

ABSTRACT

Title of Document: ALTERING THE AI-2 MEDIATED QUORUM SENSING CIRCUITRY TO QUENCH BACTERIAL COMMUNICATION NETWORKS

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The emergence of antibiotic resistant bacteria poses a global threat to human health and has been classified as a clinical super-challenge of the 21st century. This has necessitated research on new antimicrobials that inhibit bacterial virulence by mechanisms other than those that target bacterial growth or viability. Such approaches have been reported to pose less evolutionary pressure on bacteria to evolve and become resistant to antibiotics. Bacterial cell-cell communication, termed quorum sensing (QS), is mediated by signatures of small molecules. QS via these small molecules has been linked to numerous undesirable bacterial phenotypes such as biofilm formation, onset of pathogenicity, triggering of virulence genes etc. The small signaling molecules represent targets for intercepting bacterial communication (and their resultant undesirable phenotypes). We have devised two strategies that interrupt bacterial communication in multispecies bacterial cultures by targeting the

interspecies signaling molecule autoinducer-2 (AI-2), which is produced or recognized by over 70 species of bacteria. Our first approach is to bring the native intracellular AI-2 signal processing mechanisms to the extracellular surroundings to quench the QS response of bacteria. Specifically we deliver the *Escherichia coli* AI-2 kinase, LsrK, to *E. coli* populations *ex vivo* and phosphorylate and degrade the extracellular AI-2. This significantly attenuates the native QS response in *E. coli*. Similar results are obtained in a tri-species synthetic ecosystem comprising *E. coli*, *Salmonella typhimurium* and *Vibrio harveyi*. In our second quenching strategy, we explore a panel of small synthetic molecules that are analogs of AI-2 (C1-alkyl analogs). The analogs are observed to cause species-specific and cross-species quorum quenching in the tri-species synthetic ecosystems of the aforementioned strains. Some of the AI-2 analogs quench pyocyanin (toxin production) in the opportunistic pathogen *Pseudomonas aeruginosa*. Based on these observations, I used analog cocktails to quench QS *en masse* in assembled synthetic ecosystems. Finally, I tested the efficiency of the analogs in quenching pathogenic phenotypes such as biofilm formation in *E. coli*. The analogs inhibit biofilm formation and act in concert with antibiotics to reduce biofilm formation even further. Our results suggest entirely new modalities for interrupting or tailoring the networks of communication among bacteria and identifying drug targets to develop the next generation of antimicrobials based on QS.

ALTERING THE AI-2 MEDIATED QUORUM SENSING CIRCUITRY TO
QUENCH BACTERIAL COMMUNICATION NETWORKS

By

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Dedication

For my parents Madhu and Vipul who made me the person I am and are my inner strength. For my grandmothers Amma and Nani for showering me with their blessings and trying to understand what I do. For my uncle Atul and my aunt Bharti for making me a home away from home and for my husband Rohan who held my hand through every step in this journey.

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Chapter 1: Introduction

1.1 Background

1.1.1 The Need for New Antimicrobial Therapies

The emergence of antibiotic resistant bacteria poses a global threat to human health and has been classified as a clinical super-challenge of the 21st century¹. Despite the continued antibiotic resistance development in human pathogens, the emergence of new and effective antibiotic treatments has been limited^{2, 3}. Arias and Murray describe the current state of antibiotics as having “come almost a full circle since the advent of antibiotics in the 1930s and arrived at a point as frightening as the preantibiotic era: for patients infected with multidrug-resistant bacteria, there is no magic bullet”¹. There are a number of antibiotic resistant pathogens that pose a significant risk to public health: methicillin-resistant *Staphylococcus aureus* (MRSA)⁴, multidrug-resistant Gram-negative bacteria (*Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*)⁵, and multidrug-resistant and extensively-drug-resistant strains of *Mycobacterium tuberculosis*⁶. These types of “superbugs” are not exclusive nosocomial infections. A study that characterized 1100 MRSA infections, reported that although 85% of MRSA infections are hospital acquired, nearly 14% are community associated⁷. MRSA infections have been estimated to cause ~19,000 deaths/ year in the U.S. with an estimated \$3 to 4 billion/year of additional health care costs⁴.

The development of antibiotic resistance in bacteria is largely due to the ability of these organisms to respond exceptionally fast to a selective pressure in their environment by both genotypic and phenotypic means. Currently, antimicrobial therapies target bacteria by inhibiting cell growth (bacteriostatic) or causing cell death (bacteriocidal), and pose an evolutionary pressure for resistance development in order to survive. Therefore, there is a growing need to develop alternative antimicrobial therapies that are neither bacteriostatic or bacteriocidal, thus eliminating selection for microbial resistance. Amongst these, alternative therapies that target bacterial quorum sensing signaling pathways are promising^{8,9}.

1.1.2 Quorum Sensing as a Target for Development of Antimicrobials

Quorum sensing (QS) is the mechanism of cell-cell communication amongst bacteria. QS enables bacteria to establish multi-cellular level interactions and, among other phenotypes, increase their propensity for infection in humans and other hosts¹⁰. QS amplifies bacterial virulence by stimulating the expression of disease causing attributes, such as biofilm formation^{11,12}, and secretion of virulence factors^{13,14}. QS is mediated by the synthesis, sensing and uptake of small molecule chemical signals called autoinducers. These autoinducers form the chemical language by which bacteria “talk”. By sending and receiving these chemical signals, bacteria enumerate members of their own species and other bacterial species in order to determine if a sufficient “quorum” exists to modulate their behavior to evade the host immune response¹⁵. Understanding QS mechanisms will enable “talking” to bacteria in their own language to inhibit or silence their QS behavior (i.e. severely weaken bacterial

pathogenicity but otherwise remain innocuous) and thus prevent extreme selective pressure that comes from targeting bacterial survival and growth. Autoinducer molecules can be used in species-specific or multi-species communication. Amongst these signal molecules, the autoinducer AI-2 is most widely involved in multi-species QS and is often referred to as the universal autoinducer, mediating bacterial esperanto¹⁶. The AI-2 synthase gene, *luxS*, has been found in more than 70 bacterial species¹⁷ and its ubiquity makes it a potentially useful target for developing multi-species QS inhibitors. This review concentrates on inhibition of AI-2 mediated QS mechanisms in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae* and *Pseudomonas aeruginosa*, as they are clinically relevant human pathogens. We also review AI-2 inhibition in *Vibrio. harveyi* the marine organism in which AI-2 was first discovered¹⁸ and it is also the most commonly used AI-2 reporter via bioluminescence production.

The vast array of QS signal generators, receptors, transporters, regulators and the signals themselves represent multiple targets that can be “rewired” to switch QS circuits “off”. This strategy of inhibition of QS pathways by interfering with signal generation, signal relay, signal transduction or destruction of the signal entirely is also known as quorum quenching¹⁹. Here we classify quorum quenching strategies based on the QS circuit level where quenching occurs; either at the signal generator, signal receptor or the QS signal itself. To date, most QS inhibition strategies have targeted species specific autoinducers, like autoinducer I (AI-1)²⁰ and would therefore only address infection by a specific, single species. However, a QS inhibition strategy

targeting the more ubiquitous AI-2 opens avenues for broadly inhibiting and modulating QS pathways in a large number of species simultaneously.

1.1.3 Bacterial Quorum Sensing

QS extends beyond the inter-species bacterial interactions to inter-kingdom where QS facilitates interaction between bacteria and eukaryotic hosts. QS was first studied in the marine bacterium, *Vibrio fischeri*, in which QS is utilized in such a symbiotic relationship with a eukaryotic host. *V. fischeri* bioluminesces through QS in the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*²¹. The bioluminescence enables the squid to blend in with its surroundings and avoid casting its shadows on the sea-floor thus evading its predators and prey²². Many early discoveries regarding QS and its regulation were aided by investigating the relationship behind bacterial growth and regulation of *Vibrio* bioluminescence²³⁻²⁵. It was observed that light production by the bacteria was controlled by secretion and detection of small signal molecules that were observed to accumulate in the extracellular environment. Through these molecules, bacteria sense their population density. When a particular “threshold” concentration is reached (Figure 1-1a), there is an influx of signal molecules into the cell leading to gene expression changes in a population density-dependent manner (Figure 1-1a)¹⁵. Many of these gene expression changes are for specific functions that are useful for the bacteria only when carried out by a large number of cells, for example biofilm formation (Figure 1-1a)^{11, 12}, toxin and virulence secretion²⁶, swarming ability²⁷ and sporulation²⁸. Hence the process

was initially referred to “autoinduction” and the small signal molecule facilitators were called “autoinducers”²⁴.

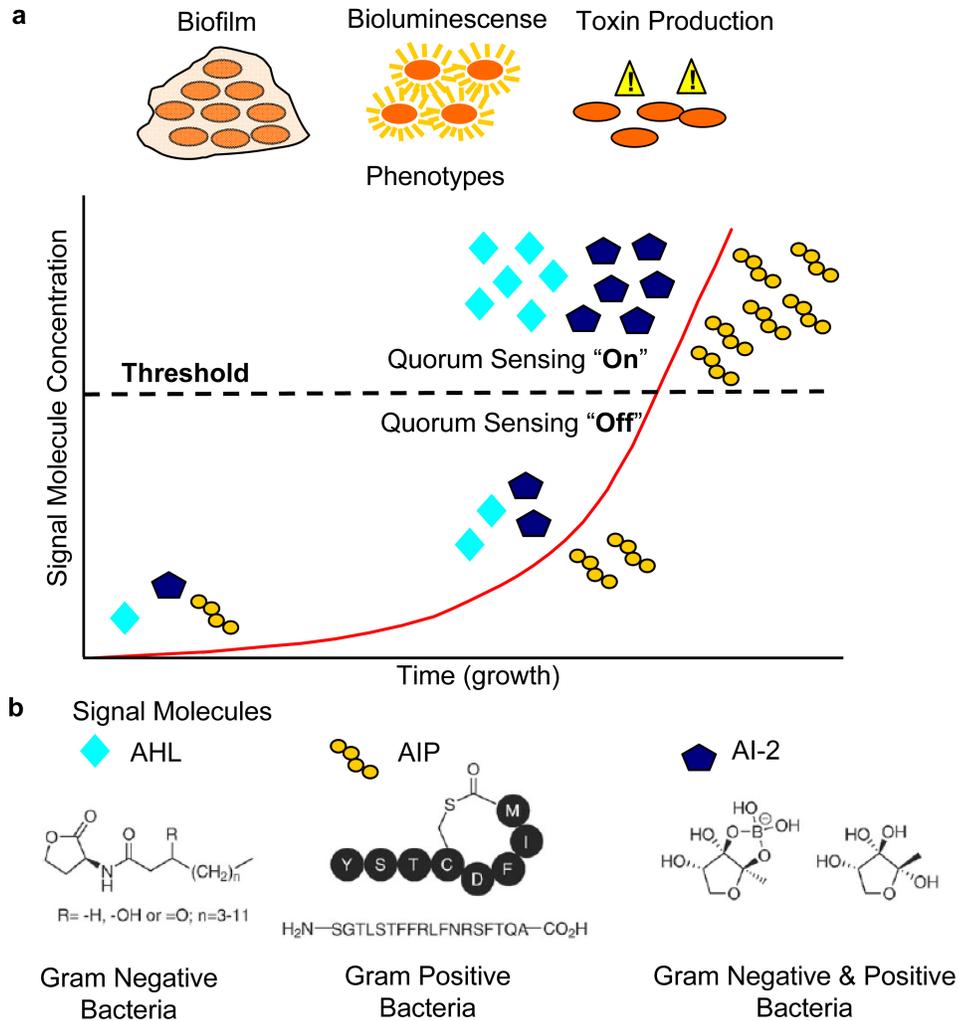


Figure 1-1 Schematic of the Quorum Sensing “Switch” and Types of Signal Molecules.

a) Signal molecules are produced by the bacteria and accumulate in the extracellular environment. Once the concentration of AI-2 reaches a particular “threshold” concentration, the QS genes and phenotypes are switched “on”. Phenotypes include biofilm formation, bioluminescence and toxin production. b) Three main families of QS signal molecules that occur in various bacteria.

1.1.4 Autoinducer Classification

Conventionally, autoinducers have been divided into two generic groups: acyl homoserine lactones (AHLs), used by Gram negative bacteria and oligopeptides, used by Gram positive bacteria (Figure 1-1b)¹⁰. However, it has been shown clearly that bacterial QS is not limited to these two classes of molecules. There are others such as the universal autoinducer (AI-2), quinolones²⁹, hydroxyl ketones^{13, 30}, bradyoxetin³¹ and others. There is also evidence of an autoinducer, AI-3, produced by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and other intestinal bacterial species³², however the structure of this molecule is currently unknown. Of the various classes of QS molecules AI-2 is particularly interesting as it is found in both gram negative and gram positive bacteria. The synthase (LuxS) which produces AI-2 is found in more than 70 bacterial species^{17, 33}. Besides these there exist other species of bacteria that do not possess their own AI-2 synthase but are capable of AI-2 transduction this helps them determine populations of various other bacteria in the surrounding environment. This review will focus on a the autoinducer AI-2³⁴, its synthesis, regulation, and modes of QS inhibition.

1.2 QS Mechanisms in Different Bacteria

1.2.1 *E. coli* and *S. typhimurium*

E. coli and *S. typhimurium* are common causes of food-borne illness³⁵. *E. coli* is common inhabitant of the lower intestine, where it is an important member of the gut microflora³⁶ and normally nonpathogenic. However, some strains acquired genes that conferred virulence factors involved in gastroenteritis, urinary infections and

neonatal meningitis³⁷. Microarray studies have shown that the AI-2 signal synthase gene, *luxS*, is a global regulator in *E. coli* O157:H7 and affects the expression of over 400 genes in this pathogen; including genes involved in the shigella toxin production, motility and surface adhesion³⁸. *S. typhimurium* is the causative agent of typhoid, a disease endemic in Asia, Africa, and South America. It also associated with enterocolitis, the second most frequent cause of bacterial food-borne disease in the US³⁵.

The QS sensing circuits of *E. coli* and *S. typhimurium* are homologous. In the AI-2 biosynthesis pathway, the precursor of AI-2, DPD, is a product of the activated methyl cycle. The metabolite S-adenosyl methionine (SAM) is converted to the toxic intermediate S-adenosylhomocysteine (SAH) via methyl transferases. DPD is synthesized from SAH via a two-step enzymatic process catalyzed by methylthioadenosine/S-adenosylhomocysteine nucleosidase (Pfs) and S-ribosylhomocysteinase (LuxS)³⁹. More specifically, SAH is converted to S-ribosylhomocysteine (SRH) and adenine via Pfs. The enzyme S-ribosylhomocysteinase (LuxS) then acts on SRH to convert it to DPD and homocysteine. Homocysteine is recycled to produce methionine. In solution, DPD is known to undergo rapid intra-molecular cyclization and exists in equilibrium with several different isoforms, known collectively as AI-2 molecules⁴⁰. Thus, the AI-2 signal itself should not be recognized as a single structure, but a family of isomers, each potentially representing a different mode of perception. Different bacteria are capable of transducing different forms of the AI-2 molecule. In *S. typhimurium*, the crystal structure of the AI-2 binding protein LsrB, was found to bind a specific

chemical form of AI-2, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran⁴¹. In *E. coli*, the exact structure of the signal molecule has not yet been determined and is currently under investigation.

The mechanism of AI-2 synthesis, secretion, uptake and signal transduction in *E. coli* and *S. typhimurium* is regulated by the *luxS*-regulated operon (*lsr*)⁴². In *E. coli*, it is formally known as the *b1513* operon, but all gene functions are completely homologous to the *S. typhimurium lsr* operon^{43, 44} (Figure 1-2a). The enzymes Pfs and LuxS convert SAH to DPD inside the cell, releasing adenine and homocysteine as intermediates. DPD cyclizes to form AI-2, and is exported into the extracellular medium by a membrane spanning protein known as YdgG. It has been shown that deletion of YdgG greatly decreases the amount of extracellular AI-2⁴⁵. AI-2 initially accumulates in the extracellular medium in response to the increasing cell population density as cell growth occurs. When the AI-2 concentration in the medium exceeds a threshold amount, a bacterial quorum is reached and a signal transduction cascade for the uptake and processing of AI-2 is triggered. As part of this cascade, AI-2 is imported into the cell via an Lsr transporter. The Lsr transporter consists of five proteins (LsrACDBFG) with LsrB exposed on the cell surface (Figure 1-2a).

Once inside the cell, AI-2 is phosphorylated by the kinase, LsrK, to form phospho-AI-2. Phospho-AI-2 activates the QS circuit by binding to the repressor LsrR. In the absence of phospho-AI-2, the repressor is bound to the promoter region of the *lsr* operon, and prevents operon expression. AI-2 binds to LsrR and causes dissociation from the promoter region and de-repression of the *lsr* operon (Figure 1-2b). The *lsr* promoter region controls the expression of the *lsr* operon genes,

lsrACDBFGE^{43, 44}. Once phospho-AI-2 induces the *lsr* operon, the circuit uses a positive feedback mechanism causing increased expression of the transporter on the cell surface, and results in increased internalization of AI-2. Also upregulated is LsrG, which is involved in the degradation phospho-AI-2⁴⁶. LsrE, which is a putative sugar epimerase, is present in *S. typhimurium* but not in *E. coli*.

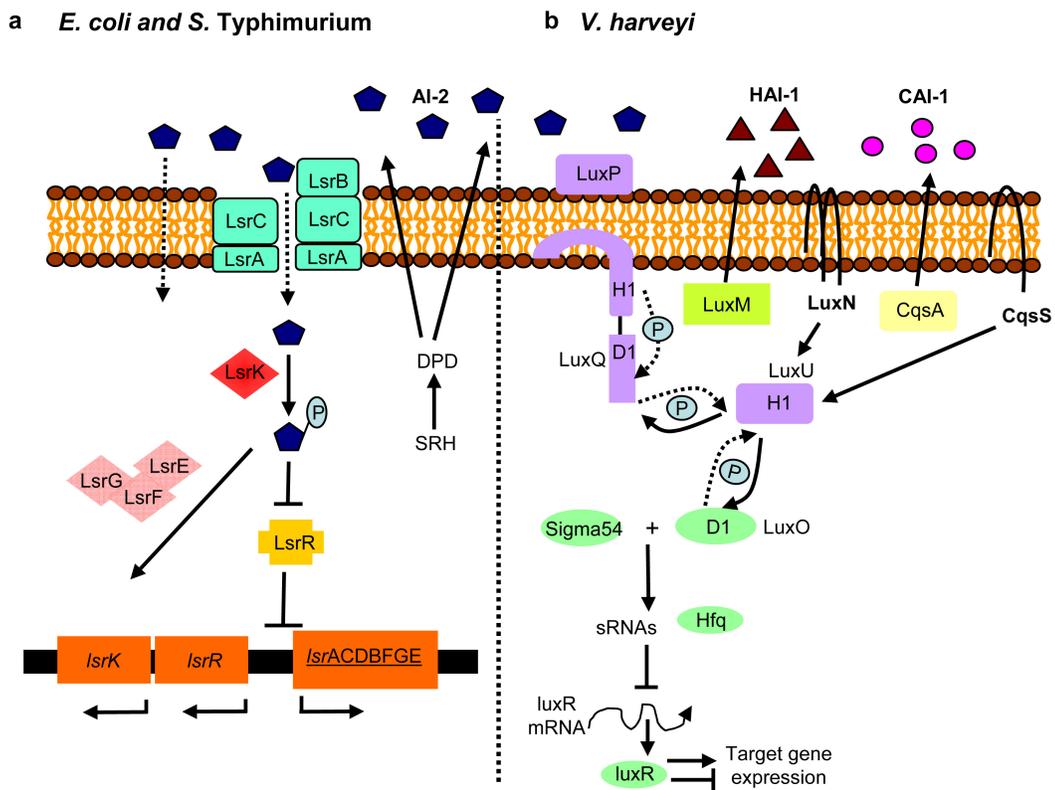


Figure 1-2 Quorum Sensing Pathways in *E. coli*, *S. typhimurium* and *V. harveyi*.

a) The analogous *lsr* QS signaling circuits in *E. coli* and *S. typhimurium*. The AI-2 signaling pathway is also present in *V. harveyi*. b) The three signals of the *V. harveyi* QS circuit - AI-2, HAI-1 and CAI-1. The three signals bind their respective membrane-bound cognate receptors that function in parallel to channel the multi-signal phosphorylation relay into a single regulatory pathway.

1.2.2 *V. harveyi* and *V. cholerae*

AI-2 was first discovered and characterized in the Gram negative marine bacterium *V. harveyi*¹⁸. Similarly to other Gram negative organisms, *V. harveyi* produces and responds to acylated homoserine lactone (AHL) autoinducer (AI-1). It was these molecules that were first linked to density-dependant bioluminescence production through the operon *luxCDABE* in *V. harveyi*. While investigating AI-1 mediated QS, Bassler *et al* noted a second density dependent, AI-1 independent signal that controlled bioluminescence¹⁸. This second signal was appropriately named AI-2, and was found to be a furanosyl borate diester when the crystal structure of the membrane sensor periplasmic protein, LuxP, was solved bound to it⁴⁷. There also exists a more recently identified third *V. harveyi* signal termed CAI-1⁴⁸. The three autoinducers and the three cognate receptors function in parallel and channel the information into a single, shared regulatory pathway (Figure 1-2b). In this circuit, AI-1 is produced by LuxM and binds to the membrane bound protein, LuxN. LuxS produces AI-2 and binds to LuxP, the LuxP-AI-2 complex then interacts with LuxQ in the membrane. CAI-1 is produced by the CqsA enzyme and interacts with CqsS. The cognate receptors, CqsS, LuxN and LuxQ, are all membrane bound histidine sensor kinases. The sensory information feeds into a two-component response regulatory pathway, which ends at the production of LuxR. The LuxR protein controls the expression of bioluminescence related genes. At low cell densities, the cognate receptors function as kinases and lead to the synthesis of phospho-LuxO via LuxU. Phospho-LuxO, in conjunction with σ^{54} , synthesizes sRNAs that interact with the RNA chaperone hfq to degrade *luxR* mRNA. At high cell densities, the membrane

sensors bind their respective autoinducers and switch from kinase to phosphatase activity, and remove phosphates from LuxO to LuxU. This allows translation of *luxR* mRNA to LuxR and expression of bioluminescence¹⁰.

V. cholerae, the causative agent of cholera, has a QS system very similar to that of *V. harveyi*⁴⁹. Like *V. harveyi*, *V. cholerae* has two QS circuits, the CqsA/S system and the LuxS/PQ system, and a third system that remains uncharacterized⁴⁹. *V. cholerae* QS has been labeled counterintuitive, in that the QS phenotype is expressed in the absence of the signal and suppressed in its presence. AI-2 and the AI-1 molecule, CAI-2, are synthesized by LuxS and CqsA, and sensed by LuxP/LuxQ and CqsS, respectively. The signal is then channeled in parallel through LuxU and LuxO. At a low cell density, a low QS signal concentration causes LuxO activation and the subsequent activation of sRNAs expression through σ^{54} , which results in the destabilization of *hapR* mRNA and prevents translation of this regulator protein^{50, 51}. By inhibiting HapR production, the genes responsible for cholera toxin, toxin co-regulated pillus, and EPS/biofilm are expressed. Conversely, at a high cell density, the QS signals inactivate LuxO and HapR is expressed and QS-related genes are repressed^{49, 52-55}.

1.2.3 *P. aeruginosa*

P. aeruginosa is unique from the other pathogens described here because it does not make its own AI-2. However, it has been shown to increase its virulence factor expression in response to AI-2 produced by other microflora⁵⁶. *P. aeruginosa* QS utilizes the LuxI/LuxR system, as do over 30 species of Gram negative bacteria⁵⁷.

Furthermore, many bacteria have additional complexities and modifications in their LuxI/LuxR systems. In this system, LuxI is the HSL synthase and LuxR, the homologue to the *V. harveyi* signal receptor protein, is a transcriptional activator protein. It is LuxR that controls expression of the particular QS phenotypes. The opportunistic pathogen *P. aeruginosa* has two LuxI/R systems, LasI/R and RhlI/R, which work in tandem or series to control the expression of various QS associated virulence factors (Figure 1-3), such as cell adhesion and biofilm formation. Both LasI and RhlI produce different AHL autoinducers, N-(3-oxododecanoyl)-HSL (OdDHL) and N-butyryl-HSL (BHL) respectively⁵⁷. These act through their corresponding transcriptional activators, LasR and RhlR, to activate a variety of target genes⁵⁸. Once activated, LasR also enhances expression of the *rhl* locus, along with *mvfR*, leading to increased production of two other QS signal molecules, 4-hydroxy-2-heptylquinoline (HHQ) and *Pseudomonas* quinoline signal (PQS). The hydrophobic PQS signal is released from the cell by pinching off of extracellular membrane, while HHQ is synthesized intracellularly. LasR regulates *rhl* and *mvfR*, and virulence factor production, such as elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, superoxide dismutases and biofilm formation⁵⁹. Although the *P. aeruginosa* QS circuit is AHL signaling based, it is capable of sensing AI-2 and is therefore susceptible to AI-2 mediated QS inhibition. *P. aeruginosa* was observed to sense AI-2 produced by the normal microflora of cystic fibrosis patients, which led to increased virulence factors expression and infection⁵⁶. The use of AHL antagonists in *P. aeruginosa* QS inhibition has been widely studied⁶⁰⁻⁶², however few studies have examined the role of AI-2 antagonists in this organism⁶³.

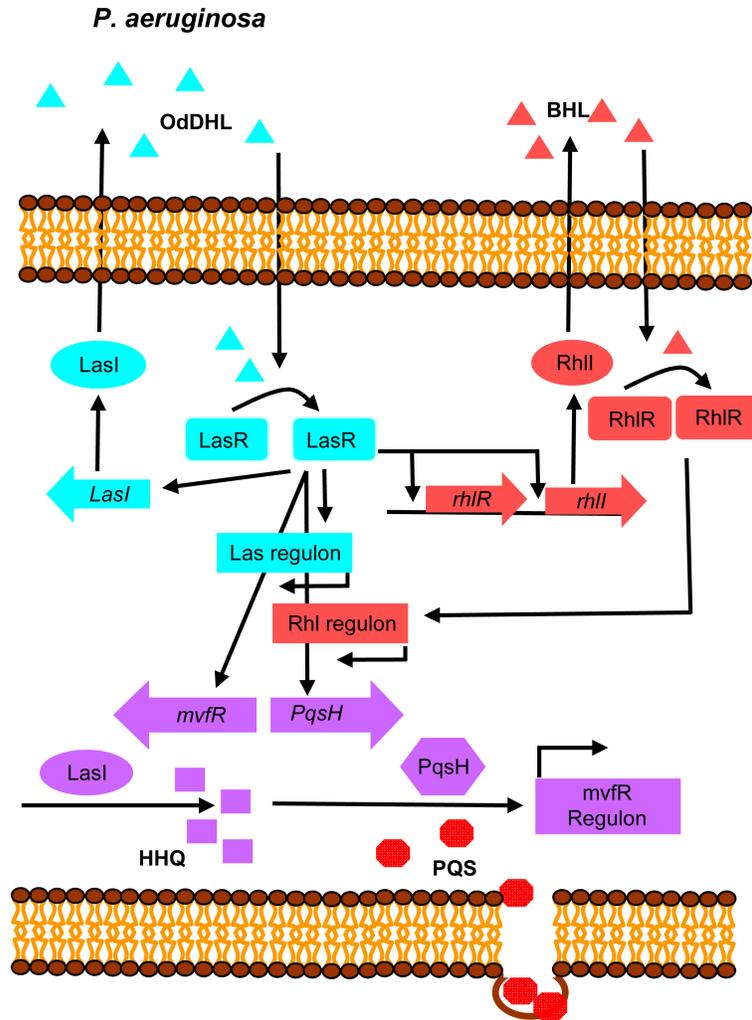


Figure 1-3 The Hierarchical Quorum Sensing Circuit in *P. aeruginosa*.

The two LuxI/LuxR QS systems viz. LasI/R and RhlI/R are shown. LasR upregulates expression of the Rhl regulon and also of mvfR & PqsH that produce other *P. aeruginosa* autoinducers HHQ and PQS. PQS is transferred to neighboring cells via extracellular vesicles that pinch off from the membrane.

1.3 Inhibiting Quorum Sensing

QS inhibition, of all types of QS circuits, is a potentially advantageous antimicrobial strategy not only because it removes the selective pressure for resistance

development, but also controls many bacterial virulence factors that facilitate human infection. Such QS regulated virulence factors have been shown to cause serious infections, some associated with high mortality⁶⁴. One of the best characterized examples of such a QS regulated trait is biofilm formation. Biofilm formation occurs in 80% of human bacterial infections⁶⁵ and pathogens contained in biofilm have a 1000- times higher tolerance to antibiotics than the same organisms in a planktonic state⁶⁶. The AI-1 autoinducers, AHLs, have been detected at significantly higher concentrations (632 μ M) in *P. aeruginosa* biofilms grown *in vitro*, as compared to effluent cells (14nM)⁶⁷. AHLs have also been detected in freshly removed bladder catheters of patients with in-dwelling catheters⁶⁸ and sputa of cystic fibrosis patients⁶⁹. Biofilm formation is also linked to AI-2 QS signaling. The direct addition of *in vitro* synthesized AI-2 to *E. coli* increased their biofilm mass 30-fold⁷⁰. Furthermore, when the membrane spanning protein YdgG was removed, intracellular AI-2 concentrations increased and, consequently, biofilm thickness and biomass significantly increased by 7000 -fold and 574-fold, respectively⁴⁵. Removal of AI-2 processing enzymes has been shown to affect biofilms for example *E. coli* strains lacking *lsrK* and *lsrR* were found to form significantly thinner biofilms with altered architecture⁷¹. These findings highlight the importance of QS in virulence phenotype expression and demonstrate that both AI-1 (AHLs) and AI-2 autoinducers are involved in exacerbation of bacterial infections. Thus inhibiting QS has become an important antimicrobial approach for the pharmaceutical industry, healthcare community and academia⁶⁴.

Traditionally, QS inhibition has concentrated on oligopeptide and AHL inhibition strategies, as AHLs are species specific and can be easily linked clearly to a particular organism's pathogenicity⁷²⁻⁷⁴. Over the past five years, AI-2 inhibition strategies have been developed for cross species quorum quenching. This review focuses on highlighting methods of AI-2 inhibition at different interception points in the AI-2 QS circuitry (Figure 1-4).

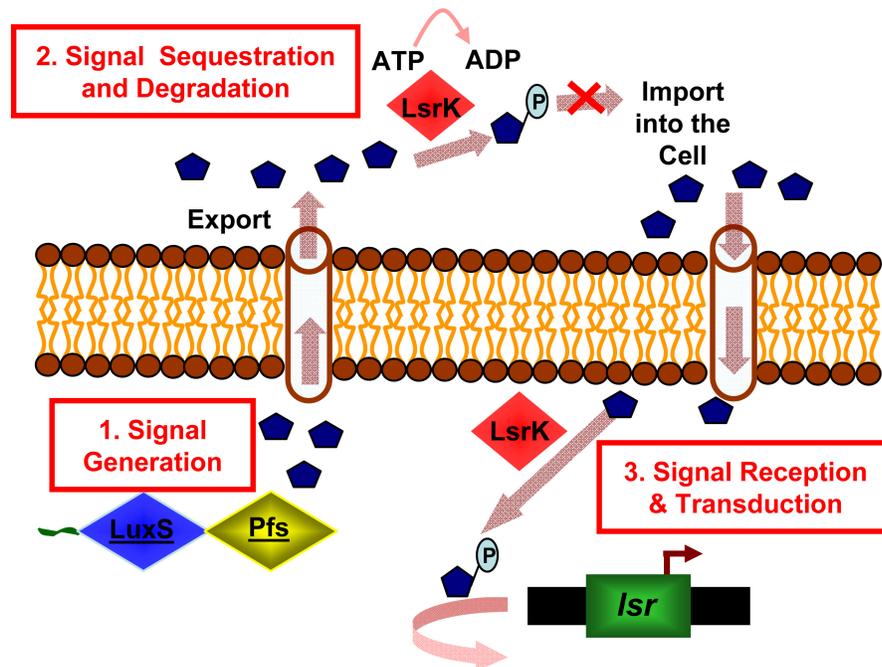


Figure 1-4 The Three Points of QS Interception Shown in Reference to the *E. coli* QS Circuit.

(1) Signal generating enzymes (LuxS and Pfs in *E. coli*). (2) Signal sequestration and degradation outside the cell (e.g. by enzymes such as LsrK). (3) Receptors and rTransducers in the signal transduction cascade.

1.4 Inhibition Target: Signal Generator

1.4.1 Inhibitors of SAH/MTA nucleosidase (Pfs)

Both AI-1 and AI-2 families of autoinducers are derived from S-adenosylmethionine (SAM) as part of bacterial 1-carbon metabolism. 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN/Pfs) catalyzes the hydrolytic deadenylation of MTA or SAH, and accumulation of either product inhibits the AI-1 or AI-2 pathways respectively. MTAN inhibitors can potentially alter both AI-1 and AI-2 signaling by disrupting signal generation⁷⁵. Guillerm *et al.*⁷⁶ first showed hydroxylated pyrrolidines to be SAH/MTA inhibitors, and speculated these compounds may be transition state analogs. Other investigations focused on substrate analogs of MTA, which are alkyl or aryl substituted, and acted as potent *Pfs* inhibitors⁷⁷. Detailed crystallographic studies in recent years have elucidated the complete structure and geometry of the binding site of *E. coli* SAH/MTA nucleosidase, enabling more complementary inhibitor design⁷⁸. Many kinetic studies were conducted using these analogs and their inhibition constants (K_i) were determined. Despite this, most were not tested for biological activity and effectiveness. Tedder *et al.*⁷⁹ and Li *et al.*⁸⁰ screened thousands of commercially available purine or indazole derived compounds against the binding site properties. Of the purine analogs, a purine benzylamine derivative with a $K_i = 1$ nM was discovered. The indazole inhibitors were found to have K_i values in the nanomolar range. Many of the purine derived inhibitors were active in antimicrobial assays and inhibited the growth of pathogenic bacteria. Although some analogs were successful

in SAH/MTA nucleosidase inhibition, the resulting growth inhibition also poses a risk for resistance development in the future.

Recently, compounds with femtomolar K_i values have been synthesized and are transition state analogs⁷⁵. Transition state analogs for *V. cholerae* MTAN, such as MT-DADMe-Immucillin-A, EtT-DADMe-Immucillin-A and BuT-DADMe-Immucillin-A, were tested for biological inhibition. They disrupted autoinducer production in a dose dependent manner without affecting cell growth. MT- and BuT-DADMe-Immucillin-A were also found to inhibited AI-2 production in EHEC. Of these transition state analogs, BuT-DADMe-Immucillin-A was able to inhibit AI-2 production in both strains for several generations and caused significant reduction in biofilm formation⁷⁵. These results suggest MTAN inhibition is a possible drug strategy as it provides “single-shot” target for both AI-1 and AI-2 QS controlled bacteria. However, inhibiting Pfs is lethal to the cell as MTA and SAH accumulation is toxic to the cell⁸¹. Excess MTA levels in cells inhibit growth processes and DNA synthesis by indirectly preventing synthesis of polyamines involved in these vital processes⁸².

1.4.2 Inhibitors of S ribosylhomocysteinase (LuxS)

Few studies have targeted S-ribosylhomocysteinase (LuxS) for the development of inhibitors. LuxS serves as a narrower spectrum antimicrobial that does not target processes essential for growth and survival of the organism. The known functions of this enzyme is DPD synthesis, SAH detoxification⁸³ and it plays a minor role in the sulfur recycling pathway⁸⁴. Thus, inhibitors of LuxS would be

highly specific QS regulators. Alfaro *et al.*⁸⁵ was the first to report on a specific LuxS inhibitor. They synthesized two substrate analogs S-anhydroribosyl-L-homocysteine and S-homoribosyl-L-cysteine, which also function as mechanistic probes. A subsequent investigation developed structural analogs of the substrate, SRH, and a 2-ketone intermediate⁸⁶. The most potent of these inhibitors, (2S)-2-amino-4-[(2R,3S)-2,3-dihydroxy-3-N-hydroxycarbamoylpropylmercapto]butyric acid, and (2S)-2-amino-4-[(2R,3R)-2,3-dihydroxy-3-N-hydroxycarbamoyl-propylmercapto] butyric acid, had K_i values in the submicromolar range. To date, the effect of these LuxS inhibitors on biological phenotypes has not been characterized.

1.5 Inhibition Target: Signal Molecule

1.5.1 Signal Sequestration or Degradation

A widely studied strategy for AHL QS inhibition is inactivation or complete degeneration of the signal molecules themselves. This type of AHL inactivation can be achieved through various mechanisms⁸⁷⁻⁸⁹. Some organisms are also known to metabolize AHL^{90, 91}. Although AHL degradation techniques have been the focus of many investigations, the degradation of AI-2 *ex vivo* to attenuate the QS response has not been demonstrated thus far.

1.6 Inhibition Target: Signal Receptor

1.6.1 Analogs of the Signal Molecule

1.6.1.1 AHL Analogs on *P. aeruginosa*

One highly effective AHL QS inhibition strategy is the prevention of signal perception, either by blocking or destruction of the receptor protein. This has been studied extensively in AHL signaling and many synthetic QSI's have been constructed and primarily evaluated using *P. aeruginosa*. One noteworthy analog, 3-oxo-C12-(aminocyclohexanone), was shown to significantly lower LasR-controlled expression from *lasI* when supplied in 100-fold excess over the native AHL molecule⁹². Exchanging the hexanone ring for a phenolic ring in this analogue resulted in generation of a more potent QSI compound that required ten times less concentration than its predecessor to result in similar knockdown⁹³.

1.6.1.2 DPD Analogs

As previously discussed, AI-2 does not have one specific defined structure, but rather is a class of molecules. The linear precursor of AI-2, DPD, cyclizes to form various isomers in solution⁴⁰ and to date, structures of only two forms have been identified^{41, 47}. Therefore, the lack of a defined AI-2 structure has presented a challenge for analog and ligand synthesis.

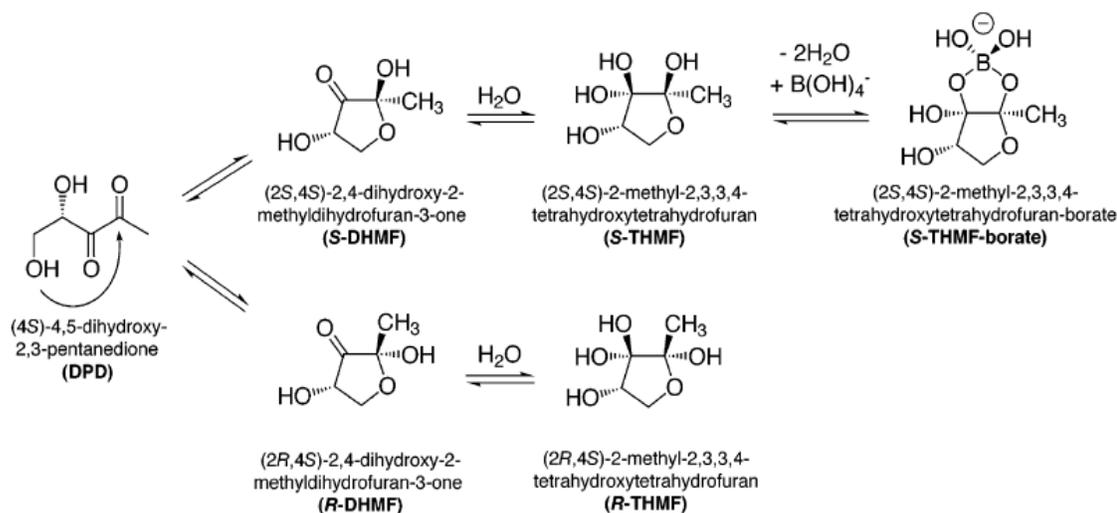


Figure 1-5 Conformations of DPD in solution.

Model showing structures of the various cyclic forms DPD adopts in solution before forming the S-THMF-borate molecule shown in the first branch which is recognized by *V. harveyi* and binds to its LuxP receptor⁴⁷. The second branch shows formation of the non-boronated R-THMF form which is recognized by the LsrB receptor in *S. typhimurium*⁴¹.

Current AI-2 analogs were synthesized as alkyl derivatives of the DPD C1 position. The first published screen of such derivatives consisted of the alkyl groups ranging from C1 to C4 and C6 along with phenyl and azidobutyl⁹⁴. In the presence of 1 μ M native DPD, these analogs had antagonistic actions in *S. typhimurium*, but agonistic actions in *V. harveyi*. The most significant inhibition of *S. typhimurium* QS, as measured by reduced *lsr* gene expression, was in the presence of propyl and butyl-DPD⁹⁴. Another investigation further diversified the shape of these linear C1-alkyl analogs, and also screened branched analogs, such as isopropyl and tertbutyl-DPD, as well as cyclic analogs, such as cyclopropyl and cyclohexyl-DPD. Despite such diversity in size and shape, all analogs caused synergistic agonism in *V. harveyi*. This suggested that the receptors involved in *V. harveyi* bioluminescence were promiscuous⁹⁵.

Another study of C1 alkyl analogs by Lowery *et al.*⁹⁶ showed reduced *V. harveyi* QS associated bioluminescence. This reduction was greater than fimbrolide, a natural brominated furanone and known potent antagonist often used as an established standard for AI-2 inhibition. In an investigation of pyocyanin inhibition, a

QS regulated *P. aeruginosa* virulence factor, linear DPD analogs, like butyl and pentyl-DPD, were shown to inhibit by ~50% pyocyanin production⁹⁷.

1.6.1.3 AI-3 Analogs in EHEC

Although little information is known about AI-3 and its pathway, some analogs have been identified. QseC is a conserved membrane histidine sensor kinase that is found in EHEC and responds to AI-3. A high throughput screening of 150,000 organic small molecules identified LED209 as a potent inhibitor of QseC. Inhibition of QseC blocks the expression of virulence factors such as attaching and effacing lesions required for establishing EHEC infection⁹⁸. The mechanism of such AI-3 analogs is poorly understood and more investigation is needed.

1.7. Inhibition Target: Various (Natural QS Inhibitors)

1.7.1 Brominated Furanones

Plants and fungi have co-existed with QS bacteria for millions of years, and many of them have evolved natural QS inhibitors to attenuate bacterial fouling and infection. Surfaces, particularly those submerged in water, are susceptible to bacterial colonization in the form of biofilm. This is then often followed by the colonization of macrofouling organisms. The red marine algae *Delisea pulchra*, has developed natural defense mechanisms to prevent microbial biofouling of its surfaces. The algae produce a number of non toxic, halogenated metabolites, such as brominated furanones, that effectively prevent biofilm formation. These compounds have been studied extensively for their ability to inhibit AI-2 mediated QS. One of the first

studies employed the natural *D. pulchra* furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone and found it to inhibit swarming and biofilm formation in *E. coli*. The furanone also inhibited AI-1 based QS in *V. harveyi* 3300-fold and AI-2 based QS 5500-fold, it also caused a significant reduction of *E. coli* QS via AI-2⁹⁹. Thus this furanone was classified as non specific inter-cellular antagonist of QS. To gain insight into genes affected by this compound and elucidate a potential mode of action, a DNA microarray study was performed. Analysis showed that 90 genes were differentially expressed by furanone treated *E. coli* (34 were induced and 56 genes were repressed). Most of the repressed genes were involved in chemotaxis, motility and flagellar synthesis. Furthermore, 80% of the genes repressed were known to be induced by AI-2. This suggested the furanone may interfere with the AI-2 regulon¹⁰⁰. An investigation of a synthetic furanone with two to six carbon atoms side chains in *S. typhimurium* was found to significantly decrease biofilm formation at non-growth-inhibiting concentrations¹⁰¹.

A furanone structural study was conducted by Han *et al.*¹⁰² to identify the most important elements for non-toxic inhibition of *E. coli* biofilm formation. Their data showed that a conjugated exocyclic vinyl bromide was required on the furanone ring. Defoirdt *et al.*¹⁰³ employed various mutant strains of *V. harveyi* to identify various furanone targets within the QS system. They found that all three QS systems were blocked by furanone addition and likely inhibits QS by preventing LuxR from binding to promoter sequences associated with QS genes. The brominated furanones have been found to protect some marine organisms from infections by QS bacterial species. Gnotobiotic brine shrimp, *Artemia franciscana*, were protected from

infection by pathogenic *V. harveyi*, *V. campbelli* and *V. parahaemolytic* by treatment with a natural furanone. The addition of the synthetic furanone (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone was also successful in preventing these organisms from colonizing the shrimp, however a higher concentration was required to prevent infection by *V. campbelli* than necessary for *V. harveyi*¹⁰⁴.

It has also been shown that a naturally occurring brominated furanone covalently modified and inactivated a recombinant LuxS protein¹⁰⁵. The effectiveness of these natural compounds may be due, in part, to their ability to target QS at multiple levels of the circuit.

1.7.2 Fatty Acids

Lu *et al.*¹⁰⁶ found that the washes of some food items were capable of inhibiting AI-2 activity while screening washes for the ability to induce bioluminescence in a *V. harveyi* reporter strain. A follow-up study found that several long chain fatty acids obtained from poultry meat washes were able to inhibit AI-2 based QS in the *V. harveyi* BB170 reporter assay. Most of the inhibitory fatty acids were 13.7 kDa or less and had hydrophobic properties. Linoleic acids, oleic acid, palmitic acid and stearic acid each caused inhibition from ~ 25 % to 99 %, which was concentration dependant in the 0.1 mM – 10 mM range. These fatty acids did not inhibit bacterial growth, as they did not lead to a significant reduction in colony counts. The fatty acids identified in this study have similar chemical structures, with differences in their chain length¹⁰⁷. The mode of action for these compounds was not

established and currently remains unknown. However, these molecules represent a distinct chemical class of potential natural QS inhibitors.

1.8 AI-2 QS Inhibition is Useful for Interrupting Polymicrobial Communication Networks

The understanding of polymicrobial communication networks is a crucial factor in the development of QS inhibition based antimicrobial therapies. The bacterial QS response is not necessarily triggered by AI-2 produced by an organism of the same species, genus, or even class. AI-2 mediated QS often occurs in bacterial communities composed of multiple species of various types of microbes. Several studies have demonstrated QS phenotypes in polymicrobial communities, including AI-2 QS mediated activities between normal microflora and pathogens. As AI-2 QS regulates pathogen virulence, in polymicrobial communication networks these provide another target for QS quenching molecules.

1.8.1 AI-2 Mediated Interactions Between Normal Microflora

The oral cavity harbors a diverse and abundant polymicrobial community as biofilms on teeth, gums, and tongues of the host. The understanding of oral microbial interactions is crucial as it is the portal of entry for both the gastrointestinal (GI) and respiratory tracts. AI-2, produced by the enzyme LuxS, is an important signaling factor in these communities, and many species cannot be cultured in the laboratory due to their metabolic interdependencies on other community members¹⁰⁸. Several oral species that were *luxS* deficient show aberrant monospecies biofilms, such as

the case for *Streptococcus mutans*^{109, 110} and *Streptococcus gordonii*¹¹¹. Furthermore, LuxS-dependent signaling was shown to be required for establishing polymicrobial biofilms of *P. gingivalis* and *S. gordonii* following the multimodal coadhesion that occurs between these organisms¹¹². This suggests that AI-2 QS plays an important role in the colonization of oral surfaces and other functions of AI-2 in this community will likely be established in the future.

1.8.2 AI-2 Mediated Interactions Between Pathogens and Native Microflora

Laboratory study of bacteria occurs in mostly as pure cultures, but they rarely exists as such outside of a laboratory, including the human body. The cumulative manifestation of a bacterial infection is not solely the result of a single particular pathogen's growth but is an outcome of a complex interaction between the pathogen, the host, and possibly the host's normal microflora. It is thought that the host's normal flora acts as a protective barrier to prevent invasion and infection of a pathogen, however there is evidence that shows the normal microflora can aid in the upregulation of a pathogen's virulence factors and worsen the infection., A study using the rat lung infection model of CF showed such an effect of avirulent oropharyngeal flora (OF) on pathogenic *Pseudomonas aeruginosa*⁵⁶. The presence of the OF were shown to enhance lung damage caused by *P. aeruginosa* infection. Furthermore, significant levels of AI-2 were detected in sputum samples collected from CF patients and in cultures of non- pseudomonad OF bacteria. A genome wide *in vitro* transcriptional analysis in a *P. aeruginosa* showed that OF generated and exogenous AI-2 could upregulate genes involved in *P. aeruginosa* pathogenicity.

These results demonstrate the potential impact of AI-2 QSI on reducing the pathogenicity of AI-2 independent organisms, such as *P. aeruginosa*. Chronic and recurring *P. aeruginosa* infections are common in CF patients, and ultimately prove fatal¹¹³. The chronic nature of such infection is due, in part to persister cell development though antibiotic treatment¹¹⁴⁻¹¹⁶. Interrupting or removing the mediator of such interspecies communication networks could reduce *P. aeruginosa* pathogenicity and allow CF to survive infection. Such strategies may be key to more effectively treating these types of infections.

1.8.3 AI-2 Mediated Interactions Between Pathogens in Polymicrobial Infections

Just as non-pathogenic normal flora can enhance an organism's pathogenicity, so can infections due to multiple pathogenic microbes. Studies have shown that communication between bacterial species promotes persistence and resistance to antibiotics. These considerations are important in the diagnosis, prevention, and treatment of polymicrobial infections, like otitis media (OM). OM is the most common childhood infection and the leading cause for antibiotic prescriptions in children¹¹⁷. OM can be chronic and recurrent in children due to the persistence of bacteria within biofilms, makes antibiotic clearance more difficult. OM infections are typically polymicrobial, that is they are caused by simultaneous infection of multiple bacterial species. *Haemophilus influenzae*, *Streptococcus pneumoniae* and/or *Moraxella catarrhalis* have been detected in patient samples with chronic OM¹¹⁸. Animal studies have showed that co-infection with *H. influenzae* and *M. catarrhalis* promotes increased resistance of to both antibiotics and host clearance due to biofilm

formation. This type of co-infection allowed *M. catarrhalis* to persist in the body longer than if the animal was infected by *M. catarrhalis* alone. Furthermore, co-infection of *M. catarrhalis* and a *luxS* deficient *H. influenzae* was found not to promote *M. catarrhalis* infection persistence¹¹⁹. The results of this study show that *H. influenzae* is able to promote *M. catarrhalis* persistence in a polymicrobial biofilm via AI-2 interspecies quorum signaling. Although this study is the only to demonstrate such a relationship, it is likely that other polymicrobial infections also utilize AI-2 mediated QS to enhance persistence and virulence of the co-infecting species.

1.9 Research Motivation

In the previous sections we presented background about AI-2 mediated QS systems in various bacteria, and the existing methods to alter AI-2 signaling at various “points” in its’ signal transduction. AI-2 inhibition techniques have been developed mostly in the past five years and there is potential for further developing AI-2 inhibition strategies to quench QS in one or more species of bacteria simultaneously.

In the current techniques for the method of targeting “the signal molecule itself” as discussed above, there currently exist no enzymes or other methods of sequestering or degrading the AI-2 signal molecule. The AI-2 processing mechanisms/enzymes in various bacteria have been studied in detail. In *E. coli* for example it is known that LsrK and LsrG phosphorylate and degrade AI-2 *in vivo*. Thus, there is opportunity for developing AI-2 processing techniques *in vitro* i.e.

synthesizing these enzymes and delivering them *ex vivo* to modify or sequester the AI-2 signal and study the resulting effect on the *in vivo* response.

Some groups have developed analogs of the AI-2 precursor DPD. There is a great opportunity to further diversify the library of functional DPD analogs. Also, besides identifying inhibitors in the AI-2 analog library, these analogs can be used to study how the AI-2 native machinery perceives and processes “AI-2 like” molecules. This will further enhance our understanding of the enzymes and receptors in the AI-2 QS pathway that the analog utilizes. Understanding the processing of the “AI-2 analogs” to be active QS inhibitors will help us tailor the analogs to be more potent QS inhibitors. Probing the mechanistics of the machinery will help us identify more drugable targets.

The existing AI-2 inhibition techniques have currently been tested in only pure cultures of bacteria. In the previous section we mentioned that in nature most bacteria exist in communication networks with other bacteria around them and AI-2 is the mediator of these inter-species interactions. Thus, it is plausible that due to the ubiquitous presence of AI-2 its inhibition strategies will have cross-species QS quenching potential. The AI-2 inhibition strategies we develop will also be tested in mixed species cultures to identify broad-spectrum QS inhibitors. Since QS responses are modified by the extracellular AI-2 concentration which changes in response to AI-2 uptake by other species in the environment and also the release of proteins and polysaccharides in the environment. Testing AI-2 inhibition mechanisms in mixed cultures will be crucial in establishing their role in real-time environments such as polymicrobial infection.

Most of the chemically synthesized analogs have not been tested to show inhibition of pathogenic phenotypes such as biofilm formation. There is potential for studying the effect of AI-2 inhibition molecules on biofilm formation individually and in a combinatorial therapy with antibiotics, where they could enable more potent QS inhibition.

1.10 Global Objective, Global Hypothesis and Specific Aims

The global objective of this dissertation is to create methods to inhibit AI-2 mediated signaling in pure and mixed cultures of bacteria. The overall hypothesis of this work is “Delivering AI-2 processing enzymes and AI-2 analogs *ex vivo* sequesters/inhibits the AI-2 based signaling and enables cross-species quorum quenching”.

The specific aims of this research are: -

1. To deliver *E. coli*'s native AI-2 processing machinery outside the cell and sequester and destroy the AI-2 signal before it can elicit an *in vivo* cell based QS response both in pure and mixed cultures of bacteria.
2. To create a vast library of AI-2 analogs and identify broad spectrum and species specific analog based quorum quenchers. The biological mechanism of action of the AI-2 analogs also needs to be elucidated.
3. Use cues from the first AI-2 analog study to diversify the AI-2 analog library and add more potent QS quenchers. Use mixtures of analogs i.e. analog

cocktails to quench QS selectively in subpopulations or *en masse* in bacterial co-cultures.

4. Monitor the effect of the developed AI-2 inhibition strategies on pathogenic phenotypes such as biofilm formation.

1.11 Dissertation Outline

Chapter 2 describes the *in vitro* expression and purification of LsrK and confirms the formation of phospho-AI-2 *in vitro*. The effect of *in vitro* synthesized phospho-AI-2 on the QS response of *E. coli*, *S. typhimurium* and *V. harveyi* is monitored in pure cultures and a self-assembled trispecies synthetic ecosystem. This chapter also investigates the result of delivering LsrK and ATP on native bacterial cross-talk by delivering them *ex vivo* in co-cultures of *E. coli* and *S. typhimurium*.

Chapter 3 describes the synthesis of a library of 14 C1-alkyl DPD analogs and monitors their effect on quenching native QS responses in *E. coli* and *S. typhimurium*. The biological mode of action of the analogs and the ability of the *E. coli* QS kinase to phosphorylate them is also investigated. A trispecies synthetic ecosystem of *E. coli*, *S. typhimurium* and *V. harveyi* is assembled to identify broad spectrum and species specific QS quenchers.

Chapter 4 describes diversification of the AI-2 analog library to include cyclic and aromatic C1-alkyl analogs of DPD. Besides, *E. coli* and *S. typhimurium* the effect of these analogs is tested on *P. aeruginosa* in pure cultures and an analog cocktail inhibits QS *en masse* in an ecosystem containing all three bacteria.

Chapter 5 describes the effect of isobutyl-DPD a broad spectrum potent QS quencher identified from the previous studies; in inhibiting *E. coli* biofilm formation. In this study a combinatorial approach of isobutyl-DPD and the antibiotic gentamicin to remove pre-existing biofilm is also investigated.

Chapter 6 summarizes the work in the previous chapters, mentions some challenges and future work that applies and/or builds on techniques devised in this work. The broader impact and significance of the work is also mentioned.

Chapter 2: Cross Species Quorum Quenching Using A Native AI-2 Processing Enzyme

2.1 Abstract

Bacterial Quorum Sensing (QS) is a cell-cell communication process, mediated by signaling molecules, that alters various phenotypes including pathogenicity. Methods to interrupt these communication networks are being pursued as next generation antimicrobials. We present a technique for interrupting communication among bacteria that exploits their native and highly specific machinery for processing the signaling molecules themselves. Specifically, our approach is to bring native intracellular signal processing mechanisms to the extracellular surroundings and “quench” crosstalk among a variety of strains. In this study, the QS system based on the interspecies signaling molecule, autoinducer-2 (AI-2), is targeted because of its prevalence among prokaryotes (it functions in over 80 bacterial species). We demonstrate that the *Escherichia coli* AI-2 kinase, LsrK, can phosphorylate AI-2 *in vitro*, and when LsrK treated AI-2 is added *ex vivo* to *E. coli* populations, the native QS response is significantly reduced. Further, LsrK-mediated degradation of AI-2 attenuates the QS response among *Salmonella typhimurium* and *Vibrio harveyi* even though the AI-2 signal transduction mechanisms and the phenotypic responses are species-specific. Analogous results are obtained from a synthetic ecosystem where three species of bacteria (enteric and marine) are co-cultured. Finally, the addition of LsrK and ATP to growing co-cultures of *E. coli* and *S. typhimurium*, exhibits significantly reduced native “cross-talk” that

ordinarily exists among and between species in an ecosystem. We believe this nature-inspired enzymatic approach for quenching QS systems will spawn new methods for controlling cell phenotype and potentially open new avenues for controlling bacterial pathogenicity.

2.2 Introduction

Signal molecules mediate information transfer between cells and drive cell function. Intra and intercellular “signaling” or “communication” guides the establishment of bacterial infections and mediates their virulence^{43, 120}. Interrupting these signaling processes can limit or halt disease progression^{13, 98}, potentially without adversely affecting bacterial growth⁹⁸. Such innovative approaches that are neither bactericidal nor bacteriostatic should minimize the emergence of antibiotic resistant strains⁸ and lead to entirely new approaches for guiding bacterial phenotype. Naturally, components of signal transduction pathways are key targets for these approaches. Signal transduction in bacteria occurs by cell-cell communication via small signaling molecules, in a process called quorum sensing (QS)²⁴. In QS pathways, the approach of interfering with or destroying the QS signal is referred to as “quorum quenching”¹²¹. Strategies for quorum quenching include out competing the native signaling processes with signal molecule analogues and limiting signal generation with synthase-specific inhibitors (as reviewed by Rasmussen and Giskov⁹). A third method, involving sequestering native signal molecules via molecular traps or degradative processes, has been proposed¹²² and is investigated here.

Bacterial autoinduction (AI) is ripe for all of these quenching strategies since the modes of signal generation and perception vary widely. Early strategies targeted intracellular communication processes typified by the acylated homoserine lactone (AHL) signal molecules, also known as autoinducer-1 (AI-1), which is specifically secreted and recognized by a variety of Gram negative pathogens^{123, 124}, while recent findings suggest these molecules could even mediate intercellular signaling events¹²⁵. Most reported quorum quenching techniques have concentrated on inactivation of the AHL molecules. These methods employ a number of enzymes, such as AHL lactonases⁸⁹ and AHL acylases⁹¹ which cleave the lactone ring or acyl chain of the AHL molecule respectively, as well as various paraoxonases (PONs)¹²⁶ that have AHL hydrolytic activity. Besides AHL degradation enzymes, there are structural mimics of the QS signal, such as halogenated furanones^{127,60} and synthetic autoinducing peptides¹²⁸ that typically interfere with signal binding to the receptor. Approaches that exploit molecular evolution of the cognate transcriptional regulators have increased the diversity and scope of candidates¹²⁹ and, in turn, led to new technologies for guiding phenotype¹³⁰. The autoinducer-2 (AI-2) signal transduction process is thought to be “universal” in that its principal synthase, *luxS*, is found in over 70 genera and AI-2 itself is actually a family of isomers, each potentially representing a different mode of perception⁴¹. Unlike the AI-1 system, molecular evolution approaches to enhance diversity of AI-2 recognition have not appeared, perhaps due to the still-yet unresolved mechanism of its signal transduction processes.

Approaches that target AI-2 processes are conceptually fertile in that candidate molecules could conceivably “quench” the communication among multiple

species simultaneously. Brominated furanones¹³¹ from the alga *Delisea pulchra*, fatty acids derived from ground beef¹³² and poultry meat¹⁰⁷; various synthetic^{94, 95} and transition state^{133, 134} AI-2 analogues represent AI-2 inhibition schemes in development. Our work aims to quench AI-2 mediated communication by exploiting the native signal processing machinery, specifically, the DPD kinase, LsrK from *E. coli* (Figure 2-1, panel a). 4,5-dihydroxy-2,3-pentadione (DPD) is the precursor of AI-2 and it exists in equilibrium with several interconvertible isoforms in solution, all of which are referred to as the family of AI-2 signaling molecules⁴¹. The QS machinery in *E. coli* consists of various *luxS*-regulated (*lsr*) genes⁴³, LsrK being the putative kinase which functions intracellularly in the native system to phosphorylate DPD (the single linear form of the AI-2 signaling molecules) to phospho-DPD (also referred to as phospho-AI-2). This molecule, in turn, is pivotal as it is the antirepressor of the QS system. That is, by binding to transcriptional repressor LsrR, phospho-AI-2 induces transcription of the *lsr* genes (Figure 2-1, panel a)¹³⁵. Phospho-DPD itself has shown to be unstable, and degrades in 16 hours to form 2-phosphoglycolic acid (PG)⁴⁶ (Figure 2-1, panel c).

In this study we add *E. coli* QS kinase LsrK *ex vivo* (outside the cell, in the extracellular medium) to phosphorylate AI-2 to phospho-AI-2, thus conferring a negative charge on AI-2 thereby restricting its transport into the cell via binding the Lsr transporter. PG that is subsequently produced is not predicted to affect *lsr*-mediated processes. Thus, LsrK targets AI-2 for degradation and “quenches” the quorum sensing response. We deliver LsrK *ex vivo* to *E. coli*, *S. typhimurium* and *V. harveyi* for the selective modification of AI-2 and cessation of AI-2 signaling in these

cell types (Figure 2-1, panel b). The signal molecule in this method is transformed outside the the cell, thus unlike analogue based competitive inhibition agents there is no compulsory cell membrane barrier to overcome.

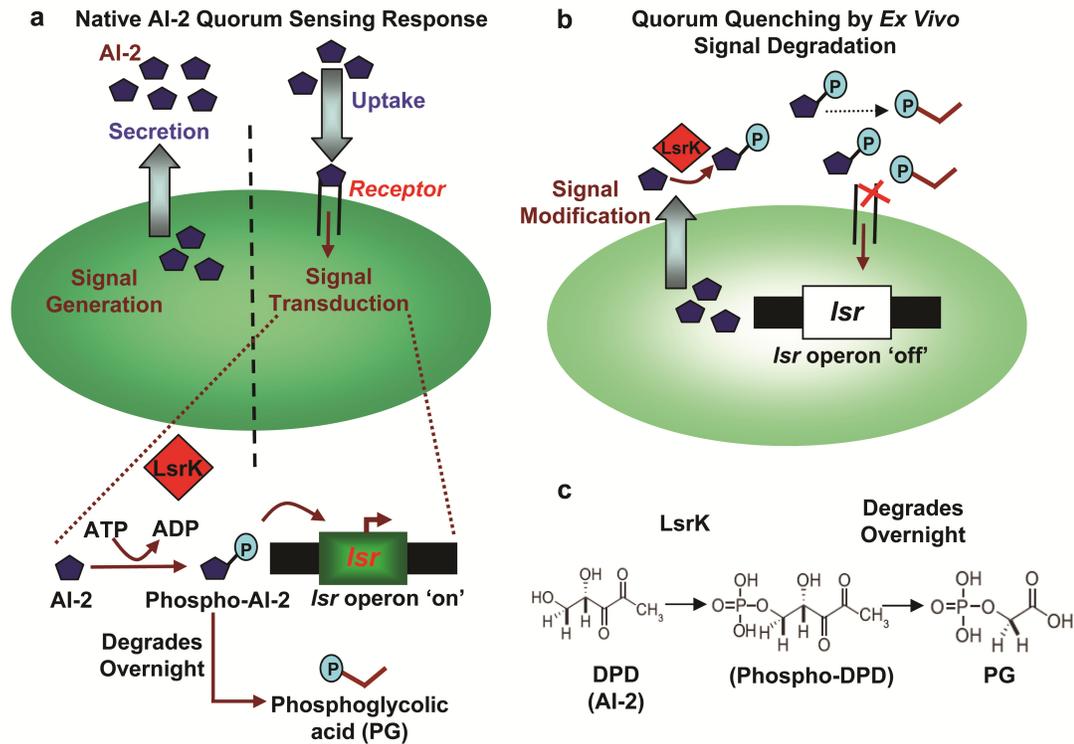


Figure 2-1 Schematic of LsrK mediated quorum “quenching”.

a) Native quorum sensing is mediated by the generation, secretion, and uptake of autoinducer-2 (AI-2) followed by signal transduction indicated by *lsr* gene expression; phospho-AI-2 degrades overnight to 2-phosphoglycolic acid (PG). b) LsrK and ATP delivered outside the cell phosphorylate AI-2; phospho-AI-2 is presumably prevented from being transported into the cells and is degraded to PG. In this scenario, the quorum sensing response is quenched. c) LsrK phosphorylates DPD (AI-2 precursor); phospho-DPD degrades to PG overnight *in vitro*.

We demonstrate the effects of LsrK treated AI-2 on phenotypic responses such as *lsr* expression in gut commensal bacteria such as *E. coli* and *S. typhimurium* and bioluminescence in the marine bacterium *V. harveyi* in both pure cultures and also in a synthetic ecosystem, where all three are co-cultured. These bacteria are chosen as they represent different modes of signal recognition and transduction. To demonstrate utility of our scheme in interrupting bacterial cross-talk in nature where one bacterium responds to the AI-2 produced by another, we co-culture *E. coli* and *S. typhimurium*, which are known to co-exist in the gut during *S. typhimurium* infections. In this co-culture, the *E. coli* produces AI-2 which is sensed by *S. typhimurium*. The addition of LsrK and ATP *ex vivo* to this co-culture is monitored. By capturing the signal molecule in one state (linear DPD, via the native processing enzyme, LsrK) and sequestering it from its equilibrium isoforms, this approach has the potential of silencing QS activity in broad based populations, and preventing associated pathogenic responses, such as the formation of biofilms.

2.3 Materials and Methods

Bacterial Strains and Growth Conditions

Table 2-1 lists bacterial strains and plasmids used in this study. *S. typhimurium* and *E. coli* strains were cultured in Luria-Bertani medium (LB, Sigma) at either 30 °C or 37 °C with vigorous shaking (250 rpm) unless otherwise noted. The *V. harveyi* strains were grown in AB medium. Antibiotics were used for the following strains: 60 or 100 µg ml⁻¹ kanamycin for *S. typhimurium* MET715, 50 µg ml⁻¹ ampicillin for *E. coli* BL21 *luxS*, 60 or 100 µg ml⁻¹ ampicillin for *E. coli* LW7, 50 µg

ml⁻¹ ampicillin and 50 µg ml⁻¹ kanamycin for *E. coli* MDAI-2 pCT6, and 20 µg ml⁻¹ kanamycin for *V. harveyi* BB170.

Table 2-1 List of bacterial strains, plasmids and primers used in this study.

Strain, plasmid or primer	Relevant genotype and/or property	Reference
<i>Escherichia coli</i> strains		
W3110	Wild type	Laboratory Stock
BL21 luxS ⁻	<i>F'</i> ompT hsdS _B (<i>r_B⁻m_B⁻</i>) <i>gal dcm ΔluxS</i> :: Kan	Laboratory Stock
LW7	W3110 <i>ΔlacU160-tna2 ΔluxS</i> :: Kan	135
MDAI2	W3110 <i>luxS</i> ::Tc ^r W3110-derived <i>luxS</i> mutant strain	136
ZK126	W3110 <i>ΔlacU169-tna2</i>	137
<i>Salmonella typhimurium</i> strains		
MET715	<i>rpsl putRA</i> :: Kan- <i>lsr-lacZYA luxS</i> :: T-POP	42
<i>V. harveyi</i> strains		
BB170	BB120 <i>luxN</i> :: Tn5 (sensor 1 ⁻ , sensor 2 ⁺); AI-1 ⁺ , AI-2 ⁺	138
MM30	BB120 <i>luxS</i> ::Tn5 (sensor-1 ⁺ , sensor-2 ⁺); AI-1 ⁺ , AI-2 ⁻	139
Plasmids		
pLsrK	pET200 derivative, <i>Escherichia coli</i> W3110 LsrK ⁺	This Study
pLW11	<i>galK'-lacZYA</i> transcriptional fusion vector, containing <i>lsrACDBFG</i> promoter region, Amp ^r	135
pCT6	pFZY1 derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region fused with <i>T7RPol</i> , Ap ^r	136
pET-GFP	pET200 derivative, containing <i>gfpuv</i> , Km ^r	
Oligonucleotide primers		
Name	Sequence	Relevant property
LsrKFrwd	5'- CAC CAT GGC TCG ACT CTT TAC CCT TTC - 3'	Upstream primer to amplify LsrK sequence from whole genomic DNA W3110. (This Study)
LsrKRev	5'-CTA TAA CCC AGG CGC TTT CCA TAA C- 3'	Downstream primer to amplify LsrK sequence from whole genomic DNA W3110. (This Study)

Construction of *E. coli* LsrK plasmid

To construct plasmid pET200-LsrK, *E. coli* *lsrK* (*ydeV*) was amplified with primers listed in Table 2-1 from *E. coli* W3110 whole genomic DNA using Vent DNA polymerase (New England Biolabs; NEB). The 1593 bp fragment was isolated and purified via the QIAquick gel extraction kit (Qiagen). This blunt ended fragment was then inserted into pET200/D-TOPO (Invitrogen), which has a hexa histidine tag at the N- terminus (Figure 2-2, panel a). The sequence of pET200-LsrK was confirmed by sequencing.

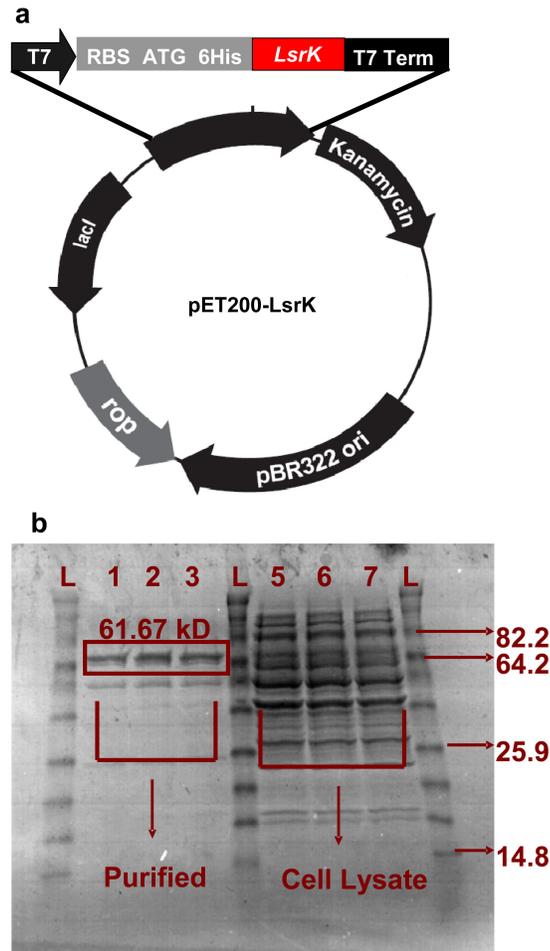


Figure 2-2 Construction and expression of hexahistidine tagged LsrK.

a) Plasmid map and b) SDS-PAGE showing LsrK expression bands at 61.67 kDa.

Overexpression and Purification of *E. coli* LsrK

The *E. coli* pET200-LsrK expression vector (Figure 2-2, panel a) was transformed into *E. coli* BL21 *luxS* and was cultured in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin at 37 °C and 250 rpm. LsrK protein expression was induced by IPTG addition and later purified as described¹⁴⁰. Purified LsrK (61,670 Da) was confirmed by loading 1 μg protein on an SDS-PAGE gel (Biorad) (Figure 2-2, panel b).

LsrK Activity

AI-2 was synthesized *in vitro* using synthases LuxS and Pfs¹⁴⁰. LsrK activity was estimated using an ATP bioluminescence assay kit (CLS II, Roche Scientific). One μM LsrK and 300 μM AI-2 were incubated with varying concentrations of ATP (5 μM , 10 μM , 20 μM). A 100 μl aliquot was mixed with 100 μl of the luciferase reagent, and light production was recorded at 2 minute intervals over a span of 30 minutes using a luminometer (EG&G Berthold). Values depicted were 10 second running averages. The concentrations of AI-2 (300 μM) and LsrK (1 μM) for the ATP assay were selected after trying a range of concentrations of AI-2 from 50-400 μM , and from 0.5 - 4 μM LsrK, to determine the combinations at which maximum ATP was consumed.

***In vitro* phosphorylation of AI-2**

LsrK treated AI-2 was synthesized by incubating LsrK (1 μ M) with 80 μ M ATP (Roche), 20 μ M AI-2, 200 μ M MgCl₂ in 25 mM phosphate buffer, pH 7.4 for either 1 or 16 hours.

Thin layer Chromatography (TLC)

LsrK treated AI-2 (1 h or 16 h) samples (as described above) were incubated with 0.2 Ci of [γ ³²P] ATP (Perkin-Elmer) after which 5 μ L of the sample was spotted onto a cellulose TLC plate (Selecto Scientific). The plate was developed using 0.8 M LiCl as the solvent, air dried and developed via autoradiography.

Measurement of the QS response (*lsr* expression)

The QS response indicated by *lsr* gene expression was analyzed in pure culture studies by culturing *E. coli* LW7 pLW11 and *S. typhimurium* MET715 overnight in LB medium supplemented with suitable antibiotics as stated previously. These were diluted into fresh LB medium supplemented with the antibiotic and grown to an OD₆₀₀ of 0.8 - 1.0 at 30 °C, 250 rpm. Cells were then collected by centrifugation at 10,000 xg for 10 minutes, and resuspended in 10 mM phosphate buffer. LsrK treated AI-2 (1 h; 16 h) or untreated AI-2 (500 μ l; 20 μ M) was added to 500 μ L of either the *E. coli* LW7 or *S. typhimurium* MET715 suspension for 2 hours at 37 °C. AI-2 dependent β -galactosidase production was quantified by the Miller assay¹⁴¹ or Fluorescein di- β -D-galactopyranoside (FDG)¹⁴².

Flow Cytometry

E. coli MDAI-2 pCT6 was cultured overnight in LB medium, diluted in fresh LB medium and grown at 30 °C, 250 rpm for 8 h. Either the 16hr LsrK treated AI-2 or untreated AI-2 (final concentrations; 10 μ M) were added at time 0 and then added again after 2.5 hours. After 7.5 hours, the cells were fixed overnight in a 1:1 ratio of cold 4 % paraformaldehyde. All samples were analyzed by flow cytometry (FACS Canto II, BD 394 Biosciences), with 20,000 gated events analyzed per sample.

Measurement of the QS response (Bioluminescence)

The effect of LsrK degraded AI-2 on QS associated bioluminescence production by *V. harveyi* was recorded by measuring the light production using reporter strain, *V. harveyi* BB170. AI-2 treated with LsrK for 16hr or untreated AI-2 both at a concentration of (2 μ M) was added to *V. harveyi* and assayed as previously described¹³⁹.

Analyzing QS Response in the Synthetic Ecosystem

The *S. typhimurium* MET715, *V. harveyi* MM30 and *E. coli* MDAI-2 pCT6 were each cultured separately overnight in LM medium¹⁸ supplemented with the appropriate antibiotic. The cultures of MM30, MET715 and MDAI-2 were diluted (1:4:8) respectively, into a single volume of 1 ml fresh LM medium without antibiotics. The co-culture was supplemented with LsrK treated AI-2 or untreated AI-2 (final concentrations; 10 μ M) initially and again after 3 and 5 hours of growth. The *V. harveyi* luminescence response was measured after 2.5 hours. The *S. typhimurium* *lacZ* (β -galactosidase) activity was measured after 4 hours. The *E. coli* response was

determined after 10 hours, by fixing the cells with 1:1 cold 4 % paraformaldehyde and using flow cytometric analysis.

Analyzing Bacterial Cross-talk in a Natural System

The *S. typhimurium* MET715 and *E. coli* ZK126 were each cultured overnight in LB medium with the appropriate antibiotic. The samples were grown to OD₆₀₀ 0.6 - 0.8 at 30 °C, 250 rpm and collected by centrifugation at 10,000 xg for 10 minutes. The pellets were then resuspended in 5ml LB. A 1:1 mixture of *E. coli* and *S. typhimurium* was prepared in fresh microcentrifuge tubes with increasing concentrations of LsrK (0.1 μM, 1 μM and 5 μM). Negative controls were also prepared without LsrK. ATP (80 μM) was added to all samples and incubated in a 30 °C water bath for 2 hours. AI-2 dependent β-galactosidase production was quantified by the Miller assay¹⁴¹.

2.4 Results and Discussion

2.4.1 LsrK Phosphorylates AI-2 *In Vitro*

The uptake of AI-2 and its subsequent processing has been described in various bacteria¹⁰. The enzyme LsrK, a kinase component of the AI-2 processing machinery in *E. coli*⁴³ and *S. typhimurium*⁴², phosphorylates AI-2 upon uptake into the cell, using ATP. Purified LsrK (YdeV) was derived from *E. coli* W3110 and assayed for activity using *in vitro* synthesized AI-2 as a substrate. An ATP luminescence assay which monitors the residual ATP present in the sample

(measured via luminescence) following phosphorylation of AI-2 by LsrK is used to indicate activity (see methods). In this system, luminescence was dependent on the ATP concentration. The luminescence decreased rapidly over time for all tested ATP concentrations in the presence of LsrK, relative to controls as shown in (Figure 2-3, panel a). These results indicate that the *in vitro* purified *E. coli* LsrK was active (depleted ATP) and observed trends were in agreement with previous studies where the homologous *S. typhimurium* LsrK was observed to phosphorylate AI-2 within 10 minutes⁴⁶.

The product of the phosphorylation reaction is phospho-AI-2, which breaks down over time to 2-phosphoglycolic acid (PG)⁴⁶. To ascertain the relative proportion of phospho-AI-2 and PG as a function of time, samples containing AI-2 and ATP were treated with LsrK for either 1 h or 16 h (overnight) and spotted on a thin layer chromatography (TLC) plate. A large amount of AI-2 was converted to phospho-AI-2 after 1 h, which appears at the bottom of the TLC plate (Figure 2-3, panel b). Samples left for 16 h showed a near complete conversion of phospho-AI-2 to PG.

Image analysis of the TLC results indicated that the 16 h phospho-AI-2 levels were 4-fold lower than the 1 h sample, while the net increase in PG in the 16 h sample was 2-fold that of the 1 h sample. Our results demonstrate that *E. coli* derived LsrK treated AI-2 formed both phospho-AI-2 and PG and that the relative ratio of phospho-AI-2 to PG after LsrK treatment decreased as a function of reaction time. Since the LsrK reaction rate is rapid and there is a 4-fold excess ATP in the reaction samples (Figure 2-3, panel b), we expect that there was nearly complete conversion of AI-2 to phospho-AI-2 in 1 h. This is consistent with investigations involving *S.*

typhimurium LsrK⁴⁶. Subsequent experiments for AI-2 activity were performed using both the 1 h and the 16 h mixtures described above.

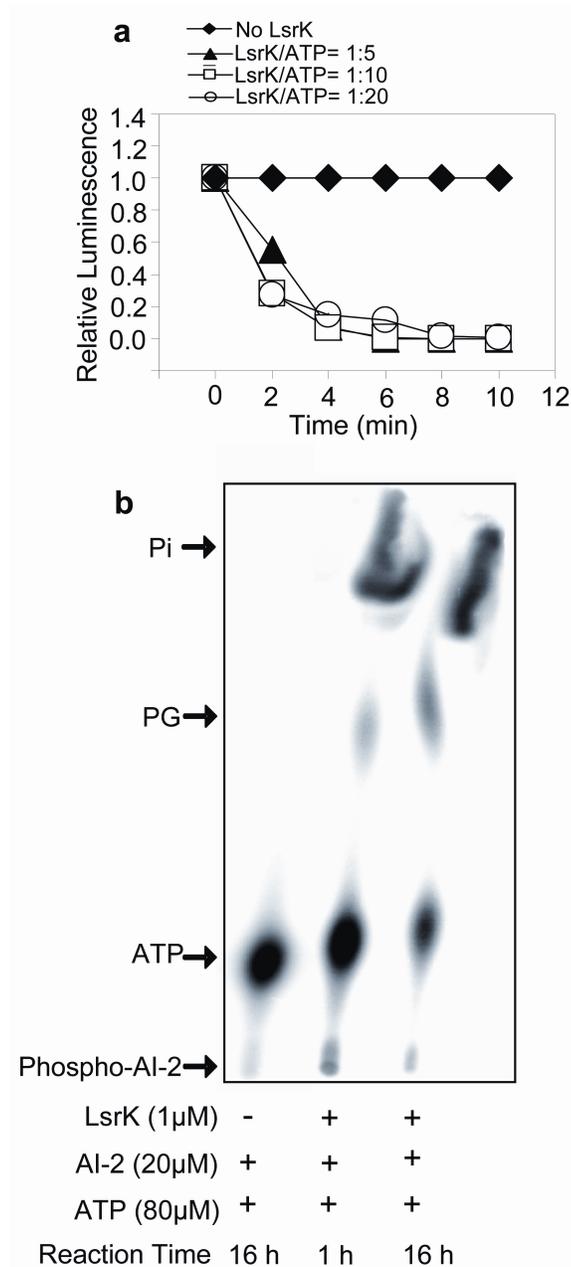


Figure 2-3 *In vitro* LsrK is active.

a) ATP consumption assay for LsrK mediated AI-2 phosphorylation. ATP consumption for varying LsrK/ATP ratios (1:5 – 1:20) in presence of AI-2. **b)** Thin layer chromatography (TLC) analysis of the LsrK mediated reaction. Lane 1: AI-2,

ATP incubated for 16 h Lanes 2, 3: LsrK, AI-2, ATP incubated for 1 h and 16 h respectively.

2.4.2 LsrK Treated AI-2 Precludes *lsr* Expression

Inside the cells, LsrK phosphorylated AI-2 normally derepresses gene expression in the *lsr* operon by binding LsrR⁴³ (Figure 2-1, panel a). The effect of addition of LsrK treated AI-2 outside the cells on the QS response of *lsr* gene expression was observed using the reporter strain *E. coli* LW7 pLW11 which is a *luxS* null mutant and cannot produce AI-2 (See Table 2-1). Plasmid pLW11 encodes the *lacZ* gene under the control of the *E. coli lsr* promoter so that *E. coli* LW7 pLW11 synthesizes β -galactosidase in response to added AI-2. *E. coli* LW7 pLW11 was incubated with fluorescein di- β -D-galactopyranoside (FDG) and either AI-2 or LsrK treated AI-2. Two-fold higher β -galactosidase activity was found in cells incubated with AI-2 relative to those with LsrK treated AI-2 (Figure 2-4, panels a and b). The β -galactosidase activity of the latter was similar to controls (without AI-2; data not shown).

To more accurately quantify the cell response, β -galactosidase activity was assayed and was reduced from 60-70 Miller units in the presence of AI-2 to 10-20 Miller units in the 1 h LsrK treated AI-2 samples, and to only 7 Miller units for the 16 h samples (Figure 2-4, panel c). The results confirm that the *lsr* expression is significantly higher when AI-2 is transported into the cell and phosphorylated internally by LsrK^{44,143}. In the case when LsrK is external to the cell and provided ATP, the resultant phospho-AI-2 is unable to stimulate the QS response (*lsr* gene

expression). This is presumably due to the prevention of AI-2 transport into the cell after phosphorylation.

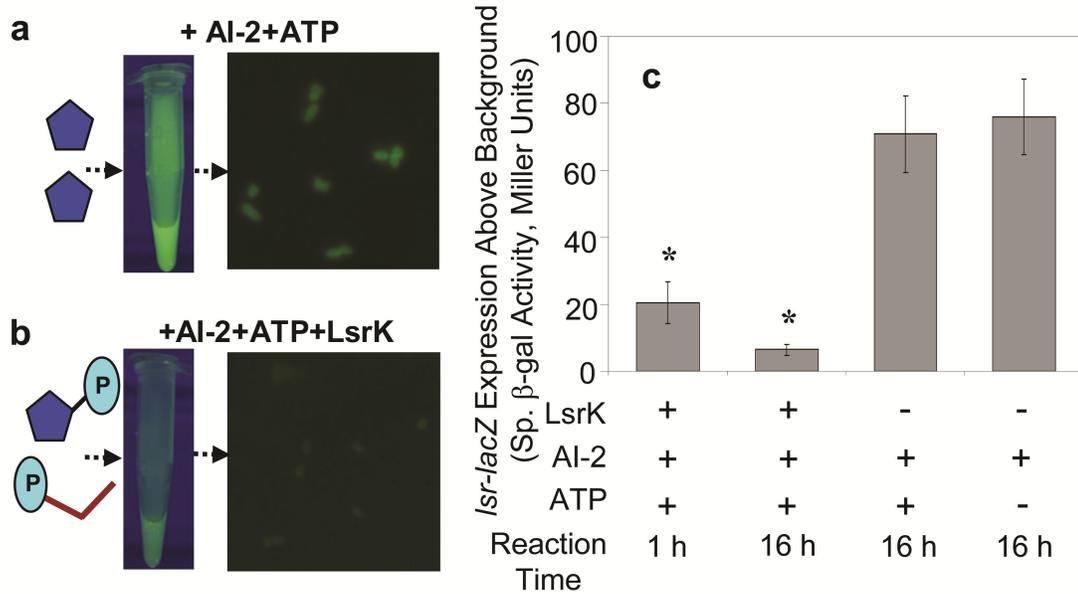


Figure 2-4 LsrK treated AI-2 “quenches” QS response in *E. coli*.

AI-2 dependent β -galactosidase production in *E. coli* LW7 pLW11 in response to a) AI-2 and ATP and b) AI-2, ATP and LsrK recorded via a fluorescent reporter - fluorescein di- β -D-galactopyranoside (FDG) c. AI-2 dependent β -galactosidase production in the same reporter in response to combinations of AI-2, ATP and LsrK and differing reaction times, quantified by colorimetric reporter o-nitrophenyl- β -D-galactoside (ONPG) (* indicates $p < 0.05$ for an unpaired t test of the experimental sample compared to the native response with AI-2 and ATP).

2.4.3 Quorum Quenching in *E. coli* Populations

In order to investigate the effect of LsrK treated AI-2 on the *lsr* expression in a cell population, LsrK treated AI-2 was added to *E. coli* MDAI2 pCT6 pET-GFP

(Table 2-1) and assayed using flow cytometry. This strain is a *luxS* mutant and produces GFP in response to AI-2¹³⁶. Experiments were performed analogously to those in Figure 2-4. Eight hours after AI-2 addition, 33% of the cell population was found to be GFP positive (Figure 2-5, panel a). Conversely, addition of LsrK treated AI-2 showed that only 1 % of the population expressed GFP, (Figure 2-5, panel b). These findings demonstrate that the QS response was quenched in the entire population. By extension, LsrK treated AI-2 enables a phenotypic “switching” behavior in the population in that an individual cell’s response could be turned “off” from “on”.

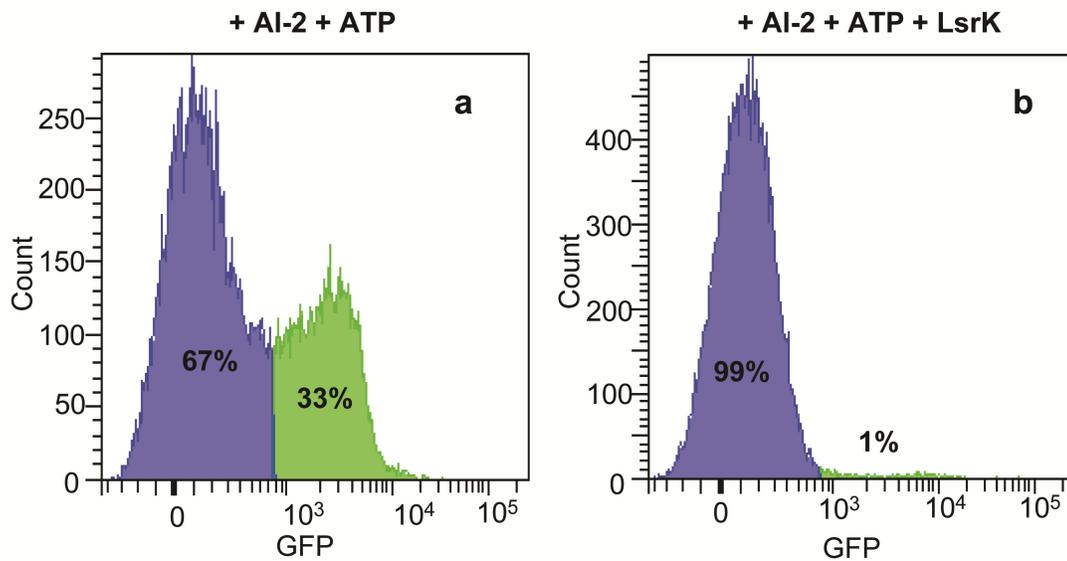


Figure 2-5 LsrK treated AI-2 “quenches” the population-wide QS response in *E. coli*.

AI-2 dependent GFP production in *E. coli* MDAI-2 pCT6 in response to a) AI-2 and ATP or b) AI-2, ATP and LsrK, measured by flow cytometry.

2.4.4 Cross Species Quenching of *lsr* Expression

Since AI-2 is presumed to be a ‘universal’ signaling molecule¹⁰, it is hypothesized that the degradation of AI-2 should quench QS expression across species and among species in mixed populations. A study was conducted to investigate the effect of LsrK treated AI-2 on two other species of QS bacteria: *S. typhimurium* and *V. harveyi*. *S. typhimurium* was selected as its AI-2 uptake and processing mechanisms are similar to that of *E. coli*⁴³ while *V. harveyi* is the most commonly used AI-2 reporter. Also, the AI-2 uptake and processing machinery in *V. harveyi* is significantly different from that of *E. coli* and *S. typhimurium* in that the signal is transduced through a protein kinase cascade and an RNA chaperone to a luminescence reporter²⁴.

In vitro AI-2 is catalyzed using *E. coli* AI-2 synthases (40), so the effects on *S. typhimurium* and *V. harveyi* are in response to *E. coli* derived AI-2. The *S. typhimurium* MET715 strain used in the study (Table 2-1) is a *luxS* mutant containing *lacZ* expressed from the *lsr* promoter⁴³. Analogous to *E. coli* experiments (Figure 2-4, panel b) LsrK treated AI-2 (1 h and 16 h) was incubated with pure cultures of *S. typhimurium* MET715. We observed a 17-fold and 100-fold reduction in response in the 1 h and 16 h samples respectively relative to the controls (Figure 2-6, panel a). The reduction in *S. typhimurium lsr* response was far more dramatic than that observed in *E. coli*. We speculate that in addition to restricted transport of phospho-AI-2, this could be due sequestration of the linearized form of the AI-2 isomers and, correspondingly, the complete elimination of the identified “active” form for *S. typhimurium*. That is, phospho-AI-2 is derived from phosphorylation of the terminal

hydroxyl group on DPD⁴⁶ so that DPD cannot cyclize to (2R,4S)-2-methyl-2,3,3,4 tetrahydroxytetrahydrofuran (R-THMF), the confirmed signaling molecule transduced by *S. typhimurium*⁴¹. Also, the distinct chemical form of the signal molecule that binds to the *E. coli* AI-2 transporter (or otherwise is transported into the cells), has not been reported thus there is ambiguity in the forms of DPD that can enter *E. coli* through the AI-2 transporter. Similar decreases in AI-2 based response (bioluminescence) were observed for *V. harveyi* incubated with LsrK treated AI-2 (figure 5, panel b). Unlike *S. typhimurium* or *E. coli* used in our study, *V. harveyi* BB170 (Table 2-1) is *luxS*⁺, however, the observed luminescence represents a response elicited by the *ex vivo* addition of the *in vitro* AI-2 or phospho-AI-2 as BB170 cells typically produce their own AI-2 at a later time; typically after 4 h of growth. The phospho-DPD, as explained above in the case of *S. typhimurium*, is also prevented from cyclizing into its known boronated AI-2 signaling molecule⁴⁷.

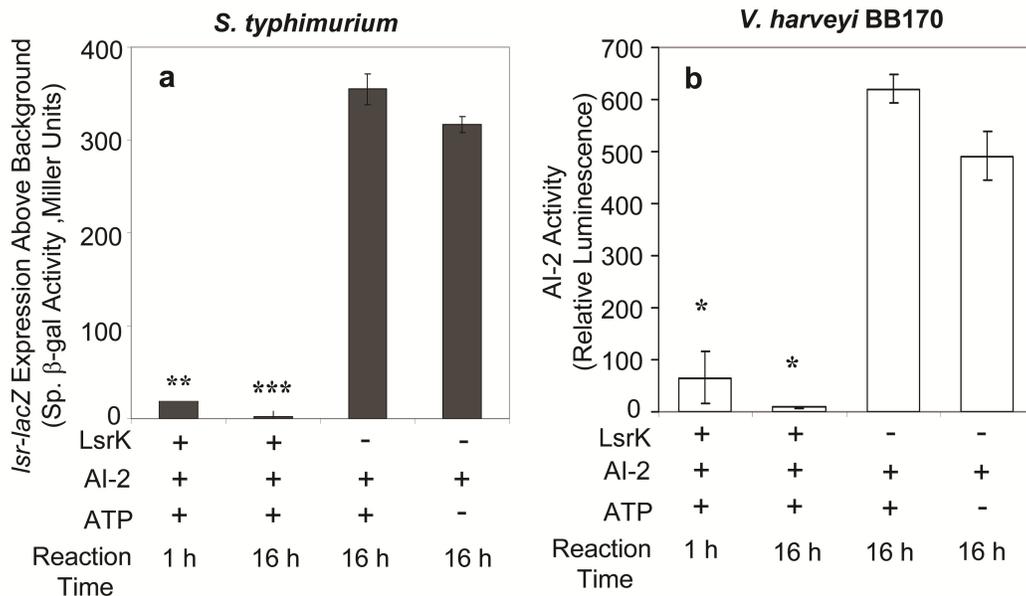


Figure 2-6 LsrK treated AI-2 “quenches” the QS response in *V. harveyi* and *S. typhimurium*.

- a) AI-2 dependent β -galactosidase production in *S. typhimurium* MET715 in response to *in vitro* AI-2 treated with ATP and LsrK (** indicates $p < 0.01$ and *** indicates $p < 0.001$ for an unpaired t test as compared to the native response with AI-2 and ATP).
- b) AI-2 dependent bioluminescence production in *V. harveyi* BB170 in response to *in vitro* AI-2 treated with ATP and LsrK (* indicates $p < 0.05$ for an unpaired t test as compared to the native response with AI-2 and ATP).

2.4.5 Quorum Quenching in a Synthetic Ecosystem

Bacteria rarely grow in isolated, pure cultures in a host organism or a natural environment. Therefore, to investigate the effects of LsrK treated AI-2 in a mixed bacterial environment; we assembled a synthetic ecosystem composed of three bacterial populations. Specifically, *S. typhimurium* MET715, *V. harveyi* MM30 and *E. coli* MDAI-2 (pCT6), each *luxS*⁻, were co-cultured in the same tubes. Under these conditions, the “native” and “quenched” responses were entirely due to *in vitro* addition of AI-2 to the cells. As each species contained a different QS reporter, β -galactosidase (MET715), bioluminescence (MM30) and GFP (MDAI-2), their responses could be differentiated.

S. typhimurium β -galactosidase activity decreased 10-fold, while *V. harveyi* luminescence dropped 2-fold (Figure 2-7, panels a and b), as compared to untreated AI-2 controls. The *E. coli* GFP response in co-culture was analyzed by flow cytometry and 12% of the *E. coli* population was GFP positive in the presence of untreated AI-2, while only 3% of the population was GFP positive in response to

LsrK treated AI-2 (Figure 2-7, panel c). The GFP positive population in co-culture was lower than that seen in the pure culture (12% vs. 33%). Direct correlation of co-culture results to pure culture is not straightforward due to many factors including; different growth media (LM¹⁸ vs LB), specific uptake of AI-2 at different rates by *S. typhimurium* and *V. harveyi*, also due to other potentially nonspecific effectors secreted or taken up by these cells. Our results clearly demonstrate that LsrK treated AI-2 resulted in an attenuated QS response from all three bacteria in the co-cultured synthetic ecosystem. We note, however, that the reduction was less dramatic in the synthetic ecosystem than in the pure cultures. In the case of *V. harveyi*, we used MM30 in the ecosystem and BB170 in the pure cultures; MM30 responds to endogenously synthesized AI-1¹³⁹, while BB170 responds to its own AI-2, both by bioluminescence. Hence any reduction in the *V. harveyi* response was viewed as significant.

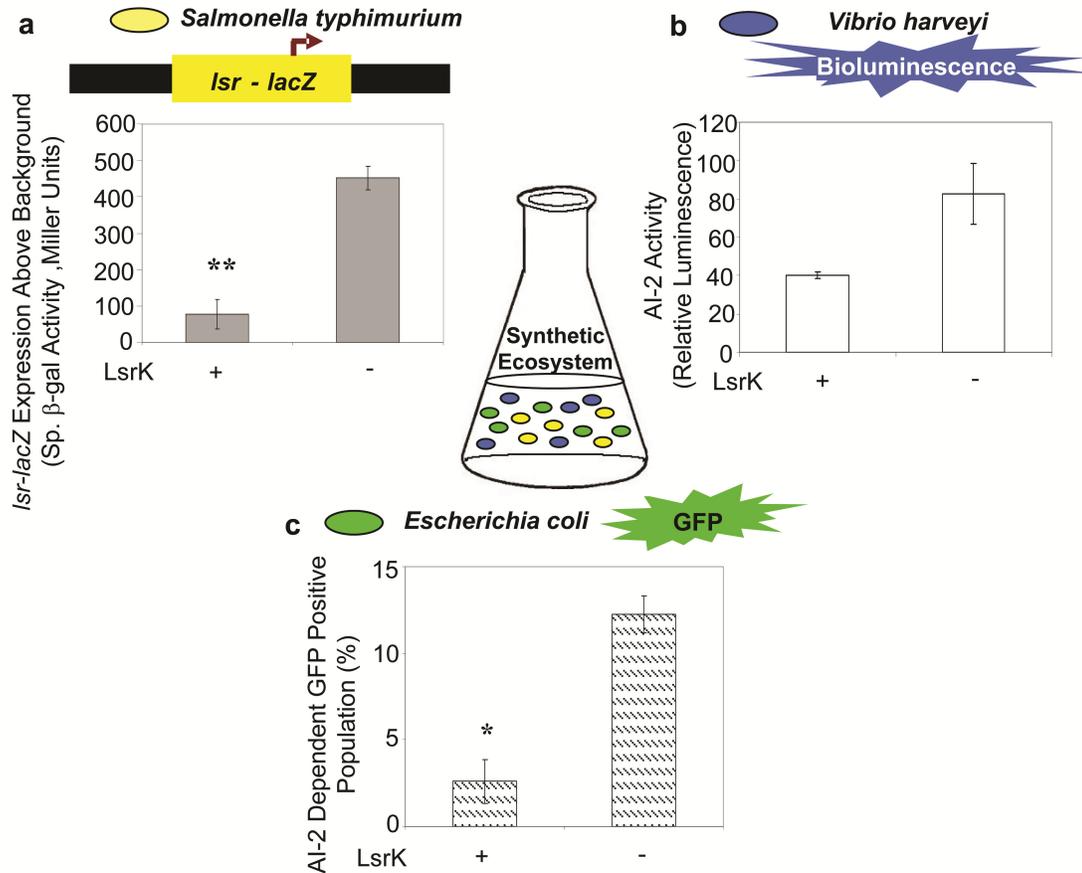


Figure 2-7 LsrK treated AI-2 quenches the QS response in a tri-species synthetic ecosystem.

a) AI-2 dependent β -galactosidase production in *S. typhimurium* MET715 (** indicates $p < 0.01$ for an unpaired t test as compared to the native response with AI-2 and ATP), b) AI-2 dependent bioluminescence production in *V. harveyi* MM30 and c) AI-2 dependent GFP induction in *E. coli* MDAI-2 pCT6 (* indicates $p < 0.05$ for an unpaired t test as compared to the native response with AI-2 and ATP) in response to LsrK treated AI-2 in a co-culture of the three species.

2.4.6 Quenching Bacterial Cross-talk

The results demonstrated thus far, show that quorum “quenching” was facilitated by *in vitro* LsrK phosphorylation of AI-2 on various *luxS*⁻ bacterial species. In order to better simulate the natural condition, the native QS response of a wild-type (*luxS*⁺) mixed bacterial population was quenched by LsrK and ATP added *in vitro*. In order to test bacterial cross-talk where one bacterial species responds to AI-2 produced by another species, gut consortia *E. coli* ZK126 (*luxS*⁺, *lacZ*) and *S. typhimurium* MET715 (*luxS*, *lacZ*⁺) were co-cultured and assayed (Table 2-1). The presence of *lacZ* expression indicates a positive response in *S. typhimurium* to AI-2 produced by *E. coli* (Figure 2-8, panel a). As shown in (Figure 2-8, panel b), *S. typhimurium* exhibited significant β -galactosidase activity in response to *E. coli* AI-2, and the response decreases significantly with increasing concentrations of LsrK added *ex vivo* to the bacterial populations (see Methods). That is, addition of 5 μ M LsrK “quenched” the “native” QS response by 3-fold relative to the control (no LsrK).

As demonstrated by our results for LsrK phosphorylation, we hypothesize that LsrK phosphorylated AI-2 that had been secreted from *E. coli* and the product of this reaction (phospho-DPD) degraded to PG, or was otherwise unable to enter the *S. typhimurium* cells and produce a response. Since QS responses are dependent on AI-2 in the environment after having reached a “threshold” concentration, this study suggests the addition of LsrK sufficiently sequesters the native AI-2 so that the “threshold” concentration is not achieved. Hence, a potentially pathogenic phenotype may be prevented.

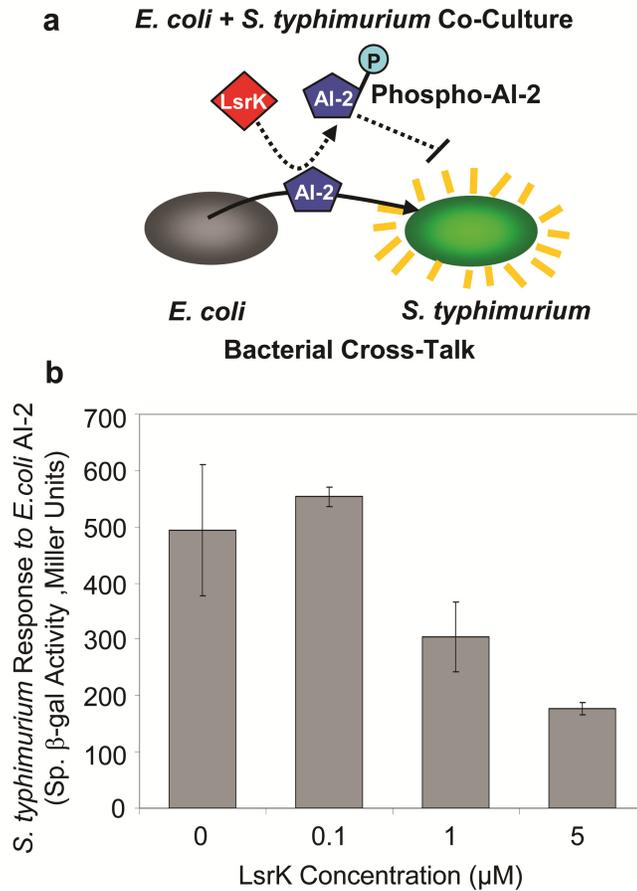


Figure 2-8 Addition of LsrK and ATP “quenches” AI-2 signal generated by *E. coli* and detected by *S. typhimurium* in a co-culture.

a) *S. typhimurium* produces β-galactosidase in response to AI-2 secreted by *E. coli* in the environment; LsrK sequesters the AI-2 in a phosphorylated form and prevents the *S. typhimurium* response. b. AI-2 dependent β-galactosidase production in *S. typhimurium* MET715 in response to increasing concentrations of LsrK added to the co-culture of 1:1 *E. coli* : *S. typhimurium* (* indicates $p < 0.05$ for an unpaired t test as compared to the control with no LsrK).

2.5 Conclusion

This investigation provides the basis for AI-2 quorum quenching using the native bacterial phosphorylation machinery. LsrK is typically viewed as the QS kinase for phospho-AI-2 generation, which, in turn, is required to switch “on” *lsr*-regulated gene expression in *E. coli* and *S. typhimurium* QS circuits. Instead, we provide an alternative view of LsrK in which it functions outside of cells to switch “off” *lsr*-regulated gene expression. LsrK based quorum quenching modulates responses in pure cultures of *E. coli*, *S. typhimurium* and *V. harveyi* as well as in a tri-species synthetic ecosystem. Moreover, LsrK based quorum quenching was observed in a simulated *in vivo* environment wherein LsrK (and ATP) were added to co-cultures of AI-2 signal generating *E. coli* and AI-2 detecting *S. typhimurium*. Thus, we have demonstrated intraspecies and interspecies quenching. This suggests the utility of LsrK as a broad range “quorum quencher” in a variety of applications. For example, we anticipate this advance will enable a more mechanistic understanding of cell-cell communication within mixed cultures where competing signals from a variety of bacteria are synthesized and perceived in an otherwise undefined network. That is, specific interruption of one signal molecule within a network of signaling stimuli will help to elucidate its role in relation to others as the population “phenotype” is being determined. Since the levels of DPD needed to affect the biofilm phenotype in natural mixed culture communities is significantly lower (0.08 nM – 8 nM)¹⁴⁴ than the levels tested here, one might this approach would be feasible and result in “inactivated” DPD. Therefore anticipated biomedical applications may include its incorporation into an antimicrobial therapy by encapsulation with ATP and

delivery to sites of infection and its use as a modulator of phenotype in environments prone to biofilm formation.

2.6 Acknowledgements

The authors would like to acknowledge the advice provided by H. O. Sintim in understanding the significance of the mixed culture studies; B. Bassler for generously providing *S. typhimurium* MET715; and T. Dunn and H.-C. Wu for their help in conducting the flow cytometry studies. Funding for this work was provided by the National Science Foundation and the R.W Deutsch Foundation.

Chapter 3: Synthetic Analogs Tailor Native AI-2 Signaling Across Bacterial Species

3.1 Abstract

The widespread use of antibiotics and the emergence of resistant strains call for new approaches to treat bacterial infection. Bacterial cell-cell communication or “quorum sensing” (QS) is mediated by “signatures” of small molecules that represent targets for “quenching” communication and avoiding virulent phenotypes. Only a handful of small molecules that antagonize the action of the “universal” autoinducer, AI-2, have been reported. The biological basis of antagonism as well as the targets for these select few AI-2 antagonists, have not been clearly defined. We have developed C-1 alkyl analogs of AI-2 that quench the QS response in multiple bacterial species simultaneously. We also demonstrate the biological basis for this action. Like AI-2, the analogs are activated by the bacterial kinase, LsrK, and modulate AI-2 specific gene transcription through transcriptional regulator, LsrR. Interestingly, addition of a single carbon to the C1-alkyl chain of the analog plays a crucial role in determining the effect of the analog on the QS response. While an ethyl modified analog is an agonist; propyl becomes an antagonist of the QS circuit. In a tri-species synthetic ecosystem comprised of *E. coli*, *S. typhimurium* and *V. harveyi* we discovered both cross-species and species-specific anti-AI-2 QS activities. Our results suggest entirely new modalities for interrupting or tailoring the network of communication among bacteria.

3.2 Introduction

Quorum sensing (QS) is a bacterial cell-cell communication system that regulates diverse phenotypes including motility, attachment, biofilm formation, and pathogenicity by the secretion and perception of small signal molecules. QS receptors, transporters, regulators, and the signals themselves (also known as autoinducers), represent a vast reservoir of targets that can be manipulated to interrupt communication^{74, 120, 145}. QS effector molecules that function to eliminate pathogenicity but otherwise remain innocuous to the cells have received significant attention. They presumably pose less evolutionary pressure on bacteria than current bacteriostatic or bacteriocidal antimicrobials that drive mutation and the emergence of drug resistant strains^{9,146, 147}. Anti-QS agents, used in combination with antibiotics, have already shown promise in clearing recalcitrant *Pseudomonas aeruginosa* biofilms¹⁴⁸. Additional approaches that “quench” QS communication will build on the emerging understanding of QS circuitry, its signal transduction process, and the sender-receiver relationship that connects one bacterium to another or to a population.

The autoinducer signaling molecules are organized into families based on their structure and mode of action. Autoinducers-1 (AI-1) such as acyl homoserine lactones (AHLs) mediate *intra*-species communication¹⁴⁹, whereas a family of cyclic furanones, collectively termed autoinducer-2 (AI-2), are utilized in *interspecies* bacterial communication¹⁰. Interestingly, both AI-1 and AI-2 families of autoinducers are derived from S-adenosylmethionine (SAM)¹⁵⁰, as part of bacterial 1-carbon metabolism. The AI-2 precursor 4, 5-dihydroxy-2, 3-pentanedione (DPD) is synthesized from S-adenosylhomocysteine (SAH) via a two-step enzymatic process

catalyzed by methylthioadenosine (MTA) nucleosidase (Pfs) and S-ribosylhomocysteinase (LuxS). Synthase inhibitors can potentially alter both AI-1 and AI-2 signaling by disrupting signal generation⁷⁵. Also, a large and diverse population of bacteria could be addressed with AI-2 inhibitors as LuxS is present in over 70 bacterial species¹⁷. Correspondingly an alternative and equally important strategy is to disrupt AI-2 QS signaling by interfering with the signal reception and transduction processes.

Thus far, the majority of these quorum “quenching” strategies have focused on developing inhibitors of AI-1 (AHL) signaling^{89, 151}, which are mostly species-specific. The ubiquitous nature of AI-2, however, opens avenues for broadly inhibiting or modulating QS communication as AI-2 modulates the behavior of many microbes including *Bacillus anthracis*¹⁵², *V. cholerae*¹³ and *E. coli* O157^{153,154} and many others. Few small molecule antagonists of AI-2 quorum sensing have been reported, however, and the molecular targets for these have not been clearly defined. Identifying these targets will lead to new inhibitor families and new strategies for treating QS modulated disease. In enteric bacteria, including pathogenic *E. coli* O157 and *S. enterica* Serovar Typhimurium, a small number of AI-2-regulated genes and proteins are known – they are denoted LxS-regulated (*lsr*). AI-2 is transported into these cells by an ABC cassette-like transporter (Lsr)¹⁵⁵, followed by phosphorylation via a kinase LsrK^{135,156}. The phosphorylated AI-2 binds to and de-represses the transcriptional regulator (LsrR), releasing it from the *lsr* (*luxS* regulated) operator, allowing transcription of the genes encoding the aforementioned AI-2 processing proteins⁴³. LsrR has been shown to play an important role in *E. coli* biofilm

maturation⁷¹, as well as regulating the expression of over 68 proteins in *E. coli*, including important virulence determinants⁴⁴. LsrR-like proteins are therefore important targets for the development of small molecule quorum sensing modulators¹⁵⁷. By targeting AI-2: Lsr interactions we have attempted to modulate QS circuitry in a variety of bacterial species.

Herein we identify, through focused chemical library design and biological and biochemical studies, the key structural components of AI-2 that are important for the development of a potent, broad-spectrum anti-quorum sensing agent. We synthesized C1-alkyl analogs of 4,5-dihydroxy-2,3-pentadione (DPD), which is the precursor of the universal autoinducer AI-2. DPD is the linear form of AI-2 and it exists in equilibrium with several interconvertible isoforms in solution, all of which are known as the family of AI-2 signaling molecules. This panel of C1-alkyl AI-2 analogs, permits detailed investigation of uptake, phosphorylation and altered AI-2 mediated gene transcription in several bacteria. Our work suggests that the antagonistic activity of C1-alkyl analogs of AI-2 is likely due to competitive binding to the LsrR transcriptional regulator. We demonstrate that, unlike other bacterial kinases^{158, 159}, LsrK from *E. coli* has broad substrate specificity and phosphorylates C1-alkyl AI-2 analogs of different shapes and sizes. In *E. coli*, the majority of the phosphorylated AI-2 analogs compete with phospho-AI-2 for binding to LsrR. Interestingly, lower alkyl chain AI-2 analogs (C1 and C2) destabilize the LsrR-DNA complex and promote *lsr* transcription whereas higher alkyl chain AI-2 analogs (C3-C7) stabilize the Lsr-DNA complex and inhibit *lsr* transcription. The Lsr proteins of *S. typhimurium* and *E. coli* share high sequence and predicted structural homologies,

yet the majority of AI-2 analogs that inhibited *lsr* expression in *E. coli* failed to do so in *S. typhimurium*; demonstrating both flexibility and specificity in QS circuitry. Thus our analogs differentiate between highly homologous quorum responses and represent tools for controlling QS systems with an additional level of selectivity and finesse. This current work therefore demonstrates for the first time that the nature of the C1-alkyl chain of AI-2 analogs may be used to either quench QS in a variety of bacteria (broad-spectrum anti-QS) or in selected or targeted^{160,161} bacteria. The ability to modulate QS *en masse* in a tri-species synthetic ecosystem or selectively has important clinical implications. For example, in the gut, the microflora is composed of various non-pathogenic and mutualistic bacteria that also utilize AI-2 signaling for non-pathogenic processes^{162,83}. Therefore, specifically targeting pathogenic bacterial species such as enterohemorrhagic *E. coli*, but not the symbionts will allow the useful bacteria to coordinate their behavior in an efficient manner, whereas the targeted pathogenic species will be out-of-sync and hence engage in “out-of-quorum” behaviors that might be evolutionarily disadvantageous.

3.3 Materials and Methods

Synthesis of Diazodiols

DBU (0.16-0.20 eq) and the requisite aldehyde (2-(*tert*-butyldimethylsilyloxy) acetaldehyde or acetaldehyde) (1-1.5 eq) were added to a solution of the diazocarbonyl in anhydrous acetonitrile (0.2 M). The reaction was stirred at room temperature under nitrogen for 4 - 8 hours and monitored by TLC. Upon disappearance of starting material, the reaction was quenched with sodium

bicarbonate. The organic layer was extracted with dichloromethane (3 x 20 mL) and dried with magnesium sulfate. The solvent was evaporated under reduced pressure. To a solution of crude product in anhydrous tetrahydrofuran (0.2M) TBAF was added (1-2 eq) at 0 °C. The solution was allowed to warm to room temperature and stirred for 1-3 hours under nitrogen. The solvent was evaporated and the crude product was purified by column chromatography. The product eluted as yellow oil with 1:3 to 3:2 ethyl acetate:hexane.

Synthesis of DPDs

Dioxirane (15-20 mL) in acetone was added dropwise to a solution of diazodiol (1 eq) in acetone (1-2 mL). The reaction was allowed to stir at room temperature (1-2 hrs) until complete disappearance of starting material was indicated by TLC (loss of UV activity). Solvent and excess reagent was evaporated under reduced pressure. NMR was taken without further purification.

Synthesis of Quinoxaline Derivatives

1, 2-phenylenediamine (1.5 eq) was added to a solution of DPD-analog. The reaction was stirred at room temperature for 10 minutes and then the reaction mixture was washed with (2M) HCl. The crude mixture was purified on silica.

Bacterial Strains and Growth Conditions

Table 3-1 lists the bacterial strains and plasmids used in this study. *S. typhimurium* and *E. coli* strains were cultured in Luria-Bertani medium (LB, Sigma)

at either 30 °C or 37 °C with vigorous shaking (250 rpm) unless otherwise noted. The *V. harveyi* strains were grown in LM medium. Antibiotics were used for the following strains: (60 or 100 µg ml⁻¹) kanamycin for *S. typhimurium* MET715, (50 µg ml⁻¹) ampicillin for *E. coli* BL21 *luxS*⁻, (60 or 100 µg ml⁻¹) ampicillin for *E. coli* LW7 pLW11. (50 µg ml⁻¹) ampicillin and (50 µg ml⁻¹) kanamycin for *E. coli* MDAI-2 pCT6 and *E. coli* SH3 pLW11 along with (20µg ml⁻¹) chloramphenicol for the latter and (20 µg ml⁻¹) kanamycin for *V. harveyi* BB170.

***In vitro* Phosphorylation of Analogs**

LsrK was purified from *E. coli* BL21 pET200-LsrK as described before¹⁵⁶. Phosphorylated analogs were synthesized by incubating (1 µM) LsrK with (40 µM) ATP (Roche), (0.2 Ci) of [P³²] ATP (Perkin-Elmer), (300 µM) AI-2, (200 µM) MgCl₂ in (25 mM) phosphate buffer, pH 7.4 for 2 hours. A (2.5µL) aliquot was then spotted onto a cellulose TLC plate (Selecto Scientific). The plate was developed using (0.8 M) LiCl as the solvent, air dried and developed via autoradiography.

Measurement of the QS Response (*lsr* expression)

The QS response indicated by *lsr* gene expression was analyzed in pure culture studies by culturing *E. coli* LW7 pLW11, *E. coli* ZK126 pLW11 and *S. typhimurium* MET708, *S. typhimurium* MET715 overnight in LB medium supplemented with appropriate antibiotics as stated previously. These cells were then diluted into fresh LB medium (with antibiotics) and grown to an OD₆₀₀ of 0.8 - 1.0 at 30 °C, 250 rpm. Cells were then collected by centrifugation at 10,000 xg for 10

minutes, and resuspended in 10 mM phosphate buffer. AI-2 (20 μ M) and the respective analog (20 μ M) were added to the *E. coli* or *S. typhimurium* suspension for 2 hours at 37 °C. AI-2 dependent β -galactosidase production was quantified by the Miller assay¹⁴¹.

Measurement of the QS Response (Bioluminescence)

The effect of isobutyl-DPD or isopropyl-DPD on QS associated bioluminescence production by *V. harveyi* was recorded by measuring the light production from the reporter strain, *V. harveyi* BB170. The analogs were added at concentrations of (20 μ M or 40 μ M) to *V. harveyi* BB170 which was assayed as described previously¹⁸.

Analyzing QS Response in the Synthetic Ecosystem

The *S. typhimurium* MET708, *V. harveyi* BB170 and *E. coli* W3110 pCT6 were each cultured separately overnight in LM medium¹⁸ supplemented with the appropriate antibiotic. *V. harveyi* BB170, *S. typhimurium* MET708 and *E. coli* MDAI-2 were diluted (1:4:8), respectively, into a single 1 ml volume of fresh LM medium without antibiotics. The co-culture was supplemented with either (20 μ M) or (40 μ M) analog initially and again after 3 and 5 hours of growth. The *V. harveyi* luminescence response was measured after 2.5 hours. The *S. typhimurium lacZ* (β -galactosidase) activity was measured after 4 hours. The *E. coli* response was determined after 8 hours, by fixing the cells with 1:1 cold 4 % paraformaldehyde and

using flow cytometric analysis. Samples were analyzed by flow cytometry (FACS Canto II, BD 394 Biosciences), with 20,000 gated events analyzed per sample.

Table 3-1 List of bacterial strains, plasmids and primers used in this study.

Strain, plasmid or primer	Relevant genotype and/or property	Reference
<i>Escherichia coli</i> strains		
W3110	Wild type	Laboratory Stock
BL21 <i>luxS</i>	<i>F'</i> ompT hsdS _B (<i>r_B⁻m_B⁻</i>) <i>gal dcm ΔluxS</i> :: Kan	Laboratory Stock
LW7	W3110 <i>ΔlacUI60-tna2 ΔluxS</i> :: Kan	135
ZK126	W3110 <i>ΔlacUI69-tna2</i>	137
LW9	ZK126 <i>Δ(lsrACDBFG)::Kan</i>	135
SH3	W3110 <i>ΔlacUI60-tna2 ΔluxS ΔlsrK</i> :: Kan; Cm	Laboratory Stock
LW8	ZK126 <i>_lsrR::Kan</i>	135
<i>Salmonella typhimurium</i> strains		
MET715	<i>rpsl putRA</i> :: Kan- <i>lsr-lacZYA luxS</i> :: T-POP	42
MET708	<i>rpsl putRA</i> :: Kan- <i>lsr-lacZYA</i>	42
<i>V. harveyi</i> strains		
BB170	BB120 <i>luxN</i> :: Tn5 (sensor 1 ⁻ , sensor 2 ⁺) ; AI-1 ⁺ , AI-2 ⁺	138
Plasmids		
pLsrK	pET200 derivative, <i>Escherichia coli</i> W3110 LsrK ⁺	156
pLW11	<i>galK'-lacZYA</i> transcriptional fusion vector, containing <i>lsrACDBFG</i> promoter region, Amp ^r	135
pCT6	pFZY1 derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region fused with <i>T7RPol</i> , Ap ^r	163
pET-GFP	pET200 derivative, containing <i>gfpuv</i> , Km ^r	163

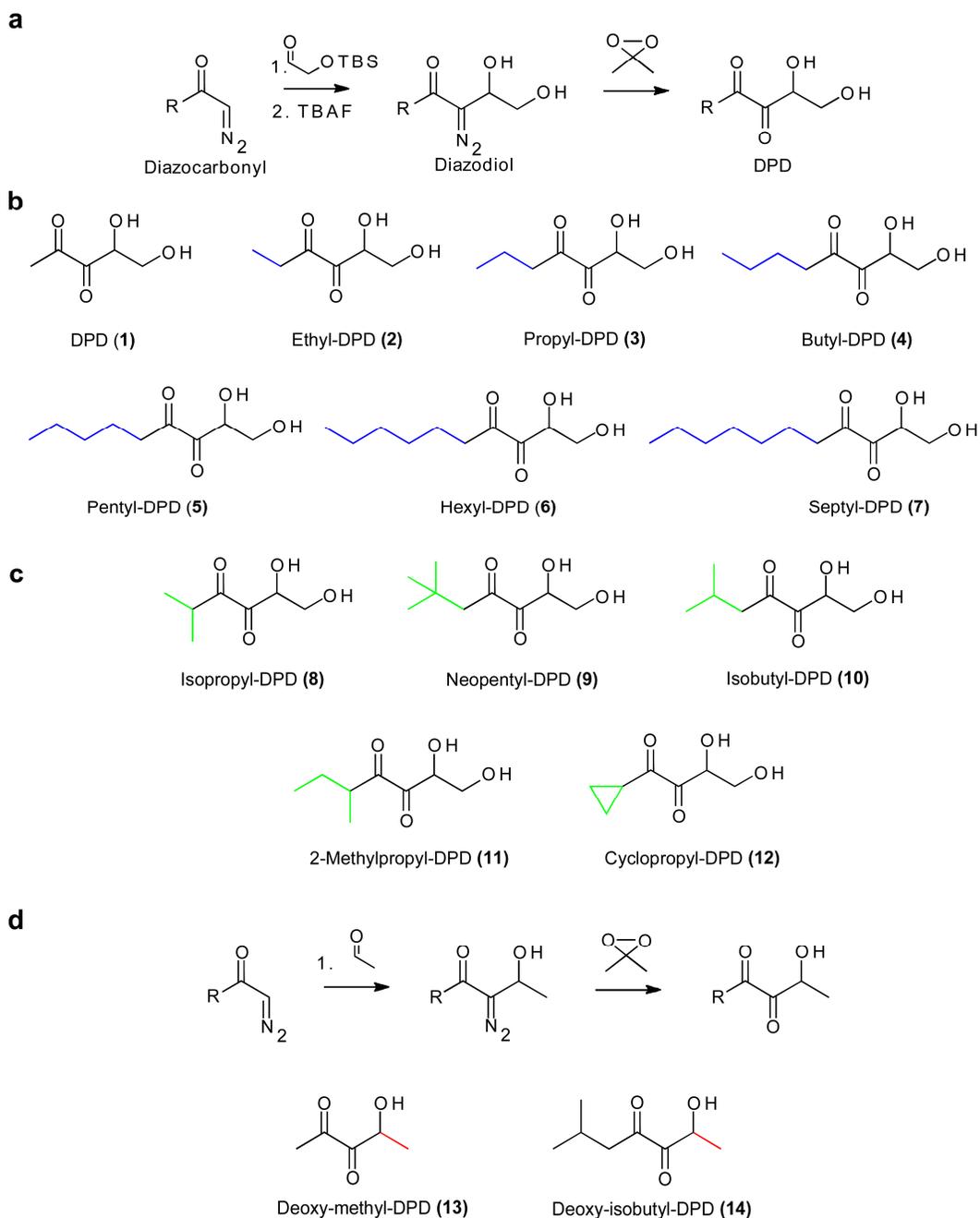


Figure 3-1 Library of 14 C1-alkyl analogs.

a) Synthesis strategy for linear and branched DPD analogs. b) Structures of the 7 linear DPD analogs. c) Structures of the 5 branched DPD analogs. d) Synthesis strategy and structures for the deoxy-DPD analogs.

3.4 Results

3.4.1 Synthesis of the C1-alkyl Analogs Panel

A panel of linear ((see Figure 3-1 b) 1-7) and non-linear DPD analogs ((see Figure 3-1c) 8-12) was prepared via a simple two-pot DPD synthesis⁹⁵. This expedient process uses diazocarbonyls as an umpolung for the dione (dicarbonyl) of DPD. Aliphatic diazocarbonyls were readily generated from acid chlorides and diazomethane. The various diazocarbonyls were then reacted with a silyl-protected oxo-aldehyde under mild, catalytic DBU-conditions. Without isolation of the resulting adduct, the silyl group was deprotected with TBAF and the diazodiols were oxidized with dimethyl dioxirane to afford DPD analogs.

Studies by Bassler and others^{46,164}, have indicated that phosphorylated DPD and not the unphosphorylated form, binds to LsrR to destabilize the LsrR/DNA complex. In order to verify that unphosphorylated byproducts of LsrK-mediated phosphorylation are not antagonists, we synthesized DPD analogs ((see Figure 3-1d) 13-14) that lacked the primary hydroxyl unit (site of phosphorylation). As these deoxy-DPD analogs cannot be phosphorylated, they serve to test whether the phosphate moiety of AI-2 analogs is important for *lsr* antagonism.

3.4.2 Identifying Quorum Quenchers in Enteric Bacteria

The panel of C1-alkyl analogs was screened to identify QS modulators in the enteric bacteria *E. coli* and *S. typhimurium*. We monitored transcription of the QS associated *lsr* operon, by using *lsr-lacZ* reporter strains (Table 3-1) which produce β -

galactosidase in response to added AI-2. In order to first establish if the analogs were agonists or antagonists in the QS circuit, the analogs were incubated with *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (see Supporting Information Figure 3-S1 a-b) both of which are *luxS*⁻. None of the analogs, except ethyl-DPD behaved as agonists for *lsr* expression. Previous studies investigating AI-2 antagonism in *P. aeruginosa*⁹⁷ or in *S. typhimurium*⁹⁴ were carried out using chemically synthesized AI-2 added to *luxS*⁻ cells. In order to simulate the natural scenario where wild-type cells produce and detect their own AI-2, our panel of C1-alkyl analogs were tested on *luxS*⁺ cells *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 (Table 3-1).

We observed that a minimum of three carbons in the C1 alkyl chain of DPD was required for QS antagonism; propyl-DPD, and all larger linear alkyl chain analogs tested (see Figure 3-2a) caused a significant knockdown in native *lsr* expression in *E. coli*. However, for the linear analogs, only butyl-DPD caused a considerable reduction in *lsr* expression in *S. typhimurium*. In the panel of non-linear DPD analogs (see Figure 3-2b), neopentyl-DPD, isopropyl-DPD and 2-methylpropyl-DPD caused substantial reduction of the *E. coli* QS response, but only isobutyl-DPD caused substantial reduction of the wild type *lsr* expression in both *E. coli* and *S. typhimurium* (see Figure 3-2b). The neopentyl, 2-methylpropyl and cyclopropyl QS analogs attenuated expression in *S. typhimurium*, but minimally. The deoxy-C1-analogs did not substantially reduce the QS response in either *E. coli* or *S. typhimurium* (see Figure 3-2b). The same trends were obtained by adding a 1:1 ratio of enzymatically synthesized AI-2 and DPD analogs to the *luxS* strains *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (see Supporting Information Figure 3-S2 a-b).

None of our tested DPD analogs were bacteriocidal or bacteriostatic as the growth of *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 growth in the presence and absence of the analogs were similar (see Supporting information Figure 3-S3 a-b).

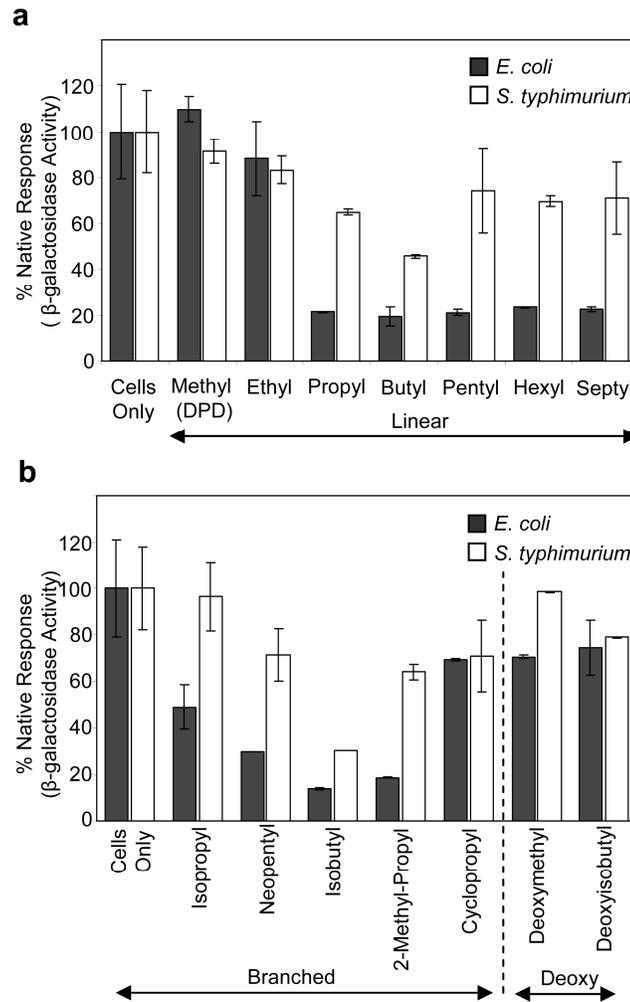


Figure 3-2 Analogs inhibit native signaling in *E. coli* and *S. typhimurium*.

AI-2 dependent β -galactosidase production in *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 both (*luxS*⁺) in response to a) linear analogs, b) branched and deoxy analogs. (100 % Native *E. coli* (ZK126) response = 1103 Miller units and 100 % native *S. typhimurium* (MET708) response = 4478 Miller units).

Interestingly, though the operon organization and processing proteins of the enteric organisms, *E. coli* and *S. typhimurium* are homologous they responded differently to the DPD analogs. The *E. coli* QS circuitry appears susceptible to silencing by a variety of DPD analogs of different shapes and sizes, whereas *S. typhimurium* was typically minimally affected (isobutyl-DPD being a noted exception). This suggests that the QS processing machinery in *E. coli* is flexible (or promiscuous) in processing different DPD analogs whereas that of *S. typhimurium* seems to be more specific. Isobutyl-DPD emerged as an effective and cross-species quencher; causing significant *lsr* expression knockdown in both *E. coli* and *S. typhimurium*.

3.4.3 Uptake and Phosphorylation of Analogs by *E. coli*

The decrease of *lsr* expression caused by the linear DPD analogs in *E. coli* seemed to be dependent on the length of the alkyl chain; a minimum C3 alkyl chain was required for significant *lsr* expression knockdown. We note that the AI-1 family of autoinducers, long chain homoserine lactones, are known to diffuse freely into the cells through the membrane and directly bind to cognate transcriptional regulators. The possibility that our C3+ AI-2 alkyl analogs might freely pass through the cells and function as antagonists was intriguing. Hence, we studied whether the *lsr* AI-2 transporter^{135,43} was necessary for analog transport by adding to *E. coli* LW9 pLW11 cells (Table 3-1) that lack the Lsr transporter. This strain also lacks phospho-AI-2 degradation enzymes, LsrG and LsrF.

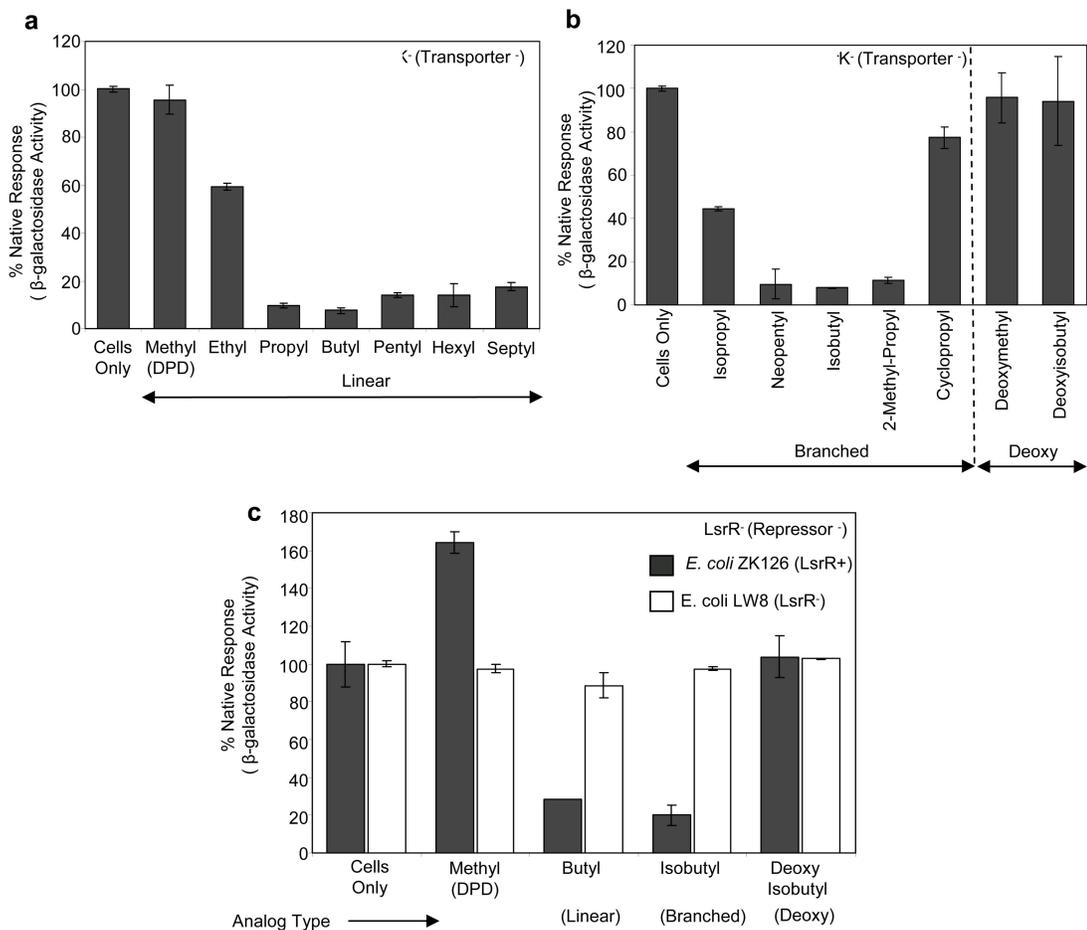


Figure 3-3 DPD Analogs inhibit native signaling in *E. coli* in the absence of the AI-2 transporter but only in the presence of the QS *lsr*-circuit repressor LsrR.

AI-2 dependent β -galactosidase production in *E. coli* LW9 pLW11 (*luxS*⁺) in response to a) linear analogs, b) branched and deoxy-DPD analogs c. AI-2 dependent β -galactosidase production in *E. coli* ZK126 pLW11 (*luxS*⁺, *lsrR*⁺) and LW8 pLW11 (*luxS*⁺, *lsrR*⁻) in response to a representative analogs from the linear branched and deoxy-analog categories (100 % Native *E. coli* (LW9) response = 3716 Miller units, 100% Native *E. coli* (ZK126) response = 1507 Miller units and 100% Native (LW8) response = 2883 miller units).

This strain is expected to be more sensitive for detecting the presence of processed AI-2 or analogs, than a strain containing both LsrF and LsrG, as the intracellular level may be higher. Results indicated nearly identical suppression for all analogs among both the Lsr⁻ and Lsr⁺ strains (e.g., transporter ⁻) (Compare Figure 3-2 a-b with Figure 3-3 a-b). There was apparently no difference due to the transporter. We had previously found that enzymatically synthesized AI-2 could enter *E. coli* and activate *lsr* transcription independent of the *lsr* transporter¹³⁵. Our new results demonstrate that AI-2 and synthesized analogs can enter bacteria via an alternative pathway; either through simple diffusion (e.g., enabled by “AI-1 like” alkyl chains) or as mediated by an unknown transporter. In order to elucidate the action pathway of the analog once inside the cells; we looked at the importance of the transcriptional repressor of the circuit LsrR and observed that the analog could not function in repressing *lsr* expression in the absence of LsrR. Thus LsrR seems to be pivotal for the action of an analog as an antagonist in the cell.

If the native AI-2 processing machinery were flexible, then analogs that are sufficiently “AI-2 like” could potentially be acted upon and “activated” for signal transduction. *In vivo*, the critical step that makes AI-2 functional in derepressing the *lsr* operon is phosphorylation by the kinase, LsrK. Thus, the ability of LsrK to phosphorylate the analogs was monitored by incubating the analogs with LsrK and an excess of ATP for 2 hours using a method adapted from Xavier *et al.*⁴⁶ All analogs, except the deoxy-C1-alkyl forms, were phosphorylated but to varied extents (see Figure 3-4 and Supporting Information Figures 3-S4 and 3-S5). A representative thin

layer chromatography (TLC) plate of DPD analog phosphorylation is shown in (see Figure 3-4), including an analog from each category (linear, branched and deoxy- C1-alkyl-DPD) and enzymatically-synthesized AI-2.

Radio-labeled phosphorylated DPD analogs have lower mobility than radio-labeled ATP, in agreement with Xavier *et al.*⁴⁶ The deoxy-C1-analogs remained unphosphorylated as the terminal hydroxyl required for DPD phosphorylation was absent; further confirming Bassler's earlier report that LsrK phosphorylates the primary and not the secondary hydroxyl unit of DPD⁴⁶. Interestingly, isobutyl-DPD was found to be a potent QS "quencher" and its unphosphorylated form, deoxy-isobutyl-DPD, silenced the *lsr* operon minimally as shown in (see Figure 3-2 a-b). This provides circumstantial evidence that phosphorylation of an analog is most likely essential for its role in QS inhibition.

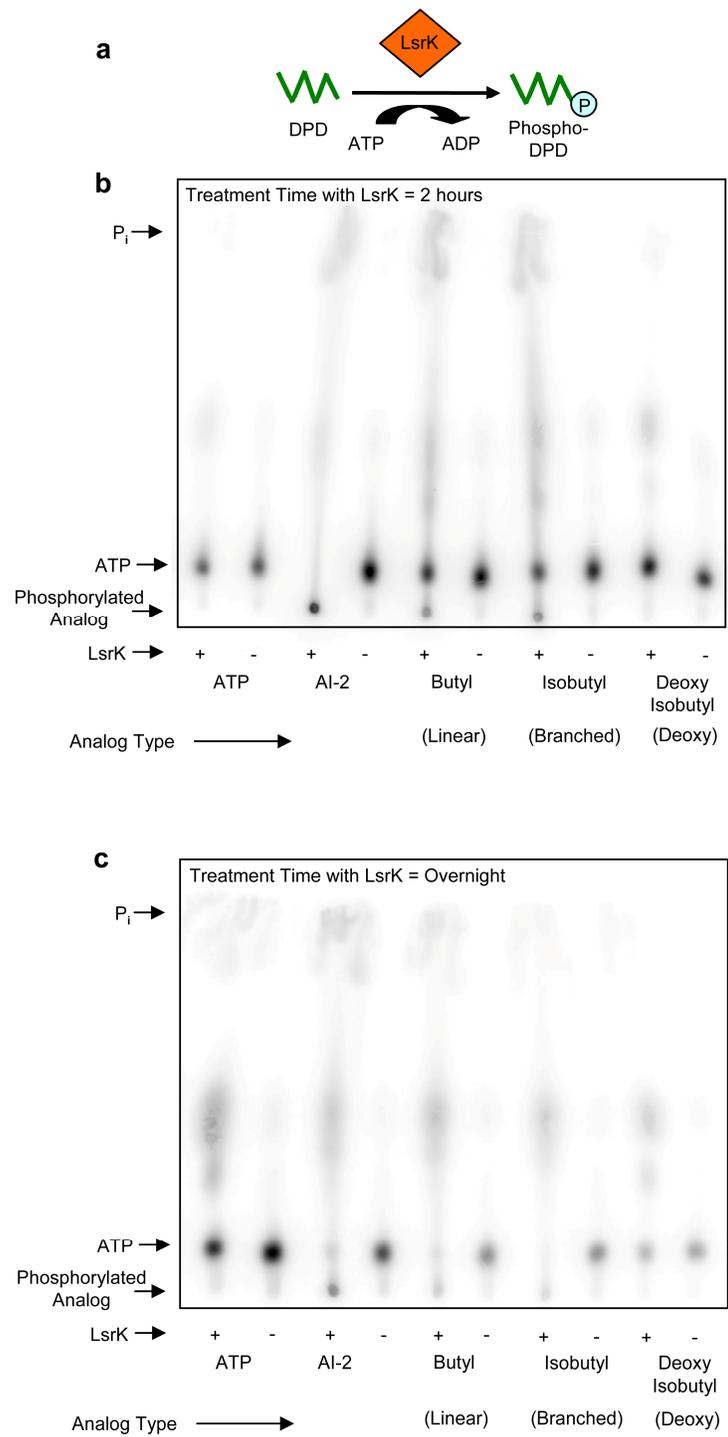


Figure 3-4 *In vitro* phosphorylation of analogs by LsrK.

a) Schematic of Phosphorylation of DPD by LsrK in the presence of ATP. Representative thin layer chromatography (TLC) analysis of the LsrK mediated analog phosphorylation. ATP, AI-2 (methyl-DPD), butyl-DPD, isobutyl- DPD and deoxy-isobutyl-DPD, treated with LsrK for b) 2hrs and c) overnight.

Phosphorylation of a DPD analog is, however, not the only determinant as to whether the analog can affect *lsr* expression. For example, cyclopropyl-DPD is phosphorylated by LsrK but is neither an *lsr* agonist nor antagonist. Significantly, isopropyl-DPD which has similar size and electrostatic molecular surface to cyclopropyl-DPD (see Figure 3-5 a-b) is both phosphorylated by LsrK and acts as a partial antagonist of *lsr* expression. Cyclopropyl-DPD is similar in size/shape to isopropyl-DPD but the hydrogen bonding capability of the carbonyl moiety in cyclopropyl-DPD is slightly different from that of isopropyl-DPD. The cyclopropyl substituent can stabilize the carbonyl moiety better than the isopropyl alkyl group. Density functional theory (DFT) calculations (see Figure 3-5 c-h) reveal that the highest occupied molecular orbital (HOMO) of both cyclopropyl- (HOMO-3) and isopropyl- (HOMO-4) DPD are close in energy to the lowest unoccupied molecular orbitals (LUMO) of the carbonyl moieties. However the cyclopropyl HOMO-3 overlaps better with the carbonyl LUMO as compared to the overlap between the isopropyl HOMO-4 and its carbonyl LUMO. These effects are subtle but the biological responses to these analogs are drastically different, suggesting that the carbonyl units in DPD analogs play an important role in modulating AI-2-based QS circuitry.

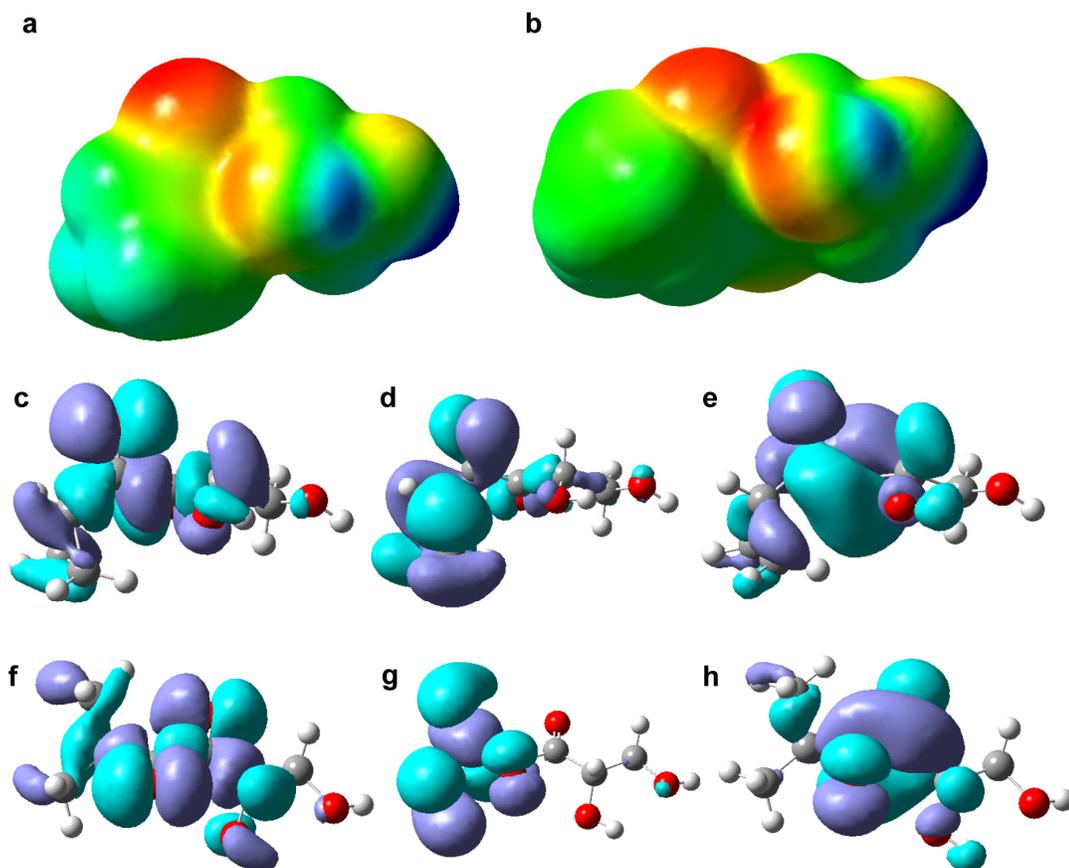


Figure 3-5 Electrostatic potential map.

Alkyl chain of cyclopropyl-DPD analog is slightly more electron deficient than isopropyl-DPD analog a. cyclopropyl-DPD b. isopropyl-DPD; Both cyclopropyl (HOMO-3 orbital) and i-Pr (HOMO-4 orbital) stabilize the adjacent carbonyl group (LUMO) via hyperconjugation. c) HOMO of cyclopropyl-DPD (-0.23 eV); d) HOMO-3 cyclopropyl-DPD (-0.30 eV); e) LUMO cyclopropyl (-0.06 eV); f) HOMO i-Pr-DPD (-0.24 eV); g) HOMO-4 i-Pr-DPD (-0.34 eV); h) LUMO i-Pr-DPD (-0.08 eV).

Ethyl-DPD also acts as a QS circuit agonist in *E. coli* but only in the presence of *in vivo* LsrK; in a LsrK knockout strain, ethyl-DPD did not initiate *lsr* transcription (see Supporting Information Figure 3-S6a). Neither AI-2 nor butyl or isobutyl- DPD could function in the absence of LsrK (see Supporting Information Figure 3-S6b). This suggests that the phosphorylation of DPD analogs by LsrK inside the bacterial cell is essential for them to effect the *lsr* QS circuit; either as agonists or antagonists. The phosphate moiety in phospho-AI-2 and analogs, therefore, seems important for binding to LsrR, but it appears that the stabilization of LsrR/DNA complex is governed by the C1 alkyl chain (C3 is the minimum requirement).

3.4.4 Cross-Species and Species-Specific Quorum Quenchers in a Synthetic Ecosystem

Bacteria, whether in their natural environments or during a host infection seldom grow in isolated pure cultures. Therefore, to investigate the effect of our DPD analogs in a mixed bacterial environment, a synthetic ecosystem composed of three different bacterial populations was assembled. Specifically, *S. typhimurium* MET708, *V. harveyi* BB170 and *E. coli* W3110 (pCT6), each *luxS*⁺ (Table 3-1), and each producing their native AI-2 were co-cultured in the same tubes. By design, we selected each species so that a different reporter probe would indicate the specific bacterium's QS response: *S. typhimurium* (MET708) expresses β -galactosidase; *V. harveyi* (BB170) elicits bioluminescence; and *E. coli* (W3110) are engineered to

synthesize GFP. In this way, their responses can be differentiated and quantified in response to AI-2.

Since isobutyl-DPD was able to suppress QS in pure cultures of *E. coli* and *S. typhimurium* and isopropyl-DPD was selective only causing *lsr* suppression in *E. coli*, these analogs were selected to monitor their specificity in the trispecies culture environment. Upon addition of isobutyl-DPD, we found *S. typhimurium*'s β -galactosidase activity sharply decreased 4-fold while isopropyl-DPD was ineffective (see Figure 3-6a). These trends were similar to those observed in pure culture studies. Interestingly, both isobutyl-DPD and isopropyl-DPD decreased bioluminescence in *V. harveyi* and this appeared to be concentration dependent (see Figure 3-6b). It has been shown that DPD analogs when added to *V. harveyi* grown in AB media actually were found to be agonists of bioluminescence^{94,95}. Our LM media results, which demonstrate antagonism of bioluminescence in *V. harveyi*, could be thought of as conflicting, but reinforce the context-dependent nature of QS responses⁴³. That is, in LM media the analogs antagonize bioluminescence in the synthetic ecosystem and also in pure cultures (see Figure 3-S7). Hence, additional media components as demonstrated by Xavier and Bassler⁴³ play a pivotal role in modulating QS signaling.

It is important to note that the AI-2 uptake and processing machinery in *V. harveyi* are distinctly different from the enteric organisms *E. coli* and *S. typhimurium*. In *V. harveyi*, AI-2 binds to cell surface bound receptor LuxP which, in turn, recognizes a boronated form of AI-2⁴⁷; the QS signal is transduced through phospho-relay mechanisms²⁴ whereas in *E. coli* or *S. typhimurium* AI-2 is first internalized and processed by *lsr* machinery before eliciting a QS response. Hence, it is not surprising

that these analogs yielded different outcomes. To ascertain the QS response in *E. coli*, we monitored the AI-2 stimulated GFP expression via flow cytometry. In the absence of isobutyl-DPD, ~ 60% of the population was QS positive (green), and after the addition of isobutyl-DPD, QS positive cells were reduced to less than 1%. Isopropyl-DPD decreased the QS response to a lesser extent (to 21% of the population) (see Figure 3-6c).

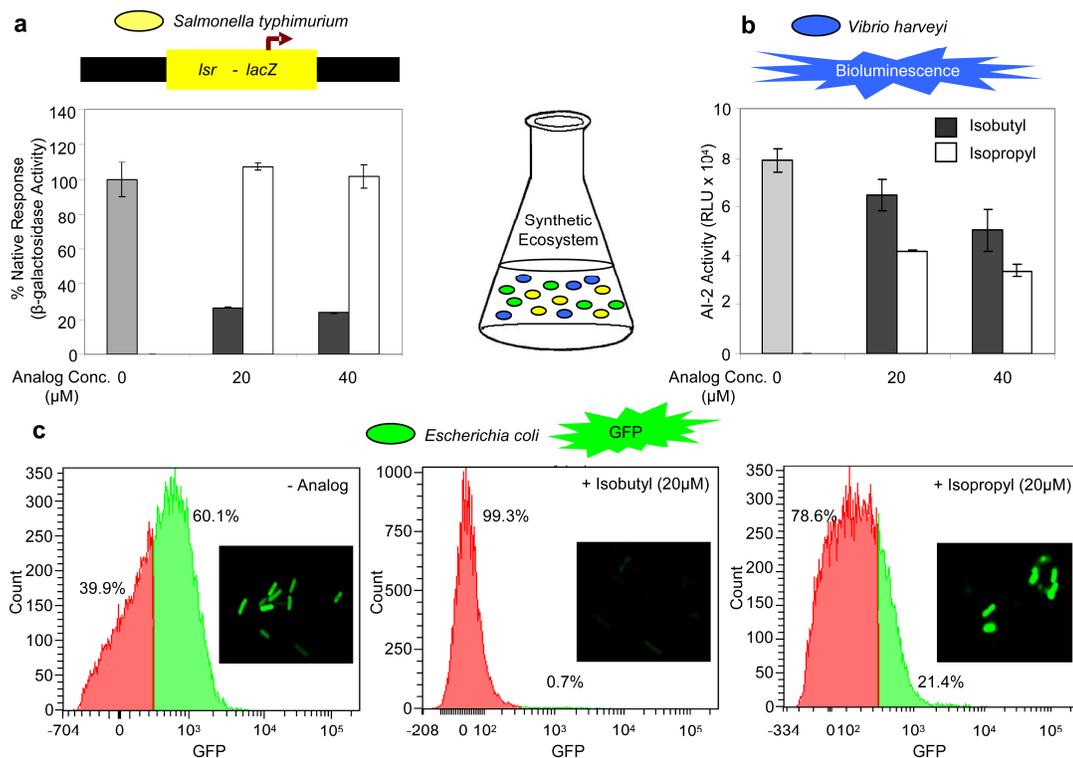


Figure 3-6 Analogs inhibit native signaling in a trispecies synthetic ecosystem.

a) AI-2 dependent β-galactosidase production in *S. typhimurium* MET708 (100 % Native *S. typhimurium* response in trispecies culture = 1063 Miller units). b) AI-2 dependent bioluminescence production in *V. harveyi* BB170 and c) AI-2 dependent GFP induction in *E. coli* W3110 pCT6 (all strains are *luxS*⁺) in response to isobutyl-DPD and isopropyl-DPD.

3.5 Discussion

It is now generally accepted that AI-2 is a universal *bona fide* quorum sensing autoinducer in several bacteria including several clinically relevant species, although in some bacteria AI-2 is also a metabolic waste¹²⁰. Both unmodified and “processed” AI-2 have been shown to bind to protein receptors and affect QS response in a variety of bacteria^{24,97}. In some bacterial species, such as *V. harveyi* and *V. cholerae*, both AI-1 and AI-2 feed into the same phospho-relay QS circuitry⁴⁹. In other bacteria, AI-1 and AI-2 circuits do not necessarily converge but, instead, affect the production of different factors yielding different phenotypes. Therefore, for the exquisite modulation of bacterial quorum sensing, a “tool kit” that contains molecules that can separately antagonize the actions of AI-1 or AI-2, or broad-spectrum molecules that can simultaneously quench the QS response of both AI-1 and AI-2 are desired. Although a handful of reports have demonstrated AI-2 antagonism⁹⁶ or synergism⁹⁵ using AI-2 like molecules, this is the first systematic study using an expanded set of AI-2-like molecules (14 total analogs) to identify or define the functional units of AI-2 that can be tweaked to yield agonists or antagonists. Also, another unresolved issue that this manuscript addresses is whether “AI-2-like” antagonists need to be phosphorylated in order to be active. Our data provides strong evidence that indeed phosphorylation is also important for AI-2 analogs that act as antagonists.

Our results show that of the panel of DPD analogs, only ethyl-DPD acts as a QS circuit agonist in *E. coli* and *S. typhimurium*. A variety of DPD analogs caused inhibition of native QS signaling in *E. coli* while only butyl-DPD and isobutyl-DPD significantly inhibited signaling in the homologous QS circuit of *S. typhimurium*.

Alignment studies (using protein blast¹⁶⁵) reveal that both *E. coli* and *S. typhimurium* LsrK and LsrR proteins show significant homology (82% and 77% identical sequences, see Supporting Information figures 3-S8 and 3-S9). The 30 bp putative LsrR binding site in *E. coli*¹⁶⁴ was aligned to the respective promoter region in *S. typhimurium* using Clustal W¹⁶⁶ and showed 83% homology (see Supporting Information figure 3-S10). Secondary¹⁶⁷ (see Supporting Information figure 3-S11) and tertiary structure¹⁶⁸ (see Supporting Information figure 3-S12) predictions show that LsrR from both *E. coli* and *S. typhimurium* have similar folds. We conclude from this work that although the AI-2 processing enzyme LsrK and LsrR-DNA binding sites in both *E. coli* and *S. typhimurium* share significant homology, subtle structural differences can result in these systems responding differently to small molecules. The instability of LsrR which also leads to its insolubility, as observed by us and also by others¹⁶⁴, has made difficult a more thorough study of LsrR binding to AI-2 analogs. Future engineering of a more stable LsrR and structural work should help shed more light on the origin of the differences in AI-2 analogs binding between the *E. coli* and *S. typhimurium* LsrR proteins.

Kinases are important enzymes for several processes in bacteria and the substrate specificity of these enzymes can be important for maintaining high fidelity of critical processes¹⁵⁹. In this paper we have demonstrated that LsrK is promiscuous; different AI-2-like molecules with structurally diverse alkyl groups can be efficiently phosphorylated. Substrates that lack primary hydroxyl functionalities are however not accepted as substrates, but the mere presence of a primary hydroxyl group is not sufficient for kinase processing, as it has already been shown that LsrK from *S.*

typhimurium phosphorylates AI-2 but not ribose or glucose, which also contain primary hydroxyl units⁴².

There are three known AI-2 processing enzymes which would be the most likely checkpoints for analog function namely LsrK, LsrR and LsrG. As, all analogs and natural AI-2 functioned irrespective of the Lsr(ACDBFG) it shows that the analogs function independent of the AI-2 transporter and LsrG. Our working model for AI-2 and analog function starts with phosphorylation of DPD analogs (see Figure 3-7) as an early pivotal checkpoint for *lsr* transcription repression; isobutyl-DPD is a potent quorum quencher while its unphosphorylated counterpart deoxy-isobutyl-DPD minimally represses *lsr* transcription (Figure 3-2a,b). We note, however, phosphorylation is not the only criterion for antagonism, as some analogs such as cyclopropyl-DPD which is phosphorylated by LsrK are still not able to repress *lsr* expression. Some of the DPD analogs, such as hexyl-DPD, are not as readily phosphorylated as is butyl DPD yet these analogs cause substantial repression of *lsr* expression. Therefore we can not completely rule out the possibility that for some of these analogs, such as hexyl-DPD, inhibition of *lsr* expression is due to the unphosphorylated form of the molecule. Future work should help resolve this issue."

A more downstream checkpoint is the binding to LsrR (see Figure 3-7) forming a stable LsrR-*lsr* DNA complex, and thus preventing derepression of the *lsr* operon by phospho-AI-2 binding. "Previous work has already demonstrated that phospho AI-2 and not AI-2 bind to LsrR^{164,46} Data from the aforementioned studies, coupled with our observation that DPD analogs do not inhibit *lsr* expression in LsrR

mutant bacteria strongly suggest that the biological profiles of these analogs are due to the binding of phospho-DPD or phosphor-analogs to LsrR.

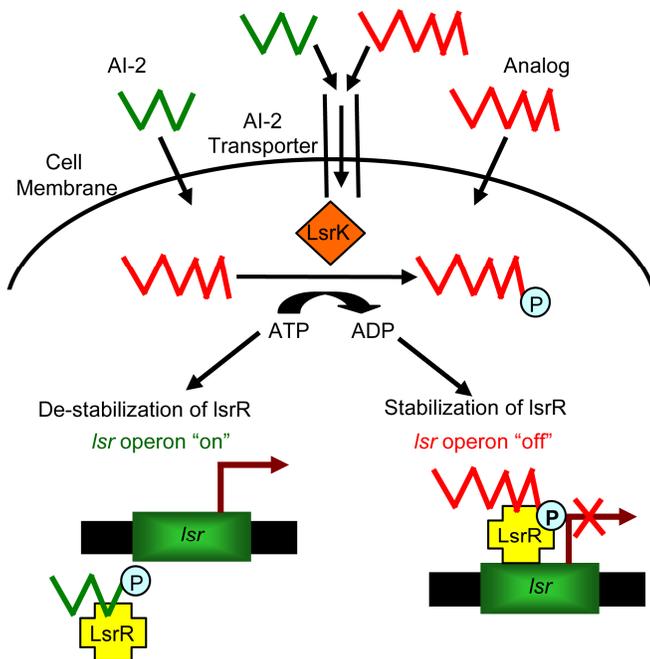


Figure 3-7 Suggested scheme for QS inhibition by a DPD analog in *E. coli*.

Analog/AI-2 enters the cell via the Lsr transporter or diffuses into the cell independent of the transporter. Analog needs to be phosphorylated by LsrK to function as an antagonist in the QS circuit. Phospho-analog also needs to compete with phospho-AI-2 for binding to the repressor protein (LsrR) in order to repress *lsr* expression.

We hypothesize that non-covalent interactions (most probably, van der Waals in nature) engage the alkyl chains of DPD analogs with certain residues in the active site of LsrR, and locks the protein into a conformation that has a higher affinity for the DNA binding sequence. The observation that a minimum of C3 alkyl chain length is required for DPD-analog antagonism lends credence to the hypothesis that a

hydrophobic pocket in LsrR plays a role in the distribution of the various LsrR conformations. Addition of a single carbon to the C1-alkyl chain of the analog seems to make a critical difference in stabilizing or destabilizing the interaction of the analog with LsrR; as while ethyl is an agonist, propyl becomes an antagonist of the QS circuit in both *E. coli* and *S. typhimurium*. The fact that isobutyl-DPD is a better antagonist than butyl-DPD (same number of carbons) or other DPD analogs, especially in *S. typhimurium* suggests that shape and not just “greasiness” (e.g., length) of the C1 alkyl chain is also important for antagonism.

It is important to emphasize that while the specific library of C1-alkyl analogs used here probes the Lsr AI-2 circuitry, additional AI-2 specific targets may be revealed by parallel studies focused on other AI-2 regulated genes revealed by our previous microarray studies⁷¹. Moreover, more comprehensive libraries of DPD analogs (e.g., substitutions at other positions and further C1 forms) may lead to new insights not currently explored.

3.6 Conclusion

This work has unveiled several important findings and refined our understanding of AI-2-based QS in both *S. typhimurium* and *E. coli*. Notably, we have demonstrated that depending on the nature of the C1-alkyl chain, phosphorylated AI-2-like molecules can either stabilize or destabilize LsrR-DNA complex. Therefore, small molecules that possess phosphate-like moieties as well as C1-alkyl chains of appropriate length and shape could become potential QS modulators in bacteria that utilize LsrR-like transcriptional factors to regulate QS

circuits. Secondly, we show that subtle differences in AI-2 processing enzymes in different bacteria allow for selective modulation of QS processes in an ecosystem. On the other hand, it is also possible to effectively modulate QS processing in a variety of bacteria that have different QS receptors or processing enzymes using a single small molecule. In the trispecies synthetic ecosystem, isopropyl-DPD could modulate QS response in *E. coli* and *V. harveyi* but not in *S. typhimurium* whereas isobutyl-DPD could modulate QS response in all three bacteria. The identification of both broad-spectrum and narrow-spectrum anti-AI-2 molecules could be important for both basic science and clinical applications whereby different scenarios might require a “conquer-all” or “conquer-selectively” approach.

Earlier observations by others revealed an unexpected switch in the modulation of AI-2-based QS in *S. typhimurium* and *V. harveyi* by AI-2 analogs⁹⁴. This switch in AI-2 perception could be explained by differences in the structure of AI-2 receptors in *S. typhimurium* and *V. harveyi* which are significantly different and do not share high homology. Additionally, the nature of the QS molecule in the *V. harveyi* case is unphosphorylated and boronated AI-2⁴⁷ whereas in *S. typhimurium*, phosphorylated AI-2 is responsible for the QS response. In this work, however, AI-2-like molecules have been shown to selectively differentiate closely related bacterial species, such as *E. coli* and *S. typhimurium*. As both the processing enzymes and the nature of the QS molecules in both species are similar, this observation is indeed remarkable and adds an additional level of sophistication to the control of bacterial quorum sensing with small molecules. The prevalence of AI-2 in controlling virulence in various pathogens such as *V. cholerae* and *E. coli* O157 suggests an

increased understanding of the AI-2 receptors and signal transduction cascades will open new avenues for guiding bacterial phenotype and modulating pathogenicity. Indeed, the AI-2 signaling molecules themselves are garnering significant attention as agents of diversity among QS-communicating populations, much like the family of AI-1 signaling molecules that mediate intraspecies communication. Our work and others related to signal perception demonstrate that *both* the generation and the perception/transduction processes are tuned by bacteria to weave a rich tapestry of communication networks and resultant phenotypes.

3.7 Acknowledgements

The authors would like to thank Dr. James N. Culver for generously providing his laboratory's facilities to conduct radioactive experiments. This work was supported by funds from the University of Maryland GRB fellowship, R.W Deutsch foundation, the Defense Threat Reduction Agency, National Science Foundation grant CHE0746446 and the National Science Foundation (EFRI Program). Jacqueline A.I Smith is a recipient of the Ministry of Education GANN fellowship.

3.8 Supporting Information

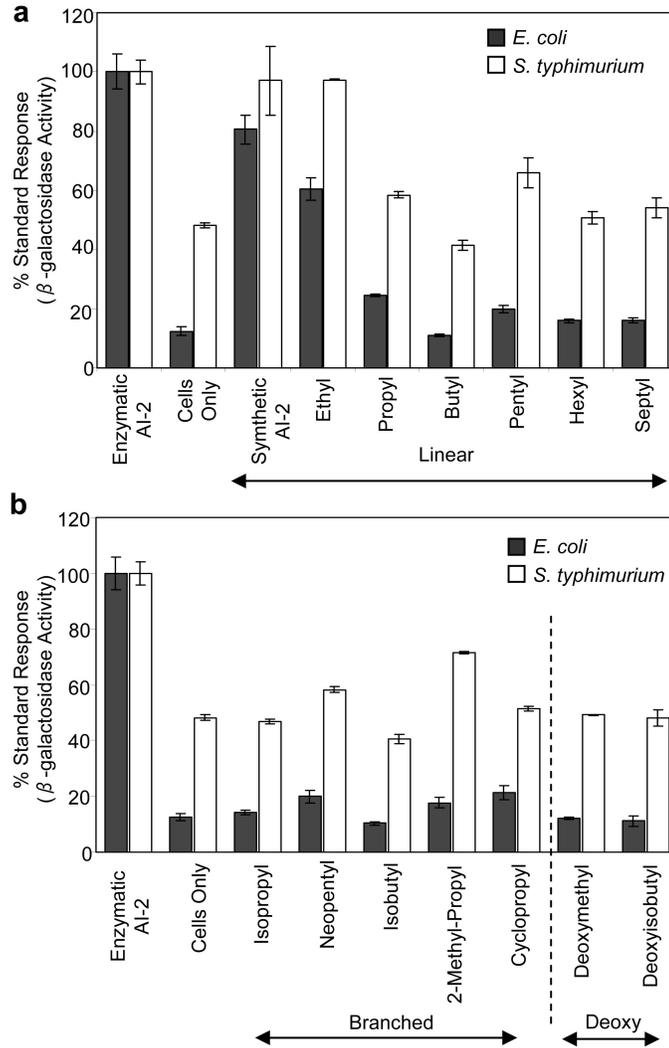


Figure 3-S1 Identification of analogs as agonists or antagonists in *E. coli* and *S. typhimurium*.

AI-2 dependent β -galactosidase production in *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (both *luxS*- and no AI-2added) in response to only the a) linear analogs, b) branched and deoxy analogs respectively (100% Standard *E. coli* (LW7) response = 1294 miller units and 100% Standard *S. typhimurium* (MET715) response = 2312 miller units both on adding enzymatically synthesized AI-2).

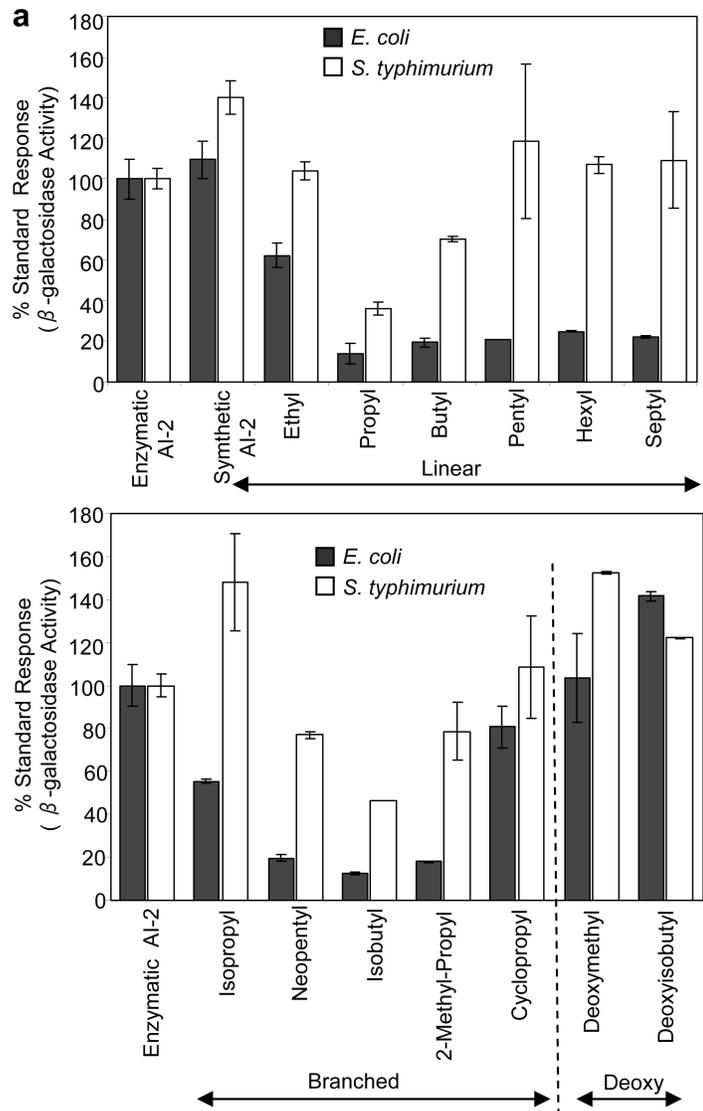


Figure 3-S2 Competitive inhibition of QS signaling by analogs in the presence of stoichiometric amounts of *in vitro* enzymatically synthesized AI-2 in *E. coli* and *S. typhimurium*.

AI-2 dependent β -galactosidase production in *E. coli* LW7 pLW11 and *S. typhimurium* MET708 (both *luxS*⁻) in response to a) linear analogs b) branched and deoxy analogs all added at 20 μ M concentrations (100% Standard *E. coli* (LW7) response = 675 miller units and 100% Standard *S. typhimurium* (MET715) response = 2921 miller units both on adding enzymatically synthesized AI-2).

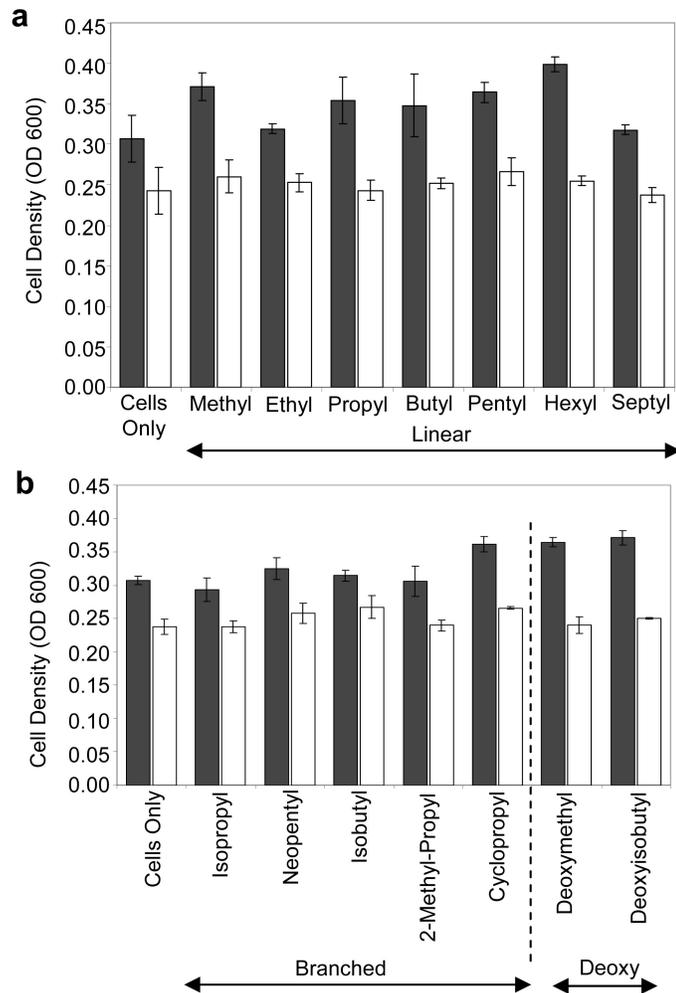


Figure 3-S3 Analog Library is not bacteriostatic or bacteriocidal in *E. coli* and *S. typhimurium*.

Cell density (OD₆₀₀) of *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 cells remains unaffected after two hours of analog treatment via a) linear analogs or b) branched and deoxy analogs, all added at 20μM concentrations.

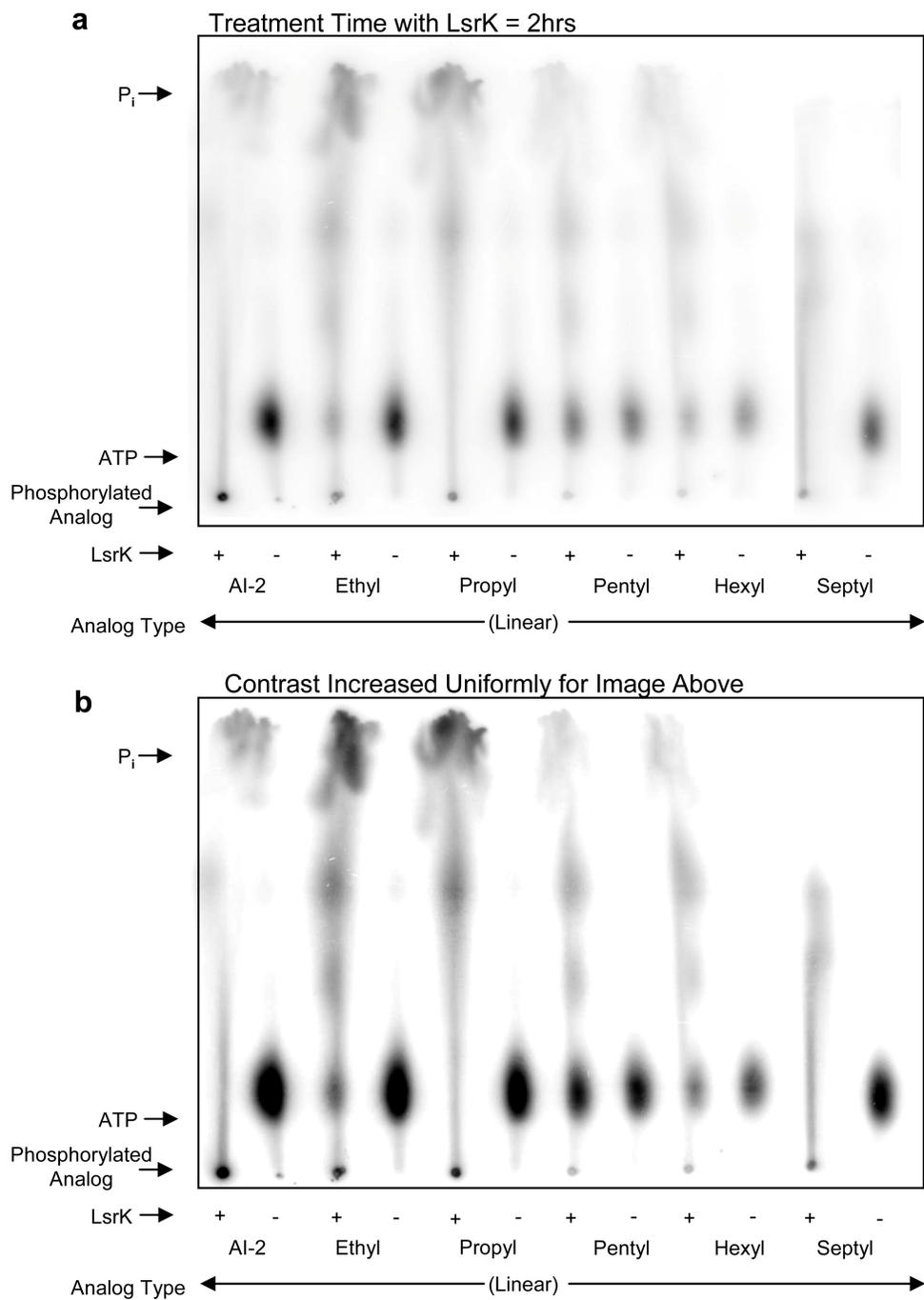


Figure 3-S4 *In vitro* phosphorylation of analogs by LsrK.

a) Thin layer chromatography (TLC) analysis of the LsrK mediated reaction for 2hrs with linear analogs. b) Contrast of 3-S4a image increased uniformly to highlight phospho-analog formation.

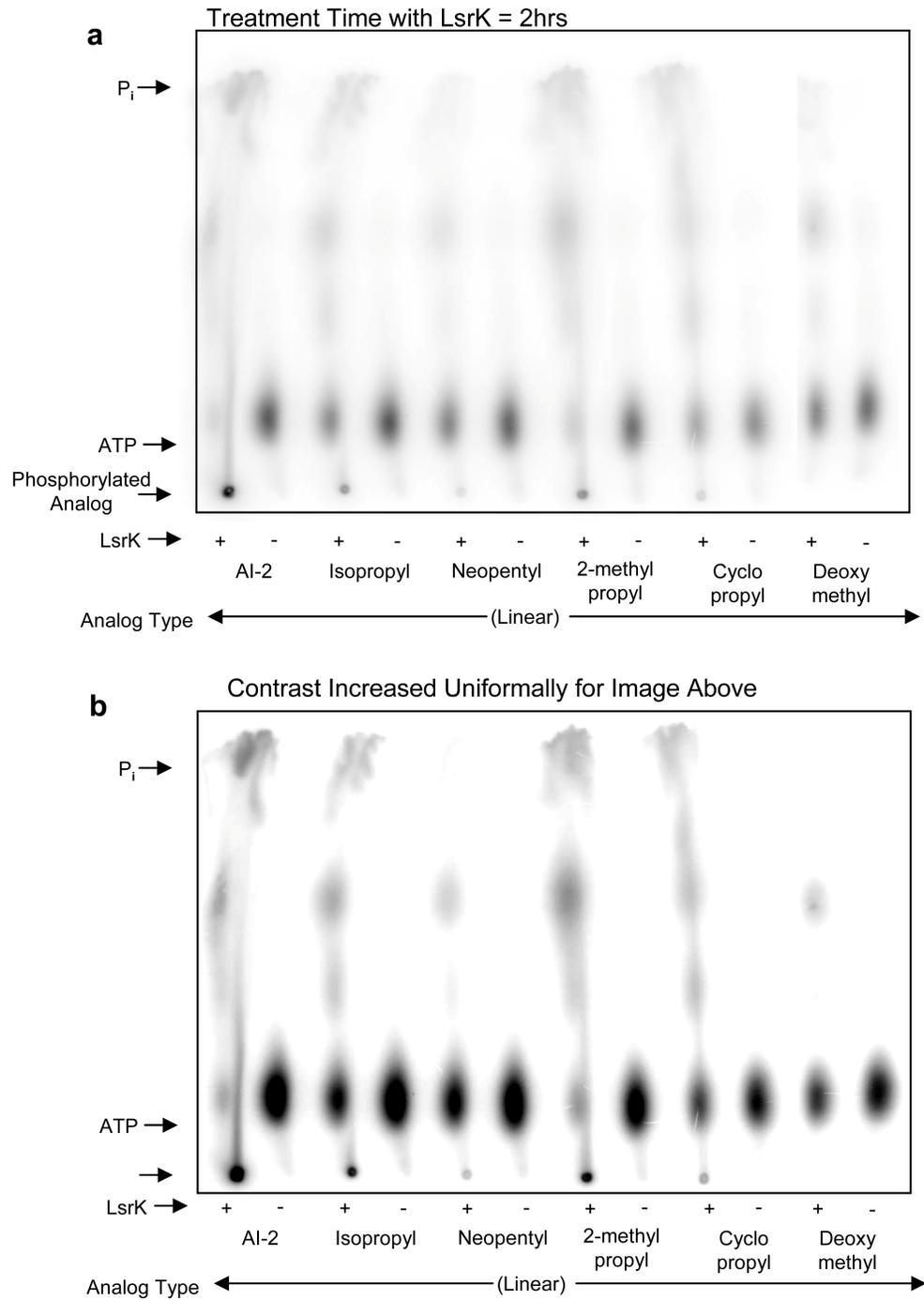


Figure 3-S5 *In vitro* phosphorylation of analogs by LsrK.

a) Thin layer chromatography (TLC) analysis of the LsrK mediated reaction for 2 hrs with branched analogs and deoxymethyl-DPD. b) Contrast of 3-S5a image increased uniformly to highlight phospho-analog formation.

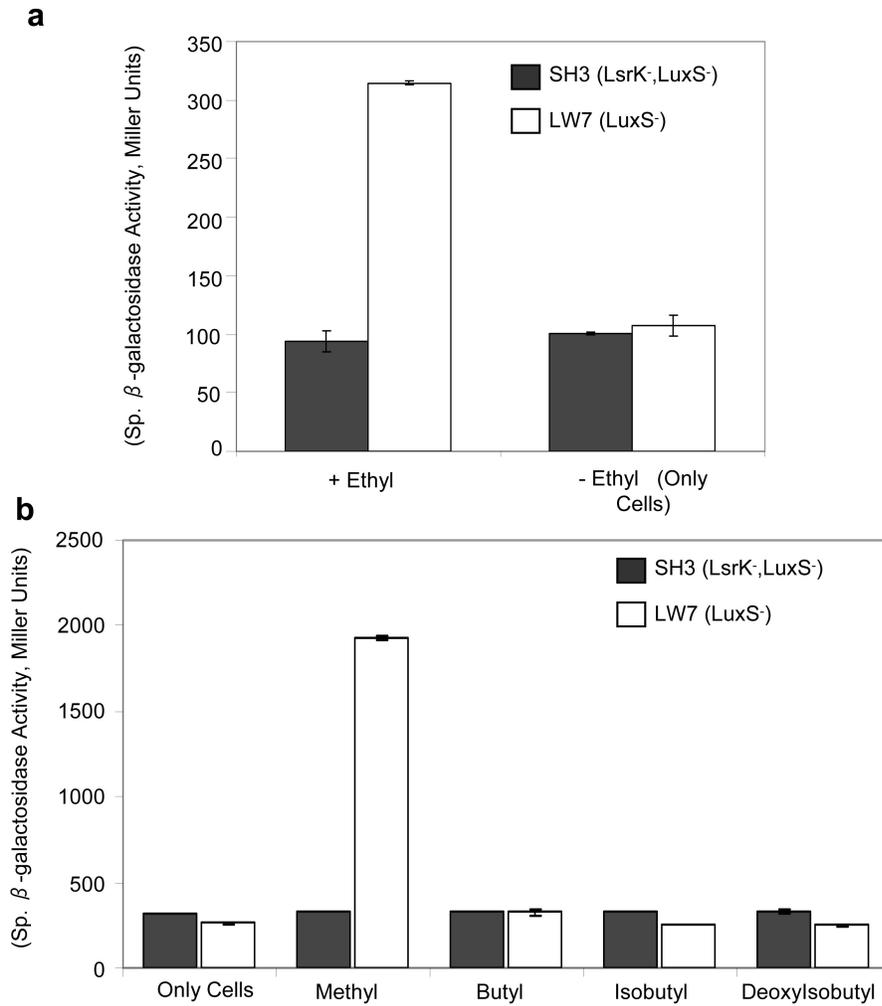


Figure 3-S6 Methyl and Ethyl-DPD require *in vivo* phosphorylation by LsrK to function as agonists of the QS response.

SH3 (*lsrK*-, *luxS*-) and LW7 (*luxS*-) are incubated in a) ethyl-DPD or b) representative analogs from the linear, branched and deoxy-DPD analog categories and the AI-2 dependent b-galactosidase response is quantified.

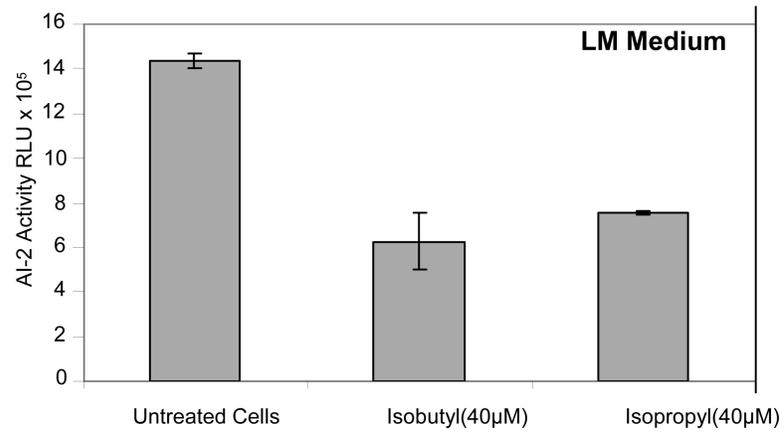


Figure 3-S7 AI-2 dependent bioluminescence production in *V. harveyi* BB170 grown in LM medium.

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Score = 905 bits (2339), Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 438/530 (82%), Positives = 481/530 (90%), Gaps = 0/530 (0%)

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Figure 3-S8 Alignment data from NCBI protein blast for *E. coli* LsrK and *S. typhimurium* LsrK (ydeV) putative kinase.

aALIGNMENT

GENE ID: 1250051_ydeW | putative regulatory protein
 [Salmonella enterica subsp. enterica serovar Typhi str. CT18]
 (10 or fewer PubMed links)

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DNA-binding transcriptional regulator [*Escherichia coli* str. K-12 substr. W3110]

Subject = *Salmonella enterica* serovar *typhimurium* as shown above

Figure 3-S9 Alignment data from NCBI protein blast for *E. coli* LsrR and *S. typhimurium* LsrR (ydeW) putative regulatory protein.

a

(i) Putative lsrR binding site in *E. coli* vs (ii) Intergenic region in *E. coli*

Homology = 100%

```
(i) _____TGAACAATTGCATTAAAGATTTAAATATGT_____ 30
(ii) GATATAAATGAGCAAGAACTGAACAATTGCATTAAAGATTTAAATATGTTCAAAGTGAAG 240
```

b

(i) Putative lsrR binding site in *E. coli* vs (ii) Intergenic region in *S.typhimurium*

Homology = 83%

```
(i) _____TGAACAATTGCATTAAAGATTTAAATATGT_____ 30
(ii) CGGTTATTTTCGGAGTGATAACTGAACAATTGTGTTAATGATTTAGAAATGTTCAAAGTAA 240
```

Figure 3-S10 Alignment of known LsrR binding site in *E. coli* to intergenic regions between the divergent genes LsrR (*ydeW*) and lsrA (*ego*) in (a) *E. coli* and (b) *S. typhimurium* respectively.

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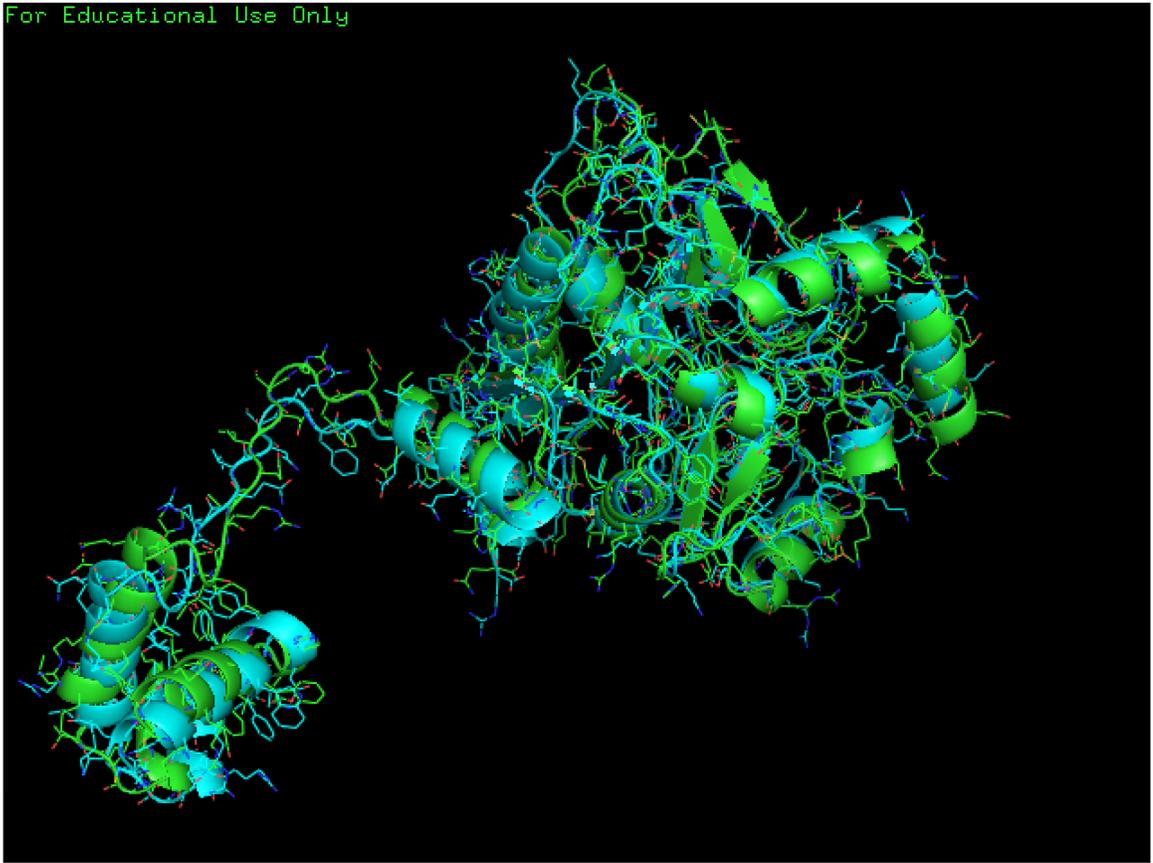


Figure 3-S12 Predicted Tertiary Structure of LsrR proteins *S. typhimurium* (Green) and *E. coli* (Cyan) provided by ESyPred3D3.

Chapter 4: Altering the Communication Networks of Multispecies Microbial Systems Using a Diverse Toolbox of AI-2 Analogs

4.1 Abstract

Small molecules are important in the investigation and treatment of diseases because of their reversible, tunable and dynamic ability to modulate biological systems. In this work, we explore the ability of small molecule analogs of the universal bacterial signaling molecule autoinducer-2 (AI-2) to selectively modulate quorum sensing (QS) in bacteria. QS is the phenomenon of bacterial cell-to-cell communication via signaling autoinducers and has been tied to pathogenic phenotypes such as biofilm formation and endotoxin production. A diverse toolbox of C-1 analogs including linear, branched, cyclic and aromatic analogs were synthesized and screened for their ability to affect QS regulated gene expression in *Escherichia coli*, *Salmonella typhimurium*. The analogs were also screened for their effect on the production of the toxin pyocyanin in *Pseudomonas aeruginosa*, which does not produce its own AI-2 but responds to it. Some analogs such as heptyl-DPD and cyclopentyl-DPD were identified to inhibit QS in *P. aeruginosa* as well as *E. coli*. Others such as phenyl-DPD inhibited *P. aeruginosa* only. Isobutyl-DPD which was previously identified as a broad species potent QS inhibitor of *E. coli*, *S. typhimurium* and *V. harveyi* was not able to inhibit pyocyanin production in *P. aeruginosa*. In addition to the individual species tests, we assembled an analog cocktail containing phenyl- and isobutyl-DPD to inhibit QS *en masse* in a trispecies ecosystem consisting

of *E. coli*, *S. typhimurium* and *P. aeruginosa*. The cocktail was observed to be more effective than the individual analogs in inhibiting the QS response of all organisms in this ecosystem. This expansive set of analogs provides a multimodal toolbox from which to alter distinct QS systems in isolation or in concert. Further, these analogs can serve as probes to identify QS processing enzymes and other mechanisms of interaction in multispecies bacterial networks which could be possible drug targets in the quest for the next generation of antimicrobials based on quorum sensing inhibition.

4.2 Introduction

Bacteria rarely exist in isolation in their natural environments and when they establish infection in a host. Therefore bacteria have developed intricate networks for communication and interaction with other bacteria around them. Most bacteria found in humans are usually non-pathogenic; they constitute the native microflora and have symbiotic relationships with the host cells. Over 205 different genera of bacteria are found on the human skin¹⁶⁹. There are over 750 taxa of known oral bacteria and it has been estimated that ~ 50% of oral bacteria have yet not been cultivated¹⁰⁸. Gut microbial flora or microbiota and their collective genomes (microbiome) is one of the richest and most diverse in the human body. The gut can be thought of as a microbial organ placed inside a human host with over 10 trillion cells. These harbor 800 different species of bacteria with over 7000 different strain numbers¹⁷⁰. In the case of oral microflora it was seen that many oral bacteria could not be cultivated in pure cultures in the laboratory due to their metabolic interdependencies, and various oral

and gut infections are polymicrobial¹⁰⁸. Thus, microbial cell-cell interactions and signaling are essential for bacterial survival and also in establishing infections in humans.

Frequently, bacteria communicate through a phenomenon known as quorum sensing (QS). QS is a form of cell-to-cell communication whereby bacteria secrete and detect chemical signals known as autoinducers¹⁰. Once a predetermined concentration of autoinducers is detected, bacteria can coordinate their gene expression and perform pathogenic processes such as biofilm formation and virulence factor production^{15, 16}. Although interfering with QS systems has been identified as a new anti-infective chemotherapy, most studies done on the inhibition of QS have been limited to single bacterial strains and their strain-specific signaling (typically through species-specific acylhomoserinelactone (AHL) analogs)^{20, 72, 73}. In this study we interfere with the intercellular autoinducer-2 (AI-2) mediated signaling mechanisms via small molecule analogs of AI-2 in mixed culture environments. AI-2, which is found in both gram-negative and gram-positive bacteria is a family of interconverting cyclic furanones derived from the linear molecule 4,5-dihydroxy-2, 3-pentadione (DPD)⁴⁰. DPD is produced by the synthase LuxS. LuxS has been found in over 70 species of bacteria and therefore AI-2 is known as the universal signaling molecule¹⁷.

We recently demonstrated that acyl analogs of AI-2 (linear or branched chain analogs) could be used to selectively modulate AI-2 signaling in three bacteria, *E. coli*, *S. typhimurium* and *V. harveyi*¹⁷¹. Interestingly, we found that, although the AI-2 signaling system in *E. coli* and *S. typhimurium* are highly homologous, they respond

differently to acyl analogs of AI-2. Our initial results prompted us to expand our study to include diverse acyl analogs of AI-2, with the expectation that increasing the diversity of the shapes and sizes of the C1-substituent of AI-2 analogs could lead to the identification of selective AI-2 modulators in diverse bacterial species. Herein, we describe the synthesis and biological evaluation of this expanded set of AI-2 analogs, which includes aromatic as well as cyclic C-1-alkyl analogs of AI-2. Cyclic analogs are of particular interest because they are less conformationally promiscuous than linear chains. Aromatic analogs are also rigid and will allow investigations into the effect of structural electronics on AI-2 activity.

The key role of AI-2 in *V. harveyi* and the homologous QS systems of *E. coli* and *S. typhimurium* have been most thoroughly characterized⁴²⁻⁴⁴. In *V. harveyi*, a cyclic borate form of AI-2 was found crystallized with the periplasmic protein LuxPQ. When AI-2 binds LuxPQ, a phospho-relay is triggered and bioluminescence is induced⁴⁷. In *E. coli* and *S. typhimurium*, the internalization of AI-2 occurs as a non-borated cyclic form. Once inside the cell, AI-2 is phosphorylated by the kinase LsrK. This phosphorylated-AI-2 then binds and de-represses LsrR, the transcriptional regulator of QS genes in enteric bacteria.

In this study, we examine the more clinically relevant bacterium, *P. aeruginosa* in addition to *E. coli* and *S. typhimurium*. *P. aeruginosa* is an opportunistic pathogen causing severe infections in immunocompromised patients. It causes extensive biofilm formation in the lungs of cystic fibrosis patients¹¹⁵. Although, *P. aeruginosa* does not produce its own AI-2 and its own QS circuit has two LuxI/LuxR type modules controlled by AHL type autoinducers, evidence suggests

that AI-2 affects QS in this organism^{10, 58}. AI-2 has been shown to effect biofilm formation in *P. aeruginosa* and a study demonstrated that AI-2 produced by the microflora of cystic fibrosis patients plays a role in *P. aeruginosa* virulence⁵⁶. We study the response of the C1-alkyl analogs in pure cultures of *E. coli*, *S. typhimurium* and *P. aeruginosa* as well as a synthetic ecosystem composed of all three (*E. coli*, *S. typhimurium* and *P. aeruginosa*). These three bacteria could co-exist naturally in human gut/upper respiratory tract infections. While *E. coli* is a part of the microflora certain strains can be enterohaemorrhagic. *S. typhimurium* is known to cause Salmonellosis while *P. aeruginosa* is associated with various upper respiratory tract infections.

AI-2 is required to establish virulence¹²⁰ in some bacteria, but in others (including our innocuous microflora) it is only a metabolic-by-product⁸³. Thus, we designed a diverse analog panel to obtain small molecules that can selectively modulate the AI-2 signaling of some targeted bacteria (but not all other microflora present) in a host. The goal is to use our analogs individually to tune AI-2 signaling in a subpopulation of bacteria in an ecosystem or in analog mixes or cocktails that alter signaling in the overall population. Ultimately, investigations with this expanded set of analogs and diverse bacteria will enable in-depth probing of the specificity or promiscuity of QS processing proteins.

4.3 Materials and Methods

Bacterial Strains and Growth Conditions

Table 4-1 lists the bacterial strains and plasmids used in this study. *S. typhimurium* and *E. coli* strains were cultured in Luria-Bertani medium (LB, Sigma) at 37 °C with shaking at (250 rpm) unless otherwise noted. Antibiotics were used for the following strains: (60 or 100 µg ml⁻¹) kanamycin for *S. typhimurium* MET715, *S. typhimurium* MET708 and (60 or 100 µg ml⁻¹) ampicillin *E. coli* LW7 pLW11, (50 µg ml⁻¹) ampicillin and (50 µg ml⁻¹) kanamycin for *E. coli* W3110 pCT6 dsRED .

Table 4-1 List of strains and cells used in this study.

Strain, plasmid or primer	Relevant genotype and/or property	Reference
<i>Escherichia coli</i> strains		
W3110	Wild type	Laboratory Stock
LW7	W3110 Δ lacU160-tna2 Δ luxS :: Kan	44
ZK126	W3110 Δ lacU169-tna2	
<i>Salmonella typhimurium</i> strains		
MET715	<i>rpsl putRA</i> :: Kan- <i>lsr-lacZYA luxS</i> :: T-POP	42
MET708	<i>rpsl putRA</i> :: Kan- <i>lsr-lacZYA</i>	42
<i>P. aeruginosa</i> strains		
PA01	Wild Type	Laboratory Stock
Plasmids		
pLW11	<i>galk'-lacZYA</i> transcriptional fusion vector, containing <i>lsrACDBFG</i> promoter region, Amp ^r	44
pCT6	pFZY1 derivative, containing <i>lsrR</i> and <i>lsrR</i> promoter region fused with <i>T7RPol</i> , Ap ^r	163
pET-dsRED	pET200 derivative, containing RFP, Km ^r	163

Measurement of the QS Response (*lsr* expression)

The QS response indicated by *lsr* gene expression was analyzed in pure culture studies by culturing the required bacteria *E. coli* LW7 pLW11, *E. coli* ZK126 pLW11 and *S. typhimurium* MET708, *S. typhimurium* MET715 overnight in LB medium supplemented with appropriate antibiotics as specified above. These cells were then diluted into fresh LB medium (with antibiotics) and grown to an OD₆₀₀ of 0.8 - 1.0 at 30 °C, 250 rpm. Cells were collected by centrifugation at 10,000 xg for 10 minutes, and resuspended in 10 mM phosphate buffer. AI-2 (20 μM) and the respective analog (20 μM) were added to the *E. coli* or *S. typhimurium* suspension for 2 hours at 37 °C. AI-2 dependent β-galactosidase production was quantified by the Miller assay¹⁴¹.

Measurement of the QS response (Pyocyanin Production)

The cells were grown with the required analog 3 ml total volume in 50 ml flasks in LB medium with continuous shaking. In between 22-24 hrs when the cells turned green after pyocyanin secretion, the pigment was extracted. The pyocyanin quantification assay was conducted as described¹⁷². 2 ml chloroform was added to the 3ml culture and pipetted up and down. 1ml of the chloroform was transferred to a separate tube and the pyocyanin was reextracted into 200 μl of 0.2 M HCl. The absorbance of the solution was measured at OD 520 nm. To calculate the concentration of pyocyanin extracted as μg/ml the OD at 520 nm was multiplied by 17.07¹⁷².

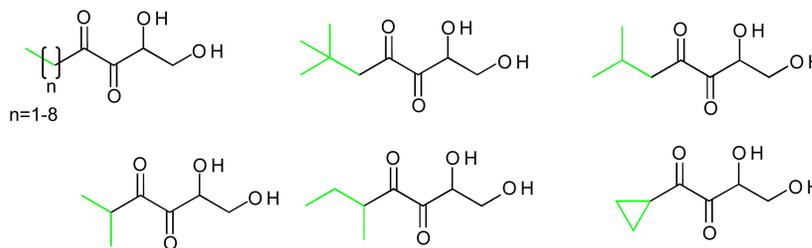
Analyzing QS Response in the Synthetic Ecosystem

The *S. typhimurium* MET708, *P. aeruginosa* PA01 and *E. coli* W3110 pCT6 dsRED were each cultured separately overnight in LB medium supplemented with the appropriate antibiotic. *P. aeruginosa* PA01, *S. typhimurium* MET708 and *E. coli* W3110 pCT6 dsRED were diluted (25 μ l:2.5 μ l:100 μ l) of the overnight culture respectively, into a single 2 ml final volume of fresh LB medium without antibiotics. The co-culture was supplemented with (40 μ M) of the respective analog or analog cocktail initially and again after 2.5, 5, 9 and 18 hours of growth. The *S. typhimurium* *lacZ* (β -galactosidase) activity was measured after 4 hours. The *E. coli* response was determined after 24 hours, by fixing the cells with 1:1 cold 4 % paraformaldehyde and using flow cytometric analysis. Samples were analyzed by flow cytometry (FACS Canto II, BD 394 Biosciences), with 30,000 gated events analyzed per sample. The Pyocyanin was also extracted after 24 hours of growth.

4.4

Results

a) Previously Synthesized C1-Analogs



b) Newly Synthesized C1-Analogs

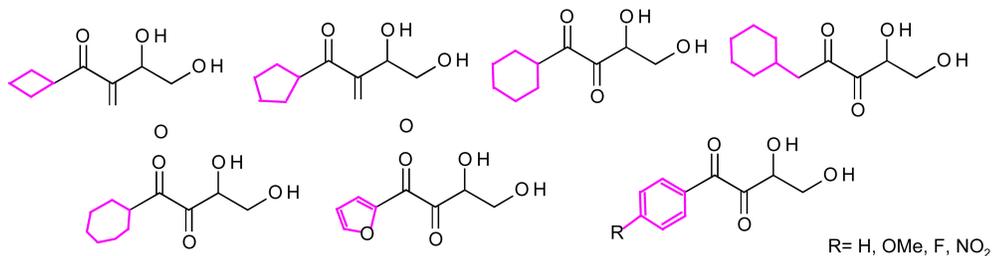


Figure 4- 1 Analog Structures.

a) Previously synthesized linear and branched analogs b) Newly synthesized cyclic and aromatic analogs

4.4.1 Synthesis of Cyclic and Aromatic AI-2 Analogs

The synthesis of AI-2 analogs (Figure 4-1a) followed the diazocarbonyl aldol methodology previously reported by us¹⁷¹. This methodology was easily amendable to a variety of cyclic and aromatic analogs, as the requisite acid chlorides were commercially available to construct the starting diazocarbonyls. Also, reaction of commercially available carboxylic acids with thionyl chloride could rapidly afford the desired acid chloride if the commercial starting material was unavailable for some

cyclic analogs. Briefly using the robust DBU-catalyzed addition of (OTBS)-acetaldehyde to the diazocarbonyl afforded the protected diazo intermediates. Without isolation of these products, TBAF-deprotection was performed to afford the diazodiol precursor to DPD in good yields. Oxidation of this product was quantitatively achieved with DMDO and excess reagent evaporated to give the desired cyclic and aromatic DPD analogs. With 10 new AI-2 analogs now added to our analog panel (Figure 4-1b), we proceeded to test their biological effects on three different bacterial strains: *E. coli*, *S. typhimurium* and *P. aeruginosa*.

4.4.2 Effect of Cyclic and Aromatic Analogs on β -galactosidase Production in *E. coli* and *S. typhimurium*

New cyclic and aromatic analogs were tested for their modulation of QS in *E. coli* and *S. typhimurium* via the β -galactosidase assay. Initial screening of analogs for agonism was conducted using *E. coli* LW7 (LuxS-) and *S. typhimurium* MET715 (LuxS-). None of the cyclic or aromatic analogs were able to initiate β -galactosidase on their own. Next, analogs were tested for inhibition in *E. coli* LW7 (LuxS-) and *S. typhimurium* MET715 (LuxS-) in the presence of 20uM AI-2 (synthetic DPD) (Figure 4-2a, 2b). Cyclopentyl-DPD was the only cyclic analog that was able to compete with AI-2 while cyclobutyl-DPD gave some knockdown and cyclopropyl-DPD gave no knockdown in *lsr* expression in *E. coli*. Larger cyclic analogs (cyclohexyl-DPD, CH₂-cyclohexyl-DPD and cycloheptyl-DPD) all did not give significant knockdown. Therefore AI-2 processing enzymes may only be able to accommodate only a certain amount of space, which is optimized by cyclopentyl-DPD. Also since 5-membered

rings are more flexible than 3- and 4- membered rings, a desired conformation maybe required for inhibition that is inaccessible to the more strained cyclopropyl- and cyclobutyl-DPD analogs.

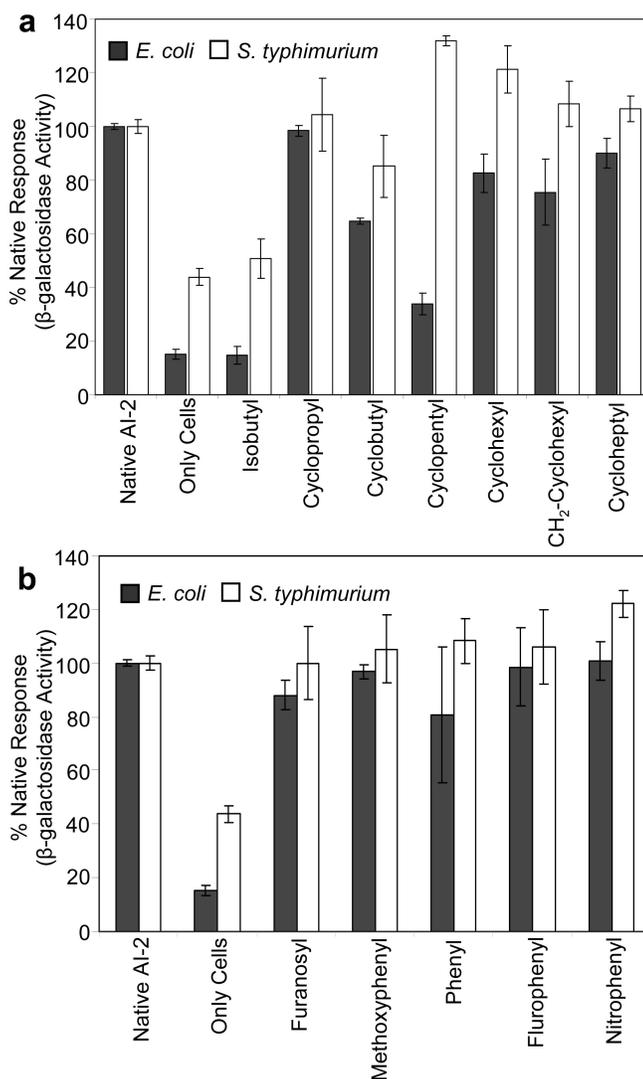


Figure 4-2 Competitive inhibition of QS signaling by analogs in the presence of stoichiometric amounts of chemically synthesized methyl-DPD in *E. coli* and *S. typhimurium*.

AI-2 dependent β -galactosidase production in *E. coli* LW7 pLW11 and *S. typhimurium* MET708 (both *luxS*-) in response to a) cyclic analogs, b) aromatic

analogs all added at 20 μ M concentrations (100% Standard *E. coli* (LW7) response = 1304 miller units and 100% Standard *S. typhimurium* (MET715) response = 3480 miller units both on adding chemically synthesized methyl-DPD).

None of the aromatic analogs were able to modulate QS in *E. coli* or *S. typhimurium*. Since the 5-membered aromatic analog, furanoyl-DPD, was ineffective, it is likely that some degree of flexibility is required at the C1 position for analogs to be processed, which the flat aromatic compounds lack. Identical results were observed in the wild-type strains: *E. coli* ZK126 (LuxS+) and *S. typhimurium* MET 708 (LuxS+); Cyclopentyl was the only new analog to significantly inhibit *lsr* expression in *E. coli* and it was ineffective in *S. typhimurium* as were all the other cyclic and aromatic analogs.(Supplementary Figure 4-S1 a,b).

4.4.3 Identifying Modulators of *P. aeruginosa* QS

Each structural class of C1 analogs (linear, branched, cyclic and aromatic) was screened for activity in *P. aeruginosa* PAO1 by monitoring pyocyanin production. Pyocyanin is a redox active phenazine compound toxin produced by *P. aeruginosa* which is shown to have antibiotic properties¹⁷³ and can be monitored by measuring the absorbance at 540 nm. It has been shown that pyocyanin is the physiological signal for upregulation of QS controlled genes in the stationary phase¹⁷⁴. As upregulation of QS intensifies *P. aeruginosa* infections, pyocyanin inhibition is an interesting drug target for anti-QS molecules. Of the newly synthesized analogs, phenyl-DPD was the most potent inhibitor while cyclopentyl-

DPD gave some inhibition of pyocyanin production (Figure 4-3). None of the linear analogs except heptyl-DPD the analog with longest alkyl chain, gave the most inhibition (Supplementary Figure 4-S2). None of the branched analogs inhibited pyocyanin production; even the previously identify broad-spectrum inhibitor, isobutyl-DPD, was ineffective.

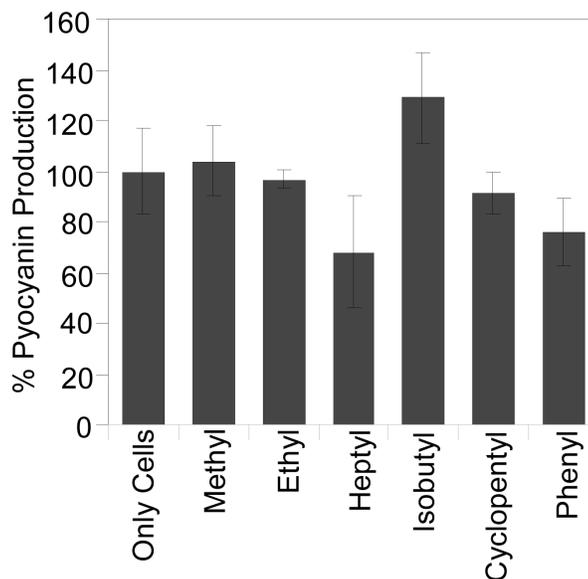


Figure 4-3 Effect of select analogs on pyocyanin production in *P. aeruginosa* PAO1.

4.4.4 Effect of Analogs in a Synthetic Ecosystem

The selective modulation of the QS systems of various organisms shown by diverse C1 analogs suggests that these analogs can tune QS response of a desired organism in mixed cell cultures. As previously mentioned, bacteria rarely exist in isolation, therefore analogs which are able to inhibit QS of one organism in the presence of many others maybe beneficial. To test this hypothesis, we screened

isobutyl-DPD and phenyl-DPD in a tri-species synthetic ecosystem consisting of *E. coli*, *S. typhimurium* and *P. aeruginosa*. In order to monitor the response from each organism, different reporter strains were used; in *E. coli* AI-2 mediated dsRED expression, in *S. typhimurium* AI-2 mediated β -galactosidase production and in *P. aeruginosa* pyocyanin production. It has previously been shown that isobutyl-DPD is able to inhibit *E. coli* and *S. typhimurium* simultaneously, but due to its lack of inhibitory effects in *P. aeruginosa*, it cannot be used as a broad-spectrum quorum silencer in this new tri-species synthetic ecosystem. The Phenyl-DPD from our new screen targeted only *P. aeruginosa* effectively and decreased pyocyanin production ~50%, but was ineffective in reducing *lsr* expression in *E. coli* or *S. typhimurium*. Therefore, to complement the effectiveness of isobutyl-DPD in inhibiting *E. coli* and *S. typhimurium*, phenyl-DPD was added to create a cocktail which is able to knockdown all three QS systems simultaneously in this ecosystem (Figure 4-4). The isobutyl-DPD and phenyl-DPD cocktail further inhibited the QS responses from *E. coli* and *S. typhimurium* in the ecosystem as compared to the inhibition caused by isobutyl-DPD on its own. Thus, with more AI-2 analogs being synthesized analog cocktails have wide applicability as broad species QS inhibitors.

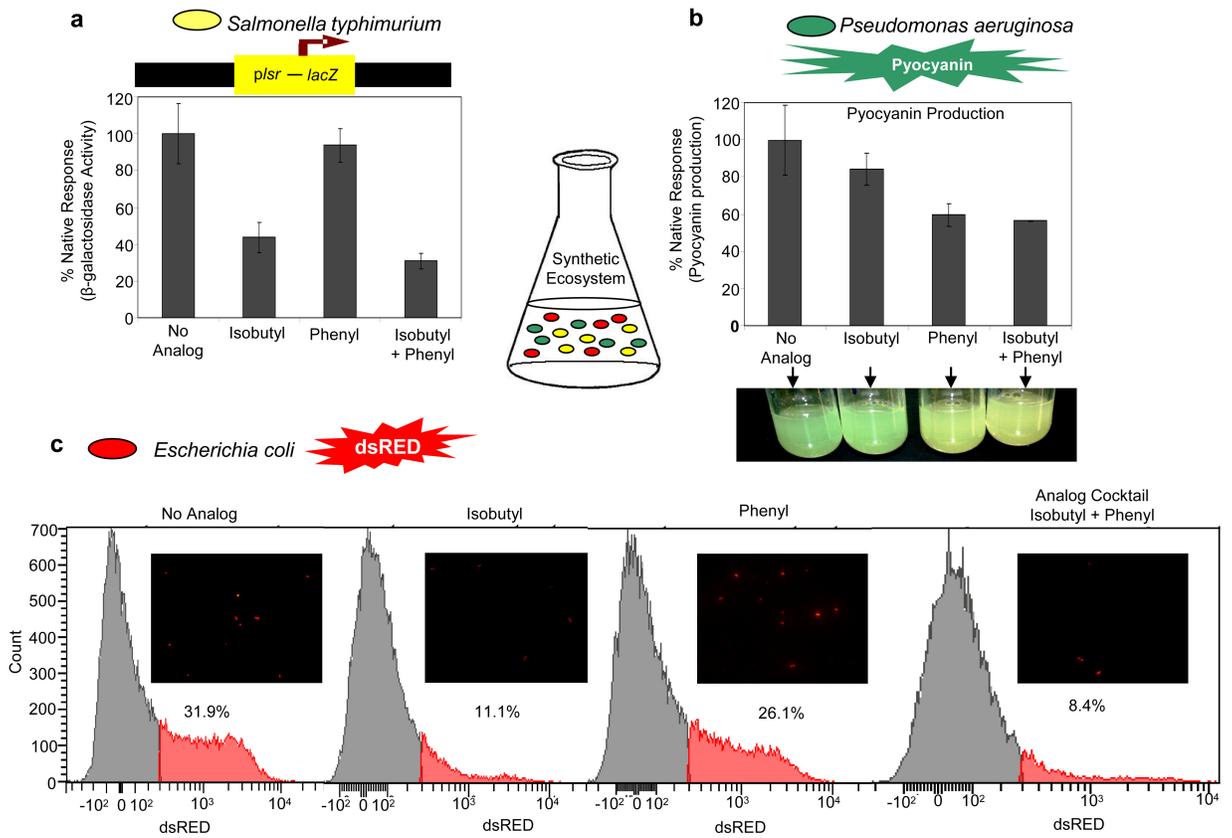


Figure 4-4 Analog cocktail inhibits QS signaling *en masse* in a trispecies synthetic ecosystem.

a) AI-2 dependent β -galactosidase production in *S. typhimurium* MET708 (100 % Native *S. typhimurium* response in trispecies culture = 475 Miller units). b) QS related pyocyanin production in *P. aeruginosa* PA01 (100 % Native *P. aeruginosa* response in trispecies culture = 2.5 μ g/ml). c) AI-2 dependent dsRED induction in *E. coli* W3110 pCT6 dsRED, in response to isobutyl-DPD and phenyl-DPD individually and a cocktail of both the analogs.

4.5 Discussion

Due to the ubiquitous nature of AI-2, it has been envisioned that an AI-2 inhibitor would offer a universal shut-down of QS due to its relevance in so many bacteria. Although this “magic bullet” may offer relief to the antibiotic resistance problem, there are many bacteria that form our microflora and are vital to our immunity and health. Ultimately anti-QS therapies must be able to offer a variety of options including both species specific- and multi-organism QS targeting.

In this work we have developed an expanded set of C1 analogs which have increased our understanding of what shapes and sizes are tolerated by AI-2 processing enzymes. In addition to the 3-carbon length minimum previously identify for *E. coli* inhibition, cyclic analogs reveal that a five-membered ring (cyclopentyl-DPD) is the maximum ring size able to affect AI-2 processing enzymes. Also, smaller rings and aromatic analogs are ineffective as QS inhibitors in *E. coli*, suggesting that some degree of flexibility is required for inhibition. Flexibility in C1-alkyl chain conformations could be the additional advantage the linear and branched C1-alkyl chain analogs had in the screen we published previously. As a result, they were able to adapt to form required conformations to cause a much larger knockdown in *lsr* expression of *E. coli* as compared to the more conformationally restricted new screen of cyclic and aromatic analogs.

Additionally in this study, our expanded set of analogs was tested for inhibition of pyocyanin production in *P. aeruginosa*. It has previously been shown that exogenous AI-2 is able to upregulate genes important for *P. aeruginosa* pathogenesis. Since several organisms which live in humans are likely to use AI-2

for signaling, *P. aeruginosa* maybe able to detect AI-2 and coordinate expression of pathogenic genes in presence of other AI-2 producing organisms in the host ⁵⁶. Although the mechanism of action by AI-2 is unknown, this study as well as others has shown that AI-2 analogs are able inhibit toxin production in *P. aeruginosa*. Furthermore, in a tri-species synthetic ecosystem consisting of organisms which are also endogenous to the human gut microflora, analogs such as phenyl-DPD were able to hit QS related expression in *P. aeruginosa* exclusively often to a greater extent than in pure cultures. Finally, by using a cocktail of isobutyl- and phenyl- DPD analogs, we silenced the QS system of all three organisms (*E. coli*, *S. typhimurium* and *P. aeruginosa*). In conclusion the synthesis of this diverse library of AI-2 analogs has allowed for further investigations into the QS systems of *E. coli*, *S. typhimurium* and *P. aeruginosa*. Identification of key structural elements that are required or not tolerated will enable new analogs to be strategically designed for altering QS in one or more required species. As it becomes clear that many infections such as oral diseases and otitis media (common childhood disease of ear infection) are polymicrobial and AI-2 increases their virulence^{109, 110, 119}, it becomes crucial to study AI-2 and other QS inhibitors in reference to bacterial networks. Testing these analogs in more diverse synthetic ecosystems will further our understanding of bacterial networks and their various QS enabled modes of virulence.

4.6 Supplementary Figures

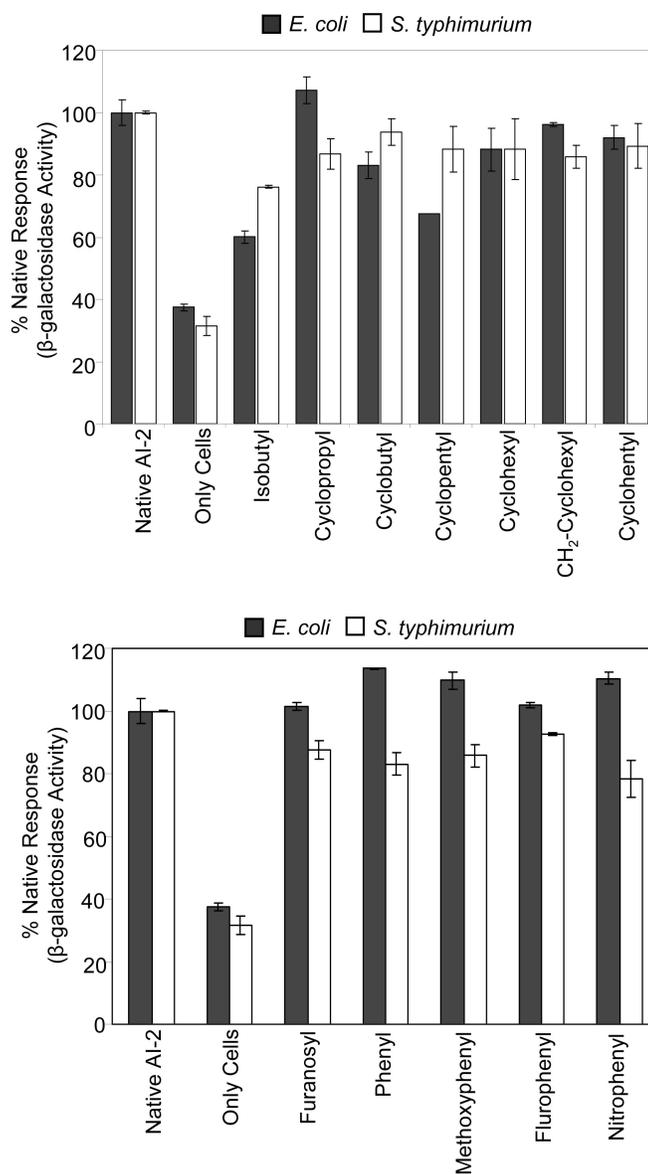


Figure 4-S1 Effect of analogs on native signaling in *E. coli* and *S. typhimurium*.

AI-2 dependent β -galactosidase production in *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 both (*luxS*⁺) in response to cyclic (top) and aromatic (bottom) analogs (100 % Native *E. coli* (ZK126) response = 597 Miller units and 100 % native *S. typhimurium* (MET708) response = 4776 Miller units).

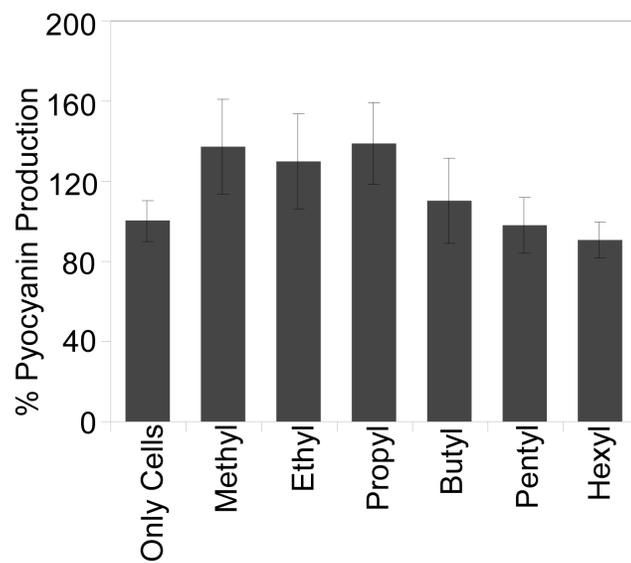


Figure 4-S2 Effect of linear analogs on pyocyanin production in *P. aeruginosa* PAO1.

Chapter 5: AI-2 Analogs and Antibiotics a Combinatorial Approach to Reduce *E. coli* Biofilms in a Microfluidic Setting

5.1 Abstract

Bacteria form complex aggregates of cells encased in thick polymeric matrices called biofilms. Biofilms are a protective cover for bacteria to evade antibacterial agents and are a significant contributor to the growing concern of antibiotic resistance. Quorum sensing (QS), the process of cell-cell autoinducer mediated signaling in bacteria facilitates biofilm formation. QS inhibitors that prevent biofilm formation and thus make bacteria more susceptible to antibiotics are a useful addition to the call for new alternative antimicrobial therapies. Autoinducer-2 (AI-2) analogs have been shown to inhibit genotypic QS responses in various bacteria. We demonstrate the ability of Isobutyl-DPD, one such AI-2 analog and an established broad species QS inhibitor, to significantly decrease growth of *E. coli* biofilms in an *in vitro* microfluidic flow cell. We also show that a combinatorial approach where Isobutyl-DPD is used with the antibiotic gentamicin is the most effective in causing near-complete clearance of pre-existing biofilms. Clearance of pre-existing biofilms is of high medical relevance as biofilms are seen in 80 % of human infections. Due to the ubiquitous presence of AI-2 in various species of bacteria and the prevalence of AI-2 mediated pathogenicity, AI-2 analogs used in combination with antibiotics have the potential of being broad spectrum pathogenic biofilm inhibitors.

5.2 Introduction

Bacteria that attach to surfaces can encase themselves in a self-synthesized hydrated polymeric matrix of polysaccharides and proteins to form slimy layers known as biofilms¹⁷⁵. Biofilms are a protected mode of growth for the bacteria and shield them from hostile environments. These structured communities enable a multicellular mode of existence quite different from planktonic bacteria¹⁷⁶. There are channels within the biofilm for maintaining flow of nutrients¹⁷⁷. Also, cells in different regions of the biofilm show different gene expression patterns¹⁷⁸. These and other attributes such as metabolic co-operativity have led to the comparison of biofilms with tissues of higher organisms¹⁷⁹. Biofilms are of high clinical relevance as biofilm formation occurs in 80% of human infections⁶⁵. Also, pathogens contained in a biofilm can have an antibiotic tolerance about 1000 times higher than that of their planktonic counterparts⁶⁶. Antibiotic therapy only kills the planktonic cells that slough off from biofilms, but are unable to eradicate the biofilm¹⁷⁶. Thus, biofilm infections typically become chronic infections, leading to continuous prescriptions of antibiotics and contributing to the clinical challenge of antibiotic resistance.

The formation of complex structures in biofilms is regulated by the exchange of chemical signals (autoinducers) in the cells via the process of quorum sensing (QS)^{123, 180}. *P. aeruginosa* is an opportunistic pathogen which forms thick biofilms on the lungs of cystic fibrosis patients. A *lasI* mutant, i.e. a mutant for the AHL (Acyl homoserine lactone) signal used for QS in *P. aeruginosa*, forms much thinner biofilms sensitive to removal by weak detergent as compared to its wild-type counterpart¹⁸⁰. The AI-1 autoinducers, AHLs, have been detected at significantly

higher concentrations (632 μ M) in *P. aeruginosa* biofilms grown *in vitro*, as compared to planktonic cells of the organism (14 nM)⁶⁷. AI-2, another type of autoinducer, is known as the universal autoinducer as its synthase (LuxS), is found in more than 70 species of bacteria³³. Several oral species such as *Streptococcus mutans*^{109, 110} and *Streptococcus gordonii*^{111, 112} that were *luxS* deficient show aberrant monospecies biofilms. Addition of *in vitro* synthesized AI-2 to *E. coli* increased their biofilm mass 30-fold⁷⁰. It was also shown that when YdgG the membrane spanning protein in *E. coli* (which is thought to be the AI-2 exporter) was removed, intracellular AI-2 concentrations increased and consequently, biofilm thickness and biomass significantly increased by 7000-fold and 574-fold, respectively⁴⁵. *E. coli* strains lacking the AI-2 processing enzymes *lsrK* and *lsrR* were found to form significantly thinner biofilms with altered architecture⁷¹. These findings show that QS and AI-2 play an important role in biofilm formation.

QS is thought to be an ideal drug target for the development of new antimicrobials as it does not target cell growth or viability. Therefore, its inhibition could prevent antibiotic resistance mechanisms such as biofilm formation. Consequently, there have been many studies on QS inhibitors and many have concentrated on AI-1 type AHL molecule analogs^{8, 9} and their effect on decreasing biofilms^{20, 181}. A few groups including us have recently developed AI-2 analogs^{94-96, 171} and shown them to be effective QS inhibitors. From our recently published screen, Isobutyl-DPD was shown to be the potent broad species QS inhibitor in *E. coli*, *S. typhimurium* and *V. harveyi*. In our study we observe the effect of different concentrations of Isobutyl-DPD on *E. coli* biofilms. The effect of the analog on the

biofilm formation is observed during its formation and growth, and also on pre-formed biofilms. We also test the affect of the analog in a combinatorial approach with the antibiotic gentamicin for clearing pre-formed biofilm. Clinically, infections are detected once biofilm formation has already occurred, thus the clearance of pre-existing biofilms is essential for an effective therapeutic approach.

Most biofilm studies are done in large flow cells¹⁸² with reactors volumes on the order of milliliters. In order to minimize the amount of analog used and perform these experiments in a high-throughput manner we conducted biofilm studies in custom-fabricated microfluidic flow cells with a volume of approximately 1 μL (Figure 5-1). In addition to minimizing the amount of reactants used, microfluidic systems provide several other advantages including inexpensive and user-customizable fabrication, highly parallel throughput, and tight control over the microenvironment for cell culture¹⁸³. While not a standard experimental platform for biofilm study, microfluidic devices are ideally suited for efficient bacterial biofilm formation and culture.

5.3 Materials and Methods

Microfluidic Device Fabrication and Assembly

Each microfluidic device consists of a coverslip serving as a base, and a molded microfluidic channel. The coverslip provides a transparent substrate that is also thin enough for high resolution confocal microscopy. The microfluidic channel is constructed from polydimethylsiloxane (PDMS) molded by photopatterned SU-8, a thick, durable photoresist. The mold is fabricated by patterning 100 μm thick SU8-50

(Microchem) on a silicon wafer using photolithography. The mold pattern used produces microfluidic channels that are 100 μm deep, 500 μm wide, and 2 cm long. PDMS (Sylgard 184, Dow Corning) is prepared in a 10:1 ratio of base to curing agent and poured over the prepared mold. The PDMS is cured over the mold in a furnace at 80 °C for 20 minutes, then peeled off the mold and cut to the size of the coverslip. Ports for interfacing the channel to fluidic tubing are drilled into the PDMS using a 2 mm dermatological punch. The PDMS is reversibly bonded to the glass coverslip by soaking the side of the PDMS to be bonded in methanol for 1 minute, then aligning and placing the section of PDMS over the coverslip.

The microfluidic channel is interfaced to external fluidic components using flexible Tygon tubing. Barbed tube fittings are inserted into the fluidic ports in the PDMS, and connected to one end of a segment of tubing. At the fluidic outlet, the other end of the tubing is connected to a syringe pump, and at the inlet, the other end is inserted into a sealed microcentrifuge tube serving as a reservoir for growth medium or other liquids to be flowed into the channel. Each microfluidic device is sterilized by flowing 70 % ethanol through the channel, and is positioned in an incubator held at 37 °C. A schematic of a device is shown in Figure 5-1.

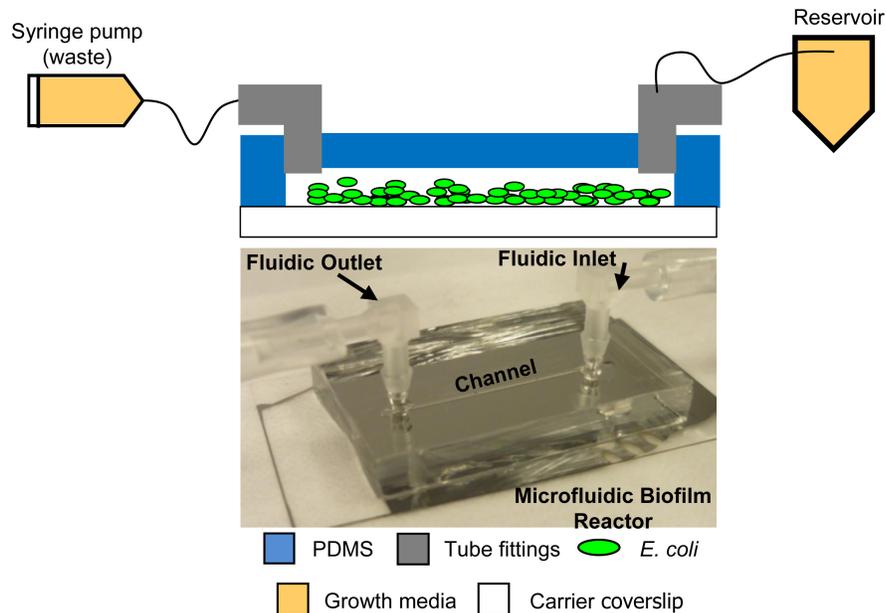


Figure 5-1 Schematic and picture of the microfluidic flow cell in which biofilms were formed.

The syringe pump was operated in withdrawal mode, providing flow of bacteria, growth media, or fluorescent dye from a reservoir through the channel. Note: the device photographed uses a coverslip with a chrome coating to aid in visualizing the microfluidic channel in the transparent PDMS.

Biofilm Formation

All the *E. coli* biofilms were formed using K-12 MG1655 ATCC (47076). The overnight culture was diluted to an OD of 0.25, flown into the microfluidic channel, and incubated with no flow at 37 °C for 2 hours. After this period, growth medium LB (Luria Bertani) was continuously flowed into the device at a volumetric flow rate of 10 $\mu\text{L/hr}$. The LB growth medium was supplemented with the required concentrations of analogs or antibiotic.

Biofilm Staining and Confocal Microscopy

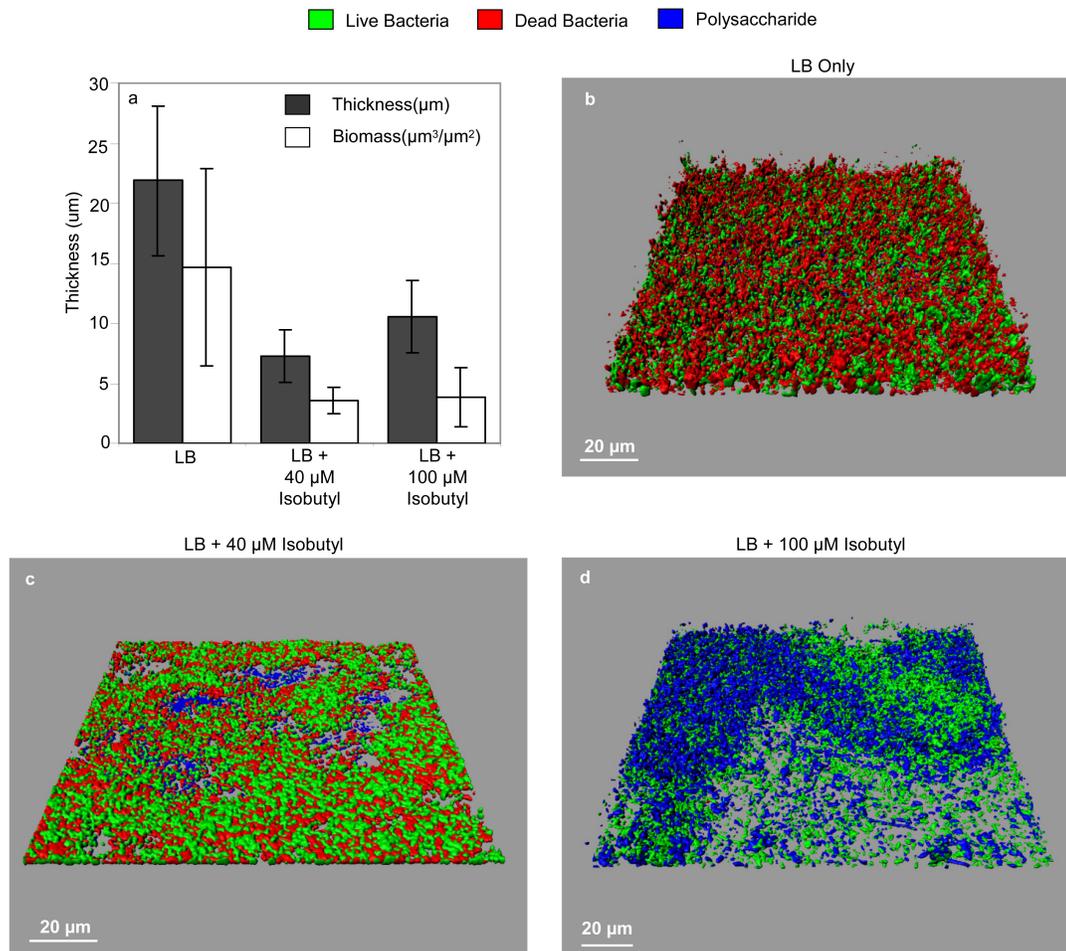
At the conclusion of a biofilm formation or treatment experiment, biofilms are fluorescently stained in preparation for microscopy. First, each biofilm is treated with a Live/Dead Bacterial Labeling Kit (Invitrogen #L7012). The two labeling components are mixed in a 1:1 ratio to a final volume of 10 μL . The dye is flowed into the channel at 10 $\mu\text{L/hr}$, the same flow rate as during biofilm growth. The Live/Dead stain is followed by 10 μL of a 100 $\mu\text{g/mL}$ calcofluor (Fluorescence Brightener 28, Sigma #F3543) stain for labeling polysaccharides contained in the biofilm matrix^{184, 185}. The dyes are fixed by flowing 3% paraformaldehyde into the channel at the same flow rate.

Labeled samples are imaged using a confocal microscope (Zeiss LSM710). Z-stacks were obtained at 7 points in each microfluidic channel, and each stack was comprised of 150 slices. The image stacks were analyzed using COMSTAT¹⁸⁶ which provides morphological information about the biofilm. The parameters provided by COMSTAT, including average thickness of the biofilm and biomass, were averaged over the image stacks imaged throughout the microfluidic channel. Additionally, surface reconstructions of the biofilms were created using Imaris (Bitplane) to aid in visualization of the resulting structures.

5.4 Results

5.4.1 Effect of Continuous Exposure of Analog on Biofilm Growth

The effect of the analog on biofilm growth was tested by continuously exposing the cells in the microfluidic flow cell to analog diluted in LB. As shown in Figure 5-2a, in the absence of any analog the biofilm grew to an average thickness of 22 μm and an average biomass of 14 $\mu\text{m}^3/\mu\text{m}^2$. The presence of 40 μM Isobutyl-DPD decreased the biofilm thickness and biomass by $\sim 70\%$ to 7 μm thick and the biomass mass to 3.5 $\mu\text{m}^3/\mu\text{m}^2$. Increasing the analog concentration to 100 μM did not increase the inhibition of the biofilm growth further. The biofilm in the case of 100 μM Isobutyl-DPD has an average thickness of 10.5 μm , and is thus slightly thicker than the biofilm formed with the lower Isobutyl-DPD concentration of 40 μM . However, repeated experiments showed that 100 μM of Isobutyl-DPD on an average decreased biofilm thickness very similarly to the 40 μM addition of the same compound. Surface rendering of the Z-stack images of the biofilm showed that in the case where LB was flown without Isobutyl-DPD the biofilm was much thicker and more structured (Figure 5-2b). The presence of analog in concentrations of both 40 and 100 μM made the biofilm less structured, i.e. extremely thin carpets of bacteria were seen as compared to the controls (Figure 5-2 c-d). The biofilm with 100 μM Isobutyl-DPD looked even more sparse i.e. (had less surface area coverage) than the biofilm formed with 40 μM Isobutyl-DPD. These results suggest that Isobutyl-DPD, which is a known inhibitor of QS responses via repressing the *lsr* operon¹⁷¹, can decrease biofilm formation in *E. coli*.



Note : - Analog was flowed in continuously while the biofilm formed for 48 hours.

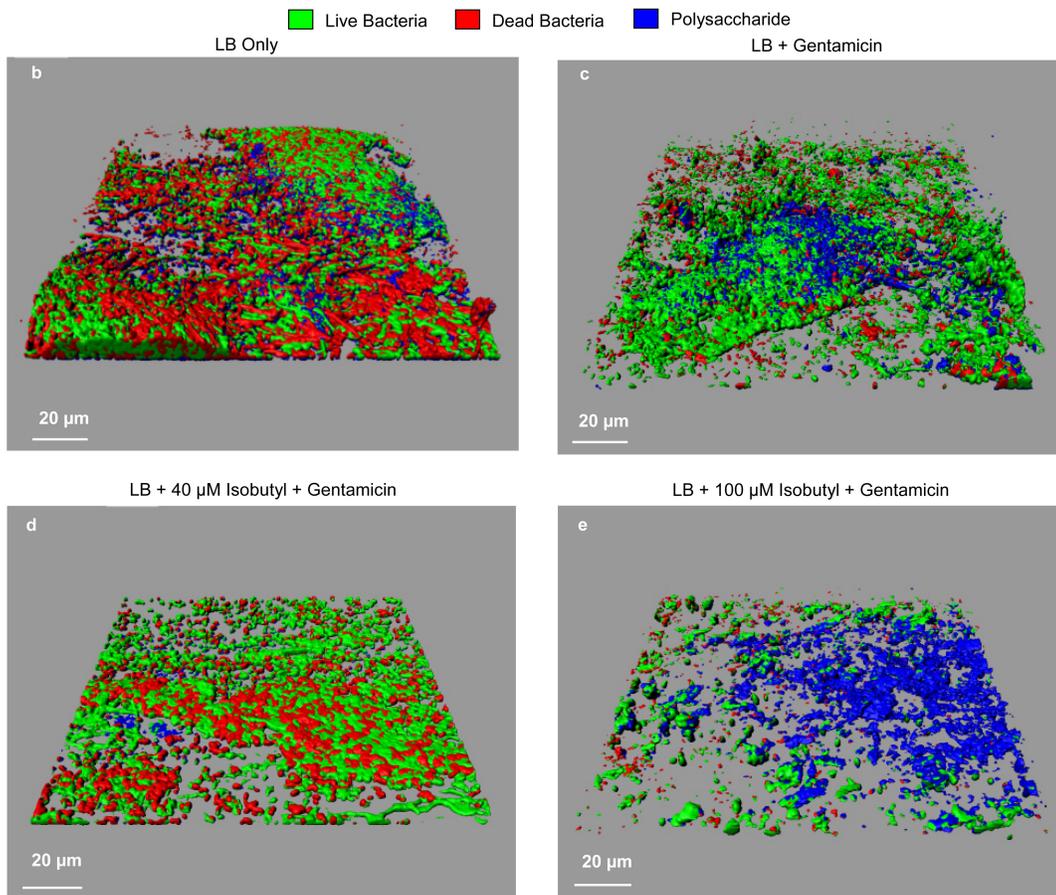
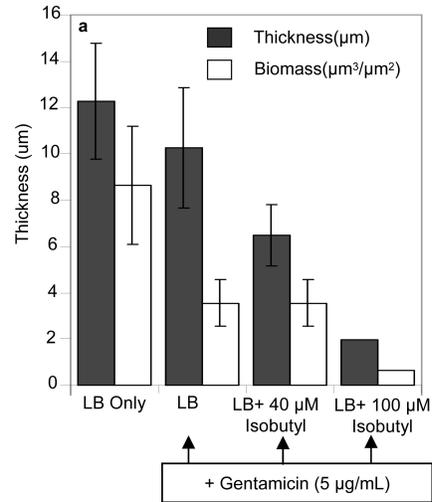
Figure 5-2 Analysis of biofilm thickness and architecture.

a) Thickness and biomass of biofilm analyzed by COMSTAT (Average of 4 different points). b-d) Representative Imaris 3-D surface reconstructions of the biofilm with b) LB Only, c) LB + 40 μM Isobutyl-DPD and d) LB + 100 μM Isobutyl-DPD

5.4.2 The Combinatorial Approach: Effect of Analog on Preformed Biofilm in the Presence of 100 μM Gentamicin

During a bacterial infection symptoms mostly present in the patient once the bacterial infection, or related biofilm has already established itself in the host. Hence, to be an effective therapy against biofilms, Isobutyl-DPD should be able to clear

preformed biofilms. An analog such as Isobutyl-DPD is an established QS quencher but it has been shown that it is neither bacteriostatic or bacteriocidal. Thus if only Isobutyl-DPD is used to treat the bacteria and the pathogenic bacterial population is not removed from the host the infection will reoccur. Hence, the more appropriate therapy would be a combinatorial approach where QS inhibitors like AI-2 analogs are used in combination with antibiotics to effectively inhibit biofilm formation and also attack bacterial cell viability. In order to test this approach, the *E. coli* biofilms which were pre-formed for 36 hours in the channel. After this, a combination of gentamicin (5 $\mu\text{g}/\text{mL}$) with increasing concentrations of Isobutyl-DPD (40 μM and 100 μM) were used to treat the pre-formed biofilm for an additional 36 hours. The control channel where the biofilm was only exposed to LB formed the thickest biofilm with an average thickness of 12 μm and with the highest average biomass of 8.5 $\mu\text{m}^3/\mu\text{m}^2$ (Figure 5-3a). Addition of gentamicin without analog decreased the biofilm partially to 10 μm thick, and the biomass by more than 50% to 3.5 $\mu\text{m}^3/\mu\text{m}^2$. Addition of the cocktail of antibiotic and 40 μM analog decreased the average biofilm thickness significantly to 6 μm . Most importantly, this study showed that 100 μM Isobutyl-DPD used with gentamicin was the most effective in clearing the biofilm, shown by the reduction in thickness by more than 80% to an average thickness of 2 μm and the biomass was nearly completely removed. The 3-D surface rendering images confirmed the morphological data (Figure 5-3 b-e), the biofilm surface thickness decreased significantly in the presence of both Isobutyl-DPD and gentamicin. At 100 μM of analog and gentamicin the biofilm was extremely sparse.



Note : - Biofilms were formed for 36 hours and then analog and antibiotic was flowed over the preformed biofilm for another 36 hours.

Figure 5-3 Analysis of effect of combinatorial approach analog and gentamicin on preformed biofilm thickness and architecture.

a) Thickness and biomass of biofilm analyzed by COMSTAT (Average of 6 different points). b-e) Representative Imaris 3-D surface reconstructions of the biofilm with b) LB Only, c) LB + 5 $\mu\text{g}/\text{mL}$ Gentamicin, d) LB + 40 μM Isobutyl + 5 $\mu\text{g}/\text{mL}$ Gentamicin and e) LB + 100 μM Isobutyl+5 $\mu\text{g}/\text{ml}$ Gentamicin.

5.5 Discussion

There are three main identified hypothesis of antibiotic resistance to biofilms¹⁸⁷. Firstly the antibiotic is unable to penetrate completely through the thick layers of the biofilm¹⁸⁸. Secondly the altered chemical environment inside the biofilm; for example the accumulation of acidic waste products in the biofilm can inactivate the antibiotic. Differences in nutrient gradient concentrations and anaerobic niches in the biofilms can lead to cells entering a dormant state whether they are neither dead or alive, somewhat similar to spores¹⁸⁹. These cells known as persister cells are highly resistant to antibacterial agents¹¹⁶. QS inhibitors can weaken thickness and all the above traits of biofilms used for antibiotic resistance. The effectiveness of a combinatorial therapy *in vivo* where a natural QS inhibitor such as garlic extract is used with the antibiotic tobramycin was shown by Bjarnsholt *et al.* to make biofilms on mice lungs more susceptible to antibiotic clearance than tobramycin used on its own¹⁹⁰. This study for the first time demonstrates the effectiveness of an AI-2 analog in decreasing the growth of an *E. coli* biofilm. Our study highlights the importance of a QS inhibitor and antibiotic combinatorial therapy as being the most effective in significantly clearing the *E. coli* biofilm. We purposely used a concentration of gentamicin (5 $\mu\text{g}/\text{ml}$) which is on the lower side of its various published MIC ranges,

because if analogs can help lower the concentration of antibiotics required to be effective inhibitors, the killing of the surrounding natural microflora can be prevented. Also if a lower concentration of antibiotic is an effective therapeutic dose it will prevent development of resistance which can be caused by continuous use of higher concentrations of antibiotics. As Isobutyl-DPD has been shown to be an effective inhibitor of QS responses in *S. typhimurium* and *V. harveyi*, this study could be extended to inhibit biofilm formation in these organisms. We have recently developed a diverse toolbox of AI-2 analogs which can have broad spectrum or selective QS inhibitory effects in mixed species culture. In the future we will use analog cocktails to inhibit mixed species biofilms. As many infections in the ear¹¹⁹, gut¹⁷⁵ and oral¹⁹¹ cavity consist of polymicrobial biofilms, this approach will test the effectiveness of AI-2 inhibitors in combating clinically relevant mixed species biofilms.

Chapter 6: Conclusions

6.1 Results Summary

In this dissertation, we developed two strategies to intercept AI-2 mediated QS signaling of bacteria in both pure cultures and mixed species co-cultures. The first method used native AI-2 processing enzymes such as *E. coli*'s QS kinase LsrK which was delivered outside the cell to phosphorylate and degrade AI-2 and hence, quench the QS response. In the second strategy a diverse library of AI-2 analogs was developed and both broad spectrum and species specific quorum quenchers were identified. We also showed the ability of the most potent analog from the screen isobutyl-DPD to inhibit biofilm formation in *E. coli*. Both of the strategies are neither bacteriostatic nor bacteriocidal and thus there is less selective pressure in the bacteria to evolve resistant mechanisms against them.

In chapter 2, the concept of using the AI-2 processing enzyme LsrK and ATP to phosphorylate and degrade AI-2 *ex vivo* was presented and tested. The concept of a synthetic ecosystem (a co-culture of different species of bacteria) with species-specific reporters to differentiate their responses was also developed in this chapter. *In vitro* synthesized LsrK with ATP was used to phosphorylate AI-2 *in vitro*. Phosphorylated AI-2 significantly quenched the QS response of *lsr* expression in *E. coli* and *S. typhimurium* and bioluminescence production in *V. harveyi* in pure cultures as well as a tri-species synthetic ecosystem of the same. *Ex vivo* delivery of LsrK and ATP also quenched native AI-2 mediated cross-talk between *E. coli* and *S. typhimurium* in a co-culture of these bacteria.

In chapter 3, a library of 14 C1-alkyl analogs of DPD (the AI-2 precursor) was developed. We screened the whole library to identify quenchers of *lsr* expression in *E. coli* and *S. typhimurium*. Even though the QS circuits of *E. coli* and *S. typhimurium* are completely homologous, *E. coli lsr* expression could be quenched by a wide variety of the analogs while only butyl-DPD and isobutyl-DPD caused potent quenching in *S. typhimurium*. Investigation of the biological mechanism of action of the analog in *E. coli* revealed that an analog could enter the cell independent of the known AI-2 transporter. It was also identified that LsrK phosphorylated most of the AI-2 analogs and phosphorylation was essential for the analog to be an effective QS inhibitor in *E. coli*. The analogs were ineffective in LsrR mutants; demonstrating that the presence of the LsrR repressor was essential for the analog to initiate QS repression. Some of the analogs were also tested in the tri-species ecosystem of *E. coli*, *S. typhimurium* and *V.harveyi* (developed in chapter 2). Isobutyl-DPD was identified as the broad species QS quencher while isopropyl-DPD was species specific.

In chapter 4, the existing analog library was expanded by the addition of cyclic and aromatic analogs. In this new analog screen only cyclopentyl caused significant QS quenching in *E. coli*. None of the analogs were effective against *S. typhimurium*. It was determined that both the shape and size of the alkyl ring were crucial for an analog to repress *lsr* expression. The opportunistic pathogen *P. aeruginosa* was also added to this investigation. Phenyl, cyclopentyl and heptyl-DPD from the new screen prevented pyocyanin production (i.e. QS associated toxin production) in *P. aeruginosa*. Isobutyl-DPD the potent QS quencher from the previous

screen was ineffective against *P. aeruginosa*. In this study we also demonstrated the concept of AI-2 analog cocktails to quench QS *en masse* in a tri-species synthetic ecosystem composed of *E. coli*, *S. tyhimurium* and *P. aeruginosa*.

In chapter 5, isobutyl-DPD was used to inhibit *E. coli* biofilm formation in microfluidic channels. Isobutyl-DPD inhibited biofilm growth in thickness by ~50%. A combinatorial approach where isobutyl-DPD was used with gentamicin was the most effective and caused near complete removal of *E. coli* biofilms.

Table 6-1 Summary of AI-2 Inhibition Strategies

Note: - The strategies in red have been developed by work done in this dissertation.

AI-2 Inhibition Strategies			
Inhibitor	Action Mode	Biological Effect	Ref
Inhibition Target: - Signal Generator			
BuT-DADMe-Immucillin-A (Transition State Analog)	Pfs inhibitor	Inhibit AI-2 production and biofilm formation in <i>V. cholerae</i> and <i>E. coli</i> O157:H7	75
Pei Compound 10 and 11	LuxS inhibitor	Not tested	86
Inhibition Target: - Signal Molecule			
<i>Ex vivo</i> addition of LsrK and ATP	Phosphorylation & degradation of AI-2	Inhibits bioluminescence in <i>V. harveyi</i> and <i>lsr</i> expression in <i>E. coli</i> and <i>S. typhimurium</i>	192
Inhibition Target: - Signal Generator			
DPD- C1- Alkyl Analogs			
(i) Linear (C1 to C4, C6), phenyl and azidobutyl	Compete with AI-2 for receptors	Agonists of bioluminescence in <i>V. harveyi</i> and antagonists of <i>lsr</i> expression in <i>S. typhimurium</i>	94
(ii) Linear (C1 to C4), isopropyl, tertbutyl cyclopropyl & cyclohexyl	Compete with AI-2 for receptors	Agonists of bioluminescence in <i>V. harveyi</i>	95
(iii) Butyl and pentyl-DPD	Compete with AI-2 for receptors	Inhibit pyocyanin (toxin) production in <i>P. aeruginosa</i> ~50%	97
(iv) Linear (C1 to C7), branched (isobutyl, 2-methylpropyl, isopropyl, neopentyl), deoxy and cyclopropyl	Compete with AI-2 for receptor; most likely LsrR	Antagonists of <i>lsr</i> expression in <i>E. coli</i> and <i>S. typhimurium</i> . Isobutyl-DPD prevents biofilm formation in <i>E.coli</i> .	171
(v) Cyclic and Aromatic alkyl chains	Compete with AI-2 for receptors	Phenyl-DPD and Heptyl-DPD inhibit pyocyanin production <i>P. aeruginosa</i> ~50%	
Inhibition Target :- Various ; Naturally Obtained QS Inhibitors			
Brominated Furanones			
(i) (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone)		Inhibited AI-2 production in <i>V. harveyi</i> 5500 fold, inhibited expression of AI-2 controlled genes in <i>E. coli</i> , protected shrimp from <i>V. harveyi</i> infections	99, 104
(ii) (Z)-4-bromo-5-(bromomethylene)-3-alkyl-2(5H)-		Decreased biofilm formation in <i>S. typhimurium</i>	101
Fatty Acids from poultry meat washes		Decreased bioluminescence from <i>V. harveyi</i>	107

6.2. Challenges and Future

The role of AI-2 QS signaling has been established in the pathogenicity of several significant human pathogens. It is the ubiquitous nature of AI-2 that makes this an excellent target for QS inhibition and as a potential antimicrobial therapy. Since AI-2 is not necessary for cell growth or survival, interference with the synthesis and processing of this molecule will likely not promote resistance development, as is the case of many current antibiotic therapies. Furthermore, the universal nature of AI-2 lends itself well for use against a broad spectrum of pathogens. With that said, it is still doubtful that QS quenching drugs will be the “magic bullet” for bacterial infection treatment. Due to the high degree of diversity amongst bacterial species, the precise role of AI-2 in QS can vary. The presence of AI-2 is essential in establishing and accentuating virulence in some organisms¹⁹³, but its absence leads to virulence in others^{49, 52-55}, while it has no effect in some bacteria¹⁶². Since QS inhibitors can attenuate phenotypic response in some pathogens, a cocktail therapy of QS quenchers and traditional antibiotics may be a promising approach. In a study of *P. aeruginosa* lung infection, the biofilms on the lungs of mice treated with a QS-inhibitory garlic extract were more sensitive to the antibiotic tobramycin¹⁹⁰. Future antimicrobial therapies will likely trend towards these types of combinatorial approaches. Nonetheless, further probing and investigation of the molecular mechanisms of QS signaling system may provide the basis for a new class of QS inhibition based antimicrobial drugs. There are other molecules, in addition to the central QS components, in hierarchical regulatory signal cascades that integrate to functionalize the central components. By extending our understanding of these

molecules, new methods can be developed to tailor microbial QS and identify valid targets for screening and developing novel signal interference drugs may emerge.

6.3 Specific Future Directions

There can be many paths forward with the developed AI-2 interception strategies to not only enhance our understanding of AI-2 inhibition but to further advance their potential as medically relevant antimicrobials. In order to incorporate LsrK based AI-2 inhibition into an antimicrobial therapy, LsrK could be encapsulated with ATP delivered to sites of bacterial infection to modulate QS phenotypes in environments prone to QS pathogenicity such as biofilm formation.

The AI-2 analogs can be tested in various mutants of AI-2 processing enzymes in *S. typhimurium* (for example, LsrB- transporter mutant and LsrR mutant) as they were tested for *E. coli* in chapter 2. This could help us in understanding why the homologous QS pathways in *E. coli* and *S. typhimurium* are not inhibited by the same analogs. Or in other words, why is the *E. coli* machinery more flexible in processing and being inhibited by different analogs while *S. typhimurium*'s is more restricted.

His-tagged LsrR was not very stable *in vitro*, and it has been difficult to do binding studies with it. *In vitro* stable LsrR could be synthesized by purifying LsrR with different methods such as GST-tagged LsrR. Stable LsrR would enable *in vitro* binding studies of analog to LsrR. Some groups are characterizing the crystal structure of LsrR. 3-D modeling of LsrR and predicted modeling of the binding of the analogs to the LsrR can be done *in silico*. Understanding the binding of Isobutyl-DPD

to LsrR will help us design a more potent analog with higher binding constants and lower IC-50 for AI-2 inhibition. The IC-50 values for analogs in the different categories are currently being characterized.

The effect of some analogs like isobutyl-DPD and phenyl-DPD which show promise for developing antimicrobials by being potent QS quenchers could be characterized on the whole genome of *E. coli* as well as the genome of some eukaryotic cells via microarray studies. It is essential to determine the effect of AI-2 analog based small molecules on eukaryotic cells before we can think of them as effective antimicrobials.

In this dissertation we studied the effect of isobutyl-DPD on *E. coli* biofilms there is potential for studying the effect of analogs on biofilms of various bacteria and also use analog cocktails to inhibit mixed species biofilms.

6.4 Broader Impact Significance of the Work

The work presented in this dissertation provides some fundamentally different ways of thinking about silencing AI-2 mediated QS mechanisms. A lot is known about the intra-cellular AI-2 processing machinery in various bacteria. In this study we used the known *E. coli* native intracellular AI-2 processing machinery to quench the AI-2 signal extracellularly. This mechanism can be extended to other known AI-2 processing enzymes in other bacteria which could be deployed *ex vivo* to quench QS. Although AI-2 analogs existed before the library in this study was created, this dissertation for the first time elucidates their biological mode of action. Probing the mechanisms of AI-2 analog action opens avenues for developing more potent AI-2

analogs and identifying more drugable targets in QS circuits. Both the strategies described in this dissertation, target DPD the precursor of all discrete structures of AI-2, thus these techniques have a potential of silencing QS in a wide variety of bacterial infections irrespective of the particular AI-2 structure or transport/sensor mechanisms used in the bacteria. Thus, this highlights the importance of this dissertation in developing broad spectrum antimicrobials which are well suited to be used in polymicrobial infections. We demonstrated the impact of the AI-2 inhibitors in silencing QS in subpopulations or *en masse* in multi-species ecosystems. Inhibitors that can target QS of particular organisms are more useful from a medical perspective as due to the ubiquitous nature of AI-2 it is also used by the good microflora. This dissertation helps enable a more mechanistic understanding of cell-cell communication within mixed cultures where competing signals from a variety of bacteria are synthesized and perceived in an otherwise undefined network. That is, specific interception of one signal molecule within a network of various signaling stimuli will help to elucidate its function in relation to others as the population “phenotype” is being determined. Our results suggest entirely new modalities for interrupting or tailoring the network of communication among bacteria.

Author Contributions

Varnika Roy designed and conducted the experiments and wrote this dissertation. The AI-2 analog panels were synthesized by Jacqueline A. I. Smith. The chemical orbital calculations in Chapter 3 were done by Jingxin Wang. The microfluidic flow cell was fabricated by Mariana T. Meyer.

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