fats. Although active modification and thus diversification of the bile acid by enteric bacteria are known, specific purposes of the activity are not clear. If the altered transcription level resulted in expressed protein levels, it would not be too much exaggerated presumption that SdiA would function in the bacterial association in the host digestive system.

Meanwhile the biggest increase by the *sdiA* deletion occurred with *glcB* (2.2), a gene associated with a glycol utilization pathway. Its product, inducible malate synthase G, converts glyoxylate to malate. In the same *glc* operon, unknown functional *glcG* was also significantly induced with a marginal increase of a divergently transcribed transcriptional activator gene, *glcC*.

Three intriguing regulon-wide changes were found; genes for sulfate assimilation, purine de novo synthesis, and motility. Reduction of SO_4^{2-} to SO_3^{2-} and H_2S is needed for cysteine and methionine synthesis catalyzed by proteins encoded as *cysDNC* and *cysJIH* operons. Although it is generally difficult to link specific amino acid pathways with the

mutation of an indirectly associated gene (Hua *et al.*, 2004), our finding of potentially increased expression of genes for sulfur reduction may have potential to be linked to the synthesis of SAM, the common substrate of AI-1 and AI-2. Importantly supporting reduced swimming motility of W2X2, 28 genes involved in flagella, chemotaxis, and motility were down-regulated. Of these genes, only the *flg* operon is located at min 24 of the chromosome, while the rest of them are clustered at min 43 of the chromosome, wherein *sdiA* is located (Fig. 15.B). The three levels of hierarchical regulation are as indicated in the figure. The two clusters of genes are separated by 11 kbp apart. Genes for purine (AMP and GMP) biosynthesis are scattered across the genome and all of the 12 genes were induced by the mutation. Exceptionally, the expression of PurR repressor (GalR/LacI family) controlling the regulon did not change in this study (Meng *et al.*, 1990). Why in the LB rich media, W2X2 induced more genes for the nucleic acid, the building block of the nucleic acid, is quite odd.

The rest of three genes (*speC*, *cvpA*, and *sodA*) in the group of metabolism and cell process could be related with the purine de novo synthesis. First, a constitutive form of ornithine decarboxylase (biodegradative, producing putrescine) encoding *speC* (1.18) is known to be positively regulated by GTP in its expression (HOLTITA *et al.*, 1972) Thus, observed induction of purine de novo synthesis pathway may have preceded the *speC* induction. Also known to raise pH in the acidic environment, possible increase putrescine may have contributed to the increased acid resistance or other shock resistance. Secondly, *cvpA* (1.0) is located within the *purF* operon and commonly regulated by PurR (Fath *et al.*, 1989). It is needed for the production of plasmid-borne small extracellular toxin protein, colicin V. Lastly, *sodA* is one of two distinct superoxide dismutases (1.2). Its

production, manganese-SOD, is only present under aerobic conditions (Touati, 2000). Also MnSOD is a part of ribonucleoside-diphosphate reductase activating system (Coves *et al.*, 1995), which is required to convert nucleotide to deoxynucleotide. Thus it is likely that the increased purine synthesis demanded the activation of ADP reductase and GDP reductase.

Metabolism and cell processes		log ₂ IR
<i>cysC</i>	adenosine 5'-phosphosulfate kinase	1.6
<i>cysD</i>	ATP:sulfurylase, subunit 2	1.8
<i>cysH</i>	3'-phosphoadenosine 5'-phosphosulfate reductase	1.3
<i>cysI</i>	sulfite reductase, alpha subunit	1.2
<i>cysJ</i>	sulfite reductase (NADPH), beta subunit	1.4
<i>cysN</i>	ATP:sulfurylase, subunit 1, probable GTPase	1.3
<i>guaA</i>	GMP synthetase (glutamine-hydrolyzing)	1.5
<i>guaB</i>	IMP dehydrogenase	2.0
<i>purC</i>	SAICAR synthetase	1.4
<i>purD</i>	GAR synthetase	1.6
<i>purE</i>	AIR carboxylase, catalytic subunit	1.2
<i>purF</i>	PRPP amidotransferase	1.2
<i>purH</i>	AICAR formyltransferase	1.7
<i>purK</i>	AIR carboxylase, CO(2)-fixing subunit	1.3
<i>purL</i>	FGAM synthetase	1.2
<i>purM</i>	AIR synthetase	1.6
<i>purT</i>	formyltetrahydrofolate deformylase	1.5
<i>pyrB</i>	aspartate carbamoyltransferase	1.0
<i>glcB</i>	malate synthase G	2.2
<i>speC</i>	ornithine decarboxylase isozyme	1.2
<i>cvpA</i>	membrane protein for colicin V production	1.0
<i>sodA</i>	superoxide dismutase, manganese	1.2
Transport and binding proteins		
<i>cysP</i>	thiosulfate binding protein	1.2
<i>sbp</i>	periplasmic sulfate-binding protein	1.1
<i>melB</i>	melibiose permease II	1.0
Others		
<i>prfC</i>	peptide chain release factor RF-3	1.4
<i>flu</i> (<i>agn43</i>)	outer membrane fluffing protein, similar to adhesin	1.0
Uncharacterized hypothetical proteins		
<i>glcG</i>	a gene within the glycolate utilization operon	1.4
<i>b2001</i>	hypothetical protein	1.1
<i>b4188</i>	conserved hypothetical protein	1.2
<i>b1287</i>	putative oxidoreductase	1.5
<i>b2789</i>	YgcZ MFS transporter	1.3
<i>b3337</i>	bacterioferritin-associated ferredoxin	1.8
<i>b3646</i>	putative membrane protein	1.5
<i>b3654</i>	YicE NCS2 transporter	1.6
<i>b1388</i>	putative phenylacetate-CoA oxygenase	1.4
<i>b1389</i>	putative phenylacetate-CoA oxygenase	1.1
<i>b1390</i>	putative phenylacetate-CoA oxygenase	1.6
<i>b1391</i>	putative phenylacetate-CoA oxygenase	1.2
<i>b1392</i>	putative oxygenase reductase	1.7
<i>b1393</i>	putative enoyl-CoA hydratase	1.6
<i>b1394</i>	putative enoyl-CoA hydratase	1.5
<i>b1395</i>	putative 3-hydroxy-acyl-CoA dehydrogenase	2.0
<i>b1396</i>	with some similarity to thioesterases	1.3
<i>b3114</i>	2-ketobutyrate formate-lyase	1.3
<i>b3115</i>	propionate kinase / acetate kinase C	1.6
<i>b3125</i>	tartronate semialdehyde reductase	1.0
<i>b3127</i>	YhaU MFS transporter	1.6

Table 6. Significant changes in ORF (upregulated).

Metabolism and cell processes		log ₂ IR
<i>flgB</i>	cell-proximal portion of basal-body rod	-1.3
<i>flgD</i>	initiation of hook assembly	-1.1
<i>flgF</i>	cell-proximal portion of basal-body rod	-1.0
<i>flgG</i>	cell-distal portion of basal-body rod	-1.0
<i>flgH</i>	basal-body outer-membrane L ring	-1.2
<i>flgI</i>	homolog of Salmonella P-ring	-1.1
<i>flgK</i>	hook-filament junction protein 1	-1.4
<i>flgL</i>	hook-filament junction protein	-1.5
<i>flgM</i>	anti-FliA (anti-sigma) factor	-1.2
<i>flgN</i>	protein of flagellar biosynthesis	-1.2
<i>fliD</i>	filament capping protein	-1.1
<i>fliE</i>	basal-body component	-1.5
<i>fliF</i>	basal-body MS-ring and collar protein	-1.2
<i>fliG</i>	for motor switching and energizing	-1.2
<i>fliH</i>	flagellar biosynthesis	-1.1
<i>fliJ</i>	flagellar fliJ protein	-1.1
<i>fliL</i>	flagellar biosynthesis	-1.1
<i>cheA</i>	proton conductor component of motor	-1.3
<i>hdhA</i>	7alpha-hydroxysteroid dehydrogenase	-2.4
Regulatory proteins		
<i>sdiA</i>	transcriptional regulator of ftsQAZ	-2.3
<i>soxS</i>	regulation of superoxide response	-1.4
<i>rpoE</i>	heat shock oxidative stress	-1.2
<i>rseA</i>	negative regulatory protein for RpoE	-1.1
<i>fliA</i>	alternative sigma factor 28	-1.5
<i>fliT</i>	repressor of class 3a and 3b operons	-1.4
<i>fliS</i>	repressor of class 3a and 3b operons	-1.4
<i>flgM</i>	anti-FliA (anti-sigma) factor	-1.2
<i>fliH</i>	regulator of flagellar biosynthesis	-1.2
<i>cheW</i>	positive regulator of CheA protein	-1.3
<i>trg</i>	ribose sensor receptor	-1.2
<i>cheZ</i>	antagonist of CheY as switch regulator	-1.0
<i>tar</i>	aspartate sensor receptor	-1.1
Transport and binding proteins		
<i>srhE</i>	glucitol/sorbitol-specific IIB component	-1.1
<i>araH</i>	high-affinity L-arabinose transport	-1.1
<i>fruA</i>	PTS system, fructose-specific transport	-1.8
Others		
<i>amyA</i>	cytoplasmic alpha-amylase	-1.2
Uncharacterized hypothetical proteins		
<i>b0250</i>	hypothetical protein	-1.2
<i>b1760</i>	conserved hypothetical protein	-1.5
<i>b1904</i>	conserved hypothetical protein	-1.7
<i>b1935</i>	hypothetical protein	-1.3
<i>b4109</i>	hypothetical protein	-1.3
<i>b1194</i>	protein involved in flagellar function	-1.3
<i>b1566</i>	hypothetical protein; gene is regulated by FliA	-1.8
<i>fliZ</i>	possible cell-density responsive regulator of sigmaF	-1.5
<i>b3525</i>	protein involved in flagellar function	-1.2
<i>b2147</i>	putative dihydrothymine dehydrogenase	-1.2
<i>b1967</i>	heat shock protein (Hsp) 31	-1.7
<i>b3570</i>	putative ATP-binding protein	-1.2
<i>b1513</i>	ATP-binding component of ABC transporter	-1.3
<i>b1515</i>	membrane component of ABC transporter	-1.5
<i>b1516</i>	periplasmic binding protein of ABC transporter	-1.1
<i>b1517</i>	putative aldolase	-1.1
<i>b1518</i>	conserved hypothetical protein	-1.3

Table 6. (contd.) Significant changes in ORF (down regulated)

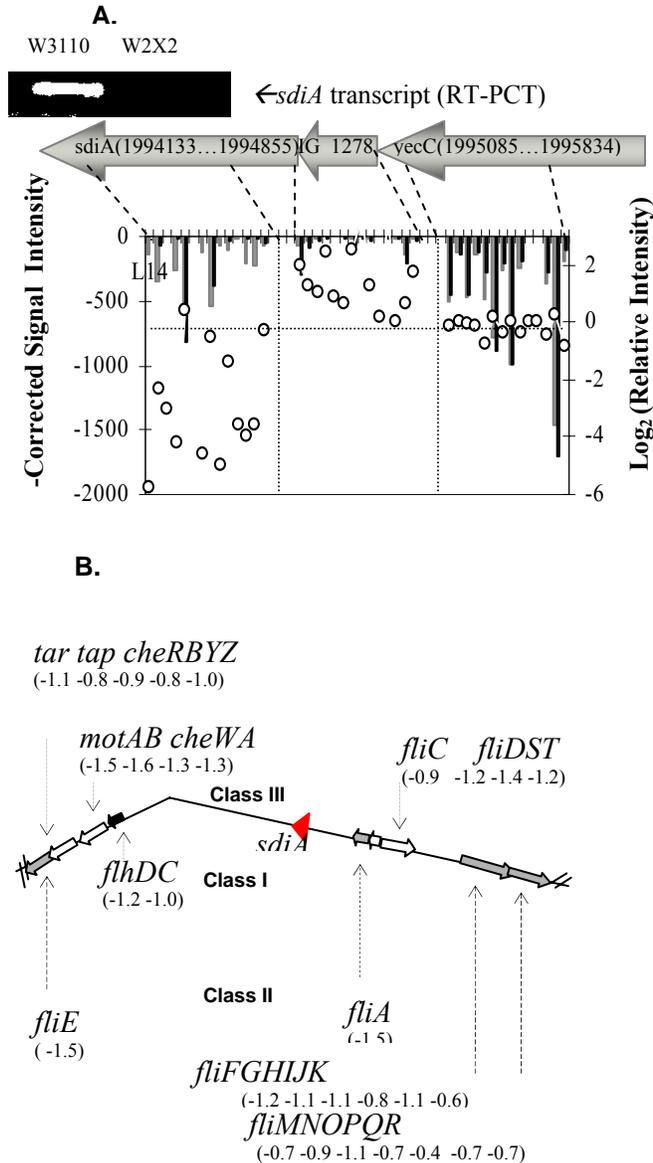


Figure 15. Motility-related genes around *sdiA* in the chromosome.

(A) Transcript changes in *sdiA* –IG-*yecC* after *sdiA* deletion. Subtranscript level changes were shown as described in Material and Methods. 42 probes are representing the region (14 of each). Open circle indicates the relative induction ratio by *sdiA* deletion (\log_2 RI_{W2X2/W3110}, right Y-axis) while bars indicate the signal intensity (PM-MM, corrected, left Y-axis). The *sdiA* deletion induced the upstream IG expression. However, the signal in the IG is not strong (absent called). Upstream gene *yecC* showed no changes by the *sdiA* mutation. Gel figure shows the absence of *sdiA* mRNA from W2X2 using RT-PCR (with 40 PCR cycles). **(B)** Genomic context of *sdiA* relating with the motility genes. The *sdiA* gene is indicated (red triangle) in the middle of flagella, motility and chemotaxis regulon (region Ila I Ib, around 1960000-2028000). The hierarchy of the regulon, (Class I, II and III) is shown along with the specific microarray \log_2 IR values for each gene.

Uncharacterized hypothetical proteins (metabolism) Among the 39 genes that were annotated as uncharacterized in the Affymetrix database, three induced groups of genes appeared as parts of operons (Fig. 16). Since the functionality of each of them have been reported in metabolism, it is appropriate to introduce them here. These are proposed to function in L-threonine degradation (*tdc*), phenylacetate degradation (*paa*), and glycolate/galactarate/glucarate degradation (*gar*).

First, the *tdc* operon (min 68.3, 7 genes of *b3112-b3118* with 3 IGs, 4 significant and 6 moderate increases, anaerobic) is composed of genes encoding threonine dehydratase (*tdcB*), permease (*tdcC*), propionate kinase (*tdcD*), 2-ketobutyrate formate-lyase (*tdcE*), hypothetical protein (*tdcF*), and serine deaminase (*tdcG*). It has two genes encoding operon-specific transcriptional activators TdcA (LysR type) and TdcR. The changes of *tdcABCDEFG* expression were evenly around 2 fold-levels, while changes in *tdcR* and downstream gene *yhaO* (may function as amino acid/proton symporter) were much smaller. Besides TdcA and TdcR, multiple transcriptional factors including CRP, IHF (integration host factor), and HU (histone like protein units) are directly involved in its promoter expression (Hagewood *et al.*, 1994).

Secondly, genes for putative phenylacetate degradation pathway (min 31.2-31.5, 11 genes of *b1388-b1398*, 9 significant and 2 moderate increases) showed induction with the last two distal genes with moderate changes. Following the operon exists its transcriptional regulator encoding gene *paaX* as a transcriptional unit, however *paaX* expression did not change by the mutation. Also seen in the PurR of purine de novo pathway, the absence of changes in the regulator in contrast to the changes in the structural genes in the operon is likely to have resulted from difference in the expression

timing and controlled level of expression (transcription for the structural vs. translation for the regulatory genes) between the regulators and structural genes or from the difference in mRNA degradation speed (*i.e.* more rapid degradation of the transcriptional regulator mRNA).

Finally, induced galactarate/glucarate degradation pathway operon (5 genes of *b3124-b3128* with 1 IG, 2 significant, 1 moderate, and 3 marginal increases) encodes two transcriptional units of *garD* and *garPLRK* encode galactarate dehydratase (*garD*), MFS transporter (*garP*), aldolase (*garL*), reductase (*garR*), and glycerate kinase (*garK*). As indicated in the Fig. 5, the changes in *garP* for MFS transporter and *garR* for tartonate semialdehyde reductase were greater than the others'. A common regulatory protein, SdaR, was identified for the both directional transcription toward *garD* and *garP* (Monterrubio *et al.*, 2000). Importantly, its third transcriptional unit, *gudPD* (*b2789-b2787*), was also induced in W2X2 [MFS transporter (1.3) and glucarate dehydratase (0.8)], proving it as another example of regulon-wide changes. Particularly, the apparent increase of the two MFS transporters (*garP* and *gudP*) is intriguing. Increased glycolate (*glc* operon, above) and galactarate utilization commonly ends in the production of 3-phosphoglycerate for TCA cycle.

Combining the some of the changes mentioned above, an interesting sequence on glyoxylate metabolism can be state. Glyoxylate is generated from both purine degradation and glycolate. If purine degradation were prior to the increased purine de novo pathway, glyoxylate level increased naturally in W2X2 (combined with *gar*) and could draw precursors from the TCA cycle (glyoxylate bypass) to glyoxylate cycle for

making building blocks, such as nucleic acids. It is noteworthy that the glyoxylate cycle is often associated with growth on acetate, which, in turn, accumulates during the late exponential phase during growth in LB. Indeed, the induction of the phenylacetate degradation may have increased acetate levels in W2X2 cytosol (*paa* operon, above). This reasoning, consistently, results in increased purine synthesis.

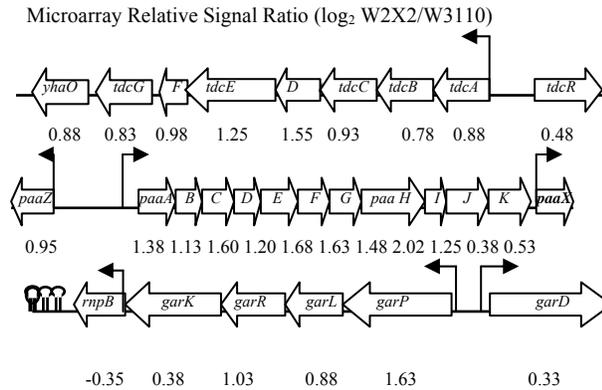


Figure 16. Operon-wide changes with putative functions (metabolism associated). Three induced operones suggested to function (still annotated as putative in Affymetrix database) are depicted and marked for the differential expression ($\log_2 IR$). These are suggested to involve in L-threonine degradation (*tdc* operon), phenylacetate degradation (*paa* operon), and glycolate degradation (*gar* operon). Notably, positive regulator of *paaX* did not change.

Transport and binding proteins Among the group of transport and substrate binding, three induced (*cysP*, *sbp*, and *melB*) and three reduced (*srlE*, *araH* and *fruA*) gene expressions were found. Induction of thiosulfate/sulfate binding proteins (*cysP* and *sbp*) can be regarded as the precedence of the increased sulfate assimilation. Their products, CysP and Sbp, are both localized in the periplasm and functionally identical interacting commonly with the components of ABC-type transporter CysATW (Sirko *et al.*, 1995). Related with sugar transport, melibiose permease and low affinity glucitol/sorbitol transporter showed operon-wide changes. Among the genes in melibiose (α -D-galactopyranosyl-(1 \rightarrow 6)-D-glucopyranose) permease operon (*melRAB*), the increase of permease gene, *melB*, was most apparent than those of a transcriptional activator and α -galactosidase encoding genes (*melR* and *melA*). Among the decreases of genes in glucitol/sorbitol transporter (phosphoenolpyruvate dependent phosphotransferase system, *srlAEBD*), the change of *srlE* (-1.1) was most clear and listed in Table 2. The operon has a transcriptional activator (*srlM*) and repressor (*srlR*) nearby (Postma *et al.*, 1993). In our study, the *srlM* was also down-regulated, however, *srlR* expression did not change. This suggests that W2X2 may have increased utilization of cytosolic glycolate (*glc* operon, mentioned above) and rather decreased its uptake, which is reasonable. Both *mel* and *srl* operons are known to be catabolically repressed by cAMP-CRP complex. We also noted interesting reduction of another transporter encoding gene clusters (*b1511-b1518*), which is discussed below.

AI-2 transporter, lsr operon Most interestingly, we noted that the ATP binding cassette-type transporter at 34.5 min of the *E. coli* chromosome, also identified as the *lsr* operon, was significantly reduced in the W2X2 cells (Fig. 17). Originally predicted as the low-affinity transporter of the two ribose transporter systems in *E. coli*, the *lsr* operon was recently characterized for AI-2 uptake and modification both in *E. coli* and *Salmonella* (Wang *et al.*, 2005; Xavier and Bassler, 2005). Transcribed opposite direction from the ABC transporter structural genes (*b1513-b1515*, *lsrACDB*) and putative AI-2 processing enzyme encoding genes (*b1517-1518*, *lsrFG*), genes encoding a negative regulator (*lsrR*, *b1512*, SorC family) and a kinase (*lsrK*, *b1511*) exist in the upstream as another transcription unit (Fig. 17.A). The *lsr* operon of *E. coli* does not have the last operonic gene, the *lsrE* counterpart of *Salmonella*, but is followed with a trans-aconitate methyltransferase encoding gene, *tam*. Unlike the previous proposition that *lsr* operon is homologous to *rbs* operon for a high affinity ribose transporter (Taga *et al.*, 2001), only one component (*rbsD*) was moderately affected (-0.6, Fig. 6.A). RbsD converts ribose from pyran to furan for accelerated utilization (Ryu *et al.*, 2004).

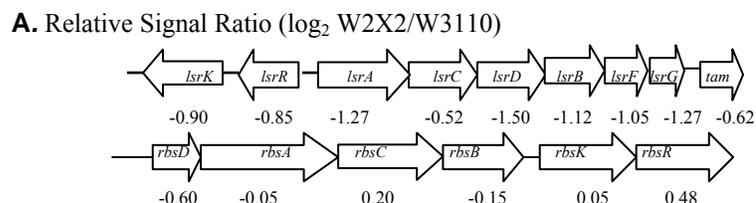
To confirm the changes in the *lsr* operon, transcriptional fusion assays for *lsrA-lacZ* fusion were performed in a *lac⁻* background (*E. coli* ZK126, Fig. 17.B). Beginning with the growth of the cells, unlike the W3110 background, ZK126 cells with an *sdiA* deletion (ZKWX2) had a lower growth yield and growth rate in the LB cultures compared to the parental control ZK126. Interestingly, adding consumable amount of glucose at the beginning of the cultures (1.11 mM, 0.1 %) reduced the difference and increase the promoter activity from both of the strains. AI-2 levels in CM from these cultures were typically higher in CM from the parental cells than the *sdiA⁻* cells (Fig.

17.C). Regardless of the presence of glucose in the media, *sdiA*⁻ resulted in lower *lsrA* transcription (~2-3 fold as measured by β -galactosidase expression), which is consistent with microarray results (Fig. 17.B). The differences were particularly noteworthy during the late exponential phase but not in the early exponential phase (2-3 hr), which may explain why this operon was not detected in the experiment for overexpression by microarray.

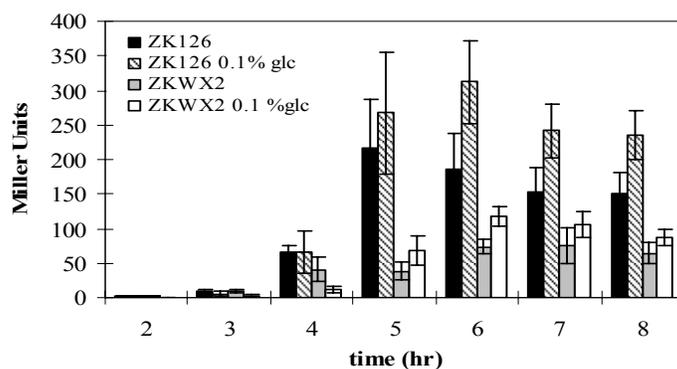
Catabolite repression is the major driving force of *lsr* structural gene transcription finely tuned by AI-2 responsive regulator (LsrR) (Wang *et al.*, 2005). The reduced *lsr* operon expression in our study showed that presence of SdiA somehow assists CRP-driven *lsr* transcription. Following the reasoning of Bassler and colleagues, increased AI-2 must have activated LsrK kinase for derepressing LsrR, which in turn induced the transcription toward *lsrA* and the following genes of the operon. Non-consumable amounts of glucose (0.8%) abolished the expression of the operon both from W3110 and W2X2.

These data demonstrate that the systems 1 and 2 of *E. coli* quorum sensing merge on the ATP-binding cassette type transporter characterized as the AI-2 specific uptake machinery. Although, in general AI-2 and AI-1 molecules share the amino acid precursor, S-adenosyl methionine (SAM), genes involved in AI-2 synthesis (*pfs* and *luxS*) were not affected. Whether there is a physical interaction with SdiA and the *lsr* promoter was not tested. Convergence of different quorum sensing pathways has been suggested in other species when there are multiple pathways present in an organism, as in the case of *Pseudomonas* and *Vibrio* species. Most recently, two different quorum regulators in

Streptococcus pneumoniae were shown to positively regulate the same ABC transporter for transporting peptide pheromones (Knutsen *et al.*, 2004)



B. Transcriptional fusion assay



C. AI-2 assay

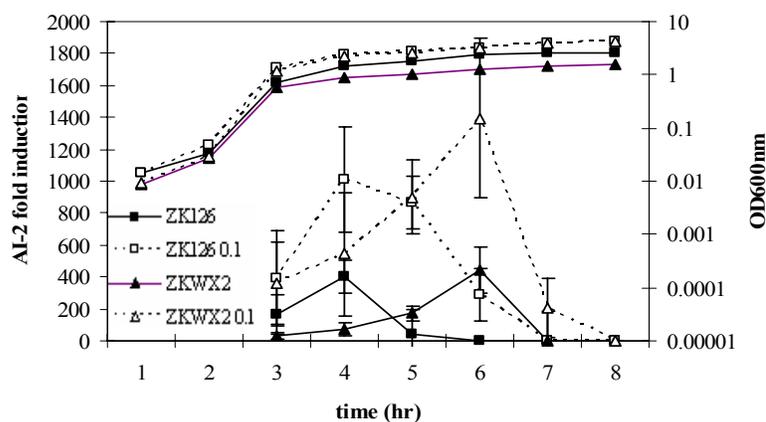


Figure 17. The positive regulation of SdiA on AI-2 uptake machinery.

(A) Divergent transcriptions from *b1511-b1518* (*lsr* operon) are depicted with the differential expression value (\log_2 IR) obtained from our microarrays. Suggested homologues *rbs* operon is also depicted. (B) Low copy vector based *lsrA*-transcriptional fusion assay was performed (Wang et al. 2005) in the presence and absence of glucose (0.1%). X-axis represents time and y-axis represents miller units. Error bars are obtained from two independent experiments. (C) Corresponding AI-2 levels in the conditioned media were indicated with the growth curves.

Ag43 and biofilm formation Importantly, a gene coding antigen 43 (Ag43), the major reversible phase-variable protein in the outer membrane, was induced (*flu*, *agn43*, 1.0). As a species-specific antigen, Ag43 is well-known to make cells aggregate (Kjaergaard *et al.*, 2000), which is in good agreement with the observable W2X2 phenotype seen in Fig. 11.F. There were no significant differences in the other possible adhesin, such as *curli* proteins or *fimbriae*. The transcription of the Ag43 is known to be regulated by differential methylation of Dam and OxyR interaction. OxyR binding on the promoter blocks the methylation thus inhibits *flu* expression. Locked-off *flu* expression by *oxyR* mutant was reported (Henderson and Owen, 1999). It is noteworthy that quorum-sensing often controls cell motility, biofilm formation, and siderophore production. There are three papers using DNA microarrays for *E. coli* biofilm formation (Beloin *et al.*, 2004; Ren *et al.*, 2004; Schembri *et al.*, 2003). Interestingly, *sdiA*, *lsr* operon, *flu* and many other genes were detected at least once to be differentially expressed in biofilm compared to the planktonic cells.

Transition to stationary phase (Table 7) The transition period to stationary phase is typically characterized by extensive reprogramming on translational- and transcriptional-machinery, nucleoid, and cell process (Ishihama, 1999). By the *sdiA* mutation, translation process seemed most affected caused by the changes in ribosomal RNA (rRNA) components as apparently shown in Table 3. The rRNAs are encoded in 7 operons, each of which makes a precursor of 16S, 23S, and 5S rRNA and at least one tRNA genes. Designated as 18 b-numbered ORFs in *E. coli* Affymetrix chips (8 for 5S, 6 for 23S, and 4 for 18S rRNA), almost all of the rRNAs transcriptionally decreased significantly or moderately keeping the tRNA expression levels equal. When small effector molecules that regulate rRNA transcription were considered as possible explanation of the differences, *relA* and *spoT* for the stringent response effector, ppGpp, did not differ between the two strains. However, as mentioned above, altered purine nucleotide synthesis is likely to have been realized as altered NTP concentration for initiating rRNA transcription (iNTP) (Paul *et al.*, 2004). The differences in rRNA expression, were confirmed by a RNA gel with equal amounts of total RNA in loaded samples (Fig. 18).

Unlike rRNA, genes encoding entire 55 ribosome protein components (22 for 30S ribosome subunit and 23 for 50S subunit) did not change much; only three moderate decreases (*rpsU*, *rpmB*, *rpmG*: -0.60, -0.80, and -0.58) and one moderate increase (*rpsQ*, 0.85) were found. Other genes related with translation were also examined. Of three translation termination factors, only RF-1 (peptide chain releasing factor, *prfC*, 1.35) was induced while keeping the other two RF-1 and RF-2 unvaried. Additionally, translation repressor operon *relBEF* slightly decreased (-0.72, -0.47, and -0.10, respectively). Interestingly, the ribosomal modulation factor (*rmf*) for dimerizing ribosomes in the

stationary phase was also reduced in W2X2. Physiological outcome of the changes in the translational machinery found from the genomic analysis needs to be defined in the future.

One of the limits in current microarray studies is the inability to distinguish the rates of transcription initiation and transcription turnover. However, considering the rRNAs and tRNAs are processed from same precursors, the reduction in 5S, 16S, and 23S rRNA content keeping the equivalent expression of tRNA and even the entire ribosomal protein component levels is a convincing evidence suggestive of rRNA destabilizing force. Impacts on translation machinery by quorum sensing are quite undefined. Previously significant changes of ribosomal protein component transcription by the *luxS*-mutation in EHEC 0157:H7 was reported (Sperandio *et al.*, 2001), and there is no report on LuxR-mediated possible translation machinery modulation to our knowledge. The decreased rRNA quantity reminds us of common growth limits, however, there were no noticeable differences in growth rate in W2X2. And arrested growth nucleotide synthesis, especially of pyrimidine, tends to decrease. However, observed induction in the purine *de novo* pathway confirmed that this phenomenon differs from the common growth arrest.

Unlike rRNA levels associated with translation machinery, the expression of transcription machinery did not show dramatic changes. Of the 9 RNA polymerase components (*rpoABCDEHNSZ*), the core RNA polymerase and constitutive sigma factor transcript levels were conserved. Exceptionally alternative sigma factor *rpoE* and *rpoS* expression decreased and are discussed below.

Finally, 12 known nucleoid-associated DNA binding proteins were examined and only stationary-phase protein IHF was found to change, with a moderate decrease. Functioning in general cell process and stress response, 25 chaperone genes (including putative genes) were also examined and marginal decreases in *hslV* and *hslU* and moderate decreases in *mopB*, *mopA*, and *htpG* were shown as seen in Table 7.

Gene	Description	log ₂ IR
Ribosomal RNA with a change call		
<i>rrlA</i>	23S rRNA of rrnA operon	-0.9
<i>rrlC</i>	23S rRNA of rrnC operon	-0.6
<i>rrlD</i>	23S rRNA of rrnD operon	-0.5
<i>rrlE</i>	23S rRNA of rrnE operon	-1.1
<i>rrlG</i>	23S rRNA of rrnG operon	-1.1
<i>rrlH</i>	23S rRNA of rrnH operon	-1.1
<i>rrsA</i>	16S RNA of rrnA operon	-0.9
<i>rrsC</i>	16S RNA of rrnC operon	-0.8
<i>rrsG</i>	16S RNA of rrnG operon	-0.9
<i>rrsH</i>	16S RNA of rrnH operon	-0.7
Chaperone with a change call		
<i>hslU</i>	heat shock protein hslVU, ATPase subunit,	-0.5
<i>hslV</i>	heat shock protein hslVU, proteasome-related	-0.5
	GroES, 10 Kd chaperone binds to Hsp60 in	
<i>mopB</i>	pres.	-0.6
<i>mopA</i>	GroEL, chaperone Hsp60, ATPase	-0.5
<i>htpG</i>	chaperone Hsp90, heat shock protein C 62.5	-0.8
All RNA polymerase components		
<i>rpoA</i>	RNA polymerase, alpha subunit	-0.2
<i>rpoB</i>	RNA polymerase, beta subunit	-0.2
<i>rpoC</i>	RNA polymerase, beta prime subunit	-0.2
<i>rpoD</i>	RNA polymerase, sigma(70) factor	-0.2
<i>rpoE</i>	RNA polymerase, sigma-E factor	-1.2
<i>rpoH</i>	RNA polymerase, sigma(32) factor	-0.0
<i>rpoN</i>	RNA polymerase, sigma(54 or 60) factor	-0.3
<i>rpoS</i>	RNA polymerase, sigma S (sigma38) factor	-0.4
<i>rpoZ</i>	RNA polymerase, omega subunit	0.1

Table 7. Examination on growth phase transition.

Differential expression of components in translation and translation machinery and cell process (by chaperones) were looked into and the genes with change calls were selected and listed. Note that significant *rrl* and *rrs* changes (bold letters) are not included in the Table 6.

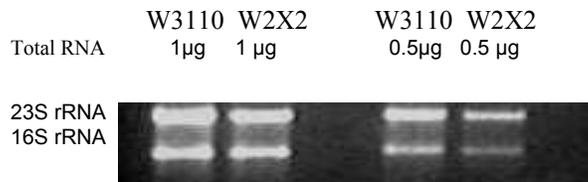


Figure 18. Reduced rRNA of W2X2 (W3110 *sdiA*⁻).

Equal amounts of total RNA (both 1.0 μg and 0.5 μg RNA) of W3110 and W2X2 were loaded onto formaldehyde (220 mM) RNA gel with 1.2 % agarose for visual comparison. Samples from W2X2 showed apparently reduced rRNA level compared to those of W3110 in the specific growth stage (late exponential).

Comparative analysis with other regulators A relatively high ratio of regulatory proteins was affected by the *sdia* mutation, possibly due to an intrinsic complicacy of transition in growth phase (as mentioned above). When we looked into known global regulators with changes, reduced extracytoplasmic stress and oxidative stress associated factors (*rpoE*, -1.23 · *soxS*, -1.35, respectively) were found, along with factors involved in starvation/anaerobic environmental adjustment (*rpoS*, -0.43 · *acrA*, -0.80) to a less extent. Intrigued with the possibility of their regulatory effects on the genes catalogued in Table2, we attempted to compile the target genes of each regulator and examined their differential ratios from expectation. Sources of the compared literature and database are listed in Table 4. [*rpoS* regulon (Weber *et al.*, 2005); *soxS* and its homologues *rob* and *marA* regulon (Barbosa and Levy, 2000; Martin and Rosner, 2002; Pomposiello *et al.*, 2001); *acrA* regulon EcoCyc; *rpoE* regulon (Dartigalongue *et al.*, 2001; Rezuchova *et al.*, 2003)].

Details of comparative studies are as follows. First, among the 140 genes expressed at the RpoS inducing conditions of stationary phase, osmotic upshift and acid stress (Weber *et al.*, 2005), 7 genes got change calls, especially with 2 significant changes of *amyA* and *hdhA*. Oddly, major stationary phase response gene, *osmY*, did not change in our study. Secondly, many genes of *soxS/rob/marA* regulon showed changes in our study, especially with three significant changes of *srlE*, *pyrB*, and *guaB*. Still, several genes showed opposite change-pattern from expectation, *e.g.* significant induction of *sodA* and 4 moderate reductions of *b1452*, *fumC*, and *pqiA*. Thirdly, dual regulator of ArcA, composing a two-component system with a sensor kinase ArcB, represses aerobic metabolism genes when respiration is not possible (such a condition of the arrested

growth during stationary phase). We found 22 genes with change calls, and most of them showed expected changes in agreement with the decrease of *soxS* expression. Finally, functioning in polypeptides folding in the envelope and lipopolysaccharide biosynthesis/transport, σ^E is known to control at least 31 promoters for 73 genes (Dartigalongue *et al.*, 2001; Rezuchova *et al.*, 2003). None of them changed significantly, however, the expression of three ORFs showed moderate changes. Unexpectedly, transcription of its downstream neighbor encoding its own anti-RpoE factor (*rseA*, -1.05) (Missiakas *et al.*, 1997) and another gene under the suppression of RpoE decreased (a growth inhibiting gene *ssnA*, -0.8). In summary, nonetheless the inconclusiveness originated from different experimental conditions independently adopted in different studies, several changes ascribable to changes in the global regulators were identified: six RpoS regulated genes were affected, three SoxS regulated genes, and three Arca regulated genes (Table 8).

In an analogous manner, our results were compared with that of *sdiA* overexpression study using PCR-based microarrays (Wei *et al.*, 2001). Surprisingly, only two genes, *yedU* and *yhiW*, showed correlated expression between the two studies *i.e.* increased expression with increased level of *sdiA* transcripts. Despite the low expression level of *yhiW* in our study (not listed in Table 2 due to being absent called), it has same a HTH domain homologous to that of *Salmonella srgC*, which was shown to be *sdiA* dependent (AraC/XylS family). If it were indeed directly SdiA-dependent, the increased acid resistance of the W2X2 strain would be glutamate-dependent (Ma *et al.*, 2002). The *yedU* encodes a heat shock protein Hsc31 (Mujacic *et al.*, 2004). Interestingly, both of

them were also identified as members of RpoS regulon (Table 8), thus, overlapped regulation of SdiA and RpoS is highly likely to exist on these genes. Also there were 6 moderate changes showing correlation, which includes *ftsQ* and *Z*, the products of the only confirmed *sdiA*- dependent transcript, *ftsQ2p*, supporting the null mutation of *sdiA*. However, *uvrY*, the gene directly downstream of *sdiA* and suggested to be SdiA-dependent from another overexpression study (Suzuki *et al.*, 2002; Wei *et al.*, 2001), did not change in our study. Similarly, genes for acridine efflux pump shown to be the reason of increased antibiotics resistance of *sdiA* overexpressing strain (Wei *et al.*, 2001) showed only marginal change only in *acrA* (-0.38). This supports and explains the absence of hypersensitivity to MMC by a *sdiA* knockout strain (Wei *et al.*, 2001). More than that, strikingly, 46 genes even showed the opposite pattern deviated from the expectation (25 significance- and 21 moderate decreases). With the exceptions of cysteine degradation and nucleotide conversion, *tnaA* and *aphA*, all of them are motility-related. The decreases of the regulatory genes for chemotaxis · motility and subsequent changes in downstream genes were depicted in Fig. 15.B. This change in transcription turned out as reduced swimming motility of W2X2 and was visually further proven as less attached flagella on W2X2 (Fig. 12). Indeed, motility must be one common target sensitive to quorum signaling [e.g. LuxS-dependent motility and attachment defects were reported (Sperandio *et al.*, 1999)].

Possible explanation for the apparent discrepancy between the two studies can be easily found from the dramatically different growth phases chosen to be compared (OD₆₀₀ 0.45 vs 1.8), or due to the general stress response resulting from protein overexpression (Bentley *et al.*, 1990). However, from more radical standpoints, SdiA may adopt

bimodular DNA binding conformation and subsequent selections of target pools depending on stoichiometry of its own concentration and the cognate signals (or even presence and absence). Still it needs to be kept in mind that the transcript level changes in the transcriptional regulator could be minor compared to changes in the post-transcriptional levels as one caveat of this comparative approach.

Global regulator gene	log ₂ IR	Literature or public database used		Target genes changed in our study		
		Ref.	Targets #	Significant	Moderate	Marginal
<i>rpoS</i> ¹	-0.43	Weber et al.	140	<u>(-)</u> <i>amyA, hdhA, yhiW, gadA, otsA, yedU</i>	(-) <i>lrp, b1519, b3100</i> (+) <i>pdhR, b1806</i>	
<i>soxS</i> ²	-1.35	Barbosa et al., Pomposiello et al.,	264	(-) <i>srlA_2</i> (+) <i>pyrB, guaB</i>	(-) <i>lldP, tnaA, mtlR, gatD, yeaQ, hupB, marA, gatC, zwf, gata, tnaL, malE</i> (+) <i>ndh, prmA, rpsQ, and yfhO</i>	(-) <i>mglB, ytfK, wrbA, tpx, mdaA, madB, yljA, and fur</i>
<i>acrA</i> ³	-0.80	EcoCyc	73	<i>sodA, glcB, glcG</i>	<i>b2997, caiF, fadAB, fumC, hybABCDE, lldP, ndh</i>	<i>aceABK, dctA, sucABC, and tpx</i>
<i>rpoE</i> ⁴	-1.23	Dartigalongue Rezuchova et al.	73		<i>htrA, ygiM, b1806</i>	
<i>sdiA</i>	-2.35	Wei et al.	137	<u><i>yedU, yhiW</i></u>	<i>ftsQ, ftsZ, glnH, pntA, pntB, and ybgl</i>	

Table 8. Literature-based comparative analysis.

Examination of cross-regulation with other well-known global regulators and comparison with the transcriptome alteration by plasmid born *sdiA* (overexpression). Absent called genes in our study are indicated by underlines.

IG: SdiA consensus sequences and sRNA expression

Affymetrix probe set formats enable users to examine the changes in the intergenic regions (IGs), which can be useful for screening putative SdiA binding motifs (in promoters) and searching the expression of novel and small RNAs. We had 319 IGs (of the 2886 IGs) with change calls and 33 of them showed more than 2 fold changes by the mutation. The induction ratio and signal intensities of 33 IGs were interrogated to a sub-transcript-level and manually grouped to be either showing congruence (20) or incongruence (12) by the relation with those of the flanking ORFs (or IGs) (Fig 20-22, Table 9). Since most of IGs in the congruent group are transcribed in a same direction with the neighbors (Fig. 20), they are likely to be in operon units or be up- or downstream transcribed but untranslated regions of neighboring genes (UTR). Here, 4 IGs could clearly fit as 5'-UTRs of proximal genes, thus they were used as inputs for searching SdiA consensus motifs below. The IGs without congruency were further divided into two groups; opposite (6, Fig. 21) or indifferent (6) to the neighbors in changes (Fig. 22). Details of our data analysis are as follows.

Consensus motif Firstly, previous biochemical analysis on *ftsQ_{p1p2}* promoters suggested “SdiA box” with inverted sequences, 5-AAAAG(N)₈GAAAA-3 (Yamamoto *et al.*, 2001). It was reported that this sequence also exists in the promoter of AI-2 regulated genes *eae* and *espA* of *E. coli* 0157:H7 and in the *sdiA* regulated gene *srgC* of *S. typhimurium* from the genome database search. Limits in single gene based model sequence are unavoidable to cover many possible SdiA dependent genes suggested either from the study of Wei *et al.* (Wei *et al.*, 2001), or from our study. In order to search

consensus sequences in promoter regions, 5'-UTR positioning IGs from three sources were examined; genes showing congruency with significant changed IG (Table 9 *ycfB*, *modA*, *purA*, and *cvp*, mentioned above), significantly changed genes with congruent IG (Table 6. *yjfN* and *hdhA*), and genes-of-interest (*yhiW* and *yedU* commonly detected from *sdiA* overexpression- and deletion, *b1513* of AI-2 uptake machinery, and the *ftsQ_{2p}* as an internal control, respectively). Details of the analysis are described in Material and Methods. The most highly scored sequences are presented in Fig. 19. and importantly, the sequence proposed inverted sequence of “SdiA box” is one of the screened (Yamamoto *et al.*, 2001). Using our results on changes of IGs in the context of proximal gene transcript changes, a search for SdiA binding motifs more in general term was performed *in silico*. Since LuxR type proteins generally bind as a dimer, we tested the two block models, giving each block of 5 bps and intervals of 8-12 bps. One of the motifs agreed with the “SdiA box” (Fig. 19), although there are other possible motifs with more weight.

Small RNA expression Secondly, extensive presence of structural or regulatory sRNAs are now confirmed (so far about 60 sRNAs are confirmed experimentally) or predicted through genome-wide searching efforts (Carter *et al.*, 2001; Chen *et al.*, 2002; Tjaden *et al.*, 2002; Wassarman *et al.*, 2001). Of the 33 IGs, 9 IGs contain a either confirmed or predicted sRNA. Studying for the IG expression, we have a notion that unexpected expression and changes in IG expression can be ascribed to small RNA if not resulted from the technical errors. Orphan IGs, being not associated with the flanking genes in the expression pattern (i.e. positioned neither in the 5'-UTR as a promoter, nor in the middle of operon, nor at 3'-UTR by incomplete termination of the precedent gene)

were selected as 13 IGs of incongruence in our grouping scheme. Notably, IGs neighboring 6 of 5S rRNA (out of 8) genes were included, which may be linked to decrease in rRNA transcripts. It is interesting that IGs neighboring the rest of two remaining 5S rRNAs were already predicted as small RNAs, although they were not significantly changed in our study (Carter *et al.*, 2001; Tjaden *et al.*, 2002). Besides, three predicted IGs from the previous studies in the incongruent group (IS094, tp18, and tk11) further supports our approach of sRNA prediction to be reasonable. However, the information on IG expression using *E. coli* antisense Affymetrix chips is limited due to probe set design biased to the neighboring ORF transcription direction. Judging from the orientation of the probes, cDNA (see Material and Methods) of IS079 (downstream of *hdhA*) is only detectable by the probes, while cDNA from C0465 and tp2 cDNA cannot hybridize with the IG probes and the orientation of tp18, tp30 and tk11 is unknown.

Regarding the signal intensity, many of IG showed lower signals than neighbors even though they were in congruent group by the induction ratio (e.g. IG_1073 of IS079-IG_1259 in operon· IG_2719 in promoter region). Same cases were found in the incongruent group (lower signal from IG_2430-IG_2599 (lower level). However, there were exceptional cases of stronger signals (IG_1512, IG_2280). Whether the signal difference across the IG and neighbors reflects real transcript level · stability of mRNA or merely resulted from different efficiency of reverse transcription remains as a question.

ID	log ₂ IR	IG start	IG length	flanking Affy.	Characteristics§	sRNA(orientation)	ref
IG_68	1.3	122857	160	pdhR/aceE	>>> incomplete termination	tp2 (<)*	Rivas et al
IG_507	-1.2	794146	166	b0762/modA	>>> promoter		
IG_715	-1	1134735	52	flgG/flgH	>>> operon		
IG_743	1.3	1191855	35	ycfC/ycfB	<<> promoter		
IG_1073	-1.8	1695077	220	gusR/hdhA	<<< incomplete termination	IS079 (<)	Chen et al
IG_1258	-1.3	1969009	45	tap/tar	<<< operon		
IG_1259	-1.4	1970716	144	tar/cheW	<<< operon	C0465(>)*	Chen et al.
IG_1260	-2.5	1975164	126	motA/flhC	<<< operon	tp30(unknown)	Rivas et al
IG_1265	-1.3	1983093	69	araG/araF	<<< operon		
IG_1283	-1.3	2004102	77	fliT/amyA	>>> (stem and loop) promoter		
IG_1291	-1.2	2010803	448	fliE/IG_1292	<<> operon, promoter		
IG_1294	-1.1	2019524	62	fliN/fliO	>>> operon		
IG_1335	1.1	2072681	114	flu/yeeR	>>> incomplete termination, operon?	C0486(<)	Tjaden et al
IG_1511	1.2	2426647	94	ubiX/purF	<<< incomplete termination		
IG_1512	1.5	2428259	36	purF/cvpA	<<< operon, strong signal		
IG_1621	1.4	2595639	212	purC/nlpB	<<< stem and loop, terminator		
IG_1636	1.4	2630556	68	guaA/guaB	<<< operon		
IG_1799	1	2886336	74	cysH/cysI	<<< operon	C0673(>)	Tjaden et al.
IG_2719	1.1	4402162	103	yjeT/purA	>>> promoter mapping		
IG_2767	1.5	4468527	33	yjgF/pyrI	<<< incomplete termination		
Incongruency 1-Opposite							
IG_132	1.1	228663	93	rrlH/rrfH	>>> small intensity compared to the flanks		
IG_2133	2.1	3421180	37	rrfF/thrV	<<< small intensity compared to the flanks		
IG_2429	1.2	3944232	92	rrlC/rrfC	>>> small intensity compared to the flanks		
IG_2430	1	3944444	52	rrfC/aspT	>>> small intensity compared to the flanks		
IG_2572	1.1	4169124	92	IG_2571/rrfB	>>>		
IG_2599	1.4	4210526	93	rrlE/rrfE	>>> small intensity compared to the flanks		
IG_1279	1.2	1994855	231	sdiA/yecC	<<<	IS094 (>)	Chen et al.
Incongruency 2-Indifference							
IG_1915	1.5	3084422	303	yqgD/metK	<<>		
IG_2280	1.4	3693591	35	yhjQ/yhjR	<<<		
IG_287	1.4	445897	142	yajR/cyoE	<<< Small intensity	tp18 (unknown)	Rivas et al
IG_1222	1.1	1910601	191	htpX/prc	<<< Small intensity	tk11 (unknown)	Rivas et al
IG_1629	1.7	2618181	85	uraA/upp	<<< Small intensity		
IG_2172	1	3487818	85	argD/pabA	<<< Small intensity		

Affy. Probe set orientation designed in antisense microarray

§ Based on information from high-resolution probe analysis

* Experimentally confirmed sRNA

Table 9. Significant changes in IGs

Spacer	Two Block Motifs			Score	Sites
8	CATT	(N)8	TCAAC	2.01	11
	GACAA	(N)8	GCAGA	2	7
	TTGTG	(N)8	AAAAG	1.96	11
	CAGTT	(N)8	TCAAG	1.96	12
	GCAAT	(N)8	ATGAT	1.95	11
	AAAAG	(N)8	GAAAA	1.95	7
9	TGCCA	(N)9	ATGAA	2.14	12
	TGACA	(N)9	ATGAA	2.1	9
	GCATT	(N)9	TCAAG	2.09	9
	GCATT	(N)9	TCAAG	2.06	9
	GCATT	(N)9	TCAAG	2.06	9
	TTTIG	(N)9	ACAGT	2.05	9
10	TGCCC	(N)10	ATGAA	2.25	13
	TGTCA	(N)10	ATGAA	2.24	13
11	TGCCA	(N)11	TGAAG	2.26	15
	TGCCC	(N)11	TCAAG	2.26	15
	TGTCA	(N)11	ATGAA	2.21	12
12	AATGT	(N)12	ATGAA	2.28	13
	TGCCA	(N)12	TGAAG	2.25	9
	TGTCA	(N)12	GAAAA	2.24	13
	TGCCA	(N)12	GAAGA	2.21	12

Weblog, Berkley



Figure 19. Searching for SdiA binding motifs.

10 chosen IGs were used as inputs for analysis with *BioProspector* (Liu *et al.*, 2002). Two block model (each block 5bp and the gap of 8 to 12 bp) was tested and most highly scored motifs are listed in the box along with the scores and the number of sites with the sequences. The most closely matching sequence to the “SdiA box” is illustrated using WebLog (Berkely.).

Congruency

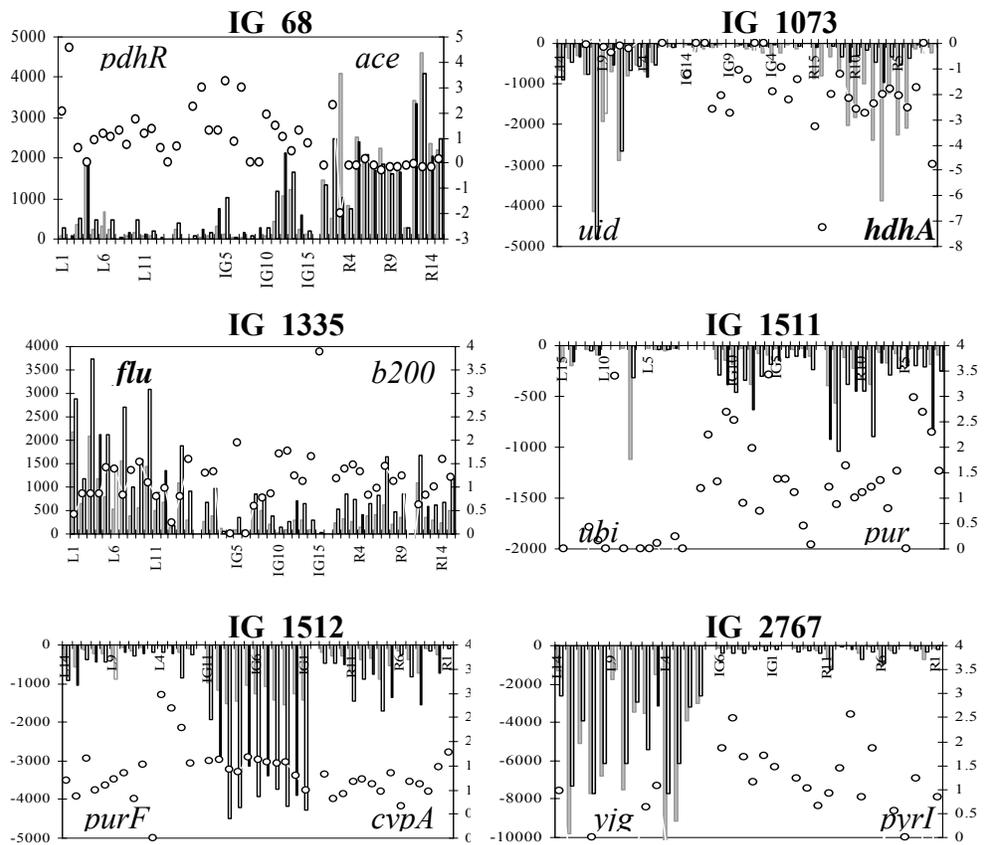


Figure 20. Significantly changed IG regions with congruency.

Probe intensities are shown as corrected probe intensity (PM-MM) for significantly changed IGs and the flanking ORFs. Individual probes are numbered as in Affymetrix probe library but aligned on the map of clockwise strand (+) (x-axis), but not distributed in physical map scale. Upward bars indicate transcripts of clockwise direction and downward bars indicate the counterclockwise directional transcripts (left y-axis). Circles show the relative expression ratio of each probe ($\log_2 IR_{W2X2/W3110}$, right y-axis). Represented IG expressions are considered to have resulted from incomplete transcription of upstream genes.

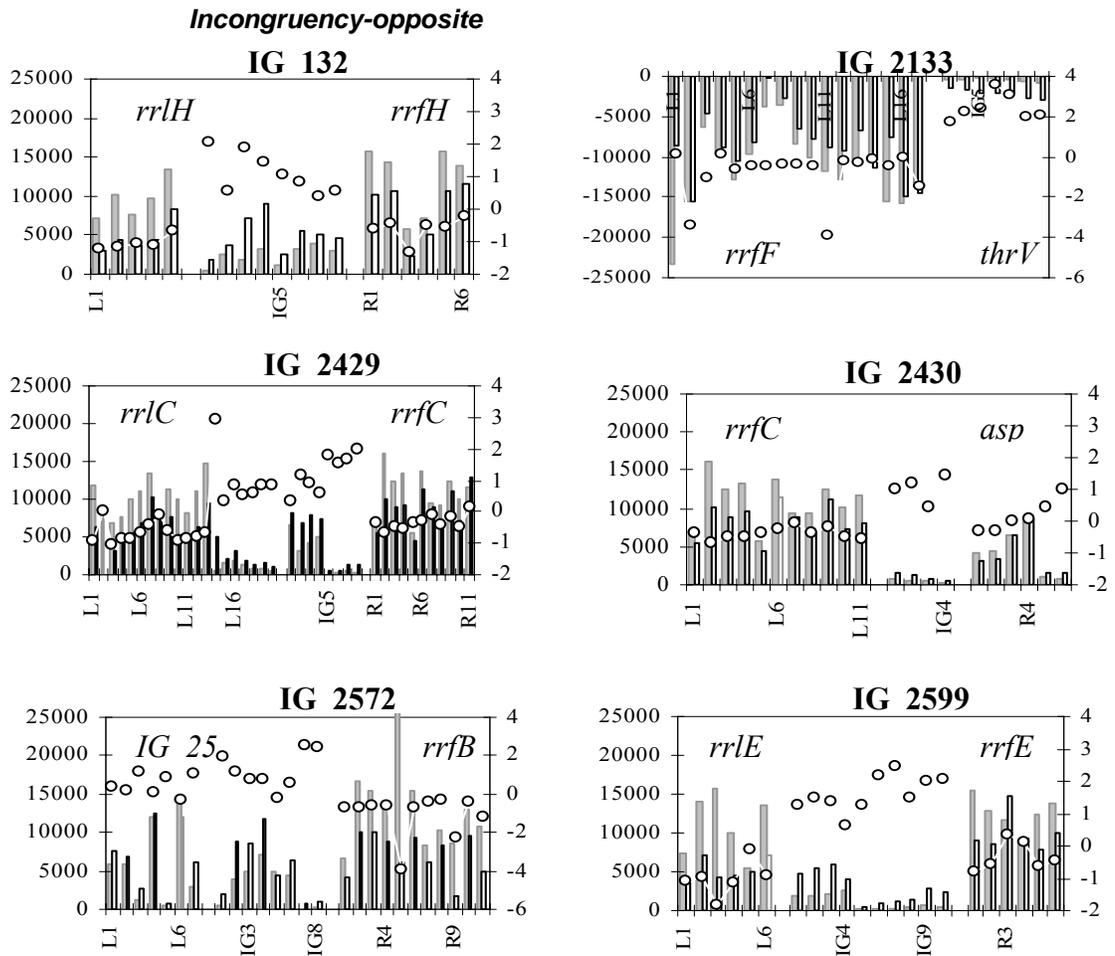


Figure 21. Significantly changed IG regions with incongruency-opposite.

Probe intensities are shown as corrected probe intensity (PM-MM) for significantly changed IGs and the flanking ORFs. Note that the five of the represented IGs are located upstream of *rrf* with the exception of IG_2430, located downstream of *rrfC*. Upward bars indicate transcripts of clockwise direction and downward bars indicate the counterclockwise directional transcripts (left y-axis). Circles show the relative expression ratio of each probe ($\log_2 IR_{W2X2/W3110}$, right y-axis). Represented IG expressions are considered to have resulted from incomplete transcription of upstream genes.

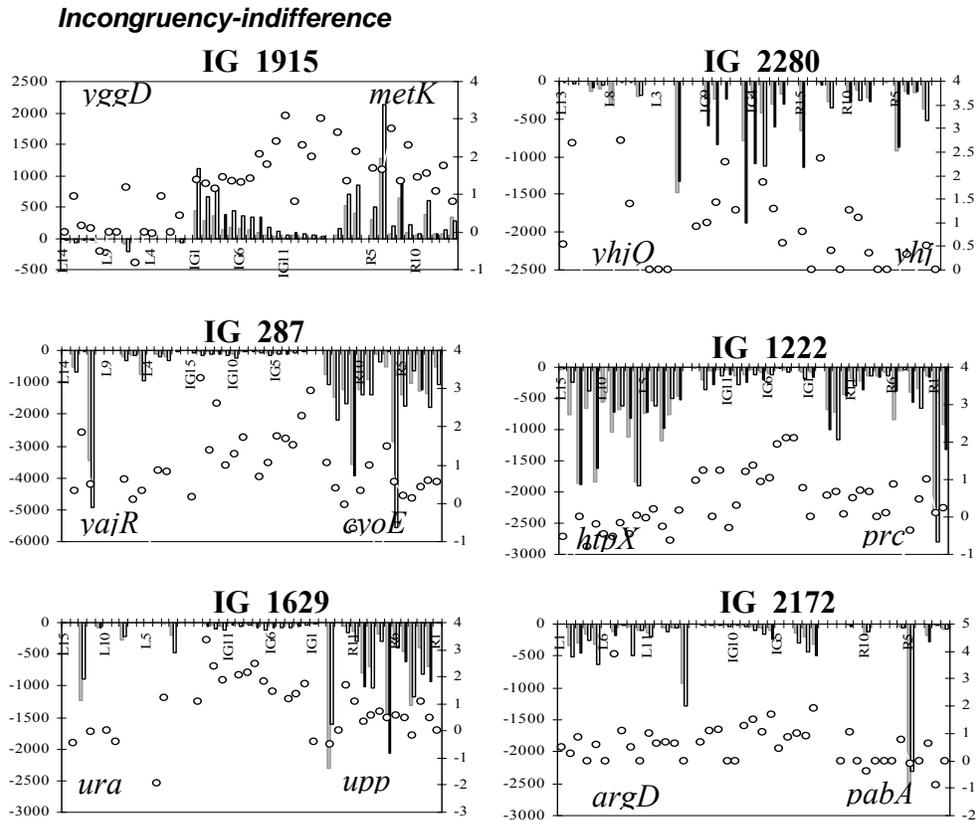


Figure 22. Significantly changed IG regions with incongruity-indifference. Probe intensities are shown as corrected probe intensity (PM-MM) for significantly changed IGs and the flanking ORFs. Notably, IG_2280 has strong signals not observed from the flanking genes. Upward bars indicate transcripts of clockwise direction and downward bars indicate the counterclockwise directional transcripts (left y-axis). Circles show the relative expression ratio of each probe ($\log_2 IR_{W2X2/W3110}$, right y-axis). Represented IG expressions are considered to have resulted from incomplete transcription of upstream genes.

Chapter 4: Conclusions

Multiple quorum signalings in one organism: testing the regulatory effect from system 2 to system 1 of *Escherichia coli*.

As introduced in Chapter 1, quorum sensing is mediated by cell signals emitted to the environment that are either diverse (species specific) and/or conserved (universal signal) and are then perceived by cells modulating activity of specific regulatory receptor components. Bacterial quorum sensing, in many cases, is composed of plural numbers of quorum sensory, which then integrate into a global network either in a parallel or in hierarchical manner for timely and effective gene expression.

Escherichia coli K-12, the experimental organism investigated in this study, also has indications of multiple-quorum signaling. We denoted one of the quorum systems in *E. coli* as system 1 represented by LuxR type protein SdiA and the other quorum system as system 2 by universal signal molecule AI-2. Importantly, we have noticed that the co-presence of *sdiA* and *pfs/luxS* in an organism is unique to γ -proteobacteriaceae of *E. coli*, *Salmonella* subspecies, *Y. pseudotuberculosis*, *V. parahaemolyticus*, and *V. cholerae* (NCBI *BLASTX* search, unidirectional homology search). Unlike *Y. pseudotuberculosis* and *Vibrio* species producing AI-1 signals, *E. coli* and *Salmonella* do not have the LuxI type proteins nor have the detectable AI-1 activity making the system 1 found in these organisms seemingly incomplete.

The unique architecture of *E. coli* quorum sensing raised questions about the functionality of *E. coli* quorum components and the relationship between the systems and their association with established global regulatory networks. I tried to answer some of these questions in this dissertation. Chapter 2 highlighted the impacts of environmental

factors on the quorum sensing system 2. In particular, cultivation temperature and heat shock were examined to delineate the apparent crosswiring of quorum system 2 with quorum system 1, and to attempt to understand how the quorum system 2 signal, AI2, is synthesized and degraded in response to environmental stresses.

To date, system 1 and system 2 have been considered independent of one another. Using a confirmed SdiA target promoter, *ftsQ_{2p}* of *E. coli*, possible AI-2 modulation (using conditioned media) of SdiA activity in *E. coli* was determined to be negative by two research groups (Michael *et al.*, 2001; Surette and Bassler, 1999). Although not described in this study as preliminary studies, we also confirmed that regulatory effects of AI-2 may not directly affect SdiA in flask cultures by using a *luxS* knockout mutant (data not shown). Also we were unable to demonstrate direct stable binding of AI-2 to SdiA when tested by mixing synthetic AI-2 with purified His₆-tagged SdiA protein (data not shown). Theoretically, the quantity of unbound and bound AI-2 could be measured by separating the appropriate receptor protein fraction from the solution. However, aggregation of SdiA and the limits of the quantitative assay for AI-2 activity were too difficult to overcome, leaving room for improvement in the future before making a conclusion.

Functionality of SdiA examined by deletion (chapter 3)

Naturally, we questioned the appropriateness of relying on the single identified target promoter (*ftsQ_{2p}*, cell division associated) as a probe of SdiA functionality. Especially since its signal responsiveness is complicated by being associated with cell division (cell divisions are affected by numerous factors). To screen more of the SdiA-

dependent genes, we performed a comparative microarray analysis between wild type W3110 and the *sdiA*⁻ mutant, W2X2. At the same time, we looked for phenotypic differences resulting from the *sdiA* deletion, such as changes in stress resistance and reduced motility.

Using the high throughput technology of microarrays, we obtained valuable information on the genomewide transcriptome changes, including all open reading frames (ORFs) and intergenic regions (IGs). We noted two intriguing classes of results signifying marked changes in expression. Namely, the *sdiA*⁻ strain had downregulated a group of genes assigned to AI-2 uptake and modification (*lsr* operon, *b1511-b1518*) and secondarily, an α -hydroxysteroid dehydrogenase for modifying bile acids (*hdh*) was also observed to be reduced in expression. Changes associated with the *lsr* operon (microarray data, transcriptional fusion assay, and AI-2 activity) are presented as conclusive evidence of cross-communication between system 1 and system 2, in which system 1 positively affects system 2 in the regulatory network. As bile acids can function as physiological ligands for the farnesoid X receptor controlling cholesterol and bile acid homeostasis (Makishima *et al.*, 1999; Wang *et al.*, 1999), the change in *E. coli hdh* expression is highly likely to associate the *sdiA*-dependent *E. coli* signaling to a mammalian signaling in the intestine. Along with the discovery of a mammalian hormone influencing enterohemorrhagic *E. coli* virulence genes (Sperandio *et al.*, 2003), symbiotic- or pathogenic interaction of *E. coli* by interkingdom signaling is more prevalent than we expected. Studies testing this idea are expected in the future.

From the altered transcriptome in the *sdiA* mutant, we noted changes in other transcriptional regulators of *arcA*, *soxS*, *rpoE* and *rpoS*. Selecting a specific target of each

regulator, which were also changed by *sdiA* deletion, left the questions as to whether the changes are solely subsequent to the changes of global regulatory proteins (ArcA, SoxS, RpoE and RpoS) or is SdiA directly regulating them in a competitive or cooperative manner with other regulators. The same question arose from the unexpected orphan expression of IGs with significant changes, which we suggest as possible sRNA. We expect that cross- or synergistic- regulatory cascades involving the other regulatory proteins or sRNA could be, when combined, a clue for explaining differences in rRNA and stress resistance developed by the mutation.

Of practical use, we performed an *in silico* study, wherein 10 IGs were searched based on several criteria, for conserved sequences with a topology of “LuxR family protein” binding sites. Experimental confirmation of the searched motifs will expand the list of SdiA-dependent targets and also provide “sensors” for quantifying SdiA activity. While our study on SdiA aimed to find its functionality in the endogenous system of pure cultures, continuous efforts towards understanding interactions between other bacteria have been reported through “eavesdropping” signals originated from other bacterial species or through competitive binding on exogenous AI-1 sensor plasmids (Smith and Ahmer, 2003)(Lindsay and Ahmer, 2005). Thus, we can say more about SdiA function, but mechanisms of action are yet to be elucidated.

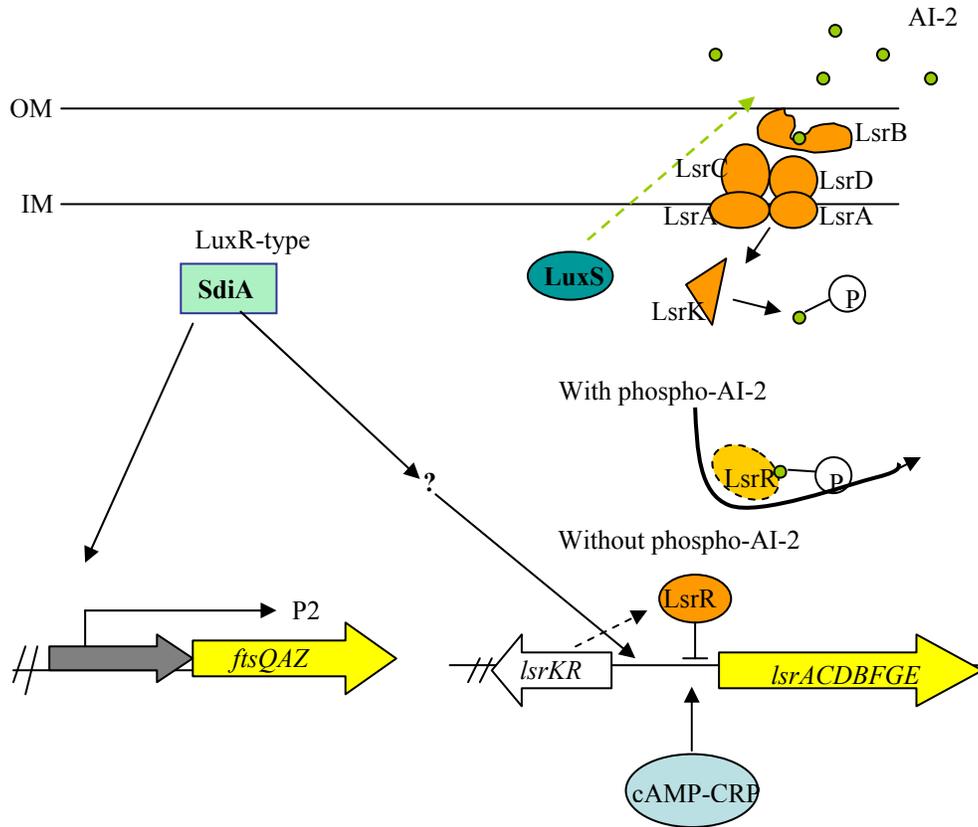


Figure 23. Suggested convergence of *E. coli* quorum system 1 and 2.

Previously divided *E. coli* quorum system 1 and 2 are now illustrated to be converged on the AI-2 uptake machinery encoding genes, *lsr* operon. Whether the positive activation of SdiA on the *lsr* operon is direct or indirect has not yet determined. The SdiA-mediated transcriptional activation on *ftsQ2p* was not affected by AI-2 in the flask culture condition (data not shown here).

Autoinducer 2 (AI-2) level is actively regulated by molecular chaperones under stress (Chapter2)

The second quorum system has the functional signal, AI-2. AI-2 production is closely linked to detoxifying SAH and recycling homocysteine. However, by using the two enzymatic conversion of SAH to homocysteine, most eubacteria can produce AI-2, which is not produced in eukaryotes. Apart from the active studies on functionality of AI-2, our work in Chapter 2 was focused on the impacts of environmental stimuli, high temperature, and the regulatory effects of molecular chaperones, DnaJKE and GroESL.

When cells have wild type GroESL and DnaJKE, high temperature (42 °C) incubation reduced AI-2 levels in glucose supplemented cultures. Interestingly, cultures of *groESL* point mutants, *dnaJ* knockouts, and *grpE280* mutants at 42 °C in the presence of glucose did not decrease AI-2 levels even after the cells entered the stationary phase, possibly due to defects in growth. From these results, we concluded that system 2 is connected to global signaling, mediated by molecular chaperones. There are several reports on the regulatory effects of environmental factors on AI-2 production (DeLisa *et al.*, 2001, 2001; Hardie *et al.*, 2003; Surette and Bassler, 1999). None of them reported the “negative” regulatory aspects of these chaperones.

The negative regulatory effects of chaperones can be described as follows: glucose drives metabolic carbon flux continuously towards AI-2 synthesis. At 42 °C, non-functional chaperones (either by point mutations or by deletion) do not exert their negative regulatory roles on their normal targets in the AI-2 production pathway, letting them produce AI-2 (possibly through the interaction with proteases).

Observations from high temperature experiments give insight into the activity of essential AI-2 synthase LuxS, which should be structurally stable and functionally active at 42 °C. Indeed, stable maintenance of active LuxS in the stationary phase cells has been reported (Hardie *et al.*, 2003; Xavier and Bassler, 2005), however its temperature dependent activity has not yet been reported. If LuxS were not the main targets of these chaperones, what protein could interact with the chaperones, directly or indirectly, to and directly govern AI-2 synthesis? One attractive candidate could be found from methionine de novo synthesis supplying the SAM for AI-2 synthesis. Thermolabile homoserine O-succinyltransferase (MetA, see Fig. 24), the first enzyme in the methionine synthesis is known to be structurally unstable, tending to aggregate at the temperature higher than 44 °C. Its interaction with DnaJ and protease ClpB has been reported (Biran *et al.*, 2000; Gur *et al.*, 2002)(Mogk *et al.*, 1999). Further biochemical analyses on protein-protein interaction and observing half-life of possible substrates of the chaperones are required to test this idea.

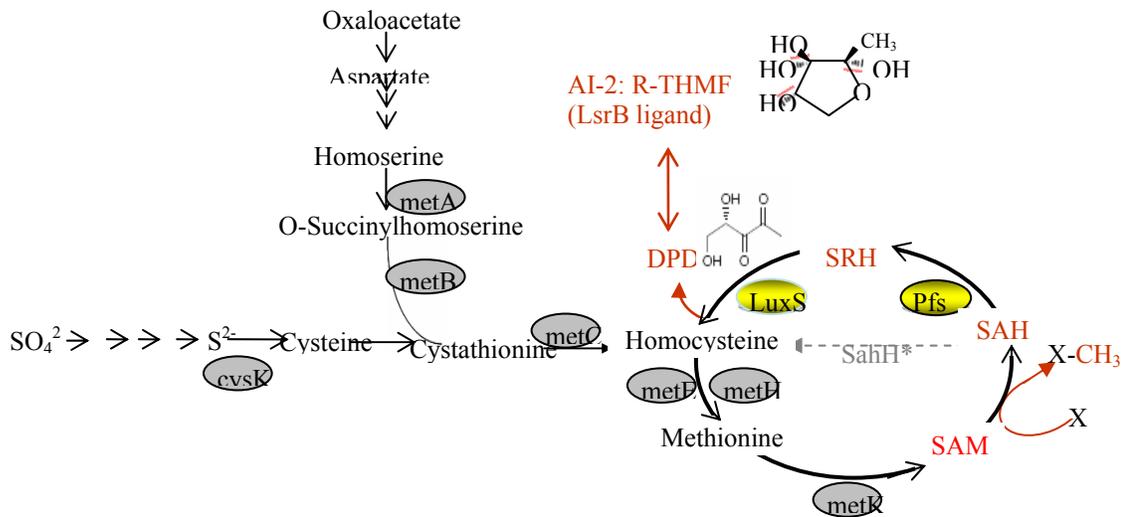


Figure 24. Metabolic flux for AI-2 synthesis.

Sulfur is assimilated to amino acids, cysteine and methionine. Homocysteine is generated from cysteine and oxaloacetate in the de novo synthesis of methionine. MetK converts methionine to S-adenosyl methionine (SAM) using ATP. Further transform of SAM to AI-2 is depicted in Figure1. While most bacteria use the two-step conversion of SAH to homocysteine (Pfs and LuxS mediated), one-step conversion also occurs in eukaryotes and some archae bacteria as depicted (SAH hydrolase, SahH*). Major enzyme encoding genes are indicated with gray ovals. The figure was modified from (Vendeville *et al.*, 2005).

Significance of the study for potential applications

The key element in controlling pathogens by manipulating quorum signaling resides the specificity of the signal action not to place unwanted selected pressure on the organism. Although the chosen *E. coli* K-12 model system is not equipped with the virulence genes for direct assessment of the manipulated quorum system 1, still, relatively small numbers of changed genes by the absence of the sensor protein expression suffices the first requirement. Remaining issues on how to augment the strength of the quorum sensory can be accomplished by searching effectors and screening the target genes, which will automatically lead to develop as “inducible system” for recombinant protein expression. The in silico study, performed based on the microarray data and literature-based comparative analysis, is going to be tested in vitro and in vivo as an important reference. Besides, associated with the intriguing phenotypic characteristics, such as the accelerated autoaggregation, screened SdiA-dependent promoter is expected to be developed as a biosensor to study further bacterial social behavior under various environmental conditions (both natural habitats and harsh conditions) and to screen inhibitory chemical substances with highly specificity on the quorum sensory (as mentioned above). This needs to be distinguished from the global regulatory networks initiated from abiotic environmental cues, which was well exemplified in Chapter 2.

Perspectives

Taken together quorum sensing of *E. coli* is in a finely tuned and regulated state are globally interconnected with other sensory systems. Unraveling this sensing mechanism is difficult in the flask cultures where various quorum inhibitory materials may accumulate over time. However difficult, study of *E. coli* quorum signaling is

progressively being understood and is likely to provide very real insight into mechanisms of bacterial virulence and pathogenicity and also commensalism.

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