



2012

BIOSENSING SYSTEMS FOR THE DETECTION OF BACTERIAL QUORUM SENSING MOLECULES: A TOOL FOR INVESTIGATING BACTERIA-RELATED DISORDERS AND FOOD SPOILAGE PREVENTION

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BIOSENSING SYSTEMS FOR THE DETECTION OF BACTERIAL QUORUM SENSING
MOLECULES: A TOOL FOR INVESTIGATING BACTERIA-RELATED DISORDERS AND FOOD
SPOILAGE PREVENTION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

BIOSENSING SYSTEMS FOR THE DETECTION OF BACTERIAL QUORUM SENSING MOLECULES: A TOOL FOR INVESTIGATING BACTERIA-RELATED DISORDERS AND FOOD SPOILAGE PREVENTION

Quorum sensing enables bacteria to communicate with bacteria of the same or different species, and to modulate their behavior in a cell-density dependent manner. Communication occurs by means of small quorum sensing signaling molecules (QSMs) whose concentration is proportional to the population size. When a QSM threshold concentration is reached, certain genes are expressed, thus allowing control of several processes, such as, virulence factor production, antibiotic production, and biofilm formation. Not only many pathogenic bacteria are known to produce QSMs, but also QSMs have been identified in some bacteria-related disorders. Therefore, quantitative detection of QSMs present in clinical samples may be a useful tool in the investigation and monitoring of bacteria-related diseases, thus prompting the use of QSMs as biomarkers of disease. Herein, we have developed and utilized whole-cell biosensing systems and protein based biosensing systems to detect QSMs in clinical samples, such as, saliva, stool, and bowel secretions. Additionally, since bacteria are responsible for food spoilage, we employed the developed biosensing systems to detect QSMs in food samples and demonstrated their applicability for early identification of food contamination. Furthermore, we have utilized these biosensing systems to screen antibacterial compounds employed for food preservation, namely, generally regarded as safe (GRAS) compounds, for their effect on quorum sensing.

Keywords: Whole-cell based biosensing systems, protein based biosensing systems, LuxP binding protein, Inflammatory bowel disease, Generally recognized as safe compounds

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I dedicate this dissertation to my mother and my father for unselfishly encouraging me throughout my Ph.D. Further, I dedicate this work to Dr. B. R. Ambedkar, the father of Indian constitution; without his vision, I will not have accomplished what I have.

ACKNOWLEDGMENTS

As a graduate student I would like to acknowledge the help and support of many people. First and foremost, I would like to express my thanks to my advisor, Dr. Sylvia Daunert, an inspiration to lead a research career. I not only learned various aspects of research under her guidance, but also learned how to work in a team and simultaneously manage it. I really thank her for all the guidance that she provided. I would also like to thank Dr. Leonidas Bachas for providing continual support and guidance throughout my time as a graduate student. Further, I would like to express my gratitude towards my committee members, Dr. Arthur Cammers, Dr. Janet Lump, Dr. Mark Lovell, and my external examiner Dr. Sue Nokes, for taking the time and effort to supervise my dissertation. I acknowledge Dr. Patrizia Pasini for her continual guidance throughout my PhD career. I acknowledge Dr. Melissa Newman (Animal and Food Sciences, University of Kentucky) for providing support while working on the GRAS compound project, and Dr. Maria Abreu (Division of Gastroenterology, University of Miami) for providing guidance while working with serum samples.

I thank my family, specially my parents, and wife for encouraging and supporting me throughout my graduate career. Finally, I would like to thank all the lab members, past and present, for all the help.

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CHAPTER ONE

INTRODUCTION

Cutting edge scientific and technological research has given rise to a gamut of new analytical tools that were previously unimaginable. With advancements in genetic research, an understanding of the workings of DNAs, RNAs, and proteins of prokaryotes as well as eukaryotes developed. Techniques of molecular biology have been used to manipulate and investigate these biological elements. A few of these techniques are Polymerase Chain Reaction (PCR) to amplify and mutate DNA, gel electrophoresis to purify DNAs and proteins, protein expression and purification techniques to produce and isolate proteins, southern and northern blotting to quantitatively detect target genes, and enzyme linked immunosorbent assay (ELISA) for analyte detection. Subsequently, nucleic acids and proteins as well as entire bacterial cells have been modified to be used in the design and development of biosensors¹⁻⁵.

A biosensor is defined as a biological element coupled with a transducer so that an analyte biospecific recognition event is translated into a measurable output. To date, a number of biosensors based on proteins and cells as the recognition elements have emerged, and a few of them have been applied as point of care devices⁶ and for environmental monitoring⁷. Although such biosensing systems offer huge promise in the management of a variety of disorders, including bacteria related conditions, such as infections, inflammatory bowel disease (IBD), and cystic fibrosis (CF), their use is still limited. Furthermore, complexities in the treatment of bacteria related conditions have

been on the rise due to the emergence of several antibiotic resistant bacteria and demand urgent attention for alternative treatment approaches.

Bacterial quorum sensing (QS) — a system of communication among bacteria that is based on small signaling molecules termed quorum sensing molecules (QSMs) — may offer an avenue of alternative therapy. In that regard, bacteria are known to employ QS to control processes, such as virulence factor production and biofilm formation, in a population size dependent manner; hence, disruption of the bacterial chatter necessary for pathogenicity may lead to a new way to treat bacterial infections. Additionally, investigations into bacteria related disorders have led to discoveries pointing to a role for QS in disease. For instance, QSMs have been identified in clinical samples of CF patients^{8,9}. Bacterial communities thrive in the human body; it has been suggested that the number of bacteria in the human body is at least one order of magnitude higher than the number of human cells. For example, bacteria colonize the gastrointestinal (GI) tract from mouth to anus and contribute to about 60 percent dry mass of feces. Usually, a symbiotic relationship exists between the host and the bacterial flora. However, evidence suggests that there may be changes in the microflora and host-microbial interactions during the course of disease. A change in the microflora would also result in alteration of the relative balance of QSMs. Therefore, detection of QSMs in physiological samples may be a valuable means to evaluate variations in bacterial composition and serve as a tool for monitoring the disease status and for investigating the disease mechanisms.

The present thesis work is focused on the development and application of biosensing systems that can be employed to detect QSMs in clinical samples. Different categories of QSMs exist as described in detail later. Specifically, we developed and utilized whole cell based biosensing systems to detect the following QSMs: long and short chain *N*-acyl homoserine lactones (AHLs) and Autoinducer-2 (AI-2). For that, several clinical matrices were investigated, including stool, saliva, bowel secretion, blood serum, and blood plasma. Each type of biological matrix is unique in its composition and, therefore, needs to be assessed for potential effects that it may exert on the analytical performance of each whole cell biosensing system.

With progress in the field of biotechnology, which led to important advancements, such as the large-scale production of recombinant protein drugs and vaccines, it has also been easier than ever to create biological threats that can be used in a bioterrorism attack and spread by means of daily utilized food products, such as milk and beef, thus affecting a larger percentage of the population. Additionally, bacteria are well known to be responsible for food spoilage. Forty eight million foodborne illnesses per year are reported in the United States, which leads to elevated health care costs, with more than a quarter million hospitalizations and more than 3000 deaths per year¹⁰. Therefore, there is a need for rapid, easy-to-use, cost-effective analytical tools for early detection of food decay and prevention of biological food contamination threats.

To that end, we evaluated the analytical performance of the developed whole cell biosensing systems in food matrices and employed them for the detection of QSMs

in food samples as a method for early detection of bacterial contamination in food. In addition, we explored a number of Generally Recognized As Safe (GRAS) compounds for their effect on bacterial QS. The selected compounds are known to inhibit bacterial growth and, due to their safety, have potential to be employed for food preservation; however, their mechanisms of action are not fully elucidated. Understanding the mechanism of action of these compounds would enable targeted preventive measures in combating bacterial contaminations as well as biological threats.

Lastly, a protein based sensor for the detection of AI-2 was designed and developed. This sensor relies on the direct interaction of a binding/recognition protein with a target analyte. Therefore, in addition to being useful for AI-2 detection in physiological and food samples, it is suitable for binding studies aimed at investigating and characterizing a variety of compounds able to interfere with QS, including the GRAS compounds mentioned above and molecules to be explored as new treatments for bacterial infections. We envision that the availability of a panel of biosensing systems for detection of QSMs will help augmenting our understanding of bacterial communities and, thus, facilitate the design of alternative strategies to combat bacteria related disorders.

Quorum sensing signaling molecules

Bacteria, although single-cell organisms, have complex behavior. One such behavior relates to the ability of bacterial cells to communicate with their neighboring cells. This phenomenon was first reported in 1965 by Tomasz¹¹ who described

“hormone like activators” that were crucial in the propagation of the Gram-positive bacterium *Streptococcus pneumoniae*, which is one of the organisms responsible for pneumonia pathogenicity in their hosts. Later, in 1970, Hastings *et al.* observed that the Gram-negative marine bacterium *Photobacterium fischeri* produces bioluminescence only when bacteria reach a certain cellular density in terms of number of cells present, and speculated that light emission was under a bacterial “control mechanism”. It is now well understood that bacteria of the same species or of different species communicate with each other by producing and sensing small chemical molecules in a process termed as quorum sensing. This phenomenon has been observed in a number of bacteria¹², and it is now understood that by means of QS, bacteria are able to control several processes, for example bioluminescence, virulence factor production, biofilm formation, antibiotic production, competence, nodulation, sporulation, clumping, and motility. The small chemical molecules that bacteria utilize for QS are known as QSMs. By detecting the QSMs, bacteria can modulate the above mentioned processes in a cell density-dependent manner. For example, in a bacterial infection, bacteria do not produce virulence factors until they reach a critical population density and, by doing so, they ensure that they can overwhelm the host’s immune response¹³.

Several groups of QSMs have been identified, which include AHLs in Gram-negative bacteria and a class of autoinducing peptides (AIPs) in Gram-positive bacteria. Whereas AHLs and AIPs are species-specific and, therefore, used for intra-species communication, a third category of molecules, AI-2, has been found in both Gram-positive and Gram-negative bacteria, suggesting a potential role in inter-species

communication within bacteria¹⁴. Table 1 lists some of these signaling molecules and the bacteria that use them in cell-to-cell communication. Briefly, AIPs are peptides ranging from 5 to 17 amino acids in length, which are post-translationally modified to yield a variety of linear and cyclic structures in Gram-positive bacteria. QSMs of Gram negative bacteria, AHLs, are composed of fatty acyl chain of 4 to 18 carbons in length attached to a homoserine lactone ring through an amide bond; AHLs present in nature have acyl chains of even numbers of carbons. Furthermore, the third carbon in the acyl chain can be fully reduced or may contain a hydroxyl or a carbonyl group. This substitution at the third carbon varies based on the fatty acid from which it is derived. Among the three types of QSMs mentioned above, AI-2 has been discovered more recently and shown to be comprised of a group of structurally related compounds; at least two of these compounds have been demonstrated to be employed as QSMs by bacteria, namely, a furanosyl borate diester form of AI-2 used by the marine bacterium *Vibrio harveyi* and a hydrated form, not containing boron, used by the intestinal pathogen *Salmonella typhimurium*¹⁵. Furthermore, some bacteria have multiple QS circuits based on more than one kind of QSM; for example, *V. harveyi* utilizes *N*-(3-hydroxybutanoyl)-L-homoserine lactone and the furanosyl borate diester form of AI-2 to control bioluminescence¹⁶. Recently, Sperandio *et al.* have discovered an additional QSM, autoinducer-3, AI-3 (unknown structure), which binds to the membrane protein QseC and activates virulence in enterohemorrhagic *Escherichia coli* (EHEC)^{17,18}. Further, it has been shown that the mammalian hormones, epinephrine and norepinephrine, are recognized by the same protein QseC and activate virulence in EHEC⁷, suggesting

involvement of the AI-3 QS system in interkingdom bacterial–mammalian cells communication.

Quorum sensing regulatory systems

The QS pathways of the three major types of bacterial QSMs listed above are outlined in Figures 1, 2, and 3. Most Gram-negative bacteria utilize AHLs as QSMs; their QS systems are similar to that of *Vibrio fischeri*, which was one of the first QS regulatory systems to be thoroughly investigated. This bacterium uses QS to regulate expression of the genes responsible for bioluminescence. It utilizes *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) as the cognate QSM, which is synthesized in the cytoplasm by the AHL synthase protein LuxI. Once formed, 3-oxo-C6-HSL diffuses out of the cell and freely enters into the neighboring bacteria, where it binds to the transcriptional regulatory protein LuxR upon reaching a threshold concentration. As the population density increases, the concentration of 3-oxo-C6-HSL also increases proportionally. By being able to synthesize and detect 3-oxo-C6-HSL, *V. fischeri* can thus estimate its population size. The LuxR protein, upon binding 3-oxo-C6-HSL, forms the LuxR–3-oxo-C6-HSL complex, which binds to the operator/promoter region of the *luxICDABE* operon to express the enzymes involved in bioluminescence. Specifically, the genes *luxA* and *luxB* encode bacterial luciferase, and the *luxC*, *luxD*, and *luxE* genes code for the enzymes required for the synthesis and recycling of the long chain aldehyde substrate for luciferase. The light output obtained is proportional to the amount of 3-oxo-C6-HSL present in the environment, which is relevant for the development of whole-cell sensing

systems based on QS regulatory systems, as highlighted in following sections of this work. While AHL based QS controls bioluminescence in *V. fischeri*, it regulates diverse functions in other bacteria by means of a variety of LuxI and LuxR homologue proteins. Notable examples are the AHL-dependent LasI/LasR and RhII/RhIR QS systems of *Pseudomonas aeruginosa*, which control production of virulence factors and biofilm formation in this microorganism. Figure 1 shows a general schematic of AHL dependent QS system.

AIPs are utilized by Gram-positive bacteria for QS. Various peptide lengths and post-translational modifications have led to diverse structures of AIPs, thus conferring selectivity and specificity. Several Gram-positive bacteria have been studied in detail and shown to follow a general theme, as outlined in Figure 2. Briefly, AIP originates from a precursor peptide through post-translational processing and modifications, and is then secreted out of the cell by an ATP-binding cassette transporter. The AIP signaling pathways are comprised of two component signaling systems. These two components include a sensing receptor protein, which binds the AIP, and a response regulatory protein, which translates the binding event into expression of target genes, including the QS regulated genes and those responsible for synthesis of the AIP precursor and release of AIP. Specifically, AIP binding to the receptor protein, which is a histidine sensor kinase, triggers autophosphorylation at a conserved histidine residue, followed by a cascade of phosphorylation events, which ultimately transfer the phosphate group to a conserved aspartate residue of the response regulatory protein. The phosphorylated response regulatory protein then binds to specific DNA regions and

activates transcription of the regulated genes. Some examples of two component systems include the ComD/ComE competence system in *S. pneumoniae*, ComP/ComA competence and sporulation system in *Bacillus subtilis*, and AgrC/AgrA virulence system in *Staphylococcus aureus*.

In contrast to QS systems utilizing AHLs and AIPs, which are usually specific to Gram-negative and Gram-positive bacteria respectively, those that utilize AI-2 have been found in both types of bacteria. Bassler *et al.* have reported that AI-2 is not only produced, but also sensed by a large number of bacterial species¹⁹. Generally, AI-2 refers to a group of isomers derived from the hydrated form of the precursor 2,3-dihydroxy-4,5-pentanedione (DPD) (Figure 3A). Although only the borate isomer derivative of DPD, *S*-THMF-borate, was previously thought to be the active form recognized by the protein LuxP and its homologues, more recently, a different isomer, *R*-THMF has been found to be the cognate molecule of the sensor protein LsrB in *S. typhimurium*. The mechanism of QS in AI-2 based systems is represented in Figure 3B. Briefly, AI-2 is synthesized in the cell by Pfs and LuxS enzymes. Pfs converts *S*-adenosylhomocysteine (SAH) to *S*-ribosylhomocysteine, which is converted to DPD by action of LuxS. Once formed, DPD undergoes instantaneous cyclization to form an isomeric mixture (Figure 3B). In the presence of borate, which is abundant in the oceanic environment, *S*-THMF-borate is formed. AI-2 diffuses out of the cell and can enter bacteria of the same or other species. The receptor of AI-2 in *V. harveyi* is the protein LuxP, which belongs to the class of periplasmic binding proteins. Upon AI-2

binding to LuxP, the LuxP-AI-2 complex regulates the expression of controlled genes through a cascade of phosphorylation/dephosphorylation steps.

Quorum sensing molecules in disease

Numerous *in vitro* and *in vivo* studies have demonstrated that expression of bacterial virulence factors responsible for infections in mammalian hosts is regulated by QS. Several such studies concerning GI tract infections have been discussed in a recent review by Kaper and Sperandio²⁰. For example, in the case of EHEC, which causes bloody diarrhea and hemolytic-uremic syndrome, involvement of AI-3 in regulating the expression of virulence factors was demonstrated in a HeLa cell infection model using wild-type EHEC and a LuxS mutant-LuxS has been shown to be involved in the production of AI-3²¹. In an animal study²², burn wounds of mice models were infected with wild-type *P. aeruginosa* and variants of the same bacterium carrying mutations in the genes encoding for AHL synthase. The objective of the study was to determine the efficiency of infection by assessing the ability of the bacteria to spread the infection and the time required for the onset of infection. It was observed that in mice infected with *P. aeruginosa* mutants unable to synthesize AHLs, the extent of infection was lower than in those infected with wild type bacterium. The evidence gathered is indicative of the relevance of bacterial communication in diseases of bacterial origin and, thus, the importance of detecting QS signaling molecules in physiological samples.

Identification of QS regulatory pathways in a number of bacteria¹² has enabled whole-cell-based biosensing systems to be engineered to detect QSMs by coupling the

genes coding for different QSM recognition and/or regulatory proteins to those of a variety of reporter genes²⁰. As an example, Winson *et al.* designed reporter plasmids by placing the reporter gene cassette *luxCDABE* under the control of the P_{lasI} and P_{RhlI} promoters from the *P. aeruginosa* QS regulatory systems LasI/LasR and RhlI/RhlR, respectively. The plasmids also contained the sequences of the LasR and RhlR proteins, which, upon binding AHLs, bind to the respective promoters, thus activating expression of the reporter protein²³. In another example¹⁶, Bassler *et al.* engineered a strain of *V. harveyi* in such a way that the bacterium's bioluminescence, which depends on expression of the *luxCDABE* gene cassette, was only triggered by AI-2, thus enabling detection of this QSM (as reported above, in wild-type *V. harveyi* expression of the bioluminescence genes is triggered by both AI-2 and an AHL, *N*-(3-hydroxybutanoyl)-L-homoserine lactone).

Most of the above cell-based systems have been used as bioassays to evaluate the ability of cultured bacteria to produce QSMs. However, only a few have been applied to the detection of QSMs in physiological and clinical samples in order to correlate the presence of pathogenic bacteria with the onset or status of disease. Two independent studies demonstrated the presence of AHLs in sputum samples from patients with cystic fibrosis^{8,9} by using whole-cell sensing systems. It is known that *P. aeruginosa* and *Burkholderia cepacia* colonize the airway passage and lungs in CF patients, leading to chronic lung infection and finally to destructive lung disease²⁴. These two species of bacteria use AHL-dependent QS regulation. In the study conducted by Middleton⁸ *et al.*, sputum samples from CF patients colonized by either *P. aeruginosa* or

B. cepacia, were extracted and then analyzed using an *E. coli* whole-cell-based biosensing system containing plasmids pSB401 and pSB1075 to detect short and long-chain AHLs, respectively. Among *P. aeruginosa*-colonized sputum samples, 71% showed the presence of short-chain AHLs and 61% showed presence of long-chain AHLs. Similarly, among *B. cepacia*-colonized sputum samples, 81% contained short chain AHLs and 50% contained long-chain AHLs. This AHL production profile was different from that of laboratory cultures of the same microorganisms isolated from sputum samples, in which long-chain AHLs were predominant. The difference in AHL profile was thought to be explained by dissimilar growth conditions, *in vitro* and in the lung. In that regard, this varied behavior of *P. aeruginosa* from lung infection had previously been observed by Singh²⁵ *et al.*, who hypothesized that *P. aeruginosa* exists predominantly as a biofilm in CF sputum. Further, LC–MS analysis of the same samples revealed the presence of short-chain AHLs, but not that of long-chain AHLs, indicating that the whole-cell-based biosensing system was more sensitive for detection of AHLs in sputum samples. Another study published the same year by Erickson *et al.* further corroborated the presence of AHLs in sputum samples from CF patients⁹. *P. aeruginosa*-based whole-cell biosensing systems containing plasmids pKDT17 (to detect long-chain AHLs) and pECP61.5 (to detect short chain AHLs), both utilizing *lacZ* as a reporter, were used.

Although the levels of AHLs detected were low, over 75% of the samples had long-chain AHLs whereas only 26% of the samples had short-chain AHLs. This finding is in contrast with the results of the study by Middleton⁸ *et al.* However, differences in the groups of patients, along with different sample extraction and/or processing methods

and the use of sensing systems based on different microorganisms might explain different results. Along the same lines, Chambers *et al.* detected a broad range of AHLs in mucopurulent respiratory secretion samples obtained from CF patients²⁶. They used *Agrobacterium tumefaciens*-based whole-cell biosensing system A136, containing plasmids pCF218 and pMV26 with *luxCDABE* as a reporter, allowing it to respond to both long and short-chain AHLs (4–12 carbon atoms). AHLs were extracted from the mucopurulent respiratory secretion samples, separated by reversed-phase fast pressure liquid chromatography (FPLC), and each fraction was then assayed with the *A. tumefaciens* A136 sensing system in 96-well microtiter plate format. Further, identities of the AHLs present in positive fractions were confirmed by comparing their retention times with those of standard AHLs. Using the whole-cell sensing system combined with FPLC, the authors were able to detect low concentrations of AHLs in small volumes of samples from nine (out of thirteen) CF patients and to identify seven different AHLs.

Our research group used whole-cell biosensing systems to evaluate QSMs in physiological samples from individuals affected by bacterial GI disorders, including IBD. Two of the major conditions of IBD, ulcerative colitis (UC) and Crohn's disease (CD), involve chronic and relapsing acute inflammation in the large and small intestine, respectively, with CD being able to affect any portion of the GI tract. Current methods of diagnosis and monitoring rely on endoscopic techniques and analysis of mucosal tissue biopsies taken from the inflamed regions. Alternative tools that can serve as non-invasive biomarkers for such diseases can prove very beneficial. To that end, we have developed and optimized *E. coli* based bioluminescent whole-cell sensing systems

containing plasmids pSB406 and pSB1075, which bear *luxCDABE* as a reporter, for detection of short and long-chain AHLs, respectively^{23,27}. These biosensing systems have then been used to evaluate AHLs in human samples, for example saliva and stool samples, which bear the advantage of being collected non-invasively. The effect of components of the sample matrix was evaluated by generating dose–response curves in spiked pooled samples after minimal processing (no sample extraction or extensive preparation). Importantly, nanomolar limits of detection were obtained in biological matrices, which is relevant in that nanomolar concentrations of QSMs are necessary to initiate cell-to-cell communication²⁷. Saliva samples from IBD and healthy individuals, and stool samples from newborns admitted to a neonatal intensive care unit (NICU) were then assayed²⁷. AHLs were detected at different levels in the specimens tested, showing for the first time the presence of these QSMs in such physiological samples. More recently, we also detected QSMs in blood samples of IBD patients (manuscript in preparation). If a correlation is established between the QSM levels in samples and the health status of a patient, it may be possible to use QSMs as biomarkers of bacteria-related disorders, which should aid in the management of the disease. Studies are currently in progress in our group in which physiological samples from selected sets of GI patients and matched controls are analyzed for their QSM content to evaluate relationships between QSM levels and disease status. One such clinical study involved stool samples that were obtained from infants admitted to the NICU for a variety of illnesses, including bowel inflammation and bacterial sepsis²⁸ (submitted manuscript).

Interference with quorum sensing

QS has evolved to assist bacteria to effectively communicate among each other and regulate expression of phenotypes that are involved in pathogenicity, such as, virulence determinant production and biofilm formation. In the past few decades, several examples of interference with bacterial communication as a means to interrupt the bacterial chatter and, thus, block pathogenicity, have been reported. Since bacterial QS depends on the presence of QSMs and their interaction with cognate receptors, any molecule or enzyme that either blocks the synthesis or accumulation of QSMs, or prevents them from binding to the receptor proteins, can interfere with QS.

QS interfering compounds include those from natural sources²⁹⁻³¹ as well as those that are synthesized^{32,33} in the lab. Jakobsen³⁴ *et al.* have analyzed over 60 extracts of vegetables, fruits, and spices, and found that extracts from horseradish, Tasmanian blue gum, and ginkgo showed a clear QS inhibitory activity in the screening assays employed. Further investigation using LC-MS and NMR spectroscopy led to the identification of iberin, an isothiocyanate (Figure 4A), as the compound responsible for the potent inhibitory effect of horseradish on QS in *P. aeruginosa*. Widmer and co-workers³⁵ studied several fatty acids from poultry meat and proved that four of them, linoleic acid, oleic acid, palmitic acid, and stearic acid, exhibited an inhibitory effect on the AI-2 based QS system (Figure 4B). Few other natural compounds that have been reported to interfere with QS include cinnamaldehyde from cinnamon³⁶ and ursolic acid from apples³⁷. Works by Koh *et. al* and by Zahin *et. al* provide further evidence of the anti-QS activities of the traditional Chinese medicinal plants³⁸ and medicinal plants from India³⁹,

respectively. These studies prompted us to investigate GRAS compounds for their effect on QS. GRAS compounds are food additives that are considered to be safe and are approved by food and drug administration¹⁰. A database of GRAS compounds is maintained by the FDA⁴⁰, and currently includes 373 compounds, with more compounds added each year.

Inhibition of QSM synthesis

Since all types of QSMs are synthesized in the bacterium by their respective synthases, it is apparent that if the synthase enzyme is inhibited, QS and the genes regulated by QS will also be affected. As a result, several compounds that interfere with QS by inhibiting the synthesis of QSMs have been identified. For example, in *P. aeruginosa* the AHL synthase RhII synthesizes *N*-butanoyl-L-homoserine lactone (C-4 HSL) by using crotonyl-acyl carrier proteins and *S*-adenosylmethionine (SAM). The compounds *S*-adenosylhomocysteine and sinefungin are analogues of SAM (Figure 5A) that inhibit the RhII catalyzed synthesis of C-4 HSL⁴¹. Similar studies have been conducted to find inhibitors that can block AI-2 synthesis⁴²⁻⁴⁴. The precursor *S*-ribosyl-*L*-homocysteine (SRH) is required for AI-2 synthesis; Zhou *et. al* reported that the SRH analogues, *S*-anhydroribosyl-*L*-homocysteine and *S*-homoribosyl-*L*-cysteine, exhibited inhibitory activity against the AI-2 synthase, LuxS⁴³ (Figure 5B). In addition, Shen *et.al* synthesized and analyzed several SRH analogs for their activity as LuxS inhibitors. They found that two analogs of SRH, (2*S*)-2-amino-4-[(2*R*,3*S*)-2,3-dihydroxy-3-*N*-hydroxycarbamoylpropylmercapto]butyric acid and (2*S*)-2-amino-4-[(2*R*,3*R*)-2,3-

dihydroxy-3-*N*-hydroxycarbamoylpropylmercapto]butyric acid, acted as potent inhibitors of LuxS⁴².

Degradation of QSMs

A well-studied example of interference with AHL-mediated QS is that of QSM degradation by AHL lactonases, which break open the lactone ring of AHLs to produce the corresponding acyl homoserines, thus reducing the activities regulated by QS⁴⁵. In addition to lactonases, there are acylases that work by breaking the AHL amide bond to produce fatty acids and homoserine lactones⁴⁵. Genome analysis revealed that acylase and lactonase homologues are present in a number of bacterial species. The fundamental knowledge of lactonases was put to use by Dong *et. al*, who introduced the *aiiA* gene encoding for an AHL-lactonase into the genome of tobacco and potato plants⁴⁶. After infecting these plants with pathogenic *Erwinia carotovora*, the transgenic plants showed a substantial reduction in the extent of infection as compared to the control plants without *aiiA*. In addition to these naturally existing interfering enzymes that exert their action by degrading the QSMs, an alternative approach was proposed by Janda⁴⁷ *et. al* who produced an anti-autoinducer monoclonal antibody that could inhibit QS by sequestration of the AIP-4 produced by group 4 *S. aureus*. Further, they utilized the anti-autoinducer antibody in a mouse model and found that it did not only suppress, but also provided complete protection against the *S. aureus* infection.

Interference with QSM receptor

Irrespective of the type of bacteria, a QSM either binds to a membrane bound receptor or passively transported in the cell where it binds to its cognate receptor⁴⁸. Therefore, molecules that bind to the QSM receptor but do not elicit subsequent biological response, can serve as inhibitors of QS and the processes regulated by the QSM receptor.

A number of molecules have been investigated that inhibit QS by binding to the QSM receptor. In the Gram positive bacteria *S. aureus*, a truncated form of an autoinducing peptide specific for a certain group of bacterial strains was able to inhibit the *agr* virulence response in all four groups of *S. aureus* strains⁴⁹; it was found that the inhibited target was the AIP receptor, histidine sensor kinase AgrC. Further studies identified several compounds that were able to show an inhibitory effect due to binding to the AIP receptors^{50,51}. QS systems utilizing AHLs as QSMs can also be inhibited by compounds that bind to the AHL receptors. Efforts have been mostly focused on finding AHL antagonists by modifying AHLs, which are comprised of a lactone ring and an acyl side chain. Therefore, inhibitors that block the AHL-receptor interaction usually are AHL derivatives with structural modifications in the lactone ring or acyl side chain or both⁵². Gram-negative bacteria have evolved to express different receptors for recognizing structurally diverse AHL molecules. While a type of AHL molecule might act as agonist in some bacterial species, it might act as antagonist in others. As an example, C-6 HSL is an agonist of CviR of *Chromobacterium violaceum*, but an antagonist of LuxR of *V. fischeri*⁵³. By using a QS inhibitor selector system³⁰, Persson *et. al* designed and synthesized a number of derivatives of AHLs that showed an inhibitory effect in the *las* system of *P.*

*aeruginosa*⁵⁴, with *N*-(heptylsulfanylacetyl)-L-homoserine lactone demonstrating the highest inhibitory activity (Figure 6A). In bacteria employing AI-2 based QS systems, two periplasmic binding proteins have been identified as AI-2 receptors thus far, that is, LuxP in *V. harveyi* and LsrB in *S. typhimurium* and *E. coli*; the former binds to the borate form of AI-2, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate), while the latter binds to (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) in *S. typhimurium*¹⁵. There is a limited amount of research to develop agonists/antagonists of the receptors LuxP and LsrB. Although Lowery *et. al* have developed several analogs of DPD⁵⁵, the precursor of AI-2, none of them were effective as agonists or antagonists. On the other hand, Ni *et. al* screened a number of boronic acids and aromatic diols that would form a complex with boric acid and found that *para* substituted boronic acids⁵⁶ and pyrogallols⁵⁷ were capable of inhibiting AI-2 based quorum sensing (Figure 6B).

Interference by other bacterial species and strains

S. aureus uses AIP and AgrCA two-component systems to regulate various activities, including production of virulence factors and biofilm formation. Among different strains of *S. aureus*, the AIP and its receptor AgrC show considerable intraspecies variations, which account for signal specificity and are used to identify four major groups of *S. aureus* strains. Interestingly, while each group AIP activates expression of virulence in bacteria of its own group, it represses the virulence in other groups of *S. aureus*. Mayville *et. al* studied this phenomenon in a mouse model. Addition

of AIP II, the AIP signal produced by group II *S. aureus*, to a lesion infected with group I *S. aureus* reduced the infection, as apparent from the reduced size of the lesion⁵⁸. In another study, Xavier *et. al* have shown that, in a mixed species community of bacteria that are able to produce and/or detect AI-2, one species of bacteria degrades the AI-2 produced by another species, thus interfering in the communication system of the latter⁵⁹.

Biosensors

The need of living organisms to recognize and respond to changes in their environment exemplifies biosensing in nature. Biosensing involves selective and sensitive molecular recognition between proteins and a target ligand analyte, even when present at very low concentrations. Researchers have tried to mimic the exquisite properties found in nature by utilizing naturally occurring recognition elements to develop biosensing systems for analytical applications. To that end, a biological recognition element capable of reversible binding to a target ligand analyte is coupled to a transducer element that converts the recognition event into a readable/measurable output. Several biological recognition elements, including proteins, nucleic acids, cell organelles, and intact cells, have been used as sensing elements in biosensors for applications in environmental, biological, pharmaceutical, and clinical analysis^{60,61}. Proteins with high specificity for analytes of interest have been extensively used in the development of biosensors. These proteins include enzymes, antibodies, and binding proteins, among others. For instance, glucose oxidase catalyzes oxidation of β -D-glucose

to D-glucono-1,5-lactone, which is further hydrolyzed to gluconic acid²⁶. A range of commercially available biosensors for blood glucose monitoring and diabetes management use glucose oxidase as the sensing element coupled to appropriate mediators and transducers⁶. Antibodies have exquisite specificity for their cognate antigens. In an immunosensor⁶², antibodies are used as recognition elements coupled with a variety of detection methods, including, electrochemical, piezoelectric, fluorescence, bioluminescence, absorbance, and surface plasmon resonance; labeled secondary antibodies can also be used in a different format for detection of target analytes.

A variety of sensing systems have also been developed by using hinge motion binding proteins (HMBPs), for example periplasmic binding proteins and the messenger protein calmodulin, with an incorporated signal-generating reporter molecule⁶³. In general, these proteins have extraordinary selectivity to their corresponding ligand and/or analyte, with affinities, K_D , typically in the sub-micromolar range—in some cases as low as in the nanomolar range—and undergo conformational changes upon binding to their ligands. As representative examples of periplasmic binding proteins⁶⁴, the K_D of the sulfate-binding protein is 10 nmol L^{-1} whereas that of the glucose-binding protein is 20 nmol L^{-1} . Specifically, upon ligand binding, two protein domains bend around a “hinge” region of the protein. Such conformational change can be used to quantify a target analyte and/or ligand by measuring the change in signal intensity of the transduction molecule, which can either be an environmentally sensitive fluorescent probe strategically conjugated to the protein or a reporter protein genetically fused to

the HMBP. The advantages of this type of protein-based biosensor are their high specificity toward their ligand analyte, thus resulting in high selectivity, low limits of detection, rapid response times, and amenability to incorporation into various analytical devices. Potential disadvantages of protein-based biosensors can be their storage conditions, transport, and shelf life. In most cases, protein biosensors must be stored and transported refrigerated, which limits their utility and compromises their shelf life when used at room temperature.

Intact cells, including bacterial, yeast, and mammalian cells, are used as sensing elements in biosensing systems. Bacterial cell-based biosensing systems use genetically engineered bacteria capable of generating a signal on selective recognition of the analyte or class of analytes of interest. The ability to produce dose-dependent detectable signals in response to the analyte, as described in the next section, enables selective determination of the bioavailable analyte or class of analytes present in a given sample. Cell-based sensing systems are relatively easy and inexpensive to prepare and store, and are robust: they tend to be stable to environmental changes, for example variations in temperature or pH. In addition, these sensing systems can provide physiologically relevant data and evaluate the bioavailability of the analyte of interest, because the target chemical must enter the cells to trigger a response. Moreover, by using different recognition element–reporter protein pairs, multiple analytes can be detected simultaneously in a sample. Cell-based biosensing systems have high-throughput features because they are amenable to miniaturization and incorporation into high-density analytical devices, thus enabling assay of large numbers of samples in a

single analytical run. This is a distinct advantage over conventional physicochemical analytical methods. However, a whole-cell biosensing system is not without limitations. In whole-cell biosensing systems, analytes must enter the bacteria by diffusion, which may, depending on the rate of diffusion, result in a slow sensor response. Additionally, because the cell biochemical machinery must be activated to produce the reporter protein, the response of a whole-cell sensing system is slow compared with that of protein-based biosensors, which is of the order of seconds or minutes. Additional drawbacks include potential interference with the sensor's response by components of the bacterial cell and high background signal when fluorescent proteins are used as reporters, because of the presence of fluorescent molecules in the cell. Cell batch-to-batch variability, which is intrinsic to living organisms, is a further aspect that may have to be taken into consideration⁶⁵.

Bacterial whole-cell-based biosensing systems

Bacterial biosensing systems can be categorized into two different types, depending on the mode of expression of the reporter protein⁶⁶. Expression of the reporter can either be constitutive or inducible. In constitutive expression systems, the reporter is expressed at high basal levels. An increase in the amount of compounds that are toxic to the cell causes its death, thus reducing the reporter protein expressed and its generation of signal. Whole-cell biosensing systems based on constitutive expression have been used to measure the general toxicity of a sample or test compound. A well-known example is Microtox[®] toxicity testing⁷, a standardized, commercially available

toxicity testing system that uses the spontaneously bioluminescent marine bacteria *V. fischeri* as bacterial sensor for detection of toxic compounds in water samples. When *V. fischeri* in the test kit is exposed to a sample containing toxic compounds, a dose-dependent reduction in bioluminescence is observed, indicating the toxicity level of the sample. The second class of whole-cell bacterial biosensing systems comprises inducible expression systems in which the cells are genetically engineered to contain a plasmid in which an inducible promoter fused to a reporter gene controls its expression⁶⁶. In the absence of analyte/inducer, the reporter gene is expressed at very low basal levels, while in the presence of analyte/inducer it is expressed in a dose-dependent manner. Inducible expression systems can be further classified as stress inducible or chemically inducible, depending on the mechanism of activation of the response. In stress inducible cell-based biosensing systems, the reporter gene is placed under the control of a promoter that is activated by stressful conditions, for example heat shock and osmotic stress. Several structurally unrelated compounds can activate these response mechanisms; therefore, such sensing systems are not specific to target compounds and are defined as semi-specific. On the other hand, chemically inducible cell-based systems harbor a plasmid that contains a specific promoter and the genes for regulatory and reporter proteins. The presence of an analyte or class of analytes activates the promoter, triggering the expression of the regulatory and the reporter proteins in a specific manner. The mechanism by which this occurs involves binding of the analyte to the recognition/regulatory protein, which then undergoes a change in conformation, subsequently activating expression of the reporter gene. Reporter gene expression can

be negatively or positively regulated. In negative regulation (Figure 7A), the regulatory protein is bound to the operator/promoter region of an operon and inhibits expression of downstream genes, including the reporter gene. When the analyte is present, it binds to the regulatory protein, which is then removed from the operator/promoter region, thus enabling expression of the reporter gene. In positive regulation (Figure 7B), the analyte first binds to the regulatory protein and the complex then binds to the operator/promoter region, triggering expression of the reporter gene. The reporters used in whole-cell sensing systems are typically proteins that can be detected by optical, i.e., colorimetry, fluorescence, bioluminescence, and chemiluminescence, or electrochemical methods. Although the recognition component is important in determining the selectivity, the reporter is crucial in determining the sensitivity of the bacterial sensor. A wide variety of reporter genes have been used in several applications, including gene expression, gene transfer, and cell signaling. The reporter proteins encoded by such genes have also been used as signal-transduction elements in bacterial sensors. These proteins include β -galactosidase^{67,68}, bacterial luciferase⁶⁹, firefly luciferase⁷⁰, and the green fluorescent protein and its variants. Table 2 lists reporters that are commonly used in whole-cell sensing systems, with their catalyzed reactions and methods of detection.

Miniaturization of cell-based biosensing systems

Cell-based bioluminescent biosensing systems have still to reach their fullest potential. Attractive technologies where these systems could find applications include

rugged, compact portable sensing platforms and instrumentation for on-site measurements of environmentally and clinically relevant analytes. To date, several important strides toward miniaturization have been achieved. An example of the progress made towards constructing miniaturized systems includes the whole-cell bioluminescent-bioreporter integrated circuit device developed by Simpson *et al.*⁷¹ In this system, a toluene-selective genetically engineered *Pseudomonas putida* bioreporter strain was incorporated onto a chip provided with an optical application-specific integrated circuit (Figure 8). Upon interaction with toluene vapor, a bioluminescence signal was generated and measured by the integrated circuit, allowing detection of toluene concentrations down to 50-10 ppb, depending on the signal integration time. The main advantage of this system lies in the direct coupling of the bioluminescent bioreporter cells to an integrated circuit designed for detecting, processing and reporting of the light signal. This eliminates the need for large detection instrumentation and optical components for light collection and transfer, thus providing a self-contained portable device suitable for on-site applications.

Miniaturized cell-based biosensing systems have also found application in genome-wide transcription analysis. In a study performed by Van Dyk⁷² *et al.*, sequenced random segments of *E. coli* DNA were inserted into plasmids as gene fusions with *Photobacterium luminescens luxCDABE* gene cassette and transformed into host cells. A group of functional gene fusions known as Lux Array 1.0 was selected, which contained a total of 689 diverse reporter strains. These strains were printed on a porous nylon membrane (8 cm x 10 cm) at 16 spots cm⁻² by means of a commercially available

automated workstation. During and after this process the membrane was kept in contact with LB growth media in a culture dish. These reporter strains were employed for simultaneously evaluating gene expression in the presence of nalidixic acid, an antibiotic that induces DNA damage stress response by causing a change in gene regulation. Specifically, in the described reporter gene assay, upregulation of certain genes translated to increased bioluminescence signals. This system showed the feasibility of obtaining high-density bioluminescent reporter cell arrays and suggests their potential use for analytical purposes. Notably, a further increase in the density of the arrays may be limited due to cross-illumination from neighboring spots.

In another attempt towards miniaturization, fiber optic based systems have been designed and developed in which whole-cell biosensing bacteria were immobilized onto an exposed core of a fiber-optic^{73,74}. In one case, reporter cells containing a gene fusion of the genotoxicant-inducible *recA* promoter of *E. coli* to the *P. luminescens luxCDABE* reporter were constructed⁷⁴. These sensing cells emitted light in a dose-dependent manner in the presence of DNA damaging (genotoxic) agents, such as mitomycin C, which can react with the DNA structure, destabilize it and potentially cause deadly genetic mutations. The optical fiber was treated with acid for proper cleaning. The sensing cells were mixed with a polymeric solution, such as sodium alginate, and the fiber optic tip was dipped into the mixture containing the cells (Figure 9). Further treatment with calcium chloride solution was performed to harden the cell-alginate matrix onto the fiber-optic core. When the cell-deposited fiber-optic tip was exposed to solutions of mitomycin C at various concentrations, a dose-dependent bioluminescence

response was triggered, and then measured by the fiber optic system. Notably, this fiber-optic system was able to achieve the same detection limit, in a shorter period of time, as its larger scale counterpart. In another work by Gil *et al.*⁷⁵, genetically engineered constitutively bioluminescent bacteria were deposited on an optical fiber, employing a solid matrix of glass beads and agar, to develop a biosensor for the detection of toxic gases. Specifically, the presence of toxic chemicals reduced the cells' bioluminescence intensity. Addition of glass beads increased both the porosity of the cell matrix, which facilitated the diffusion of vapors through the cell matrix layer, and the contact surface area of the cells with the gases, thus resulting in improved sensing ability of the bacterial sensor. This sensor is not as specific because it measures cell death, which can be caused not only by gases but also by other toxic compounds present in the sample. Technologies such as biological laser printing (BioLP™) have been reported for the rapid deposition of biomolecules and live bacterial sensing cells onto various surfaces⁷⁶. Forward transfer BioLP™ uses laser pulses to transfer material from a carrier support onto a receiving substrate (Figure 10). The carrier support is an absorption layer (mostly quartz coated with metal oxides) on which properly grown bacterial sensing cells are spread prior to printing. In the reported example, the sensing strain was *E. coli* harboring a plasmid-borne fusion of the *recA* gene promoter to the red fluorescent protein gene from *Discosoma*, capable of responding to genotoxicants like nalidixic acid. Then, the sensing cells on the carrier support were printed onto a receiving surface composed of a LB agar plate or a sterile glass slide with a thin film of LB agar. The laser pulse was focused on a spot in the absorption layer. The laser-

material interaction produced photo-absorption, propelling a three-dimensional pixel of biomaterial towards the receiving substrate through photomechanical and/or photothermal effects. This method of printing was reported to be precise, with an average spot diameter of $70 \pm 6 \mu\text{m}$ and an approximate volume of 5 μL . An alteration in the bioluminescence emission was observed and attributed to the genotoxicity caused by nalidixic acid. This BioLP™ technique may be applied to several diverse sensing cells to produce miniaturized chip-based sensing systems that can be used in a laboratory setting or in the field.

During the last two decades, there has been considerable interest in and efforts made to miniaturize conventional bench-top analytical techniques and incorporate them into microfluidic chip-based platforms¹ as well as to integrate multiple analytical processes into a single chip⁷⁷. The physical principles that govern mass transfer and fluid flow at the microscale level allow for rapid mass transfer and kinetics as well as high surface-to-volume ratio, which endow microfluidic systems with unique characteristics when compared to conventional volume analytical systems. Microfluidic devices such as micro-total analysis systems (μTAS) and lab-on-a-chip platforms have been developed for several analytical tasks, including whole-cell based biosensing. Generally, computer numerical control (CNC) machining and lithography techniques are used to fabricate these devices, employing polymeric materials such as poly(methyl methacrylate) (PMMA), glass and silicon. Various microfluidic structures can be fabricated that incorporate features such as micro reservoirs, microchannels, mixing devices, filtration, fractionation and separation devices and microvalves. The choice of structures

incorporated into a microfluidic device depends on the specific application desired⁷⁸. Propulsion of fluids on microfluidic platforms is accomplished by employing varied instruments such as syringe and peristaltic pumps, or by applying acoustic, magnetic or centrifugal forces⁷⁹.

Microfluidic platforms employing centrifugal forces can be designed in the form and size of a compact disk (CD). This kind of centrifugal microfluidic device has been used for cell-based detection systems. Specifically, bacterial biosensing cells containing the gene for green fluorescent protein (GFP) under the transcriptional control of the promoter and regulatory genes of the *ars* operon were employed⁸⁰. The biosensing system relies upon the recognition and binding of the target analytes arsenite/antimonite by the transcription regulatory protein ArsR, and the resulting expression of the reporter protein GFP inside the cytoplasm. The biosensing cells were incorporated into a CD microfluidic platform made of PMMA for detection of arsenite and antimonite. In this application, miniaturization significantly reduced the assay time (30 min versus < 1 min) along with the volumes of reagents used, while retaining similar micromolar detection limits and dynamic ranges, when compared to the benchtop assay. The decrease in detection time is due to faster diffusion of the analyte into the cells, thus increasing the reaction kinetics. The sensing system proved to be highly selective for arsenite and antimonite when incorporated into the microfluidic platform. Owing to precise manufacturing, all the structures have identical physical characteristics, making these platforms a very attractive solution for multiple parallel assays with potential for high-throughput screening as well as on-site monitoring. This

type of sensing platform is suitable for the development of simple instrumentation based on readily-available, cost effective hardware consisting of a drive motor, a power supply, a controller, lens optics and compact CCD cameras for the detection and quantification of emitted light. These components along with software for system control and data acquisition, processing and analysis can be easily integrated into a portable system. Figure 11 shows an example of centrifugal CD microfluidic platform. Multiple structures consisting of an arrangement of reagent reservoirs, burst valves and microchannels leading to detection reservoirs are shown. Fluid release from the reagent reservoirs is controlled by burst valves, located a very short distance from the reservoirs. Sufficient centrifugal force is needed to overcome the capillary force holding the liquids into the reservoirs and allow their flow to the detection chamber. Such force is generated by appropriate frequency of rotation (burst frequency) of the disk.

Whole-cell based biosensing systems employing firefly luciferase as a reporter for the detection of genotoxicants have been integrated into a chip-based three-dimensional microfluidic device, which was obtained by placing a silicon substrate between two poly(dimethylsiloxane) (PDMS) layers. Microchannels (volume 3 μL) in the two PDMS layers were connected via perforations in the silicon layer that served as microwells (volume 0.25 μL) to hold the sensing strains, thus forming a three-dimensional microfluidic network. The sensor strains were immobilized onto the microwell array of the silicon chip by gelation upon injection of a cell/agarose mixture through the microchannels of one of the PDMS chips. Luciferase gene expression was then induced by passing sample genotoxicant solutions through the microchannels

present on the second PDMS chip (Figure 12). Bioluminescence was triggered when a solution of luciferin/ATP was passed through the channels and detected by means of a CCD camera. The bioluminescence response obtained with this miniaturized microfluidic system (1 h) was significantly faster than the assay using test tubes (4 h). Low-cost materials were employed to make these platforms and low volumes of reagents were consumed, thus rendering the system very cost-effective. In addition, multi-analyte detection in multiple samples can be performed simultaneously by immobilizing different sensor bacteria on a single chip.

Paper strip based whole cell sensors

While a number of biosensing systems are available that can detect a number of analytes by employing various detection principles, most of these biosensing systems cannot be used outside of the laboratory due to required bulky instrumentation. This is particularly limiting for on-site environmental and biomedical applications. In addition to the miniaturized devices described in the previous section, another approach towards development of biosensors that could be used on site has been demonstrated by Stocker *et. al* who created paper strip based biosensing systems to detect arsenic in polluted water⁸¹. β -Galactosidase was employed as a reporter and detected by means of a chromogenic substrate generating an insoluble colored product that could be visualized on the paper strips. Five water samples obtained from Bangladesh indicated arsenic contamination to much higher than permissible levels (10 $\mu\text{g/L}$) (Figure 13A). Our group developed paper strip based whole-cell sensors that could be used for on-site

monitoring of AHLs present in biological and environmental samples⁸². *E. coli* cells harboring plasmid pSD908, which is based on the *lasR/lasI* regulatory system of *P. aeruginosa* with *lac-Z* encoding β -galactosidase as the reporter gene, were employed. These sensing cells were liquid-dried in vacuum on filter paper strips. A dose-dependent development of color was seen when the paper strips were incubated with various concentrations of *N*-dodecanoyl-DL-homoserine lactone followed by addition of the chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). The applicability of the sensor was validated by employing the paper strips to detect AHLs present in saliva of healthy and diseased individual (Figure 13B). The sensor can be employed not only to detect AHLs in samples, but also to evaluate molecules that interfere with AHL based QS.

Binding proteins as recognition elements in biosensors

Proteins constitute the working machinery of every living organism. Proteins perform a variety of tasks, including recognizing specific molecules, transferring messages across the cell, catalyzing biochemical reactions, and participating in immune responses. Hinge-motion binding proteins (HMBPs) are a group of proteins with high selectivity and high binding affinity towards their respective ligands, with K_d values down to the nanomolar level⁶⁴. HMBPs are generally composed of two domains connected by a hinge region, with the ligand binding site present at the interface of these domains. Ligand binding results in a change in conformation of the protein, consisting in bending of the two domains around the hinge region. The unbound or

analyte free conformation is generally referred to as “open” form and the analyte bound conformation is referred to as “closed” form. Hinge-motion binding proteins include various types of proteins, such as, periplasmic binding proteins, transcriptional regulatory proteins, enzymes, and the messenger protein, calmodulin. These proteins bind to the respective analytes with high specificity and selectivity. This property makes them appealing for use in sensing applications. As an example of HMBP, in *E. coli* glucose is transferred from the periplasmic space to the cytoplasm through binding to the periplasmic protein, glucose binding protein (GBP). Binding of the sugar to the protein occurs through various interactions, such as van der Waals, ionic, salt-bridges, and hydrogen bond formation with amino acids in the binding pocket, which causes the protein conformation to change from the open to the close form (Figure 14). The change in the conformation of a protein upon binding to a ligand can be harnessed to create protein based sensors for target analytes. In order to make a biosensor from a hinge-motion binding protein, the change in conformation due to ligand binding needs to be translated into a readable output. This can be achieved by several methods, including chemical conjugation of an environment sensitive fluorophore near the ligand binding site, genetic fusion of a fluorescent or bioluminescent protein to either the C or N terminus of the binding protein, and genetic insertion of the binding protein between two fragments of a fluorescent or bioluminescent reporter protein to form a molecular switch. Irrespective of the approach used to create a protein based biosensor, binding of the ligand to the specific protein generates a dose-dependent change in the reporter

molecule signal, thus allowing for the biosensor to be utilized for quantitative detection of the target ligand/analyte.

A commonly used strategy to make protein based biosensors employs strategic placement of environment sensitive fluorophores, such as, 5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid (IEDANS), 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC), acrylodan, and Alexa-fluor near the ligand binding site of the protein. Conjugation of the fluorophore at a site within the binding pocket would not be a good choice as it could disrupt analyte binding. Sites on the protein that experience a substantial conformational change upon ligand binding are determined from X-ray crystal structure, NMR, and modeling studies. These sites are ideal for fluorophore conjugation in that the analyte-induced conformational change is likely to perturb the microenvironment surrounding the fluorophore. Amino acids in the protein that are amenable to direct conjugation of the fluorophore include cysteine (through sulfhydryl group), lysine (through amine group), and aspartic and glutamic acid (through carboxyl group). When such amino acids are not present at the required fluorophore attachment site, genetic mutations can be performed to introduce the amino acid of interest. In our lab, a range of such fluorophore labeled binding proteins have been constructed as biosensing systems to detect environmental pollutants⁸³ and clinically relevant analytes⁸⁴. Salins⁸⁴ *et. al* utilized the glucose/galactose binding protein from *E. coli* to develop a glucose biosensor based on the principle outlined above. Specifically, cysteines were introduced into the structure of GBP via mutagenesis at three different positions. A set of four

fluorophores were conjugated at these cysteines and evaluated, with MDCC providing the maximum response and limits of detection in the submicromolar range. More recently, Siegrist *et. al* utilized fluorescently labeled glucose recognition polypeptide elements to detect glucose AT physiologically relevant millimolar levels⁸⁵. Similar strategies were used to develop protein biosensors for the detection of environmental pollutants, such as inorganic phosphates⁸⁶, nickel⁸⁷, and sulfates⁸³.

Partial or full protein sequences can be joined together via recombinant DNA technology resulting in fusion or hybrid proteins. An important application of fusion protein technology is in the field of protein purification. As an example, GE Healthcare Life Sciences offers a series of vectors that encode for glutathione-S-transferase (GST). The DNA of the protein of interest can be cloned in these vectors, thus allowing expression of a fusion of the GST tag and target protein, which can then be purified by affinity chromatography⁸⁸. In fusion protein constructs for sensing, an inherently fluorescent or bioluminescent protein is attached to either the N or C terminus of a binding protein. When the analyte binds to the binding protein, a change in conformation of the binding protein leads to altered light emission from the reporter protein. Using this principle, Dikici⁸⁹ *et. al* developed a fusion protein using calmodulin (CaM) as the recognition element to detect phenothiazine, and enhanced green fluorescent protein (eGFP) as the reporter protein. When excited at a wavelength of 488 nm, eGFP emits at 510 nm. CaM is a calcium binding protein and when it binds to calcium, a change in conformation of CaM exposes a hydrophobic pocket that interacts with the antidepressant phenothiazine. When CaM binds to antidepressants, a further

change in conformation of CaM induces a change in microenvironment of eGFP, thus altering the emission intensity in a dose-dependent manner. Dikici *et. al* demonstrated a system that serves as model to develop binding assays that employ binding proteins as drug-recognition element. Based on the concept of fusion protein, a few fluorescence resonance energy transfer (FRET) based biosensors have been reported⁹⁰⁻⁹³, in which the binding protein is sandwiched between two fluorescent proteins acting as members of a FRET donor-acceptor couple. Analyte binding leads to the fluorescence resonance energy transfer between the two proteins.

Another approach in engineering protein based sensors is the development of molecular switches. A protein molecular switch is comprised of a binding protein with two fragments of a reporter protein fused to either end. In the absence of the analyte, the two split fragments of the reporter protein stay apart and the switch is in “off” mode, while in the presence of the analyte the binding protein undergoes a conformation change that brings the two fragments of the reporter protein together, thus forming an active protein and turning on the switch readout signal. A novel molecular switch for detecting glucose was designed and developed by Teasley Hamorsky *et. al*, employing the photoprotein aequorin as the reporter⁹⁴. In brief, by using genetic engineering tools, aequorin was split in two fragments and each of the two fragments was fused to either the N or C terminus of GBP. In the presence of glucose, the change in the conformation of the binding protein allowed the two fragments of the aequorin to come together and triggered a dose-dependent light emission in the presence of coelenterazine and calcium (Figure 15).

Miniaturization of protein-based biosensing systems

Efforts have been made to further augment the utility of protein based biosensing systems by incorporating them in miniaturized devices, thus facilitating on-site applications. An approach to incorporate protein-based biosensing systems into miniaturized devices was demonstrated by Puckett⁹⁵ *et. al.* A fusion protein comprised of calmodulin and the enhanced green fluorescent protein was integrated into a microfluidic compact disk based μ TAS (figure 16). This miniaturized device was employed to detect phenothiazine, an antidepressant, which binds to the protein CaM. The microfluidic platform contained μ L-size reservoirs in which solutions of sensor protein and analyte were added. When the disk was spun at a certain velocity, the solutions flew through micro channels into a detection reservoir, due to centrifugal force. As shown in Figure 16, the phenothiazine solution was contained in reservoir 3, while the dried sensing protein was contained in reservoir 2. The flow of solutions was controlled by passive valves, which opened only at a specific angular velocity. By controlling the angular velocity, water was initially released from reservoir 1 into the protein chamber to reconstitute the protein, and later, at a higher angular velocity, the phenothiazine solution and reconstituted protein flew to the detection chamber. The binding between CaM and phenothiazine resulted in fluorescence emission from the reporter protein eGFP, which was measured using a fiber-optic based detection system. Such microfluidic platforms are easy and inexpensive to manufacture, and are amenable to multiplex and high-throughput analysis in that each disk may contain multiple

identical microfluidic structures comprised of the required microchannels and reservoirs.

Figure 1. General schematic of AHL based quorum sensing system. Upon entering the bacteria, AHL binds to LuxR type protein, and the LuxR-AHL complex regulates expression of target genes, including AHL synthase. The label LuxR indicates LuxR types proteins.

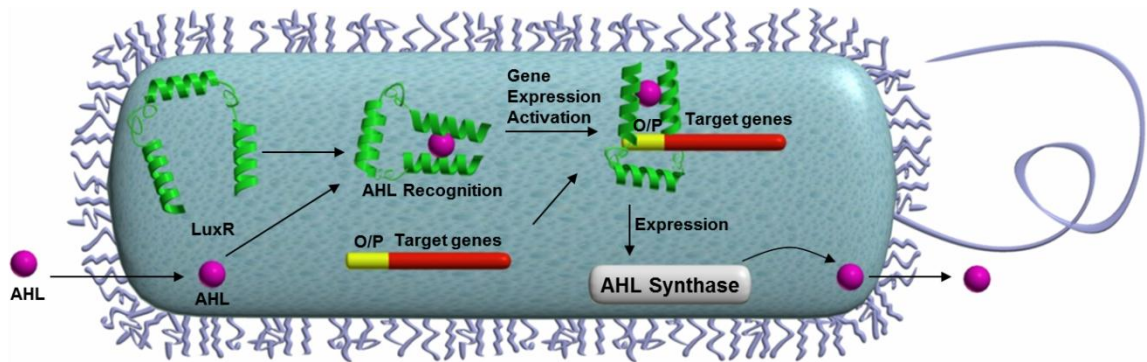


Figure 2. General schematic of AIP based quorum sensing system. Initially, a precursor peptide is produced, which undergoes modification to form a mature AIP and transported out of the bacteria by ABC transporter. AIP is recognized by sensor kinase protein, and via a cascade of phosphorylation/dephosphorylation steps, quorum sensing regulated genes are expressed. Adapted from Xavier *et al.*¹⁹

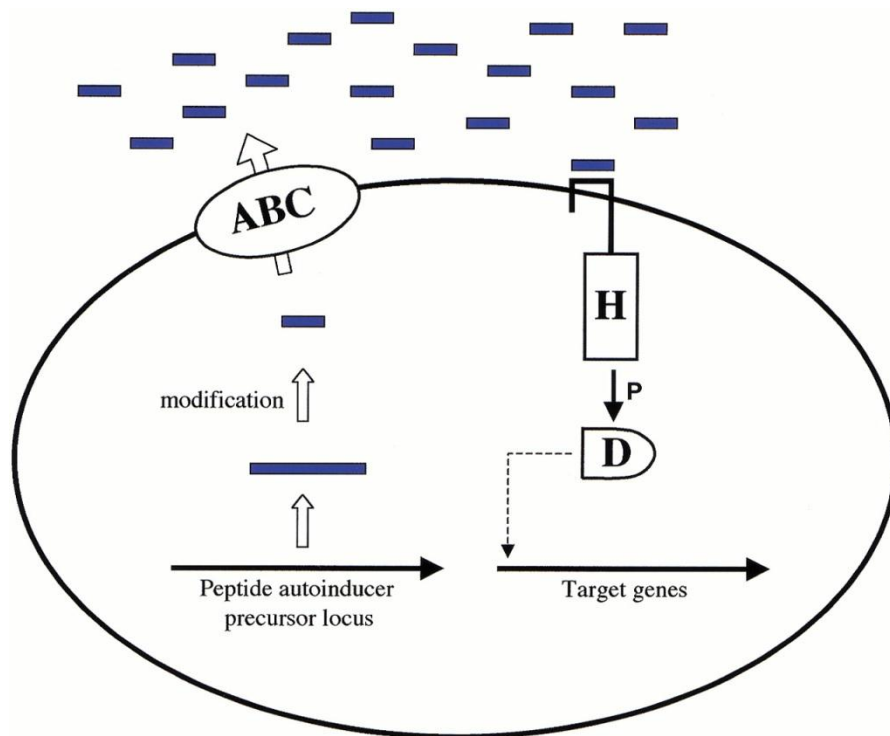


Figure 3. Autoinducer-2 and quorum sensing circuit in *Vibrio* spp. (A) Isomers deriving from DPD cyclization. Copyright from Tavender *et al.*⁹⁶ (B) Schematic of AI-2 based quorum sensing system. Copyright from Vendeville *et al.*⁹⁷

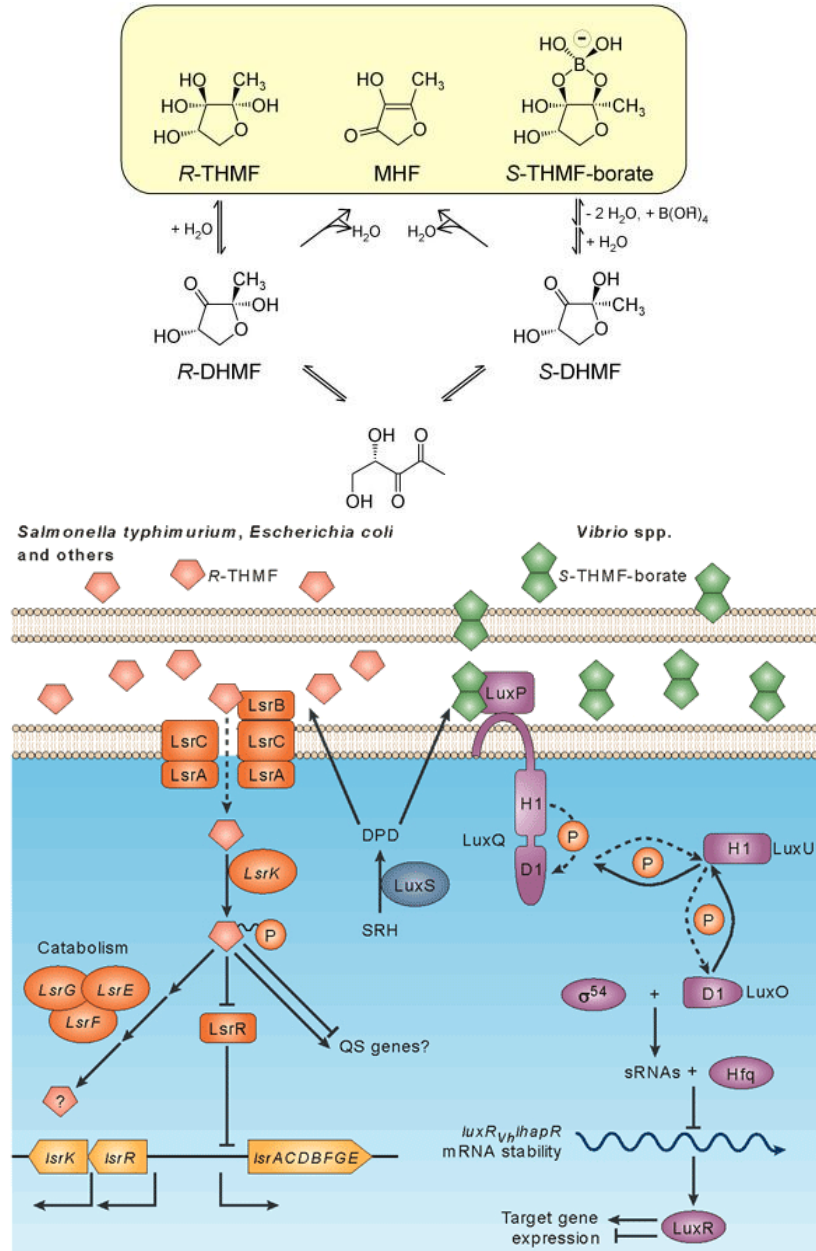
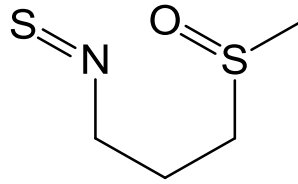
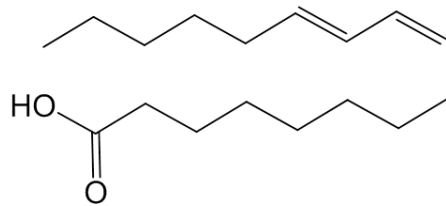


Figure 4. Natural compounds as quorum sensing inhibitors.

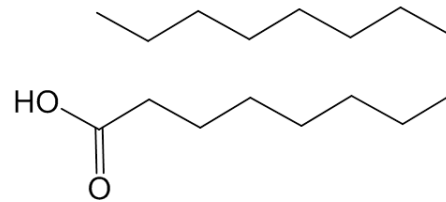
Iberin inhibits AHL based QS in *P. aeruginosa*



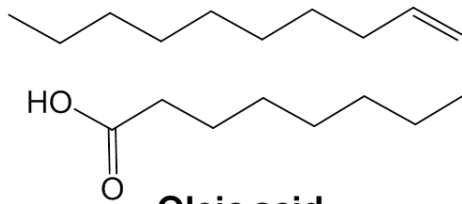
Fatty acids that inhibit AI-2 based QS in *V. harveyi*



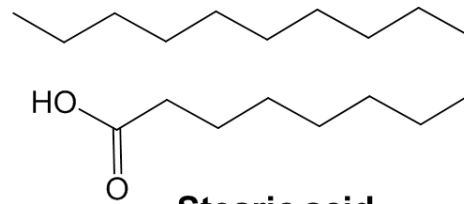
Linoleic acid



Palmitic acid



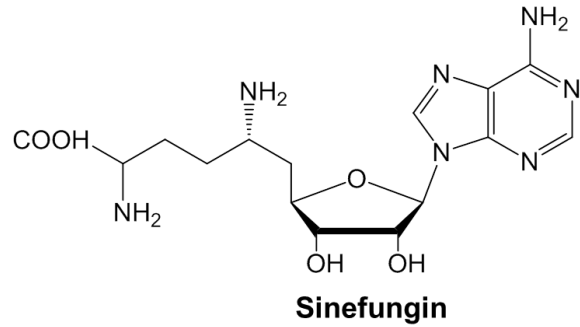
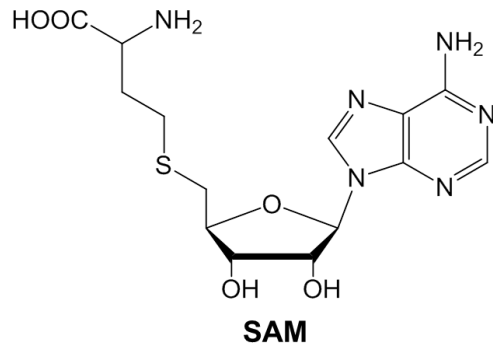
Oleic acid



Stearic acid

Figure 5: Inhibition of QSM synthesis.

(A) Compounds that inhibit AHL synthesis.



(B) Compounds that inhibit AI-2 synthesis.

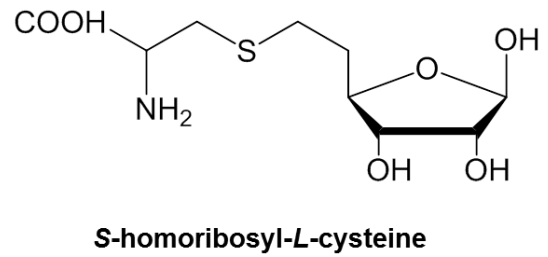
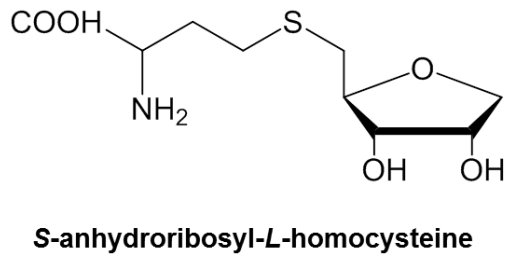
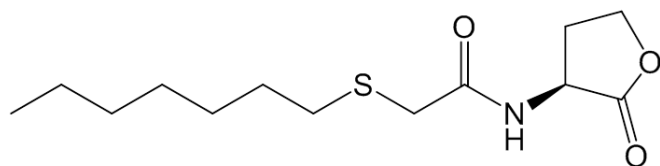


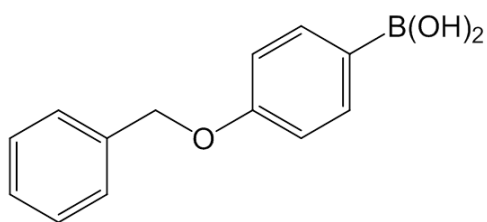
Figure 6: Interference with QSM receptors.

(A) Analogue of AHL demonstrating inhibitory activity

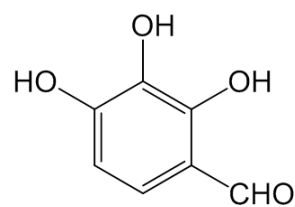


N-(heptylsulfanylacetyl)-L-homoserine lactone

(B) Analogue of AI-2 demonstrating inhibitory activity



[4-(phenylmethoxy)phenyl] boronic acid



2,3,4-trihydroxy benzaldehyde

Figure 7. Chemically inducible whole-cell biosensing systems.

(a) Negative regulation of reporter gene expression. (b) Positive regulation of reporter gene expression. Adapted from Struss *et al.*⁶⁵

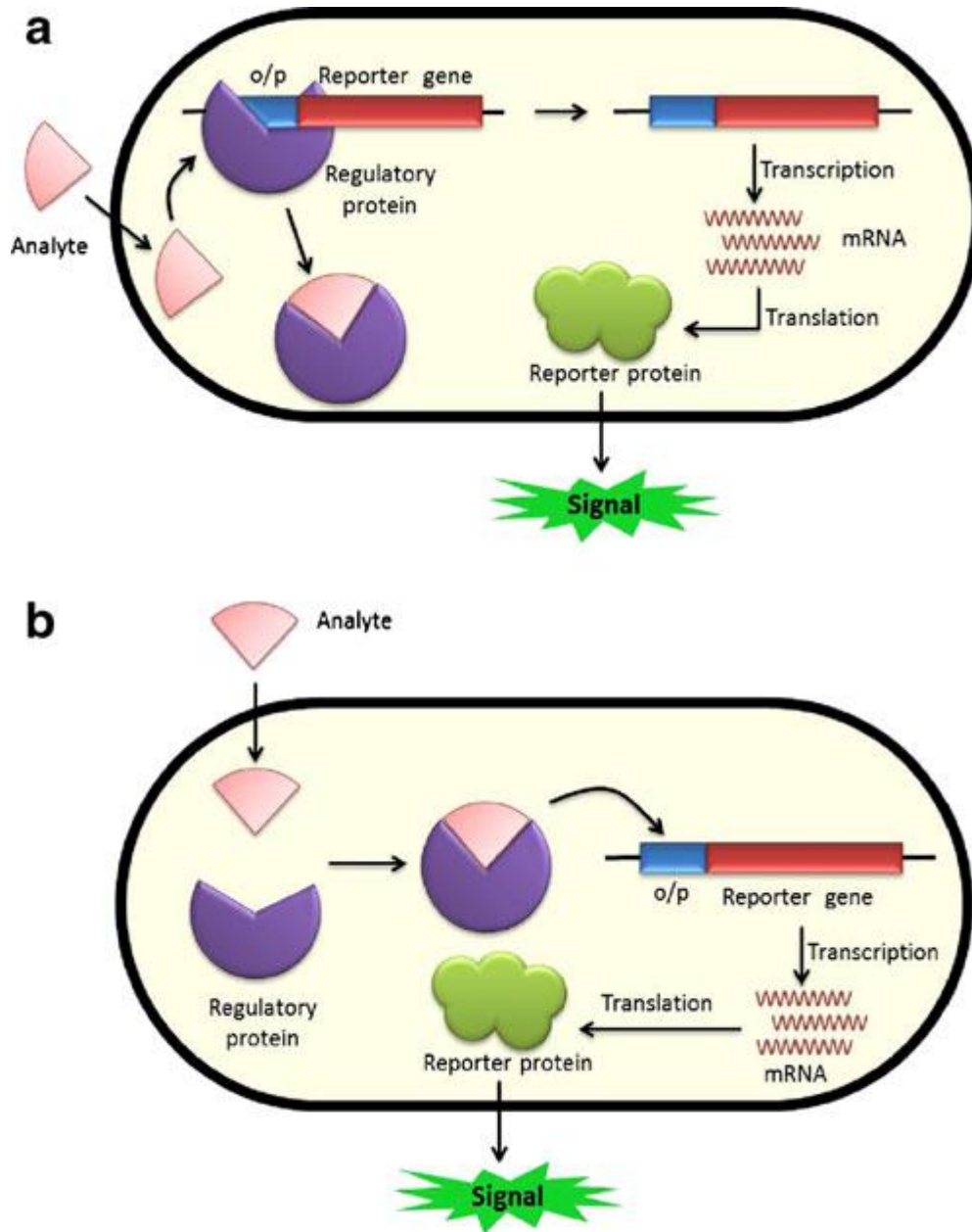


Figure 8. Bioluminescent-bioreporter integrated circuit featuring (i) enclosure containing bacterial biosensing cells and optical application-specific integrated circuit (OASIC); (ii) enclosure mounted on the chip; and (iii) the enclosure showing bacterial biosensing cells on agar plug. A tight seal between chip and enclosure is maintained by using an O-ring.

Figure copyright of Simpson *et al.*⁷¹

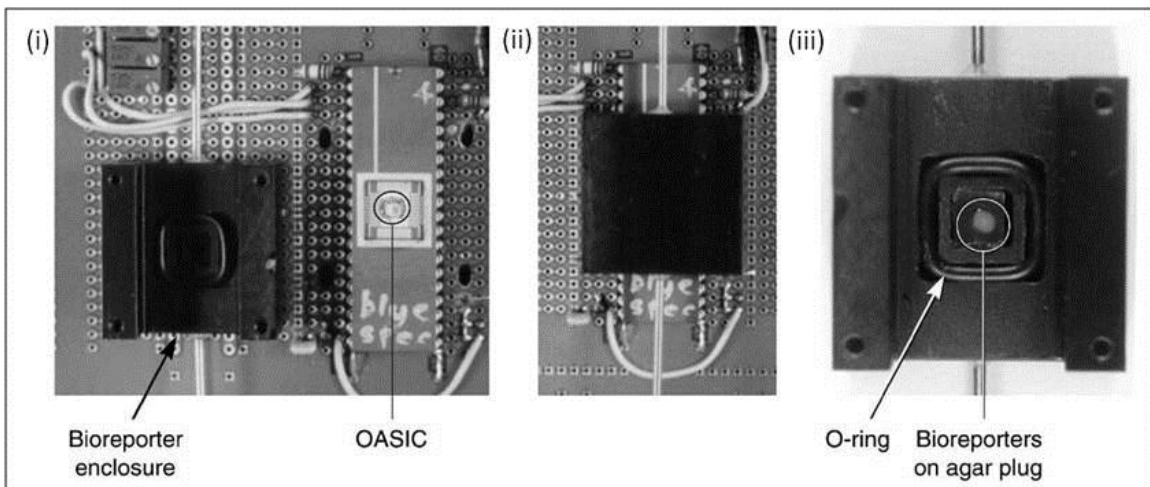


Figure 9. Fiber optic-based miniaturization. An optical fiber is placed in a conical tube containing the sample solution. The fiber optic tip is coated with an alginate matrix that incorporates the sensing bacterial cells. Bioluminescence is triggered when the analyte enters the cells and is recognized by the biosensing element. The bioluminescence emitted travels to the detector through the optical fiber. Adapted from Polyak *et al.*⁷⁴

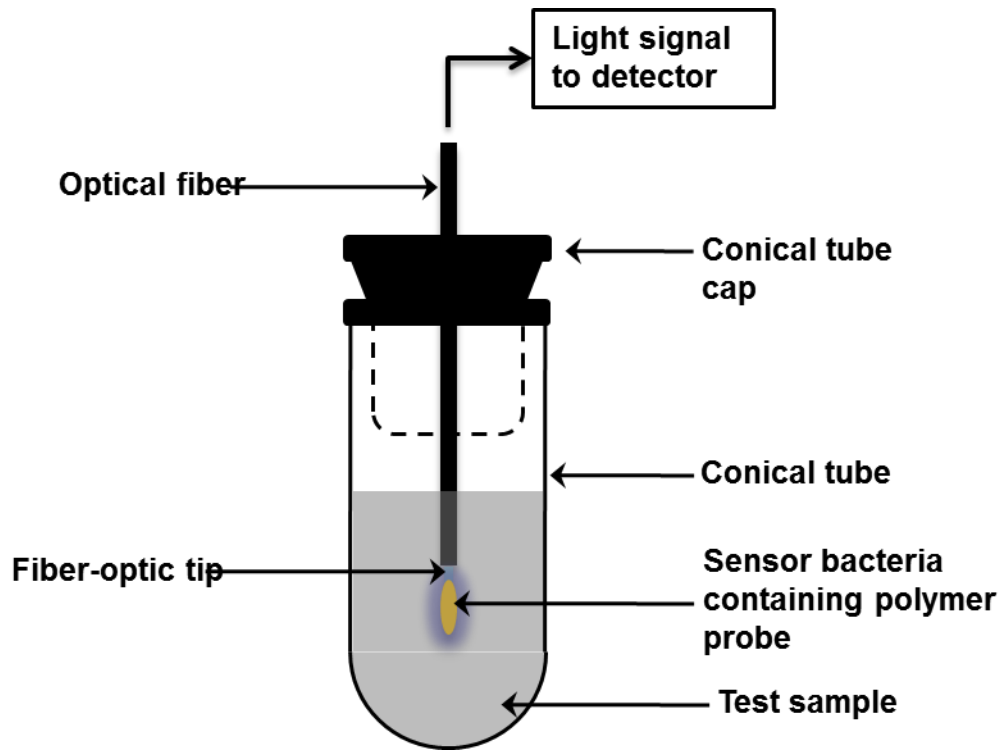


Figure 10. Schematic showing a BioLP™ device. A laser beam is focused on a spot at the interface of the support and absorption layers, causing ejection of the sensing cells containing material, which is then deposited on the substrate. Figure adapted from Barron *et al.*⁷⁶

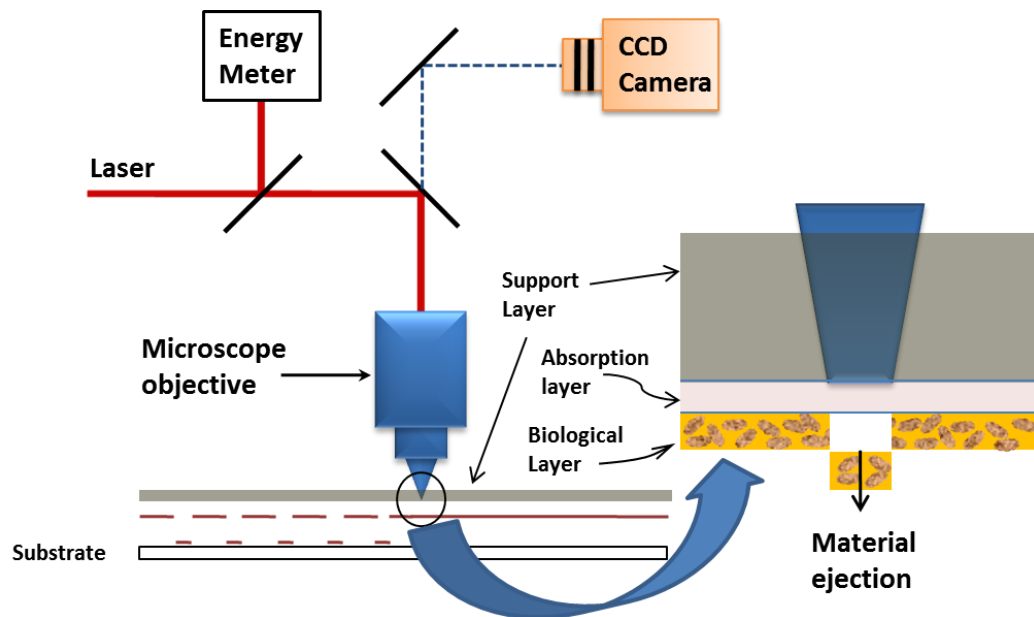


Figure 11. Example of centrifugal CD microfluidic platform. Reagent reservoirs contain the necessary reagents, which flow through the mixing channels when the platform is spun at high rpm. Finally the two solutions arrive in the detection reservoir where measurements are made using a suitable detector. Copyright from Turner *et al.*⁹⁸

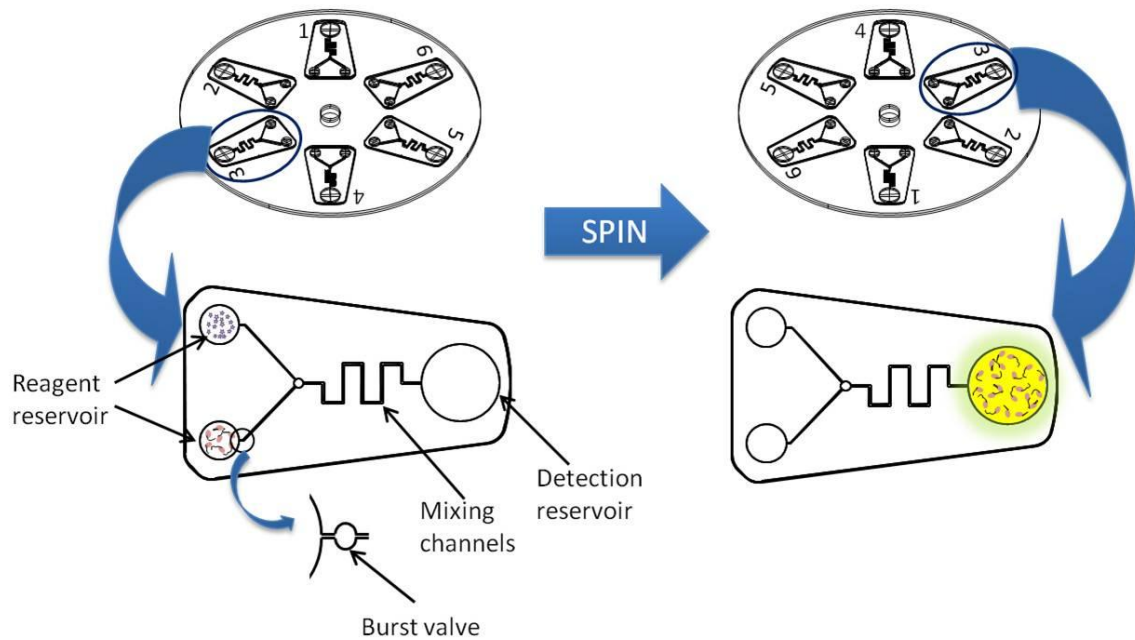


Figure 12. Three dimensional microfluidic network. (i) The solution of sensor cells is immobilized in the wells of chip 1. (ii) Analyte solutions are introduced in channels of chip 2. (iii) Schematic of the 3 dimensional structure of the platform. Copyright from Tani *et al.*⁹⁹

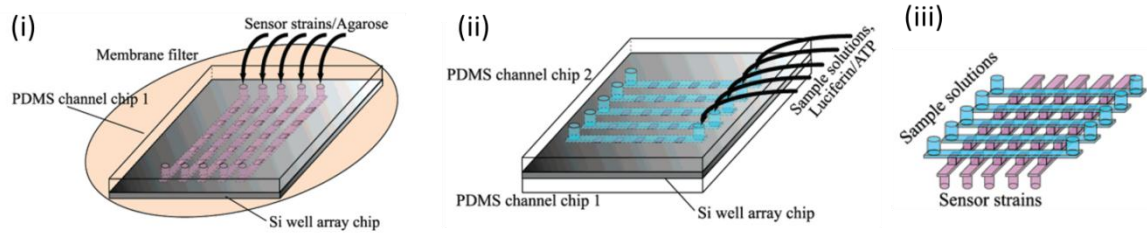


Figure 13: Immobilization of whole-cell based biosensing system on paper strips. (A) Detection of arsenic in water samples from Bangladesh. Total arsenic concentration ($\mu\text{g/L}$) measured by atomic fluorescence spectroscopy is indicated in parentheses. Copyright from Stocker *et al.*⁸¹ (B) Detection of AHLs in saliva samples using the filter-paper-based strip biosensor containing the plasmid pSD908. Samples 1–5 were from five healthy volunteers and sample 6 was from one patient with Crohn’s disease. Copyright from Struss *et al.*⁸²

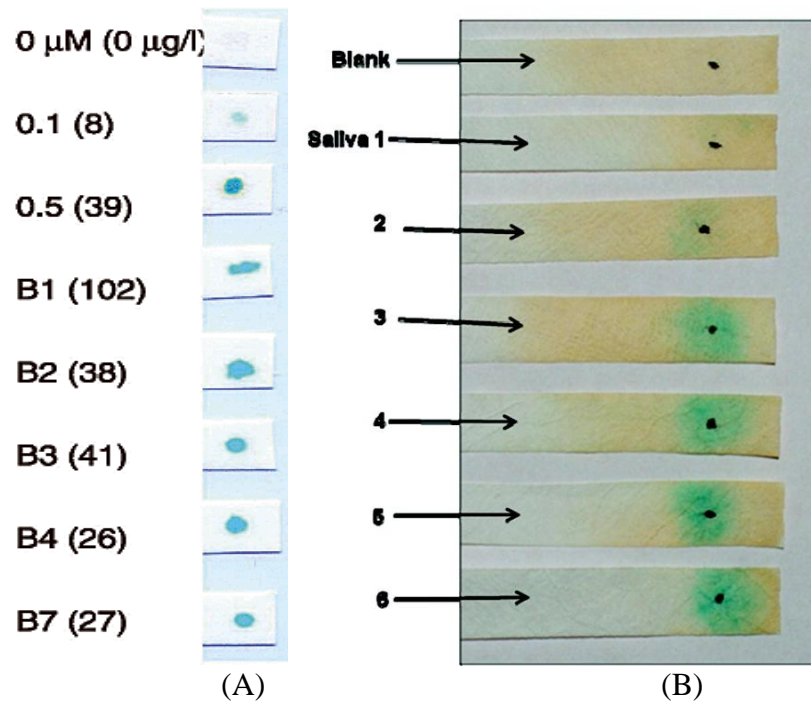


Figure 14. Crystal structure of GBP without glucose (A, open form; PDB: 2FWO) and with glucose (B, closed form; PDB: 2FVY).

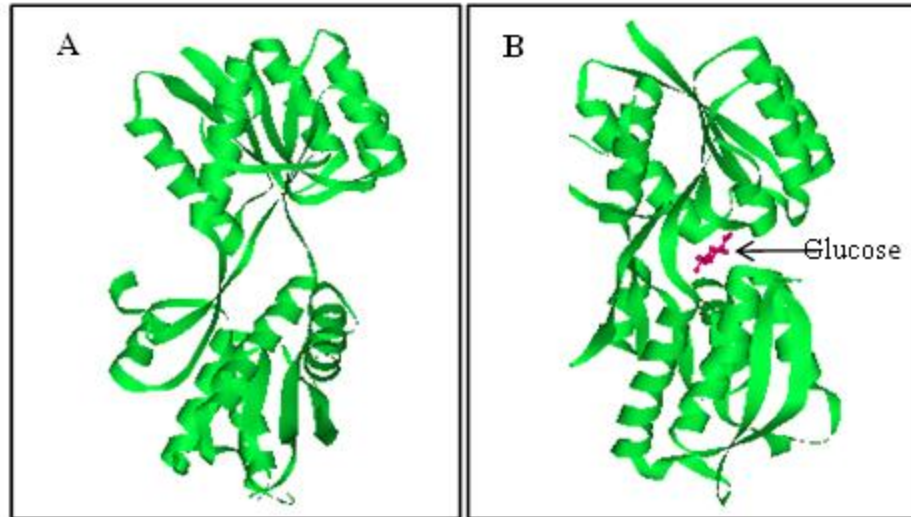


Figure 15. Protein biosensor based on molecular switch approach. The reporter protein aequorin is split into two fragments; each fragment is fused to the two ends of the recognition protein GBP. In the absence of glucose, the two fragments of aequorin are separated from each other and no emission of light is observed (off mode). In the presence of glucose, the two fragments of aequorin are brought close to each other due to conformational changes in GBP, and emission of light is observed (on mode).

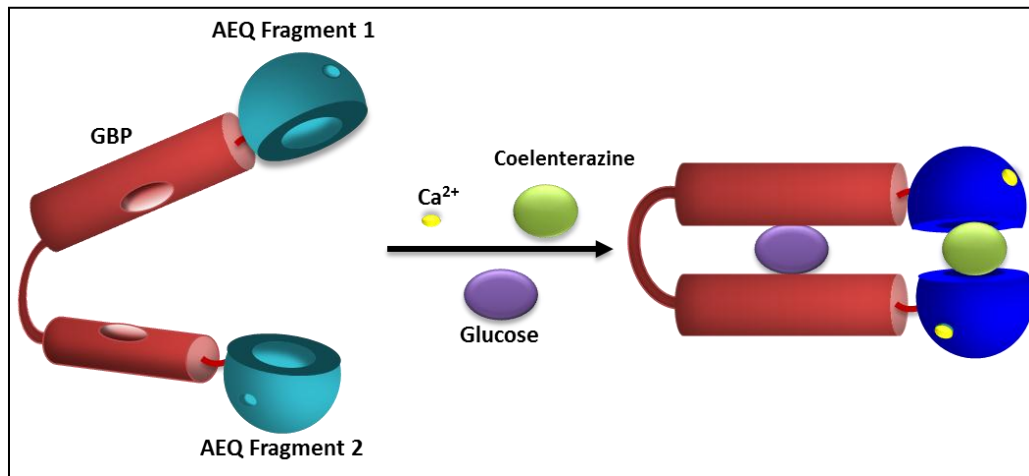


Figure 16. Microfluidic architecture utilized in the protein-based assay. Copyright from Puckett *et al.*⁹⁵

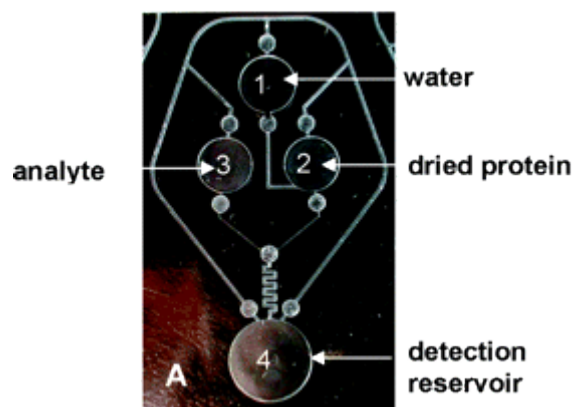


Table 1: Quorum sensing molecules utilized by various bacteria. (a) AHLs employed by Gram-negative bacteria. (b) AI-2: borate diester form employed by *V. harveyi* and hydrated form, not containing boron, employed by *S. typhimurium*. (c) Linear and cyclic peptides employed by Gram-positive bacteria. The underlined tryptophan residue (W) in the ComX pheromone indicates isoprenylation.

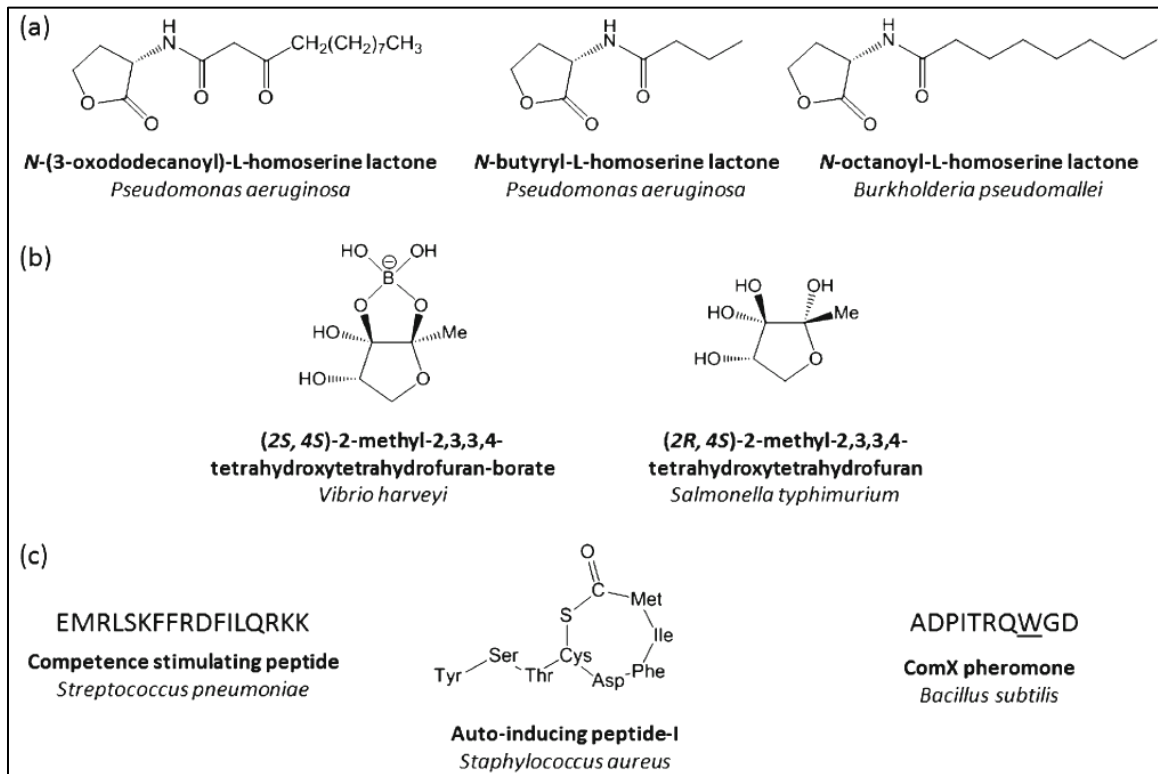


Table 2: Reporter proteins commonly used in whole-cell sensing systems. Copyright Struss *et al.*⁶⁵

Reporter proteins commonly used in whole-cell sensing systems		
Reporter protein	Catalyzed reaction	Detection method
β -Galactosidase	Hydrolysis of β -galactosides	Chemiluminescence, bioluminescence, fluorescence, colorimetry, electrochemistry
Bacterial luciferase	$\text{FMNH}_2 + \text{R-CHO} + \text{O}_2 \rightarrow \text{FMN} + \text{H}_2\text{O} + \text{RCOOH} + \text{h}\nu$ (490 nm)	Bioluminescence
Green fluorescent protein	Post-translational formation of the internal chromophore <i>p</i> -hydroxybenzylideneimidazolinone	Fluorescence
Firefly luciferase	Firefly luciferin + O_2 + ATP \rightarrow oxyluciferin + AMP + P_i + $\text{h}\nu$ (550–575 nm)	Bioluminescence

STATEMENT OF RESEARCH

Bacteria communicate with members of the same species or other species by production, secretion, and detection of small signaling molecules termed quorum sensing molecules (QSMs), whose concentration is proportional to the bacterial cell density. This system of cell-to-cell communication is named quorum sensing (QS) and allows bacteria to modulate their behavior in a population-size dependent manner. When a certain QSM threshold concentration is reached, certain specialized genes are expressed that are involved in a variety of phenotypes, including, virulence factor production, biofilm formation, motility, sporulation, and bioluminescence. In general, Gram-negative bacteria utilize *N*-acyl homoserine lactones (AHLs), while Gram-positive bacteria utilize autoinducing peptides (AIPs) as QSMs. Autoinducer-2 (AI-2) is a third category of molecules that are employed, both, by Gram-negative and Gram-positive bacteria and are believed to be involved in interspecies communication.

Bacteria have been associated with the pathogenesis of various disorders, such as, inflammatory bowel disease (IBD), a chronic inflammatory condition of the intestine with recurrent bouts of acute inflammation. Since QS is known to modulate various bacterial functions, many of which are critical for bacteria to successfully colonize a host and evade the host's defense system, we aimed at studying the role of QS in relation to bacterial conditions, mainly IBD. Specifically, we hypothesized that QSMs may serve as biomarkers for the diagnosis and monitoring of bacteria related disorders, as well as for the evaluation of treatment efficacy. To that end, we designed and developed

biosensing systems, based on engineered cells and proteins, for the detection of QSMs in physiological samples from IBD patients and control subjects. Moreover, bacteria are known to be involved in the spoilage of food by forming biofilms and producing food degrading enzymes, whose expression is often regulated by QS. Hence, we developed methods for the detection of QSMs in food samples in order to investigate the relationship between food spoilage and QS. Finally, we employed the developed biosensing systems to test target compounds for their ability to disrupt QS and evaluate their potential use as food preservatives.

In chapter two, we developed an analytical method for the detection of QS AI-2 molecules in saliva, stool, and intestinal samples. For that, we developed a whole-cell biosensing system, which is based on the QS regulatory circuit of the marine bacterium *Vibrio harveyi* and uses the LuxP periplasmic binding protein as the sensing/recognition element and *luxCDABE* gene cassette as the reporter element. This method enabled the quantitative and sensitive detection of AI-2 in the types of clinical samples mentioned above. To the best of our knowledge this is the first reported cell-based sensing system for quantitative detection of AI-2 molecules. This analytical approach has significant advantages over physical chemical methods in terms of simplicity, rapidity, and cost-effectiveness.

In chapter three, we developed, optimized, and applied whole-cell biosensing systems to the detection of QSMs in blood serum. We utilized a panel of three biosensing systems able to detect short-chain AHLs, long chain AHLs, and AI-2

molecules, respectively. In order to validate our method, we confirmed the presence of QSMs in serum by employing a conventional physical chemical technique, LC-MS-MS. Furthermore, using a mice animal model of colitis, which represents an experimental model of human IBD, we demonstrated altered serum levels of QSMs in colitic mice as compared to the controls. This indicates that the QSM levels in serum may reflect the known altered composition of the intestinal microflora in IBD (less diversity and modified relative abundance of species), thus supporting our hypothesis of employing QSMs as biomarkers of disease. To the best of our knowledge this is the first time that the presence of QSMs in serum has been demonstrated. This is an important finding due to the ready availability and minimally invasive collection of serum, as well as its extensive use as the biological fluid of choice for a number of clinical laboratory investigations.

In chapter four, we developed methods based on whole-cell sensing systems for the detection of AHLs and AI-2 molecules in food samples. Ground beef and milk were chosen as representative food matrices. After the method optimization, the biosensing systems were employed to detect QSMs in simulated spoiled foods, obtained by artificially contaminating the food samples. A wide variety of microorganisms are responsible for food spoilage, thus rendering contamination difficult to identify; on the other hand, QSM production is a feature shared by many bacteria, thus supporting the use of QSMs as general quantitative markers of bacterial presence. To that end, the analytical methods developed herein should allow for the early detection and

prevention of food contamination, thus helping to minimize the related foodborne illnesses and reduce the costs associated with the waste of degraded food.

In chapter five, we employed the developed whole-cell sensing systems for AHL and AI-2 detection to test a number of compounds, originating from Generally Recognized As Safe (GRAS) substances, for their ability to interfere with QS. The GRAS compounds chosen in this study had previously been demonstrated to possess an antibacterial effect, although their mechanisms of action had not been elucidated. To that end, these compounds were evaluated for their effects on the QS circuits of the sensing bacteria, in terms of ability to induce a sensor's response and to interfere with the sensor's response to cognate QSMs. In addition, effects on the QSM synthesis by QSM-producing bacteria were analyzed. Quorum sensing inhibition has been proposed as an alternative approach to combat bacterial infections, especially in light of the frequent occurrence of antibiotic resistant microorganisms. Along the same lines, the identification of GRAS compounds with QS inhibitory properties may lead to their safe and successful employment as food preservatives, in the form of food additives and/or components of food packaging materials.

In chapter six, we designed and developed a protein based biosensing system for the detection of AI-2 molecules. A fusion protein, comprised of the LuxP recognition/binding protein and enhanced green fluorescent protein (EGFP), was designed using molecular cloning techniques. The sensing system worked on the principles of Förster Resonance Energy Transfer (FRET) and utilized the fluorophore 7-

diethylamino-3-[*N*-(2-maleimidoethyl)carbamoyl]coumarin (MDCC) as donor and EGFP as acceptor in the FRET pair, based on the ability of MDCC to bind to LuxP. This biosensing system allowed us to quantitatively detect AI-2 molecules with high sensitivity, selectivity, and rapidity (3 min incubation time with the analyte). It was then applied to detect AI-2 in saliva and serum samples. A protein based sensing system could also be employed as a rapid tool for the investigation and characterization of agonists and antagonists, with potential to be utilized as QS disruptors.

In chapter seven, we discussed the conclusions and future prospects of the work presented in this thesis.

CHAPTER TWO

WHOLE-CELL SENSING SYSTEM FOR DETECTION OF THE QUORUM SENSING UNIVERSAL SIGNAL AUTOINDUCER-2 IN PHYSIOLOGICAL SAMPLES

Introduction

Bacteria have long been considered as independently living organisms focused on survival and propagation of single cells. However, in the past few decades it was discovered that they can communicate with and respond to neighboring bacteria of the same species or of other species by means of small signaling molecules^{12,14}. This system of communication among bacteria is termed quorum sensing (QS). Quorum sensing allows bacteria to regulate behaviors as diverse as bioluminescence, horizontal transfer of DNA, sporulation, formation of biofilms, as well as production of pathogenic factors, antibiotics, and metabolites^{12,100}. Bacteria synthesize quorum sensing signaling molecules and release them in the surrounding media, where they are recognized by other bacteria. The signaling molecule concentration increases as a function of cell density, thus allowing bacteria to sense their population size. When a threshold concentration of signaling molecules is reached, bacteria express certain specialized genes and, thus, coordinate their behavior in a cell-density dependent manner. Interestingly, bacterial communication is often involved in establishing relationships of bacteria with their respective hosts, both in beneficial and detrimental bacteria-host interactions. Well-characterized examples of such relationships include the symbiosis between the bioluminescent marine bacterium *Vibrio fischeri* and the Hawaiian bobtail

squid *Euprymna scolopes*, as well as the pathogenesis caused by the human opportunistic pathogen *Pseudomonas aeruginosa*, which colonizes the lungs of individuals affected by cystic fibrosis¹⁰¹. Furthermore, recent research has shown that bacterial signaling molecules enable interkingdom communication and are involved, for instance, in the relationship of the intestinal microbial flora, both commensal and pathogenic, with human and other mammalian hosts^{102,103}. Bacteria employ various chemical compounds as signaling molecules for quorum sensing communication. Some of them are species-specific, while others are used by different species of bacteria. Quorum sensing signaling molecules can be categorized based on the types of bacteria that produce them. In general, Gram negative bacteria use *N*-acyl homoserine lactones (AHLs) and Gram positive bacteria employ autoinducing peptides (AIPs) as quorum sensing molecules. AHLs and AIPs are used for communication between bacteria of the same species. Another group of molecules, collectively called autoinducer-2 (AI-2), are found in culture supernatants of both Gram positive and Gram negative bacteria and are used for inter-species communication¹².

The term AI-2 refers to a group of interconverting molecules formed from a common precursor, 4,5-dihydroxy-2,3-pentanedione (DPD), whose production in bacterial cells is catalyzed by the LuxS enzyme. LuxS has a crucial function as it is responsible for metabolism of the toxic intermediate *S*-adenosyl-L-homocysteine (SAH), which is formed from *S*-adenosyl-L-methionine (SAM) upon the release of methyl groups to be employed in the methylation of proteins, nucleic acids and metabolites. A two step enzymatic conversion of SAH by Pfs and LuxS enzymes produces DPD as a

byproduct. DPD undergoes spontaneous cyclization to generate either the *R* or *S* form of the thermodynamically favorable compound, 2,4-dihydroxy-2-methyldihydro-3-furanone (DHMF). In the presence of water and borate, *S*-DHMF forms *S*-THMF-borate (Figure 1), a furanosyl borate diester, which is the form of AI-2 employed for QS regulation of bioluminescence in *Vibrio harveyi*¹⁰⁴. AI-2 diffuses out of the cells and, upon entering other cells and binding to the periplasmic binding protein LuxP, triggers a cascade of phosphorylation/dephosphorylation events ultimately leading to the expression of luciferase and production of light⁴⁸.

Bacterial quorum sensing molecules, namely AHLs, have been identified in physiological samples, such as sputum samples of patients with cystic fibrosis^{8,25,26}. Studies have shown that not only quorum sensing molecules activate bacterial virulence systems and bacterial proliferation at a particular defined cell density, but also modulate the host immune system to the advantage of bacteria¹⁰⁵. Bacteria are known to play an important role in many diseases, from infections to chronic inflammation. Therefore, quorum sensing is involved in a variety of disorders of bacterial origin or where bacteria play a crucial pathogenic role. One such condition is inflammatory bowel disease (IBD), a chronic inflammation of the gastrointestinal (GI) tract that includes debilitating diseases such as ulcerative colitis (UC) and Crohn's disease (CD). The inflammation in ulcerative colitis is concentrated in the colon as has a continuous distribution, whereas Crohn's disease encompasses inflammation in the ileum and other regions of the alimentary tract in patches¹⁰⁶. Both diseases are characterized by periods of increased inflammatory activity that can occur against a background of chronic ongoing

inflammation. The causes of IBD are not fully elucidated. However, studies carried out in animals and humans have established that gut bacterial flora play an essential role in these inflammatory states of the bowel. Specifically, IBD appears to be caused by an overly aggressive immune response to commensal enteric bacteria in genetically predisposed individuals¹⁰⁷. Hence, monitoring of quorum sensing molecules over the course of time can give an idea of the bacterial load, extent of inflammation and progress of the disease. Recently, our group has developed whole-cell sensing systems for the detection of AHLs and shown that these signaling molecules are present at distinct concentrations in saliva and stool samples from CD patients and infants with various types of intestinal inflammatory illnesses. These results suggest that quorum sensing molecules may be potential non-invasive biomarkers of gastrointestinal inflammatory disease²⁷.

A bioassay based on the *V. harveyi* strain BB170, which is able to emit bioluminescence in the presence of AI-2 molecules, was developed by Bassler *et al.*¹⁶. This bioassay has mainly been employed to screen cell culture supernatants to identify and investigate bacteria able to produce AI-2; as such, it has not been characterized in terms of analytical performance and standardized for detection of AI-2 in clinical samples. Herein, we present the design, development, optimization, standardization, and characterization of a biosensing system based on *V. harveyi* BB170, as well as its application for the quantitative detection of AI-2 in saliva, stool and intestinal samples from IBD patients and control subjects.

Experimental Section

Materials: Sodium chloride and anhydrous magnesium sulfate were purchased from Sigma (St. Louis, MO). Vitamin free casamino acids were purchased from BD Scientific (Franklin Lakes, NJ). Autoinducer-2 was purchased from Omm Scientific (Dallas, TX). Reverse osmosis (RO) filtered water (Milli-Q water purification system, Millipore, Bedford, MA) was utilized for all experiments. Sterile 14-mL culture tubes were purchased from BD Biosciences (San Jose, CA). 1.5-mL microcentrifuge tubes were purchased from Eppendorf (Westbury, NY). 96-well polystyrene microtiter plates were purchased from Costar (Corning, NY). The orbital shaker incubator was from Fisher Scientific (Fair Lawn, NJ). Bioluminescence measurements were performed using the FLUOstar Optima microplate reader (BMG Labtech, Durham, NC).

Bacterial Strain and Culture Conditions: *V. harveyi* strain BB170 was purchased from ATCC (Manassas, VA). The BB170 strain is genetically modified to express luciferase and emit light only in response to the autoinducer-2¹⁶. The autoinducer bioassay (AB) media described by Greenberg *et al.* was used to grow cells¹⁰⁸. Cells were grown overnight in AB media in the orbital shaker at 30 °C, 250 rpm and glycerol stocks were prepared by adding 250 µL glycerol to 750 µL cell culture; the stocks were then stored at -80 °C. Fresh cell cultures were grown from the glycerol stocks as needed by incubation in the orbital shaker at 30 °C, 250 rpm until an optical density at 600 nm (O.D._{600nm}) of 0.01-0.02 was reached.

Sample Collection and Preparation. Saliva, stool and intestinal samples were assayed for the presence of AI-2 molecules. Saliva and intestinal samples were obtained from patients with inflammatory bowel disease in the Gastroenterology Clinic of the University of Kentucky Medical Center (Lexington, KY). Intestinal samples were comprised of rectal samples, which can be assimilated to stools, and ileal as well as duodenal washings collected during endoscopy. Saliva samples were also obtained from healthy volunteers. Additionally, stool samples were obtained from infants in the Newborn Intensive Care Unit (NICU) of the University of Kentucky Medical Center. Saliva samples were collected in the morning immediately after brushing the teeth in order to minimize collection of oral bacteria and debris. They were then processed by centrifugation in sterile microcentrifuge tubes at 13,000 rpm for 7-8 minutes to facilitate settling of any debris and particles to the bottom of the tubes. Supernatants were then stored in sterile tubes at -80 °C till needed. For analysis, saliva samples were diluted 1:10 (v/v) and 1:100 (v/v) using RO filtered water. Stool and intestinal washing samples were frozen right after collection and stored at -80 °C until assayed. Stool samples were prepared for analysis by weighing certain amounts of specimens and then suspending and diluting them in RO filtered water to a final dilution of 1:750 (w/v). Washing samples were analyzed directly without any sample preparation.

Dose-Response Curves. A commercially available 3.9 mM stock solution of AI-2 in water was employed. Just before use, the stock solution was serially diluted with RO filtered water to prepare AI-2 standard solutions at concentrations ranging from 1×10^{-4} M to 2.5×10^{-8} M. RO filtered water served as blank. 10 μ L of each AI-2 standard solution and

blank was added in triplicate to the microtiter plate wells containing 90 μL of cell culture grown to an $\text{O.D.}_{600\text{nm}}$ of 0.010. The plate was incubated in the orbital shaker at 30 $^{\circ}\text{C}$, 175 rpm for 2.5 hours. Bioluminescence was recorded in the microplate reader and measurements were expressed as relative light units (RLU) and plotted using Microsoft Office Excel 2007.

Dose-Response Curves in Saliva and Stool Matrices. Dose-response curves were generated in saliva and stool matrices. A pool of saliva was prepared by mixing equal volumes of processed saliva samples obtained from healthy volunteers. Pooled saliva was then diluted 1:100 (v/v) with RO filtered water. The 3.9 mM stock solution of AI-2 was serially diluted with 1:100 (v/v) pooled saliva to prepare AI-2 solutions at concentrations ranging from 1×10^{-4} M to 2.5×10^{-8} M. A solution containing 1% RO filtered water in 1:100 (v/v) pooled saliva served as blank. 10 μL of each AI-2 solution and blank was added in triplicate to the microtiter plate wells containing 90 μL of cell culture, and the assay was performed as described above. A dose-response curve without matrix was also obtained in the same analytical run, which served as a reference. In order to prepare a pool of stool samples, 10 samples from infants admitted to the NICU were weighed in equal amounts, mixed together, and then suspended and diluted 1:750 (w/v) in RO filtered water. The 3.9 mM stock solution of AI-2 was serially diluted with 1:750 (w/v) pooled stool to prepare AI-2 solutions at concentrations ranging from 5×10^{-4} M to 5×10^{-8} M. A solution containing 1% RO filtered water in 1:750 (w/v) pooled stools served as blank. 10 μL of each AI-2 solution and blank was added in triplicate to the microtiter plate wells containing 90 μL of cell culture, and the

assay was performed as described above. A typical dose-response curve without matrix was also obtained in the same analytical run as a reference.

Analysis of Physiological Samples. Collection and processing of saliva, stool and bowel secretion samples is described above. A final volume of 10 μL of each of the processed samples was added in triplicate to the microtiter plate wells containing 90 μL of cell culture, and the assay was performed as described above. Each analytical run included a dose-response curve generated using standard AI-2.

Results and Discussion

Several recent reports implicate intestinal microorganisms in the pathogenesis of IBD. Studies on animal models have shown that IBD does not occur in germ free environments and that enteric bacteria are essential for development of colitis¹⁰⁹. A number of bacteria have been postulated to play a role in the pathogenesis of Crohn's disease, including *Mycobacterium avium* subspecies *paratuberculosis*, *Pseudomonas* species, adherent-invasive *Escherichia coli*, and Streptococci¹⁰⁶; however, no definitive evidence exists to support any specific microorganism as the causative agent of IBD. Additionally, some Crohn's patients subjected to prolonged treatment with antibiotics have shown improvement¹¹⁰, indicating that bacterial flora must play a crucial role in IBD. Importantly, it has been demonstrated that the bacterial composition of IBD inflamed intestine differs from that of healthy intestine, with decreased microbial diversity and increased relative abundance of certain taxa in IBD^{111,112}.

The above mentioned reports, along with other published data point to the presence of bacterial quorum sensing molecules in human samples; however, only a few analytical studies have been performed to identify and quantify these signaling molecules in physiological specimens. Kumari *et al.* have utilized whole-cell based biosensing systems for detecting and quantifying nanomolar levels of AHL molecules in complex clinical samples, like saliva and stool²⁷. Sperandio *et al.* report that fecal filtrates contain AI-2, although quantitative analysis has yet to be performed¹¹³. Recently, Campagna *et al.* have utilized liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to quantify AI-2 molecules in saliva of healthy individuals¹¹⁴. The method required analyte derivatization, which is time consuming and may result in inaccurate measurements caused by low efficiency of the derivatization reaction due to the complex nature of the biological matrix.

Herein we developed, optimized, and characterized a whole-cell sensing system based on *V. harveyi* BB170 for the quantitative detection of AI-2 in physiological samples, i.e., saliva, stool and intestinal fluids, from healthy individuals as well as IBD patients. The bioluminescent bacterium *V. harveyi*, in its natural wildtype form has multiple parallel quorum sensing circuits that regulate bioluminescence. Among those, there is a circuit that responds to AHLs and another one to AI-2 (Figure 2). The *V. harveyi* BB170 variant employed in this study was, in contrast, genetically modified to only respond to AI-2. When AI-2 enters the cells, it binds to the periplasmic binding protein LuxP, which undergoes a change in conformation upon binding to AI-2 and initiates the transcription of the *luxCDABE* cassette, which codes for bacterial luciferase and other

enzymes that catalyze the synthesis of the luciferase substrate. Thus, presence of AI-2 triggers the expression of luciferase, its substrate, and ultimately results in the generation of bioluminescence. In the optimized conditions described in this work, the light signal derived from the luciferase-dependent reaction is proportional to the AI-2 present in the environment of the sensing cells. Therefore, the whole-cell sensing system is appropriate for monitoring the levels of AI-2 in saliva, stool, and intestinal washing samples. It is well established that the concentration of biomolecules and drugs in saliva reflects their systemic concentration because of the ability of many substances to reach saliva via intracellular and extracellular routes¹¹⁵. On the other hand, stool and rectal samples as well as intestinal washings are considered to be representative of the environment present in the bowel¹¹⁶.

As mentioned above, the *V. harveyi* strain BB170 has been employed as the detection element in a bioassay aimed at investigating the ability of a variety of bacterial species and strains to produce AI-2. For that, the bacteria under examination are grown for a period of time and the culture supernatants are then exposed to the reporter strain for induction of bioluminescence due to the presence of AI-2. The conventional experimental protocol employed in these studies is time-consuming (4 hours) and only provides qualitative data^{117,118}. In that regard, the goal of this work was to develop a whole-cell sensing system based on *V. harveyi* BB170 for the quantitative detection of AI-2 molecules in physiological samples. To that end, we first developed a sensing system for the detection of AI-2 quorum sensing molecules. We then optimized and

standardized the experimental protocol for effective and efficient use of the biosensing system in clinical applications.

Modifications to the conventional assay protocol allowed for a 40% reduction in time to perform the entire assay (2.5 h vs. 4 h). By exposing the sensing cells to standard solutions containing various concentrations of AI-2, we were able to establish a dose-response curve with a detection limit of 2.5×10^{-8} M AI-2 and a dynamic range of 2.5×10^{-8} M to 1×10^{-5} M. The limit of detection (LOD) was defined as sum of the average of the blank signal and three times its standard deviation. The sensing system proved to be precise and reproducible, with intra- and inter-assay percent relative standard deviation (%RSD) values less than 8%. It is noteworthy to point out that the limit of detection obtained with our whole-cell sensor is well below the levels of AI-2 found in human saliva (244-965 nM) by Campagna *et al.* using LC-MS/MS¹¹⁹. This further supports the applicability of the cell-based sensing system to the analysis of physiological/clinical samples.

When the whole-cell biosensing system was used for detection of AI-2 in stool, significant quenching of the bioluminescence signal was observed. Dilution studies were then performed in order to determine a proper stool dilution that did not show any matrix interference (data not shown). A 1:750 (w/v) dilution of stool with RO filtered water was found to be required to eliminate matrix effect. A dose-response curve was constructed in 1:750 diluted pooled stool samples and compared with a standard dose-response curve (Figure 3). As observed in the figure, the two dose-

response curves virtually overlapped and exhibited similar analytical characteristics (Table 1). Thus, a 1:750 (w/v) dilution was chosen to test unknown stool samples. A matrix effect was also observed when saliva samples were analyzed directly, without dilution. A dose-response curve was obtained by adding AI-2 solutions at known concentrations to a pool of saliva samples, which showed signal intensities lower than those observed when the dose-response curve was generated with known AI-2 standards in water. Optimization studies determined that a 1:100 (v/v) dilution of saliva was able to eliminate the matrix effect and allowed for the generation of analytically sound dose-response curves (Figure 4). The analytical parameters of dose-response curves obtained in different matrices are reported in Table 1.

After optimizing the assay conditions, we employed the whole-cell sensing system to detect AI-2 levels in individual saliva, stool, and intestinal samples collected from IBD patients and healthy subjects (Table 2). The obtained data proved that AI-2 was detectable in all three different types of samples. Although only a few samples were analyzed, it is interesting to observe that distinct levels of AI-2 were detected in the tested samples. Specifically, AI-2 levels varied considerably in stool samples from IBD patients, which may support the hypothesis that variations in the concentrations of QSMs in those samples may reflect perturbation of the microflora in the inflamed intestine^{111,112}. To the best of our knowledge, this is the first time that the levels of AI-2 are quantified in human stool and intestinal samples. The results obtained point out to a potential use of our method as a rapid and inexpensive tool for diagnosis and monitoring of bacterial-related conditions, including IBD, where bacteria is thought to

play an important role. We postulate that correlation of AI-2 levels in physiological samples with the status of the disease may have implications in the potential use of AI-2 as a biomarker of disease. Work toward the verification of this hypothesis by performing clinical studies is underway in our laboratories.

Conclusion

In conclusion, we have designed, developed and optimized a whole-cell based biosensing system that allows for quantitative detection of AI-2 quorum sensing molecules in human samples. When employed for the detection of AI-2 in physiological specimens, the method proved to be rapid, reproducible, and sensitive, with a detection limit of 2.5×10^{-8} M. Additional advantages of the method lie in the requirement of small unprocessed sample amounts (approximately 1 μ L of saliva and less than 1 mg of stool) and the potential for high-throughput analysis which are important for clinical applications, such as monitoring of IBD patients for their AI-2 levels over a period of time. This sensing system should also be applicable to other bacteria-related conditions. Furthermore, the analytical features of this method make it amenable to investigation of compounds that may act as agonists or antagonists in the AI-2-mediated QS system; this may lead to the identification of QS inhibitors that could be employed as drugs in an alternative approach for the treatment of bacteria-related disorders.

Figure 1. Synthesis of DPD in bacteria. By the action of methyltransferases, *S*-adenosylmethionine (SAM) is converted to *S*-adenosylhomocysteine (SAH). SAH is metabolized to adenine and *S*-ribosylhomocysteine (SRH) by Pfs enzyme. SRH is the substrate for the enzyme LuxS catalyzing the synthesis of 4,5-dihydroxy-2,3-pentanedione (DPD). (b) DPD undergoes spontaneous cyclization and forms (2*R*,4*S*)- and (2*S*,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*R*- and *S*-DHMF, respectively). *R*- and *S*-DHMF undergo hydration to form (2*R*,4*S*)- and (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*- and *S*-THMF, respectively). In the presence of borate, *S*-THMF converts to (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate), i.e., the autoinducer-2 form utilized by *Vibrio harveyi*. *R*-THMF is the autoinducer-2 form utilized by other bacteria, such as, *Salmonella typhimurium*. Figure copyright from Xavier *et al.*¹⁹

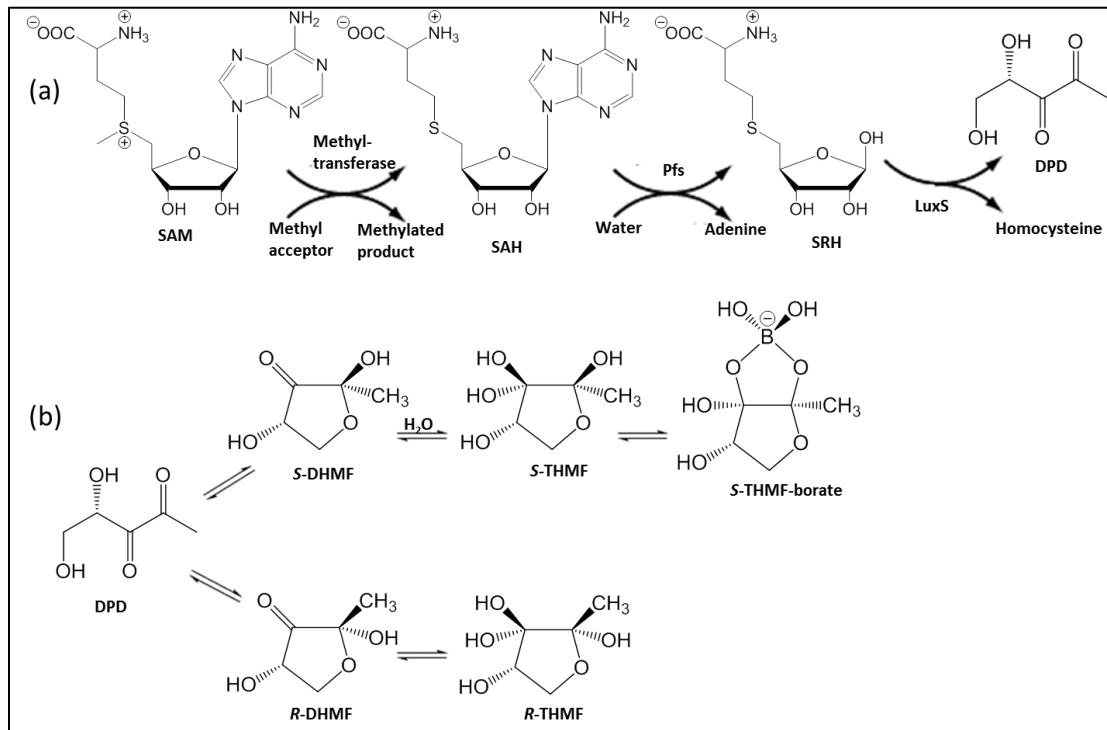


Figure 2. Quorum sensing regulation of bioluminescence in *V. harveyi* BB170. System 1, which uses an AHL as quorum sensing molecule, has been silenced. Therefore, production of bioluminescence only depends on AI-2 (System 2).

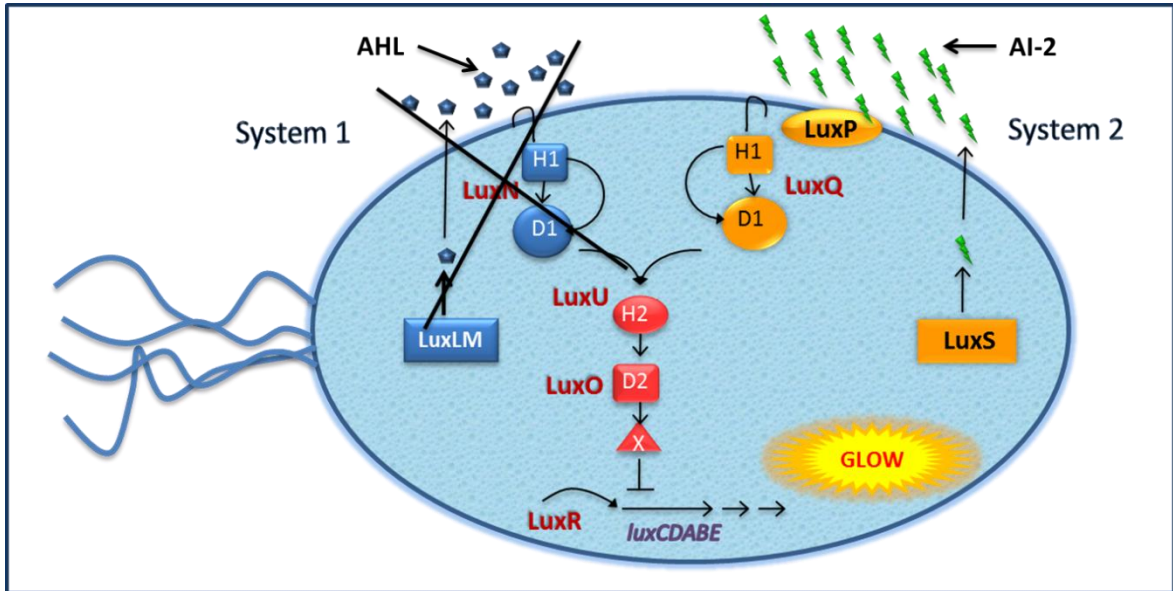


Figure 3. Standard dose-response curve (black) compared with dose-response curve in 1:750 (w/v) stool (red).

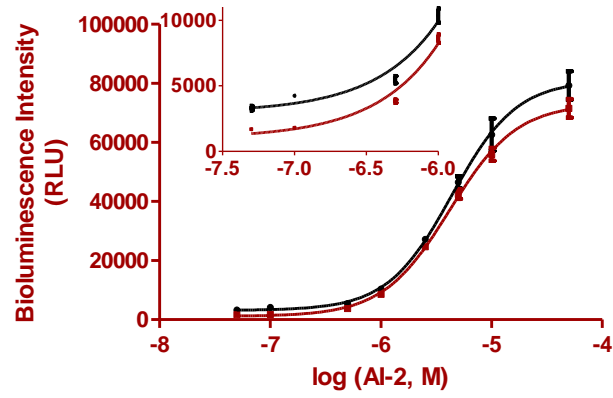


Figure 4. Standard dose-response curve (blue) compared with dose-response curve in 1:100 saliva (red).

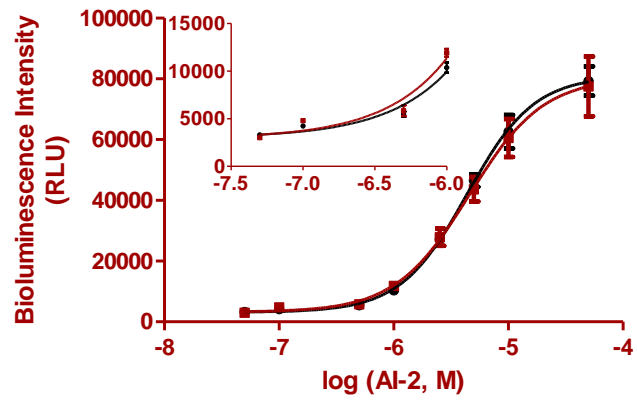


Table 1. Analytical parameters of the whole-cell biosensing system in buffer and physiological matrices.

	Signal Intensity with 5×10^{-5} M AI-2 ($\times 10^4$ RLU)	Signal Ratio	LOD (M)	Dynamic Range (M)
Buffer	7.9	1	2.5×10^{-8}	$2.5 \times 10^{-8} - 5.0 \times 10^{-5}$
1:750 (w/v) Stool	7.1	1.11	5.0×10^{-8}	$5.0 \times 10^{-8} - 5.0 \times 10^{-5}$
1:100 (v/v) Saliva	7.7	1.03	2.5×10^{-8}	$2.5 \times 10^{-8} - 5.0 \times 10^{-5}$

Table 2. Detection of AI-2 in clinical samples from IBD patients (white) and healthy subjects (gray).

Samples	Signal Intensity ($\times 10^4$ RLU)	%RSD
Stool 1	1.8	6
Stool 2	3.8	7
Ileal washing	2.5	7
Saliva 1	2.5	6
Saliva 2	2.4	10
Saliva 3	1.9	1
Saliva 4	1.2	6
Saliva 5	1.3	5
Saliva 6	2.3	6

CHAPTER THREE

DETECTION OF QUORUM SENSING MOLECULES IN BLOOD SERUM

Introduction

Bacteria are known to cause a variety of disorders from acute and chronic infection to inflammation, within and on the surface of the human body. Bacterial infections can be caused by a variety of microorganisms and result in mild to life-threatening conditions. Furthermore, certain bacteria are involved in chronic diseases, such as, inflammatory bowel disease (IBD)¹²⁰ and cystic fibrosis²⁶. A notable feature of bacteria is their ability to form biofilms. Biofilms were first reported by J. W. Costerton in 1978; using light and electron microscopy, he demonstrated the presence of biofilms on inanimate surfaces in an aquatic environment¹²¹. The presence of biofilms in cystic fibrosis lung infections has been shown by Singh *et al.*²⁵. Bacterial infections are generally treated by antibiotic administration. Formation of biofilms contributes to pathogenicity and makes the treatment less effective as bacteria are encased in a matrix composed of polysaccharides and proteins that reduces the contact between bacteria and antibacterial agent¹²². In addition, due to extensive usage of antibiotics, several pathogenic bacteria have gained antibiotic resistance through mutations and lateral gene transfer. Indeed, alternative strategies against bacterial infections that involve new classes of antibiotics are needed more than ever. In that regard, compounds that are inhibitors of quorum sensing, the mechanism by which bacterial cells communicate and ultimately leads to biofilm formation, provide with a new approach to combating

bacterial infection. Other bacterial behaviors such as virulence factor production, swarming, and motility are also modulated through quorum sensing. Bacterial quorum sensing circuits operate via synthesis and secretion of small organic molecules, termed as quorum sensing molecules (QSMs), which are recognized by bacteria of the same species (intra-species communication) or of other species (inter-species communication). Once these molecules enter the bacterial cell, they initiate a cascade of events leading to the expression of certain target genes. A general schematic of the quorum sensing circuits in the pathogenic bacterium *Pseudomonas aeruginosa* is shown in Figure 1. The expression of the aforementioned genes is regulated in a cell-density dependent manner. When cell density is low, the amount of QSMs in the environment around bacteria is low and the expression of genes is repressed. As the cell density gradually increases, the amount of QSMs in the environment also increases proportionally and, when a certain QSM threshold concentration is reached, the genes are expressed¹². Gram-negative bacteria utilize *N*-acyl homoserine lactones (AHLs) and Gram-positive bacteria utilize autoinducing peptides (AIPs) for intra-species communication. A third category of molecules, known as autoinducer-2 (AI-2), is used for interspecies communication as the genes coding for the proteins that either synthesize or bind AI-2 are present in both Gram-negative and Gram-positive bacteria. Recently, Sperandio *et al.* postulated that a new type of QSM, autoinducer-3, is involved in interkingdom communication between bacteria and their hosts¹⁷. Figure 2 lists few examples of QSMs and the bacteria that synthesize them.

Reports relating quorum sensing to bacterial infections have emerged in the last decade. Singh *et al.* reported the presence of biofilms of the pathogenic bacterium *P. aeruginosa* in the lungs of cystic fibrosis patients. It has been demonstrated that the presence of biofilms reduce the effectiveness of even aggressive antibiotic treatment²⁵. Two AHLs, *N*-butyryl-L-homoserine lactone (C-4 HSL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C-12 HSL), used by *P. aeruginosa* to control biofilm formation as well as virulence determinant production, were identified in sputum samples from these patients. The significance of quorum sensing in burnt wound infections in mice was demonstrated by Rumbaugh *et al.* who found that mice infected with mutant strains of *P. aeruginosa* that were unable to synthesize AHLs had lower levels of virulence expression as compared to those infected with wild type *P. aeruginosa*²². Another study also highlighted the importance of quorum sensing using a mouse model with ascending urinary tract infections¹²³. These studies demonstrate the relevance of detecting and quantifying QSMs. If a relationship between levels of QSMs in physiological specimens and status of the disease is established, QSMs could be used as biomarkers of bacteria-related disorders.

The most common techniques to detect QSMs include various physical-chemical methods and biosensing systems. Physical-chemical methods, such as, GC-MS^{124,125}, HPLC-UV¹²⁶, and LC-MS-MS¹²⁷ require extensive sample preparation steps, often including derivatization¹¹⁹, expensive instrumentation, and specialized laboratory personnel, along with being time consuming and low throughput. While protein-based biosensing systems to detect QSMs are rapid, cost-effective, and easy to use, they are

limited by the stability of the sensing proteins in biological matrices and relatively high limits of detection¹²⁸. Our group developed and employed two bioluminescent bacterial whole-cell biosensing systems based on the recognition and signal generation afforded by plasmids pSB406 and pSB1075, respectively, to detect with high sensitivity, selectivity, and reproducibility short and long chain AHLs in human saliva and stool²⁷.

Bodily fluids such as saliva and blood are known to contain a variety of small molecules and proteins, many of which have been investigated for potential utility as biomarkers of diseases. We have previously demonstrated that AHLs are present in saliva and stool specimens²⁷. However, until now blood has not been studied for the presence of QSMs. Blood transports oxygen and nutrients to cells and takes metabolic waste away from cells. In addition, it helps maintain body temperature, regulates pH, and allows protection against foreign agents by the action of white blood cells. Furthermore, blood represents one of the most commonly used fluids where clinically relevant molecules and drug metabolites are detected.

In this study we aimed at investigating blood for the detection of QSMs. To the best of our knowledge, this is the first time that the presence of AHLs in blood serum is reported. Moreover, we validated our findings, which were achieved in human blood serum employing whole-cell sensing systems, by analyzing the serum samples using LC-MS-MS. Furthermore, we demonstrated that AHLs can also be detected in animal serum. Specifically, in order to support the hypothesis of employing QSMs as biomarkers of disease, we report the results of a pilot study investigating the effects of experimentally induced colitis on the serum and stool levels of QSMs in mice models.

Experimental section

Materials. *N*-hexanoyl-DL-homoserine lactone (C-6 HSL), *N*-dodecanoyl-DL-homoserine lactone (C-12 HSL), sodium chloride, anhydrous magnesium sulfate, Luria Bertani (LB) media, ampicillin, and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methylene chloride used in all experiments were of HPLC grade and were purchased from VWR Scientific (Pittsburgh, PA). AI-2 was purchased from Omm Scientific (Dallas, TX). The 96-well microtiter plates were purchased from Costar (Corning, NY). Vitamin free casamino acids were purchased from BD Biosciences (Franklin Lakes, NJ).

Plasmids, bacterial strains, and culture conditions. The plasmids pSB406 and pSB1075 were originally provided by Dr. Paul Williams (University of Nottingham, Nottingham, UK) and previously transformed in *Escherichia coli* JM109 cells. The transformed cells were then stored at -80 °C as glycerol stocks. Fresh cell cultures were obtained from the glycerol stocks and grown in LB media (100 µg/mL aAmpicillin) overnight in an orbital shaker at 37 °C, 250 rpm, refreshed in the morning, and allowed to grow until an optical density at 600 nm (OD_{600nm}) of 0.45-0.50 was reached. *Vibrio harveyi* MM32 cells were purchased from American Type Culture Collection (ATCC), and glycerol stocks were stored at -80 °C. Fresh cell cultures were obtained by growing cells in autoinducer bioassay (AB) media with 30 µg/mL of kanamycin overnight in the orbital shaker at 30 °C, 250 rpm, and then refreshing the cultures in the morning to obtain an OD_{600nm} of 0.01-0.02. The procedure to prepare AB media has been described elsewhere^{16,129}.

Dose-response curves. Commercially available *N*-acyl-homoserine lactones were dissolved in acetonitrile to obtain 1×10^{-2} M stock solutions, which were serially diluted with RO filtered water to obtain standard solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. A 1% (v/v) solution of acetonitrile in RO filtered water (0.1% (v/v) acetonitrile after adding to the sensing cell suspension) was used as blank. This amount of acetonitrile was not toxic for the sensing cells. A volume of 10 μ L of each of these solutions was added in triplicate to a 96-well white polystyrene microtiter plate containing 90 μ L/well of the corresponding *E. coli* cell culture grown to an OD_{600nm} of 0.45-0.50. C-6 HSL solutions were employed for the whole-cell sensor with pSB406, while C-12 HSL solutions were employed for the whole-cell sensor with pSB1075. The microtiter plate was then incubated in an orbital shaker at 37 °C at 175 rpm for two hours. Commercially available aqueous AI-2 solution (3.7×10^{-3} M) was serially diluted using RO filtered water to obtain solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. RO filtered water was used as blank. A 10 μ L volume of each AI-2 solution and blank in triplicate was incubated with 90 μ L of *V. harveyi* MM32 cell culture grown to an OD_{600nm} of 0.01-0.02, in a microtiter plate. The produced bioluminescence from all the sensing systems was then measured using the FLUOstar Optima microplate reader (BMG Labtech, Durham, NC). The light intensity was expressed in relative light units (RLU).

Human blood serum samples. Blood serum samples were obtained from IBD patients and healthy volunteers (Division of Gastroenterology, Department of Medicine, University of Miami Miller School of Medicine). Individual serum samples were stored at

-80 °C until analyzed. To prepare a pool of serum for matrix effect studies, equal volumes of serum samples from various healthy individuals were mixed together in a 14-mL tube and vortexed for 5 minutes. Several aliquots of pooled serum were prepared and stored at -80 °C until needed.

Serum dilution study. One aliquot of pooled serum was removed from -80 °C, thawed, and equilibrated to room temperature. To test for potential matrix effect, the serum was serially diluted with RO filtered water to obtain 1:10, 1:100, and 1:1000 dilutions. Each standard QSM (C-4 HSL, C-12 HSL, or AI-2) was added to aliquots of undiluted and diluted serum to achieve a final 1×10^{-6} M concentration. A 10 μ L volume of each of these spiked and non-spiked serum samples at various dilutions was added in triplicate to 90 μ L of the appropriate sensing cells, and the assay was performed as described above. A dose-response curve with the respective QSM was also included in each analytical run.

Dose-response curves in serum matrix. 1×10^{-2} M stock solutions (prepared in acetonitrile) of C-6 HSL and C-12 HSL were serially diluted with 1:10 serum/water solution to obtain AHL concentrations ranging from 1×10^{-4} to 1×10^{-8} M. Acetonitrile at 1% in the 1:10 serum/water solution was used as blank. Similarly, the available stock of AI-2 (aqueous solution at 3.7×10^{-3} M) was serially diluted with 1:10 serum/water solution to obtain AI-2 concentrations ranging from 1×10^{-4} to 1×10^{-8} M. A 10 μ L volume of each of these solutions was added in triplicate to the wells of a microtiter plate, followed by addition of 90 μ L of cell culture of the respective biosensing system.

The assay was then performed as described above. A reference dose-response curve was included in each analytical run.

Serum storage study. Aliquots of pooled serum were spiked with C-6 HSL to obtain final concentrations in serum of 1×10^{-6} M and 1×10^{-7} M. Three sets of spiked serum samples were prepared; the first set was assayed immediately, the second set was kept at room temperature for six hours and then assayed, and the third set was kept at room temperature for two hours, later stored at -80 °C for 48 hours, and then assayed. Final concentration of Serum containing RO filtered water, instead of C-6 HSL solution, was used as blank for each storage condition. Aqueous solutions of C-6 HSL at 1×10^{-6} M and 1×10^{-7} M concentrations served as controls. The serum samples were diluted 1:10 using RO filtered water, 10 μ L of each diluted sample was added in triplicate to a microtiter plate containing 90 μ L/well of the whole-cell biosensing system bearing pSB406, and the assay was carried out as described above.

Analysis of human serum samples. After optimizing the experimental conditions, individual serum samples were diluted 1:10 using RO filtered water. A 10 μ L volume of each diluted sample was added in triplicate to the wells of a microtiter plate and incubated with 90 μ L of cell culture of each of the biosensing systems for detection of short chain AHLs, long chain AHLs, and AI-2, respectively. The assays were performed as described above.

LC-MS-MS analysis of serum samples.

Preparation of AHL standard solutions. Commercially available standard *N*-acyl-homoserine lactones were dissolved in acetonitrile to obtain 1000 μ g/mL solutions.

Twenty microliters of each of these 1000 µg/mL solutions of *N*-butyryl-DL-homoserine lactone (C-4 HSL), *N*-hexanoyl-DL-homoserine lactone (C-6 HSL), *N*-(3-oxohexanoyl)-DL-homoserine lactone (3-oxo-C-6 HSL), *N*-octanoyl-DL-homoserine lactone (C-8 HSL), *N*-decanoyl-DL-homoserine lactone (C-10 HSL), *N*-dodecanoyl-DL-homoserine lactone (C-12 HSL), and *N*-tetradecanoyl-DL-homoserine lactone (C-14 HSL) was added to 860 µL of methanol/water (35:65, v/v) with 0.1% formic acid, in order to obtain a stock mixture solution containing 20 µg/mL of each of the seven AHLs. From this stock solution, AHL standard mixture solutions, which contained 50, 25, 10, 5, 1, and 0.5 ng/mL of each AHL, respectively, were prepared for calibration. *N*-heptanoyl-DL-homoserine lactone (C-7 HSL) was used as an internal standard (since C-7 HSL is not produced by bacteria) for all the standard mixtures. A specific volume of 1 µg/mL solution of C-7 HSL in methanol/water (35:65, v/v) with 0.1% formic acid was added to each of the standard mixture solutions to achieve a final internal standard concentration of 25 ng/mL. These standard mixture solutions with the added internal standard served as laboratory control spike (LCS) solutions. Similarly, a specific volume of 1 µg/mL internal standard solution was added to water to obtain a final C-7 HSL concentration of 25 ng/mL. The resulting solution served as blank.

Extraction of AHLs from serum samples. An extraction protocol similar to that used by Kumari *et al.* to extract AHLs from saliva samples¹³⁰ was employed with modifications in this study. Briefly, an equivalent volume of acetonitrile was added to a pool of serum to precipitate the proteins out. The precipitate formed was centrifuged and the supernatant separated. The supernatant was then extracted using methylene chloride,

evaporated to dryness, reconstituted using mobile phase to about 100 μ L and C-7 HSL was added as internal standard. The LC-MS-MS analysis was performed at the Environmental Research Training Laboratories (ERTL) of the University of Kentucky as previously described¹³⁰.

Animal Study. Wild type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbour, Maine). A total of nine mice, between 13 and 17 weeks of age, were used. In the course of study, mice were fed manufacturer prescribed diet (LabDiet® 5K52/5K67). Over a period of seven days, five mice were given a 3% aqueous solution of dextran sulfate sodium (DSS) through drinking water (5 mL per day) to experimentally induce colitis. Four mice that received equivalent amount of water, without DSS, served as controls. Serum and stool samples were collected from both animal groups on day 0 (before DSS administration) and on day 7. Serum and stool samples were frozen at -80 °C immediately upon collection. Serum samples were processed and analyzed as described above for human serum. Stool samples were processed by following the procedure described by Kumari *et al.*²⁷. Briefly, a 20 mg/mL suspension of stool was prepared using 25:75 acetonitrile/water solution and diluted using RO filtered water to obtain a 1:1600 stool suspension. For analysis, a 10 μ L volume of this stool suspension in triplicate was then incubated with 90 μ L of cell culture of each of the whole-cell sensing systems. The assays were performed as described above.

Results and Discussion

A biosensing system consists of a biological recognition component coupled to a transducer component. A whole-cell biosensing system consists of the intact bacteria as the recognition component; the bacteria are genetically engineered to generate a readable output upon selective recognition of the analyte. The biosensing system produces a dose-dependent readable output in response to the analyte. The whole-cell biosensing systems carrying plasmids pSB406 and pSB1075 respond to short and long chain AHLs, respectively. Plasmid pSB406 contains the gene *rhIR*, which codes for the recognition/regulatory protein RhIR, and the reporter gene cassette *luxCDABE*, which is under the transcriptional control of promoter P_{rhII} . Similarly, plasmid pSB1075 bears the gene *lasR*, which codes for the recognition/regulatory protein LasR, and the reporter gene cassette *luxCDABE*, which is under the transcriptional control of promoter P_{lasI} . The gene cassette *luxCDABE* encodes for the bioluminescent enzyme luciferase and enzymes that catalyze the synthesis of the luciferase substrate. In the presence of AHLs, the reporter is expressed in a dose-dependent manner and the produced bioluminescent signal is proportional to the AHL concentration present inside the cell. In a similar manner, the whole-cell biosensing system based on *V. harveyi* MM32 responds to AI-2 by producing bioluminescence in a dose-dependent fashion. *V. harveyi* is a spontaneously bioluminescent marine microorganism that controls light emission through various quorum sensing systems, which employ various QSMs. Specifically, the bacterium uses QS circuits that utilize *N*-3-hydroxybutanoyl homoserine lactone (HAI-1), (*S*)-3-hydroxytridecan-4-one (CAI-1), AI-2, and recently discovered nitric oxide (NO). *V. harveyi* strain MM32 is genetically modified in such a way that it only emits

bioluminescence in response to AI-2. Additionally, it lacks the gene *luxS* encoding for LuxS protein, which synthesizes AI-2¹⁵; in this way the cells do not produce their own AI-2 and they can only produce bioluminescence in response to exogenous AI-2. Therefore, this strain can be employed as a sensor that responds with emission of light to AI-2 present in the environment of the cells. In this work we optimized, characterized, and utilized *E. coli* pSB406 and pSB1075 based whole-cell biosensing systems, and *V. harveyi* MM32 based whole-cell biosensing systems, to detect AHLs and AI-2, respectively, in blood serum samples.

In our studies, we collected individual blood serum samples from healthy volunteers and mixed them together to obtain a pool of serum in order to take in account possible variations between samples. Serum electrolytes, antibodies, hormones, and exogenous substances can affect the performance of biosensing systems, and therefore, serum needs to be evaluated in terms of its potential matrix effect on the determination of the AHLs in this sample. For that, dilution studies were undertaken by preparing serial dilutions of serum with water followed by spiking studies in which a known concentration of analyte was added to serum. Serum 1:10, 1:100, and 1:1000 dilutions were prepared using RO filtered water and incubated with the whole-cell biosensing systems employed in this study. RO filtered water served as control. When undiluted pooled serum was incubated with the whole-cell biosensing systems, reduced bioluminescence was observed as compared to that of the control; the control bioluminescence was due to the background signal of the sensing cells. However, signals equivalent to that from the control were obtained from all the serum dilutions tested,

which showed that the quenching/inhibiting matrix effect was eliminated at even a very low dilution of the serum, i.e., 1:10 dilution of serum in water. These data were confirmed by spiking the undiluted serum and above serial serum dilutions with a known concentration of QSM and employing the corresponding whole-cell biosensing system to detect the AHLs as described in the Methods Section. As an example, Figure 3 shows the results of the matrix effect studies performed using the whole-cell biosensing system containing pSB406. Dose-response curves using 1:10 dilution of the serum were then obtained. Standard solutions of QSMs were prepared by serial dilution in either 1:10 serum or water and incubated with the sensing cells. As shown in Figure 4 for detection of short chain AHLs with the pSB406-based sensing system and AI-2 with the *V. harveyi* MM32-based sensing system, the two dose-response curves of each sensing system overlap, thus confirming that a 1:10 dilution of serum eliminates the matrix effect. Similar results were obtained with the sensing systems for detection of long chain AHLs. Limits of detection of 1×10^{-9} M for short and long chain AHLs, and of 5.0×10^{-8} M for AI-2 were obtained in 1:10 diluted serum.

Following optimization and characterization of the sensing systems for use in serum by evaluating and eliminating matrix effect through serum dilution, and defining the analytical parameters of the sensing systems in serum, we analyzed the blood serum samples obtained from IBD patients and control individuals. Inflammatory bowel disease is a chronic inflammatory condition of the intestine, which is characterized by recurrent acute inflammation episodes (flare-ups) over chronic persistent inflammation. The most common forms of IBD are Crohn's disease (CD), which mainly affects the small

intestine as well as other parts of the gastrointestinal tract, and ulcerative colitis (UC), which affects the colon. The causes of IBD are not well understood; it is believed that several factors, including bacteria, play a role in the etiology of the disease. Specifically, there is evidence that an overly aggressive immune response towards the intestinal microflora occurs in genetically predisposed individuals¹³¹. Additionally, altered gut bacterial composition, with less diversity and changed relative abundance of species, has been demonstrated in IBD patients as compared to healthy subjects¹³². For this study, we obtained 6 samples from control individuals, 10 samples from UC patients, and 8 samples from CD patients. The serum samples were analyzed after a 1:10 dilution with RO filtered water. When the whole-cell biosensing system containing plasmid pSB406 was employed, short chain AHLs were detected in a number of samples. Specifically, all control samples, except one, exhibited undetectable levels of AHLs; on the other hand, AHLs were detected in the majority of IBD samples, with UC patient samples showing a trend of higher bioluminescence signal intensities as compared to CD patient samples (Figure 5). Each sample was tested at least twice to confirm the above observation; consistent results were achieved, with good reproducibility as expressed by percent relative standard deviation (%RSD) values ranging from less than 1% to 10%. Although a limited number of samples were analyzed as proof of principle, the obtained data suggest that the differences in intestinal bacterial composition between IBD and healthy subjects, as reported in the literature, may be revealed by differences in QSM levels in physiological samples. This would support the use of QSMs as biomarkers of bacteria-related diseases. However, more samples derived from larger numbers of IBD

patients and controls need to be analyzed in order to confirm the observed trend. When the whole-cell biosensing system containing plasmid pSB1075 was employed, long chain AHLs were not detected in any of the tested samples. Additionally, when samples were analyzed using the MM32-based sensing system, none of them showed AI-2 levels in our detection range. One could speculate that this may be due to limited stability of certain QSMs, e.g., AI-2 in serum. On the other hand, stability of long chain AHLs in human serum should not be an issue, as shown by the LC-MS-MS study discussed below. Further investigations are required to clarify the obtained data.

Since no studies have previously reported the presence of QSMs in serum, we deemed it necessary to confirm our findings using a conventional physical-chemical technique, such as LC-MS-MS. Recently, our group developed an LC-MS-MS method to detect AHLs in saliva¹³⁰. In this work, using HPLC coupled to tandem mass spectrometry and the above protocol with slight modifications, we were able to separate and quantitate AHLs present in standard mixture solutions, with nanomolar limits of detection. We subsequently analyzed pooled serum samples. Unlike saliva, serum forms an emulsion if methylene chloride is used for AHL extraction. Therefore, we first precipitated the proteins from serum by adding acetonitrile. This step ensured that AHLs were solubilized in acetonitrile. After separating supernatant from precipitate, methylene chloride extraction was performed; the extract was then evaporated, dried, and reconstituted with mobile phase to run the sample on LC-MS-MS. As shown in Figure 6, C-8 HSL, C-10 HSL, C-12 HSL, and C-14 HSL were detected in extracted serum samples. The daughter ion analysis (Table 1) further confirmed the identity of AHLs that

were present in serum. These results are significant because they support that AHLs are actually present in serum and validate the use of whole-cell sensing systems for detection of AHLs in this type of physiological sample.

Sample storage conditions are crucial when collecting and analyzing clinical samples. To that end, we carried out a study where we spiked pooled serum samples with C-6 HSL to obtain two final concentrations of, 1×10^{-6} M and 1×10^{-7} M, and then stored these spiked samples in various conditions before assaying them with the whole-cell biosensing system containing pSB406. The storage conditions tested were meant to represent those actually employed when collecting patient serum samples in hospital settings. After spiking, sets of serum samples were either assayed immediately, or after being kept at room temperature for 6 hours, or after being kept at room temperature for 2 hours followed by storage at -80 °C for 48 hours. We found that (Figure 7), among these spiked serum samples, only those that were assayed immediately showed a bioluminescence signal intensity similar to that of the control (same AHL concentrations in water. This indicates that AHLs rapidly degrade in serum at room temperature; hence, serum samples should not be left at room temperature and should rather be frozen as soon as possible after collection.

While no reports are available with regard to stability of AI-2 in blood, few studies have indicated that blood of mammals carries enzymes, known as paraoxonases (PONs), which are capable of degrading AHLs¹³³⁻¹³⁶. In humans, three kinds of paraoxonases have been identified – PON1, PON2, and PON3. PON1 and PON3 are synthesized in the liver and secreted into the blood. PON2, which has the highest

activity for AHLs hydrolysis, is expressed in many tissues, including brain, kidney, liver, and testis, but not in blood. While PON1 and PON3 are mostly involved in hydrolysis of organophosphates, both of them are active lactonases, although with activity much less than that of PON2^{134,136}. The presence of PON1 and PON3 in blood may affect the stability of lactones, including AHLs, and thus affect the amount of AHLs detected. Interestingly, one report has indicated reduced levels of PON1 enzymes in the serum of CD and UC patients as compared to controls¹³⁷. This reduced lactonase activity may explain, at least in part, the higher levels of short chain AHLs that we observed in IBD patients with respect to controls. In healthy individuals, higher levels of PON enzymes may degrade AHLs more effectively, while in IBD patients lower levels of PON enzymes are unable to degrade AHLs effectively, leading to accumulation in serum.

In order to further test the hypothesis that intestinal inflammation may be associated with changes in the gut microflora, which can be detected by changes in QSM production, we employed a well-established animal model of colitis and monitored the QSM levels. Specifically, we used mice that were orally administered dextran sulfate sodium (DSS) for 7 days to induce colitis^{40,138}. DSS-induced murine colitis represents an experimental model for human IBD. A group of five mice were treated with DSS, while a group of 4 mice served as control. Serum and stool samples from all mice were collected at day 0 and day 7. Serum samples were analyzed by using the protocol developed in this work. Stool samples were analyzed by using a protocol that was previously developed in our lab²⁷. Figure 8 shows the comparison of long chain AHL levels between day 0 samples and day 7 samples in control and DSS treated mice. At day 0, before the

beginning of DSS treatment, the bioluminescence signals were nearly the same for most samples, without distinctions between the two groups of animals, as expected. At day 7, the control mice samples exhibited bioluminescence signals either at the same level or higher than those at day 0. On the other hand, four out of five DSS treated mice showed a marked decrease in bioluminescence signals. Further, it is important to note that a similar trend was observed in all animals, with the exception of mouse DSS4, when comparing the changes in AHL levels in stool and serum samples; for example, if the serum AHL levels increased between day 0 and day 7 in a certain mouse, the stool AHL levels also increased in that same mouse. These results further support the feasibility of analyzing serum samples for AHL molecules. Although a very limited number of mice were employed in this study, the data clearly suggested that, under the conditions of colitis, changes in the levels of AHLs occurred that might be related to changes in bacterial flora.

Conclusion

In conclusion, we successfully utilized whole-cell biosensing systems to detect AHL molecules in serum samples of IBD patients and animal models. The method was standardized using a pool of serum and 1:10 dilution allowed to overcome the serum matrix effect. Nanomolar limits of detection were obtained in water as well as in 1:10 diluted serum. Similar slopes of the dose-response curves obtained in water and 1:10 diluted serum, indicating that whole-cell biosensing systems can be utilized to detect AHLs in serum samples without loss of analytical performance. While we did not yet

analyze a substantial number of samples, doing so may enable us to confirm our observation that there is a difference between the levels of AHLs in control and IBD samples, and between the levels of AHLs in the two types of IBD conditions, UC and CD. As we analyze more serum samples, a relationship between the levels of AHLs in serum and the status of the disease may be established, thus pointing out at the potential of employing AHLs as biomarkers of IBD. Additionally, the ability to detect AHLs in serum may also serve in studies aimed at investigating the role and relationship between the levels of AHLs in serum and the concentration and activity of paraoxonase enzymes in individuals suffering from IBD, which have been suggested to have quorum-quenching activity¹³⁷. These studies should aid in understanding the role of bacteria and, in general, the microbiome in IBD.

Figure 1. Schematic of LasI/LasR and RhII/RhIR quorum sensing circuits in *P. aeruginosa*.

LasI catalyzes synthesis of 3-oxo-C-12 HSL (triangle), which accumulates in the environment. At a threshold level, 3-oxo-C-12 HSL binds to LasR; 3-oxo-C-12 HSL bound LasR activates transcription of several virulence genes along with the *rhIR* gene, which encodes for RhIR, thus initiating the RhII/RhIR quorum sensing circuit. RhII catalyzes synthesis of C-4 HSL (pentagon), which accumulates in the environment and, at a threshold level, binds to RhIR. C-4 HSL bound RhIR activates transcription of target genes including a subset of virulence genes. Adapted from Miller *et al.*¹².

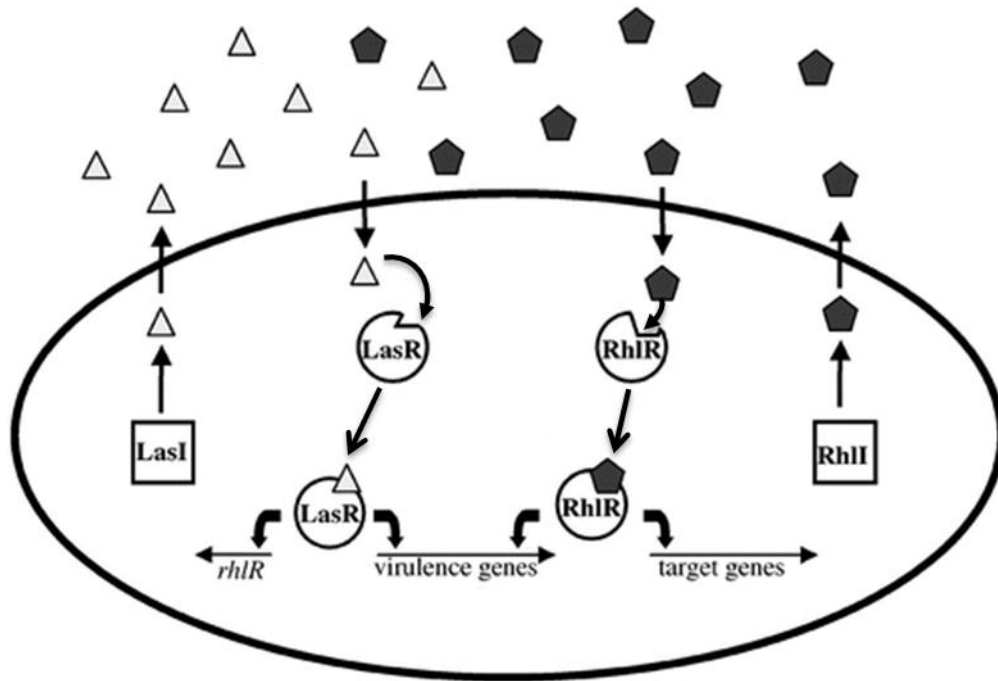
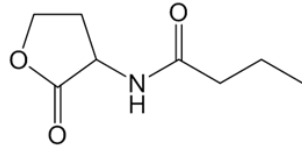


Figure 2. Quorum sensing molecules. (A) *N*-acyl-homoserine lactones. (B) Autoinducer-2.

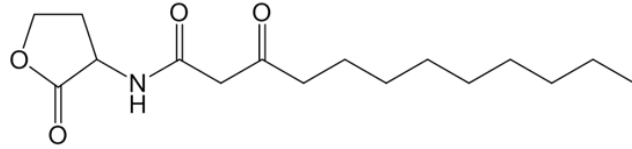
(C) Quinolone signaling molecule. (D) Autoinducing peptides (AIPs).

(A)



N-butyryl-homoserine lactone

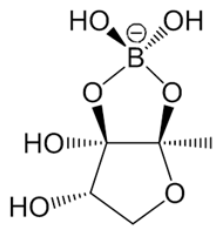
Pseudomonas aeruginosa



N-(3-oxododecanoyl)homoserine lactone

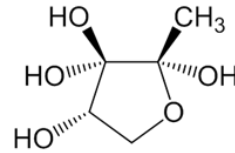
Pseudomonas aeruginosa

(B)



(2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate

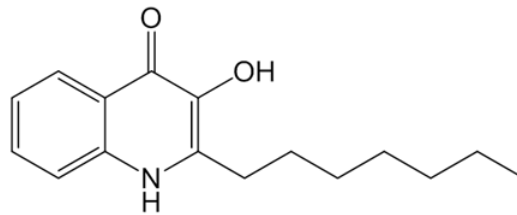
Vibrio harveyi



(2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran

Salmonella typhimurium

(C)



2-heptyl-3-hydroxy-4(1*H*)-quinolone

Pseudomonas aeruginosa

Figure 3. Undiluted and diluted serum samples were incubated with whole-cell biosensing system containing plasmid pSB406 (yellow). Undiluted and diluted serum samples were spiked with 1×10^{-7} M C-6 HSL and incubated with whole-cell biosensing system containing plasmid pSB406 (green). Blank 1 contains RO filtered water. Blank 2 contains RO filtered water spiked with 1×10^{-7} M C-6 HSL.

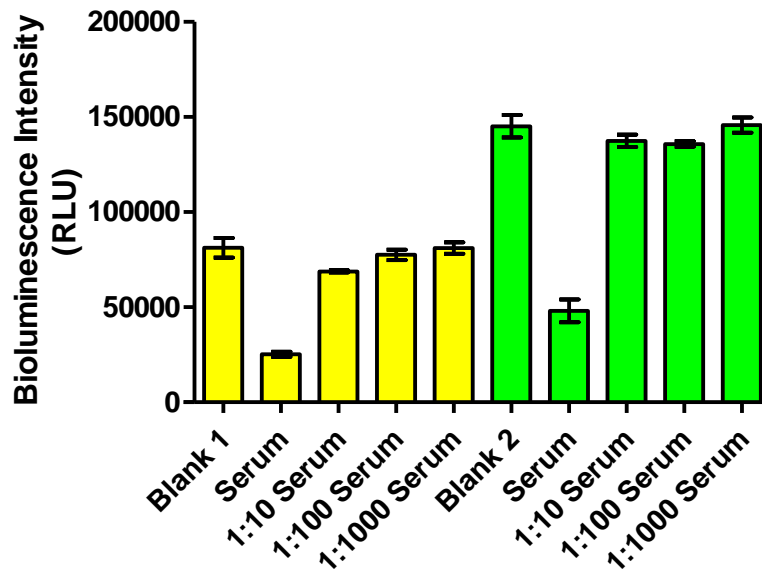
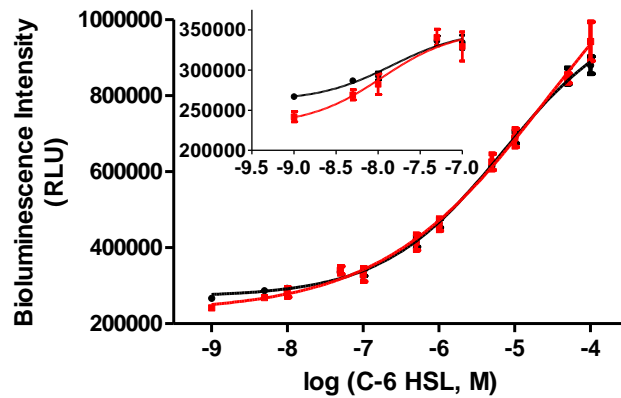


Figure 4. Comparison of dose-response curves in clinical samples with standard dose-response curve. (A) Using solutions of C-6 HSL, a dose-response curve was obtained in pooled serum (red) and compared with that obtained in water (black) in the same analytical run. C-6 HSL solutions were incubated with whole-cell biosensing system containing plasmid pSB406. (B) Using solutions of AI-2, a dose-response curve was obtained in pooled serum (red) and compared with that obtained in water (black) in the same analytical run. AI-2 solutions were incubated with *V. harveyi* MM32-based whole-cell biosensing system.

(A)



(B)

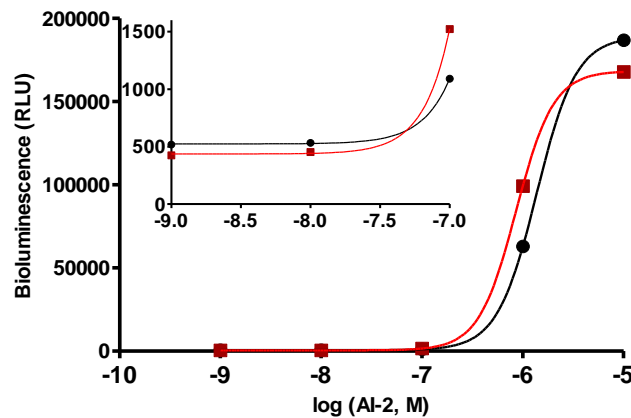


Figure 5. Analysis of serum samples from IBD patients and controls. Yellow – Serum samples obtained from healthy volunteers. Red – Serum samples obtained from UC patients. Green – Serum samples obtained from CD patients. Short chain AHLs were detected using the whole-cell sensing system containing plasmid pSB406. The reported signal values are blank-subtracted.

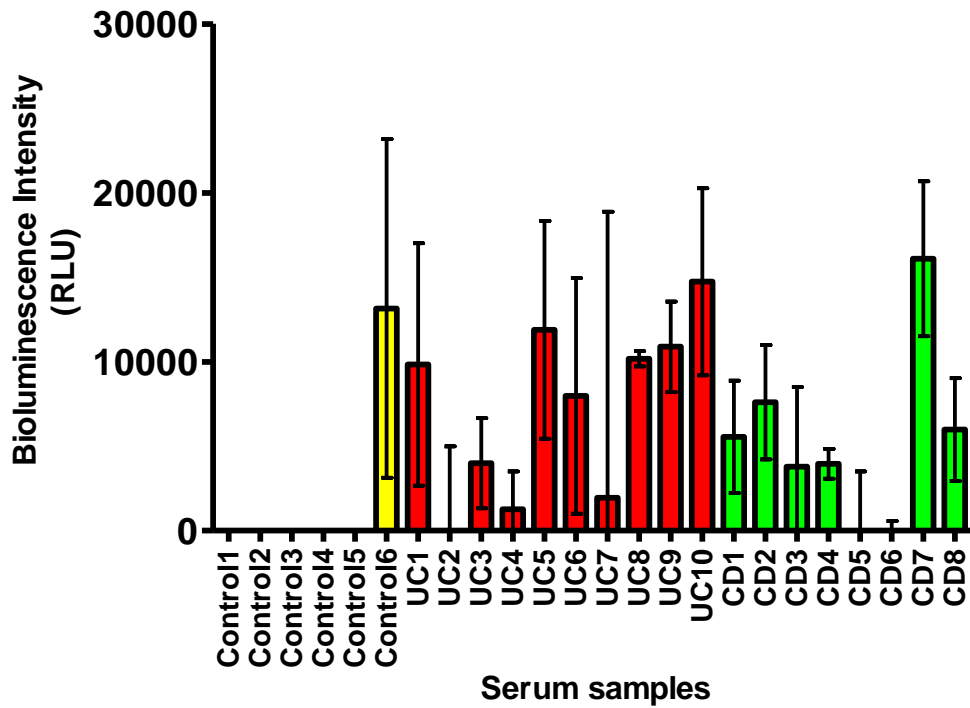


Figure 6. LC-MS-MS analysis of serum. (A) A standard mixture of AHLs (1 ng/mL each). (B) Mobile phase alone. (C) Pooled serum was extracted using acetonitrile/methylene chloride followed by reconstituting in the mobile phase.

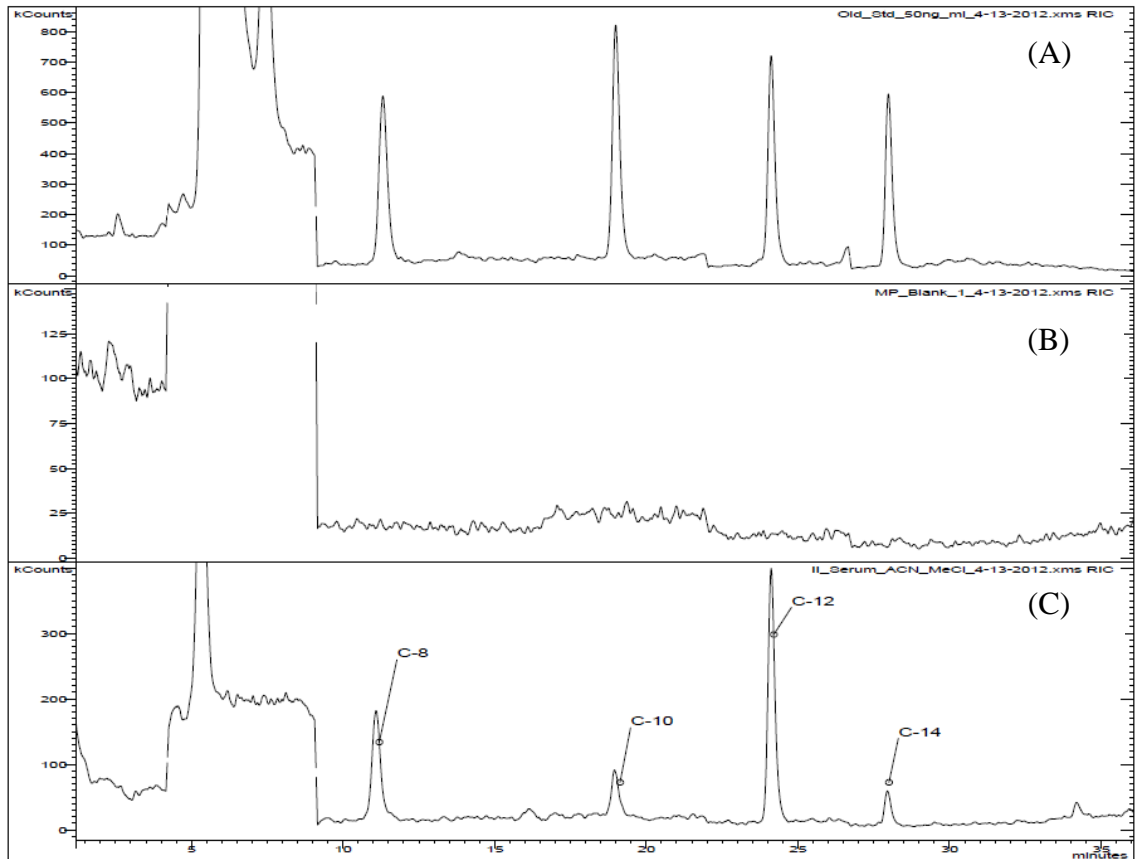


Figure 7. Stability of C-6 HSL in serum stored under various conditions. Serum was spiked with (1) 1×10^{-6} M C-6 HSL (yellow) and (2) 1×10^{-7} M C-6 HSL (blue). Control 1 is serum spiked with 1×10^{-6} M C-6 HSL and control 2 is serum spiked with 1×10^{-7} M C-6 HSL.

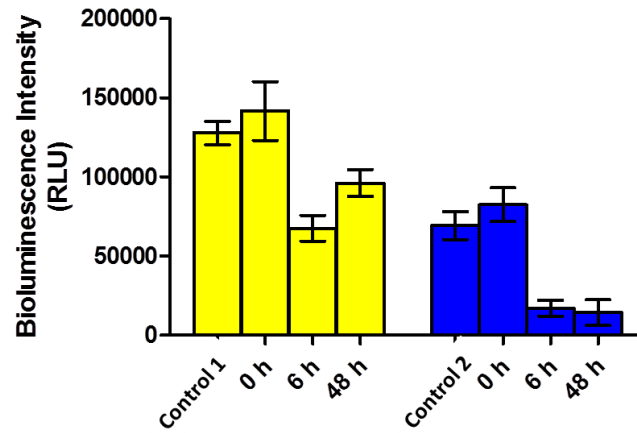
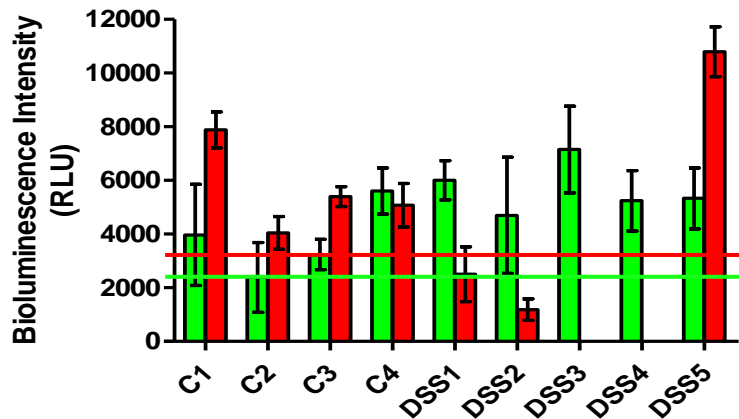


Figure 8. Mice treatment with colitis inducing agent dextran sulphate sodium (DSS). (A) Comparison of long chain AHL levels in serum samples before DSS treatment (green) and after a 7 day DSS treatment (red). The plotted values are blank-subtracted. Horizontal lines indicate the cutoff for each set of values. The cutoff is determined as the signal corresponding to 3 standard deviations of the blank. (B) Comparison of long chain AHL levels in stool samples before DSS treatment (green) and after a 7 day DSS treatment (red). The plotted values are blank-subtracted. Horizontal lines indicate the cutoff for each set of values. The cutoff is determined as the signal corresponding to 3 standard deviations of the blank.

(A)



(B)

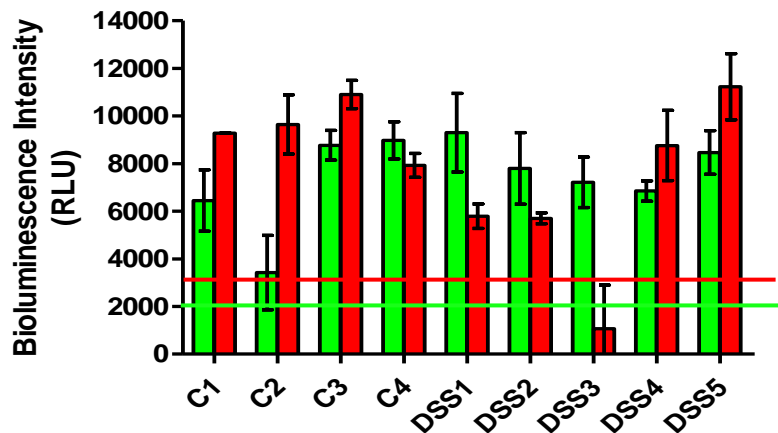


Table 1. LC-tandem MS detection of AHLs

AHLs	[M + H-101]⁺ (m/z)	[M – H₂O]⁺ (m/z)
C-7 HSL	113	196
3-oxo-C-8 HSL	141	224
C-8 HSL	127	210
C-10 HSL	155	238
C-12 HSL	183	266
C-14 HSL	211	294

CHAPTER FOUR

DETECTION OF BACTERIAL QUORUM SENSING MOLECULES IN FOOD MATRICES

Introduction

Foodborne illnesses are a major health issue affecting over 15% of the US population and resulting in 3000 deaths each year¹³⁹. Most of these diseases are due to infections caused by viruses, bacteria, and parasites, which enter into the gastrointestinal tract by consumption of contaminated food. While diseases caused by pathogens, such as *Yersinia*, *Escherichia coli* O157:H7, and *Listeria* have decreased over the past few years, those caused by *Salmonella* and *Vibrio* have been on the rise¹⁴⁰. None of these bacterial-related foodborne illnesses have been eradicated, and rather, multistate foodborne outbreaks have increased consistently over the past several years¹⁰.

Food contamination can occur at any stage, from farms and slaughter houses to distribution at restaurants and shopping centers. The list of edibles that are known to carry bacterial contamination includes milk and milk products (such as cheese, yogurt, and ice cream), meat products (such as beef, pork, chicken, and turkey), vegetables (such as broccoli, lettuce, tomato, and sprouts), eggs, and sea products. In the past, the time lag between food production and consumption was relatively short, and therefore, chances of food contamination were limited. Current market demands that food be stored for longer durations of time. To limit bacterial growth, a number of food preservation techniques have emerged. For example, milk is pasteurized by heating to

high temperatures for short periods of time, and vegetables and meat are packaged under a variety of controlled conditions.

Initial processing by heat treatments reduces the initial bacterial content considerably, and the recontamination thereafter determines shelf-life. The most commonly employed approach to improve shelf-life of foods involves focusing on packaging techniques that extend the viability of the food. Various methods are employed that utilize air, vacuum, or modified atmosphere packaging of food. A method is chosen depending on the food product and market requirements. For example, packaging with high oxygen content helps preserve the color of retail cut meat; however, shelf-life is only slightly improved¹⁴¹. On the contrary, storage of meat products in 100% carbon dioxide ensures maximum shelf life, but can cause discoloration¹⁴². Furthermore, different packaging methods differently affect the growth of various species of bacteria. In aerobic packaging a variety of bacteria can grow, including *Pseudomonas* spp., *Brochothrix thermosphacta*, *Lactobacillus* spp., and many *Enterobacteriaceae*, while in vacuum and anaerobic packaging a few bacteria can survive, the most common being *Lactobacillus* spp. Other factors impacting the growth of bacteria include temperature and pH. Many foods are amenable to storage at low temperatures, which, in most cases, extends the shelf-life by decreasing the rate of bacterial growth. Temperature studies involving Jalapeño peppers¹⁴³, shell eggs¹⁴⁴, pork¹⁴⁵, and minced beef¹⁴⁶ have shown that the bacterial count was lower when food was stored at low temperature as compared to when it was stored at higher temperatures. Moreover, while a decrease in pH may favorably reduce the growth of

certain bacteria, that is not the case for the *Lactobacillus* spp. , which produce lactic acid, and thus, contribute to a reduction of the pH¹⁴⁷.

The economic and health consequences, caused by food spoilage, have fueled efforts to detect early food spoilage and contamination. The bacterial count is one of the indicators that food may either be spoiled or prone to spoilage. The number of bacteria is often counted by determining the colony forming units (cfu) per gram of food under consideration; when the cfu value from a food sample is higher than that of standard/unspoiled food, it is indicative of potential food spoilage. The determination of cfu has been used in a number of applications, ranging from evaluation of food spoilage to assessment of the effectiveness of an agent for prevention of food spoilage^{148,149}. However, cfu determination cannot be considered accurate as it neglects the bacterial population that cannot grow under the conditions of the cfu assay. Hence, other ways to count bacteria are employed, which include epifluorescence, an ATP bioluminescence assay, impedance measurements, and spectroscopic methods. When using epifluorescence, bacteria present in the food sample are concentrated on a filtration membrane, stained with a fluorescent dye, and enumerated using epifluorescence microscopy^{150,151}. Indirect measurement of bacterial load in the sample can be obtained by measuring ATP concentration. Highly sensitive ATP determination is achieved using firefly luciferase, which in the presence of adenosine 5'-triphosphate (ATP), converts the substrate luciferin to oxyluciferin emitting a photon of light. Non-microbial ATP present in food is first removed either by enzymatic degradation¹⁵² or by filtration¹⁵³, and microbial ATP is then detected.

The bacterial load can also be estimated by measuring the change in current in the microbiological media as bacteria grow, consume nutrients, and release such highly charged molecules as fatty acids, amino acids, and organic acids. Impedance measurements have been used to measure bacteria in raw milk¹⁵⁴, frozen vegetables¹⁵⁵, meat¹⁵⁶, and fish¹⁵⁷. Bacteria also release a range of volatile compounds during their growth that are different depending on the bacterial species. Thus, the volatile compounds can be considered a fingerprint of the type of bacteria present in a sample; by using an array of sensors responsive to various volatile compounds, Blixt *et al.* evaluated the degree of spoilage of vacuum packaged beef¹⁵⁸. Methods such as Fourier transform infrared (FT-IR) and short-wavelength-near-infrared (SW-NIR) spectroscopy have been used to discriminate among pathogens¹⁵⁹ and to detect overall food spoilage¹⁶⁰, respectively. Furthermore, molecular methods, such as the polymerase chain reaction (PCR)¹⁶¹ and real-time PCR¹⁶², have been employed to detect food spoilage caused by specific microorganisms.

Bacteria are known to communicate with one another by producing, releasing, and responding to small signaling molecules¹⁴. When these molecules reach a critical threshold concentration corresponding to a given cell density, certain specialized genes are expressed. This type of cell-to-cell communication, termed quorum sensing (QS), enables bacteria to regulate specialized phenotypes, including virulence factors production and biofilm formation, depending on their population size. Among the quorum sensing molecules (QSMs) employed for bacterial chatter are *N*-acyl homoserine lactones in Gram-negative bacteria, autoinducing peptides (AIPs) in Gram-

positive bacteria, and autoinducer-2 (AI-2) in both Gram-negative and Gram-positive bacteria.

Bacteria are involved in the spoilage of food by producing food degrading enzymes, whose synthesis and release are regulated by the QS circuitry in many bacteria. Hence, bacterial QS might play a crucial role in food spoilage. Another significant QS regulated function is biofilm formation, which enables bacteria to propagate and establish themselves on the contaminated surfaces by generating complex three-dimensional structures where bacteria are included and protected from other bacteria and antibiotics¹⁶³. A biofilm is defined as “microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription”¹⁶⁴. In the biofilm mature state, the extracellular matrix is composed of exopolysaccharides, proteins, dead bacteria, and DNAs. Biofilms can be comprised of single bacterial species; however, mixed species biofilms dominate under most environmental conditions^{165,166}. Some features of biofilms include primitive homeostasis, metabolic cooperation, exchange of genetic material, and circulatory systems to facilitate waste disposal. Such biofilms can be present on the surface of foods as well as on food processing surfaces and equipment. Examples of food-related biofilm-forming bacteria include *Bacillus* spp. from dairy processing plants¹⁶⁷ and *Salmonella* from poultry processing plants¹⁶⁸, among others. In few foodborne pathogens, biofilm formation has been linked to quorum sensing. Specifically, wild type

Hafnia alvei, a milk and meat pathogen that employs the AHL-based QS signaling for communication, regularly forms biofilms, while a mutant from the same bacterial species *Hafnia alvei* that is unable to synthesize AHLs, cannot form biofilms¹⁶⁹.

In light of the evident important role that QS plays in food contamination and spoilage, we deemed necessary to further explore the relationship between food spoilage and QS. Methods that can detect QSMs in food samples should allow for the early detection and prevention of food contamination. Conventional physical-chemical methods, such as separation techniques coupled to various detection principles, are cumbersome, time-consuming, and often not sufficiently sensitive to detect QSMs at low concentrations. In addition, most of these techniques require extensive sample preparation, expensive instrumentation, and specialized technical personnel¹⁷⁰. On the other hand, whole-cell biosensing systems are sensitive, with limits of detection in the micromolar to nanomolar ranges²⁷, rapid, easy to use, cost-effective, and require simple instrumentation and minimal or no sample preparation. Furthermore, they are amenable to multiplexing and high-throughput analysis, as well as on-site monitoring when incorporated into portable devices.

To that end, we utilized cell-based biosensing systems to develop an analytical method for the quantitative detection of QSMs in food matrices such as milk and ground beef. Specifically, we employed *Vibrio harveyi* MM32-based whole-cell biosensing system for the detection of AI-2, and two *E. coli*-based whole-cell biosensing systems, one containing plasmid pSB406 and the other containing plasmid pSB1075, for

the detection of short chain AHLs and long chain AHLs, respectively. Most food products are either in liquid or solid form. We chose milk as an example of a liquid food, and ground beef as an example of a solid food. Initially, the whole-cell biosensing systems were optimized for use in these food matrices. The effect of the sample matrix on the performance of the sensing system's response was evaluated by performing recovery studies. In addition, the limit of detection, dynamic range, and reproducibility afforded by the sensing system in the two food samples were determined. In order to simulate food spoilage conditions, we employed food contaminating bacteria that naturally produce QSMs. For this purpose, we contaminated milk and ground beef samples with *Escherichia coli* AB1157 strain and *Hafnia alvei* 718 strain. The former communicates through and produces AI-2, while the latter employs the short chain AHL regulatory system. To demonstrate the validity of our proposed method in the detection of bacterial contamination, we then employed our cell-based biosensing systems in the quantitative determination of both types of quorum sensing molecules in milk and ground beef.

EXPERIMENTAL SECTION

Materials. AHLs, *N*-hexanoyl-DL-homoserine lactone (C-6 HSL) and *N*-dodecanoyl-DL-homoserine lactone (C-12 HSL), ampicillin, and kanamycin were purchased from Sigma (St. Louis, MO). AI-2 was purchased from Omm Scientific (Dallas, TX). Luria Bertani (LB) broth, nutrient broth, and vitamin free casamino acids were purchased from Difco (Sparks, MD). Acetonitrile used in all experiments was of HPLC grade and was from VWR Scientific (Pittsburgh, PA). The 96-well microtiter plates were purchased from Costar (Corning, NY). Lean ground beef (93% lean), fat free milk (skim milk), 2% fat milk, and whole milk were purchased from a local grocery store. Reverse osmosis (RO) filtered water (Milli-Q water purification system, Millipore, Bedford, MA) was utilized in all experiments as needed.

The microcentrifuge was purchased from Eppendorf (Westbury, NY). The orbital shaker incubator was purchased from Fisher Scientific (Fair Lawn, NJ). Bioluminescent measurements were performed using the FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC). The Spectronic 21D spectrophotometer used to measure optical density of the bacterial cultures was purchased from Artisan Scientific (Champaign, IL).

Plasmids, bacterial strains, and culture conditions. The plasmids pSB406 and pSB1075 were originally provided by Dr. Paul Williams (University of Nottingham, Nottingham, UK) and previously transformed in *E. coli* JM109 cells. The transformed AHL-sensing cells were then stored at -80 °C as glycerol stocks. Fresh cell cultures were obtained from the glycerol stocks, grown in LB media (100 µg/mL ampicillin) overnight, in the orbital shaker at 37 °C, 250 rpm, and then refreshed and allowed to grow until an optical

density at 600 nm (OD_{600nm}) of 0.45-0.50 was reached. *V. harveyi* MM32 (AI-2-sensing strain), *E. coli* AB1157 (AI-2-producing strain), and *H. alvei* 718 (AHL-producing strain) were purchased from American Type Culture Collection (Manassas, VA). Cultures of *V. harveyi* MM32 cells were setup in autoinducer bioassay (AB) media containing 30 $\mu\text{g}/\text{mL}$ kanamycin and grown overnight in the orbital shaker at 30 °C, 250 rpm. Overnight cultures were then diluted 1:100 to obtain an OD_{600nm} of 0.01-0.02. The procedure to prepare AB media has been described elsewhere¹²⁹. *E. coli* AB1157 cells were grown in LB media (no antibiotic) at 37 °C, 250 rpm, while *H. alvei* 718 cells were grown in nutrient media (no antibiotic) at 30 °C, 250 rpm, in the orbital shaker.

Dose-response curves. Commercially available *N*-acyl-homoserine lactones were dissolved in acetonitrile to obtain 1×10^{-2} M stock solutions, which were serially diluted with RO filtered water to obtain standard solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. A 1% solution of acetonitrile in RO filtered water was used as blank (upon addition to the bacterial culture, the acetonitrile final concentration of 0.1% did not result toxic for the sensing cells). A volume of 10 μL of each of these solutions was added in triplicate to a 96-well white polystyrene microtiter plate containing 90 $\mu\text{L}/\text{well}$ of cell culture grown to an OD_{600nm} of 0.45-0.50. C-6 HSL solutions were employed for the whole-cell sensor with pSB406, while C-12 HSL solutions were employed for the whole-cell sensor with pSB1075. The microtiter plate was then incubated in the orbital shaker at 37 °C, 175 rpm for two hours. The produced bioluminescence was then measured using the microplate reader. The light intensity was expressed in relative light units (RLU).

Al-2 aqueous stock solution (3.7×10^{-3} M) was serially diluted with RO filtered water to prepare Al-2 standard solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. RO filtered water was used as blank. A volume of 10 μ L of each of these standard solutions and blank was added in triplicate to a 96-well black polystyrene microtiter plate containing 90 μ L/well of *V. harveyi* MM32 cell culture at an OD₆₀₀ nm of 0.01-0.02. The microtiter plate was then incubated in the orbital shaker at 30 °C, 175 rpm for three hours. The produced bioluminescence was then measured using the microplate reader. The light intensity was expressed in relative light units (RLU).

Food sample collection and processing. Food samples used in this study were lean ground beef (93% lean), fat free milk, milk with 2% fat, and whole milk. They were purchased from a local grocery store. The ground beef sample was further ground, with slow addition of water, using a high performance blender to form a uniform suspension. The volume of water was then adjusted to prepare a 10% w/v suspension, which was stored as 20 mL aliquots at -80 °C. When needed, beef suspension aliquots were thawed at room temperature and diluted using RO filtered water. For simplicity, 10% w/v beef suspension is referred to as beef suspension in the rest of the manuscript. All milk samples were stored at -80 °C in 15 mL aliquots. When needed, milk aliquots were thawed at room temperature and diluted using RO filtered water.

Food sample dilution study. To test for potential matrix effect of the food samples on the whole-cell sensing systems response, an aliquot of 10% w/v beef suspension was serially diluted with RO filtered water to obtain 1:10, 1:100, and 1:1000 dilutions. At

each dilution step, proper care was given to vortex the suspensions thoroughly to avoid settling of meat particles. Similarly, a fat free milk aliquot was serially diluted with RO filtered water to obtain 1:10, 1:100, and 1:1000 dilutions. To test the beef and skim milk samples with the *E. coli* whole-cell biosensing systems containing plasmids pSB406 or pSB1075, a 10 μ L volume of the above beef suspensions and milk solutions was added in triplicate to a microtiter plate followed by addition of 90 μ L/well of the sensing bacterial cells grown to an OD_{600nm} of 0.45-0.50. Similarly, to test the beef and skim milk samples with the *V. harveyi* MM32 whole-cell biosensing system, a 10 μ L volume of the above beef suspensions and milk solutions was added in triplicate to a microtiter plate followed by addition of 90 μ L/well of the sensing bacterial cells at an OD_{600nm} of 0.01-0.02. A reference dose-response curve with the respective QSM was included in each analytical run. The assays were performed as described above for standard dose-response curves.

Evaluation of food matrix effects in the presence of QSMs. Beef suspensions and skim milk solutions at various dilutions, prepared as described above, were spiked with a fixed concentration of QSM, namely 1×10^{-6} M C-6 HSL, 1×10^{-6} M C-12 HSL, or 1×10^{-5} M AI-2. A 10 μ L volume of each of these spiked samples was added in triplicate to a microtiter plate followed by addition of 90 μ L/well of the corresponding sensing cell suspension. The assays were then performed as described above.

Dose-response curves in sample matrix. Acetonitrile 1×10^{-2} M stock solutions of C-6 HSL and C-12 HSL were serially diluted with 1:10 beef suspension or undiluted skim milk

to obtain AHL solutions of concentrations ranging from 1×10^{-4} to 1×10^{-9} M. A 1% acetonitrile solution in each of the above food sample dilution was used as blank. Similarly, 3.7×10^{-3} M aqueous stock solution of AI-2 was serially diluted with 1:10 beef suspension or undiluted skim milk to obtain AI-2 solutions of concentrations ranging from 1×10^{-4} to 1×10^{-9} M. 1:10 beef suspension and undiluted skim milk served as blanks. A 10 μ L volume of each of the obtained solutions and blanks was added in triplicate to a microtiter plate followed by addition of 90 μ L/well of cell culture of the respective biosensing system. The assays were performed as described above. A reference dose-response curve was included in each analytical run.

Monitoring of QSM production by *E. coli* AB1157 and *H. alvei* 718. *E. coli* AB1157 was grown in LB media (no antibiotic) overnight, in the incubator shaker at 37 °C, 250 rpm. *H. alvei* 718 was grown in nutrient media (no antibiotic) overnight, in the incubator shaker at 30 °C, 250 rpm. Overnight cultures were diluted 1:100 and 1:15, respectively, with fresh media and allowed to grow in the above conditions. One-milliliter fractions of culture media were collected at each hour for 8 hours, followed by collection of an overnight fraction. The collected fractions were centrifuged at 13,000 rpm for 5 minutes at room temperature and the supernatants were stored at -20 °C until analyzed. To measure the QSMs produced, 10 μ L of each supernatant sample was added in triplicate to the wells of a microtiter plate followed by addition of 90 μ L of the proper sensing cell suspension, prepared as described above. For *E. coli* AB1157 culture media fractions, *V. harveyi* MM32 sensing cells were used, while for *H. alvei* 718 culture media fractions,

the whole-cell sensing systems containing pSB406 and pSB1075 were employed. The assays were performed as described above.

Food contamination study. To investigate QSM production in food matrices, we contaminated beef and skim milk with AHL producing *H. alvei* 718 and AI-2 producing *E. coli* AB1157, respectively. An overnight grown culture of *E. coli* AB1157 was added into 10% w/v beef suspension to obtain a 1:100 dilution of the original culture and allowed to grow in the incubator shaker at 37 °C, 250 rpm. Similarly, an overnight grown culture of *H. alvei* 718 was added into 10% w/v beef suspension to obtain a 1:15 dilution of the original culture and allowed to grow in the incubator shaker at 30 °C, 250 rpm. Procedures similar to those used for contaminating beef suspension were employed to contaminate skim milk. One-milliliter fractions of media from each of the contaminated food samples were collected at each hour for 8 hours, followed by collection of an overnight fraction. The collected fractions were centrifuged at 13,000 rpm for 5 minutes at room temperature and the supernatants were stored at -20 °C until analyzed. To measure the QSMs produced, the supernatant samples were assayed using the respective whole-cell biosensing systems as described above.

Results and discussion

The purpose of our work was to demonstrate the feasibility of employing whole-cell biosensing systems to detect bacterial presence as a measure of spoilage and contamination of foods. Three different specific biosensing systems were employed in the determination of two different classes of quorum sensing molecules, AI-2 and AHLs (long and short chain), in ground beef and milk. The AI-2 quorum sensing regulatory system is used by both Gram-negative and Gram-positive bacteria, while AHL-based communication is employed by Gram-negative bacteria. The role of QS in food spoilage was validated by identification of QSMs in spoiled foods^{171,172}. For example, bacterial presence and AHL molecules were detected in vacuum packed meat samples¹⁷¹. In addition, AI-2 has been identified in food samples such as fish, tomato, carrots, tofu, and milk¹⁷². The methods employed in both of the above studies were qualitative and time consuming due to a need for processing steps and the fact that detection relied on color development by reporter cells upon exposure to AHLs¹⁷¹. To the best of our knowledge, this is the first application of cell-based biosensing systems in the quantitative detection of QSMs in food samples aimed at aiding in the early detection of food contamination and prevention of food spoilage.

In this work, we employed *E. coli* whole-cell biosensing systems containing plasmids pSB406 and pSB1075 to detect short and long chain AHLs, respectively. For AI-2 detection, we used a whole-cell biosensing system that is based on *V. harveyi* MM32. The plasmids pSB406 and pSB1075 bear recognition and regulatory elements that are

originally derived from *Pseudomonas aeruginosa* AHL-dependent RhIR/RhII and LasR/LasI QS systems²³. Plasmids pSB406 and pSB1075 carry the promoters P_{rhII} and P_{lasI} , respectively, as well as the genes *rhIR* and *lasR*, encoding for the recognition/regulatory proteins RhIR and LasR, respectively. In addition, both plasmids contain the *luxCDABE* cassette that is under transcriptional control of the respective promoters. The *luxCDABE* genes encode for bioluminescent bacterial luciferase and the enzymes catalyzing the synthesis of the luciferase substrate (Figure 1a). The plasmids are lacking the *rhII* and *lasI* genes that code for the AHL synthase enzymes, thus, exogenous AHLs need to be supplied in order for the sensing cells to produce bioluminescence. When AHLs are present in the environment of the sensing cells, they bind to the recognition/regulatory proteins, triggering the expression of luciferase and other enzymes part of the *luxCDABE* cassette. This results in the production of bioluminescence in a manner proportional to the concentration of AHLs present. The whole-cell biosensing systems based on plasmid pSB406 and pSB1075 had been previously characterized in our laboratory and employed to detect AHLs in saliva and stool samples²⁷. *V. harveyi* MM32 is a mutant of wild-type *V. harveyi* BB120, a marine bioluminescent bacterium that controls light emission through multiple QS systems. Strain MM32 was genetically modified to only emit bioluminescence in response to AI-2 and not to produce its own AI-2¹⁵; therefore, light emission is only triggered by AI-2 present in the environment of the cells. When AI-2 binds to its recognition element, the periplasmic binding protein LuxP, the binding event triggers a cascade of phosphorylation and de-phosphorylation processes in a number of proteins that control the expression of *luxCDABE*, responsible for bioluminescence,

along with other genes (Figure 1b). Thus far, *V. harveyi* MM32 has been used as a reporter strain in bioassays for a number of applications – to evaluate QS regulated functions¹⁷³, to screen compounds for agonistic and antagonistic activities⁵⁷, and to identify bacteria that produce AI-2¹⁷⁴. Herein, we employed *V. harveyi* MM32 to develop a whole-cell biosensing system for quantitative detection of AI-2. Due to the high sensitivity and selectivity demonstrated by the above whole-cell biosensing systems for AHL and AI-2 detection, we utilized them as sensitive and rapid tools for the analysis of QSMs in various food matrices. The levels of QSMs should correlate with the extent of bacterial contamination and serve as indicators of food spoilage.

In our study, milk and ground beef were chosen as food representative models to investigate whether whole-cell biosensing systems could be used with liquid as well as solid foods. Initially, the analytical parameters of the biosensing systems were determined using standard solutions of QSMs in RO water (Table 1). The limits of detection for each analyte were found to be 1×10^{-9} M. In addition, dynamic ranges of at least three orders of magnitude were obtained with all biosensing systems. At least three sets of experiments were performed to verify reproducibility, both with standard solutions and in food matrix.

Next, we evaluated the effect exerted by the food sample matrices on the whole-cell biosensing systems response. Being solid, ground beef was mixed with RO filtered water and using a grinder, a 10% w/v suspension (referred to as beef suspension) was prepared. As can be seen from Figure 2a-2c, when beef suspension was

incubated with whole-cell biosensing systems, bioluminescence signals lower than those of the controls were observed. Such decrease was probably due to components of the sample matrix diminishing the ability of the system to emit bioluminescence. The matrix effect was eliminated when beef suspension was diluted 1:10, 1:100, and 1:1000. At these dilutions, bioluminescence signals were similar to those of the controls, confirming that dilution was necessary and that a 1:10 dilution was sufficient to eliminate the matrix effect. Recovery studies were performed by spiking the beef suspension with a known concentration of a QSM standard solution. A complete signal recovery was observed when employing a 1:10 dilution of beef suspension (Figure 3a-3c).

To evaluate the performance of the biosensing systems in the beef matrix at various concentrations of QSMs, dose-response curves using a 1:10 dilution of the beef suspension were obtained and compared to the dose-response curves obtained in water during the same analytical run (Figure 4). The slopes of the dose-response curves obtained in the 1:10 beef suspension were nearly identical to those of the reference dose-response curves for all three sensing systems. Therefore, we concluded that short chain AHLs in the range of 1×10^{-5} M to 1×10^{-9} M, long chain AHLs in the range of 1×10^{-6} M to 1×10^{-9} M, and AI-2 in the range of 1×10^{-5} M to 1×10^{-9} M can be detected in 1:10 beef suspension using our whole-cell biosensing systems. The results obtained prove that these biosensing systems could be employed to detect QSMs in solid foods, such as beef, with minimal sample processing.

Similar studies were carried out to investigate the effect of matrix on QSM detection in skim milk. When undiluted skim milk was incubated with the whole-cell biosensing systems, we observed an emission of bioluminescence signal similar to that of the controls. Further, dilutions of skim milk did not alter the bioluminescence signals (Figure 5). However, when milk was spiked with fixed concentrations of QSMs, followed by incubation with the respective sensing cells, increases in bioluminescence were observed in spiked milk samples as compared to the controls; the controls consisted of aqueous solutions with the same fixed QSM concentrations (data not shown). Dose-response curves obtained in skim milk also showed higher bioluminescence emission than those obtained with reference dose-response curves in the same analytical run (Figure 6). The observed increase in bioluminescence could be due to the presence of very small amounts of fat (<0.5%) and slight acidity of the skim milk, which might increase the solubilization and stability of the QSMs. Fatty acids present in the milk may help better solubilize AHLs, thus enhancing their bioavailability to the sensing cells. This effect would be more pronounced as the hydrophobicity of AHLs increases. This conforms to our observation that a larger increase in bioluminescence was observed with long chain AHLs than with short chain AHLs.

To further evaluate the effect of fat content in milk, we spiked and analyzed two additional types of milk (whole milk, 2% fat milk), along with skim milk, which differ in fat content. We observed that as the fat percentage increased, bioluminescence also increased, thus supporting improved AHL solubility and bioavailability in the presence of

fat. As the pH effect is concerned, lower pH values are known to enhance the stability of AHLs, by reducing lactonolysis¹⁷⁵.

To further prove that our whole-cell biosensing systems can be employed in food analysis, the next step of the study was to detect QSMs in simulated spoiled food samples that were subjected to bacterial contamination. We chose *E. coli* AB1157 and *H. alvei* 718 as model organisms, given that they produce AI-2 and AHLs, respectively, to artificially contaminate skim milk and beef suspension. First, we characterized the two bacterial strains in order to find out the amount of time required for maximum production of QSMs. When grown in LB media at 37 °C, *E. coli* AB1157 produces maximum concentration of AI-2s in six hours, while maximum production of AHLs in *H. alvei* 718 was observed after five hours growth in nutrient media at 30 °C.

To perform spoilage studies, overnight separate cultures of *E. coli* AB1157 and *H. alvei* 718 were added into a beef suspension and skim milk, respectively. A 1 mL-volume sample was collected hourly, centrifuged to remove debris, and the supernatant analyzed for the presence of QSMs using the whole-cell biosensing systems. The results obtained with beef suspension contaminated with *H. alvei* 718 indicating the ability of the employed sensing system to detect the produced short chain AHLs are shown in Figure 7a. Only minor production of long chain AHLs was observed, which is consistent with the fact that *H. alvei* produces and uses short chain AHLs as QSMs¹⁷¹. Similarly, when the beef was contaminated with AI-2 producing *E. coli* AB1157, our *V. harveyi* MM32 whole-cell biosensing system was able to detect the AI-2 produced in beef

(Figure 7b). The same trend was observed when skim milk was contaminated with *E. coli* AB1157 and *H. alvei* 718, respectively. In sum, these data demonstrate that the whole-cell biosensing systems can be successfully utilized to detect different QSMs in various contaminated food matrices.

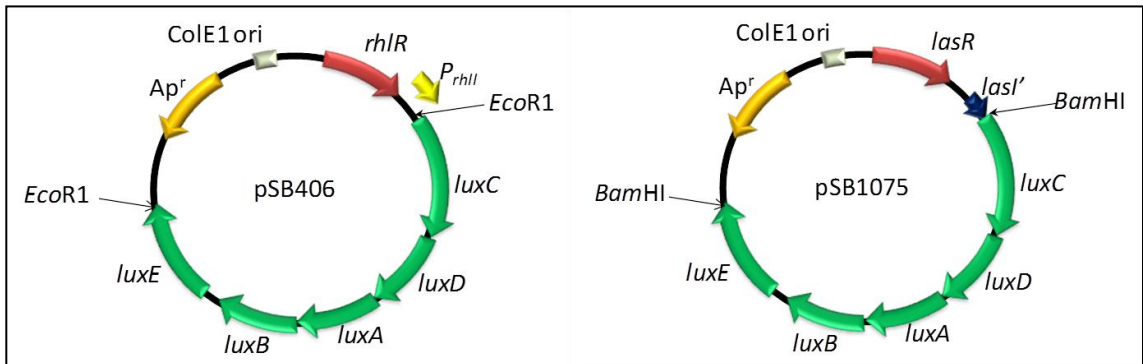
Conclusion

The incidences of food spoilage and foodborne illnesses have been on the rise, with more multistate outbreaks reported each year. There are numerous bacteria responsible for the outbreaks and foods that spread the illnesses, thus making the source of contamination difficult to identify. However, a feature common to many bacteria, including foodborne pathogens, is the production of QSMs. Herein, we investigated the feasibility of employing QSMs as quantitative markers of bacterial presence in foods. To that end, we have developed an analytical method to detect QSMs for early detection of food contamination and prevention of food spoilage in two different food matrices, namely beef and milk. The proposed method is based on the use of whole-cell biosensing systems that are sensitive, easy to use, rapid cost-effective, and amenable to miniaturization, thus exhibiting potential for high throughput and on-site analysis. The optimized method allowed for detection of QSMs in a sensitive manner with limits of detection down to nanomolar levels. Additionally, food spoilage studies proved that our whole-cell biosensing systems can be successfully employed to

detect QSMs in spoiled food, thus suggesting potential usefulness in the early detection of food spoilage and prevention of foodborne illnesses.

Figure 1. (A) Plasmids pSB406 and pSB1075 contained in *E. coli*-based whole-cell sensing systems for detection of short and long chain AHLs, respectively. (B) *V. harveyi* AI-2-based quorum sensing regulatory pathway. *V. harveyi* MM32 lacks LuxS, thus not producing its own AI-2 and only responding to exogenous AI-2.

(A)



(B)

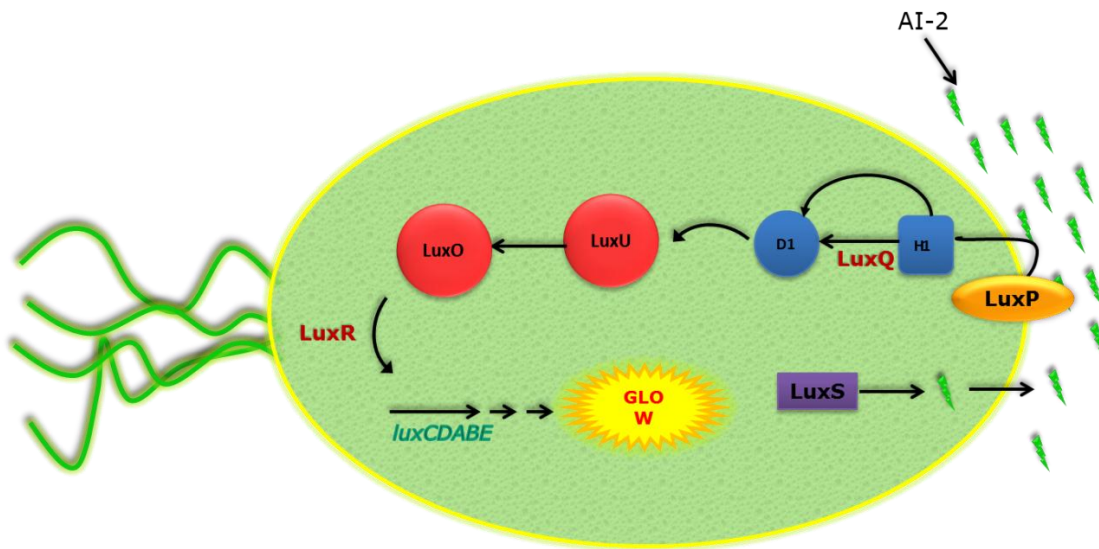
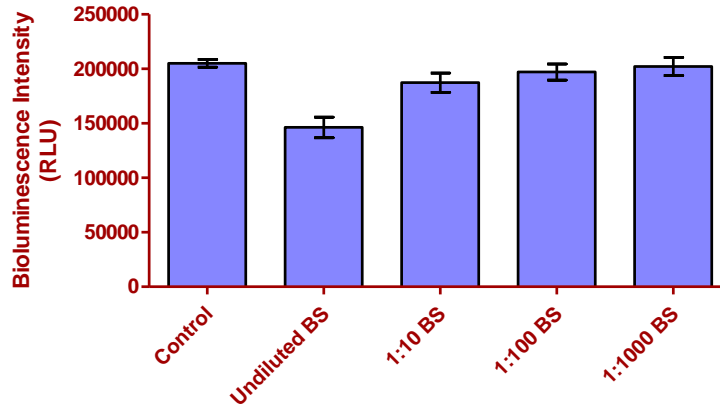
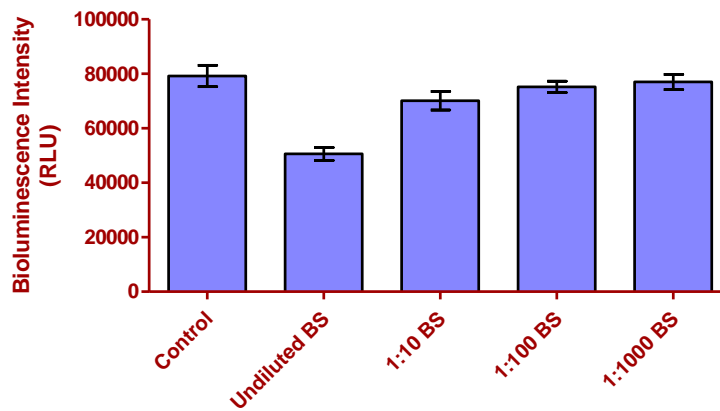


Figure 2. Beef suspension (BS) and its dilutions were incubated with whole-cell based biosensing systems. Data shown is the average \pm one standard deviation (n=3).

(A) Whole-cell biosensing system containing plasmid pSB406 for detection of short chain AHLs.



(B) Whole-cell biosensing system containing plasmid pSB1075 for detection of long chain AHLs.



(C) *V. harveyi* MM32-based whole-cell biosensing system for detection of AI-2.

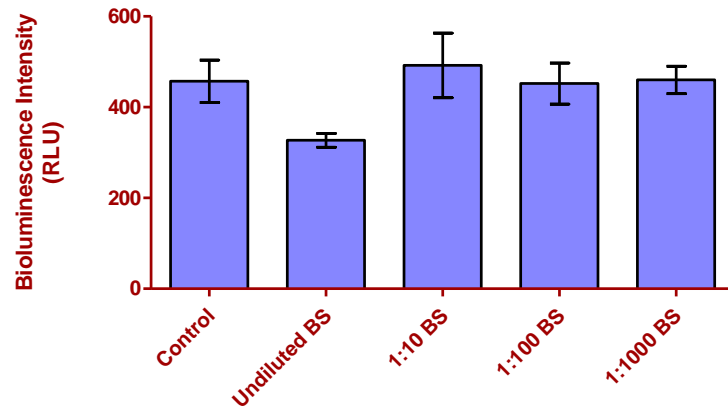
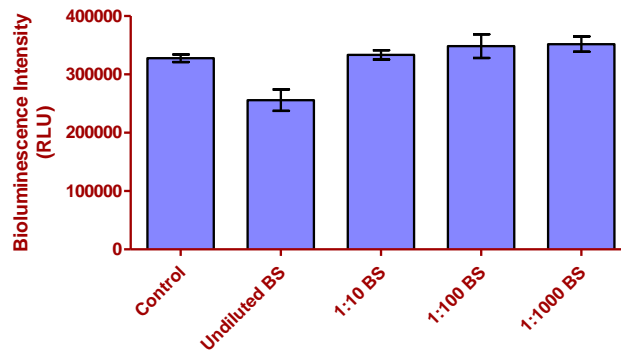
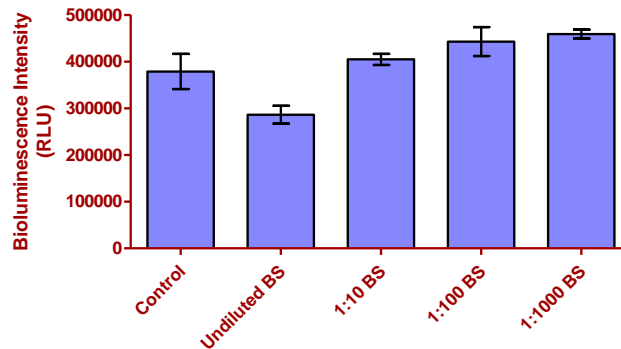


Figure 3. Effect of beef matrix on the whole-cell sensing systems response in the presence of QSMs. Beef suspension and its dilutions containing 1×10^{-6} M of C-6 HSL, 1×10^{-6} M of C-12 HSL, or 1×10^{-5} M of AI-2 were incubated with whole-cell biosensing system containing pSB406, whole-cell biosensing system containing pSB1075, or *V. harveyi* MM32 biosensing system, respectively. Data shown is the average \pm one standard deviation (n=3).

(A) Whole-cell biosensing system containing plasmid pSB406 for detection of short chain AHLs.



(B) Whole-cell biosensing system containing plasmid pSB1075 for detection of long chain AHLs.



(C) *V. harveyi* MM32-based whole-cell biosensing system for detection of AI-2.

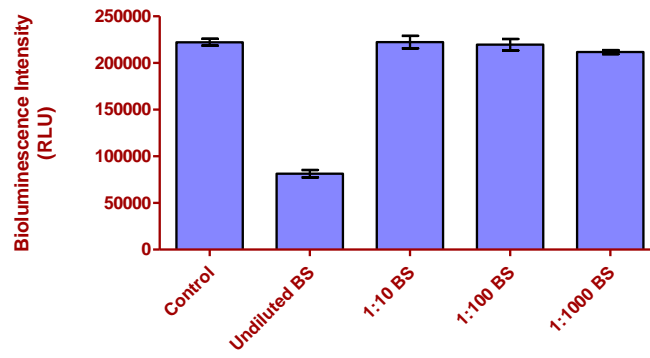
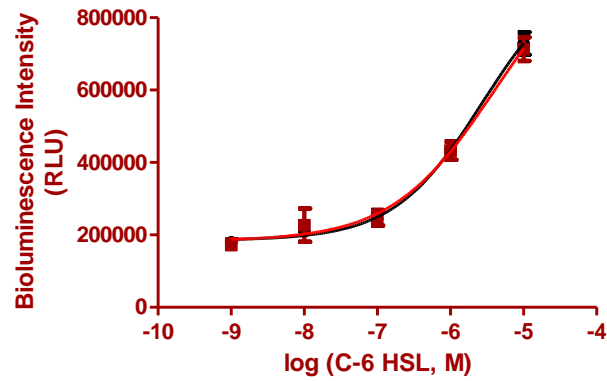
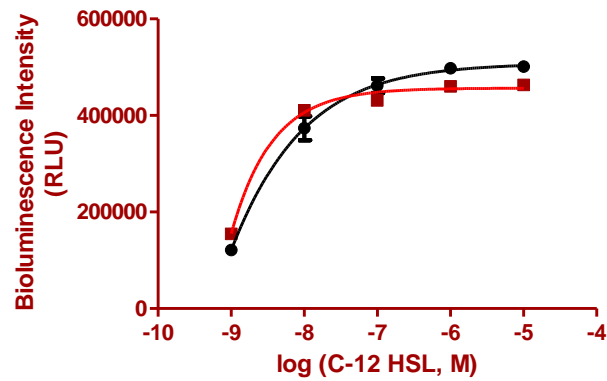


Figure 4. Standard dose-response curve (black) compared with dose-response curve in 1:10 beef suspension (red) obtained using (A) whole-cell biosensing system containing plasmid pSB406, (B) whole-cell biosensing system containing plasmid pSB1075, and (C) *V. harveyi* MM32-based whole-cell biosensing system.

(A)



(B)



(C)

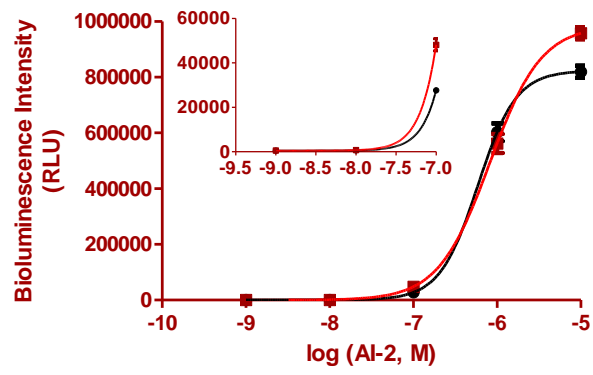
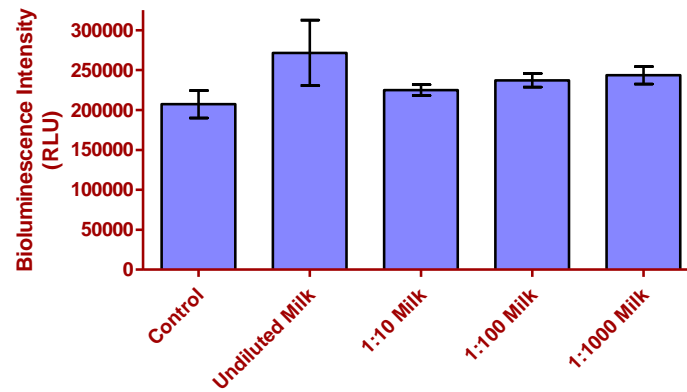
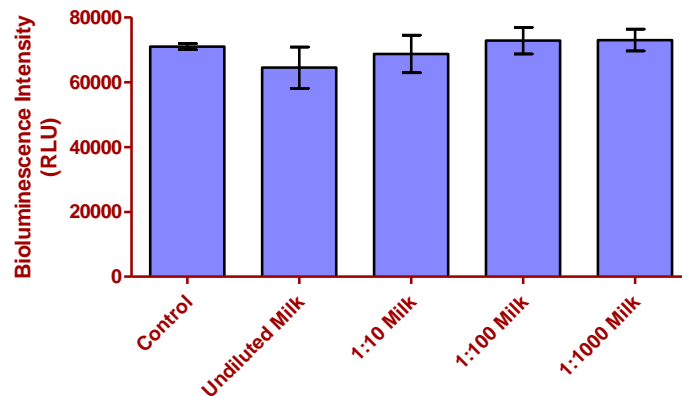


Figure 5. Skim milk and its dilutions were incubated with whole-cell based biosensing systems. Data shown is the average \pm one standard deviation (n=3).

(A) Whole-cell biosensing system containing plasmid pSB406 for detection of short chain AHLs.



(B) Whole-cell biosensing system containing plasmid pSB1075 for detection of long chain AHLs.



(C) *V. harveyi* MM32-based whole-cell biosensing system for detection of AI-2.

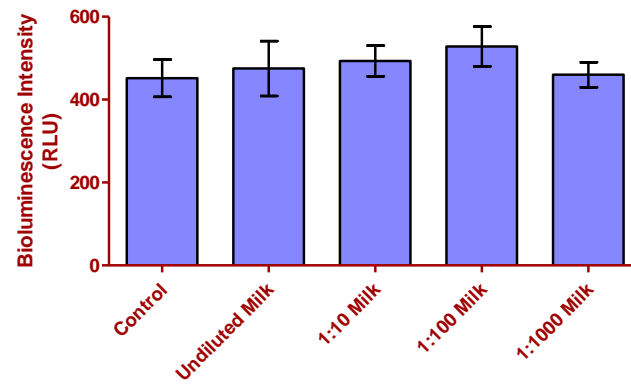
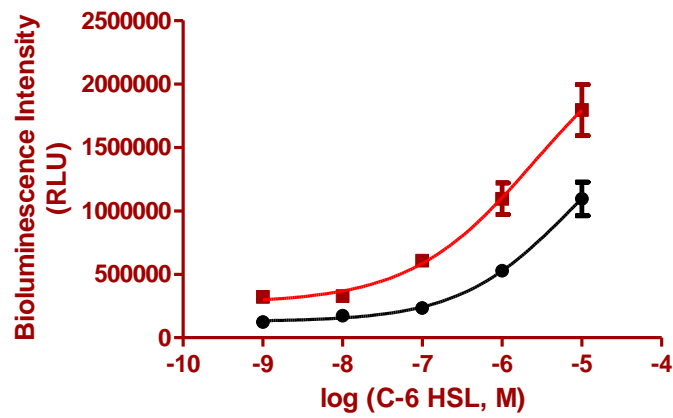
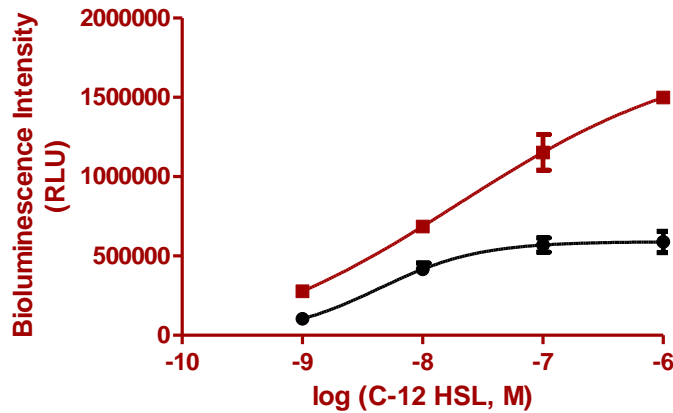


Figure 6. Standard dose-response curve (black) compared with dose-response curve in undiluted skim milk (red) obtained using (A) whole-cell biosensing system containing plasmid pSB406, (B) whole-cell biosensing system containing plasmid pSB1075, and (C) *V. harveyi* MM32-based whole-cell biosensing system.

(A)



(B)



(C)

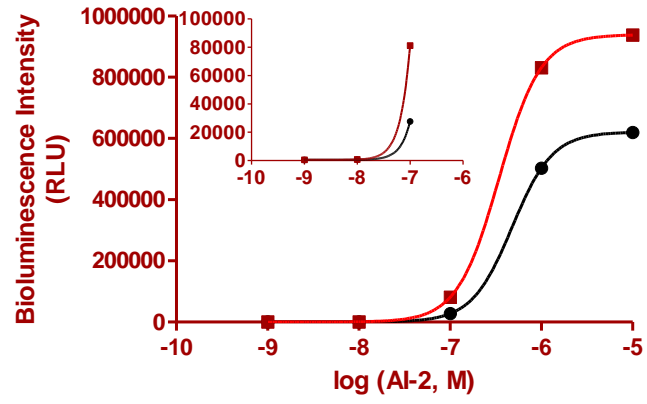
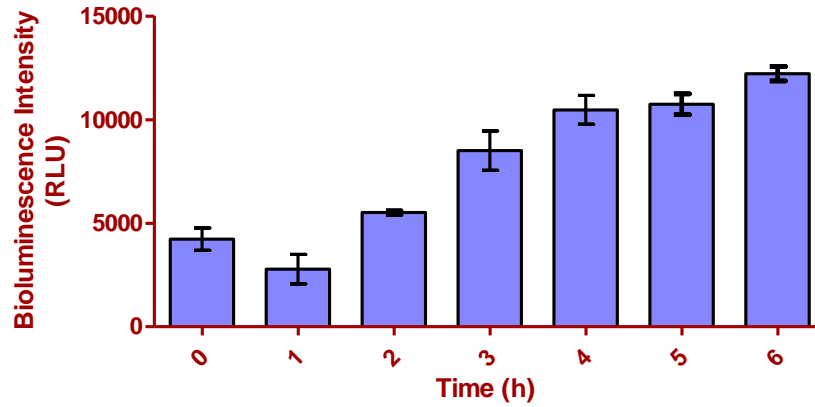


Figure 7. Contamination of food matrix with QSM producing bacteria.

(A) Short chain AHL production in beef suspension contaminated with *H. alvei* 718, as monitored with whole-cell biosensing system containing plasmid pSB406.



(B) AI-2 production in beef suspension contaminated with *E. coli* AB1157, as monitored with *V. harveyi* MM32 whole-cell biosensing system.

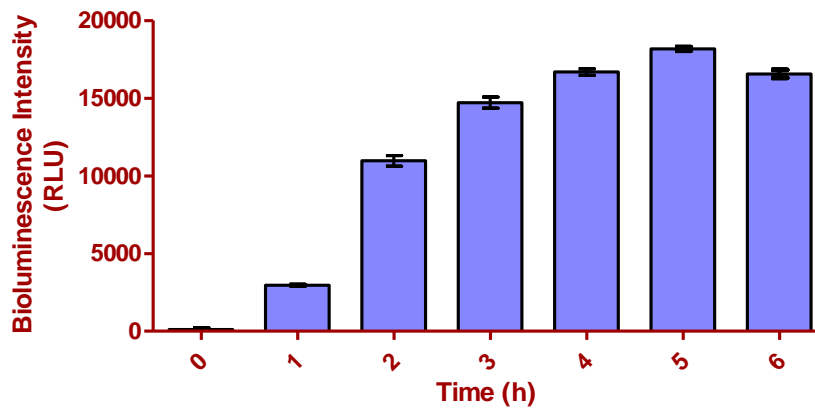


Table 1. Analytical parameters of the whole-cell biosensing systems.

Biosensing system	Limit of detection (M)	Dymanic range (M)
<i>E. coli</i> pSB406	1×10^{-9}	$1 \times 10^{-5} - 1 \times 10^{-9}$
<i>E. coli</i> pSB1075	1×10^{-9}	$1 \times 10^{-6} - 1 \times 10^{-9}$
<i>V. harveyi</i> MM32	1×10^{-9}	$1 \times 10^{-5} - 1 \times 10^{-9}$

CHAPTER FIVE
INTERFERENCE OF GENERALLY RECOGNIZED AS SAFE (GRAS) COMPOUNDS WITH
BACTERIAL QUORUM SENSING

Introduction

The discovery of bacterial communication was paradigm shifting in terms of bacteria being viewed as organisms that exert their effects as a community, not as independent single cell organisms. Cell-to-cell communication occurs via the synthesis and release of small signaling molecules, termed quorum sensing molecules (QSMs) that are transported out of the cell and can be detected by bacteria of the same (intra-species communication) or different species (inter-species communication). Such QSMs include *N*-acyl homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2), which are utilized by Gram-negative, Gram-positive, and both Gram-negative and Gram-positive bacteria, respectively. This system of communication, which bacteria employ to modulate their behavior in a cell-density dependent manner, is termed quorum sensing (QS). Specifically, when the cell population reaches a critical size reflected by a critical threshold QSM concentration in the environment surrounding the cells, certain specialized genes are expressed. Hence, this communication process allows a population of bacteria to change the community behavior in response to changes in its size. More recently, it has been reported that certain QSMs not only mediate bacterial communication, but also interkingdom communication – communication between microorganisms and their hosts^{17,103}. Figure 1(a) depicts the aforementioned QS phenomenon. QS is utilized by both pathogenic and non-pathogenic

Gram-positive and Gram-negative bacteria^{25,176,177}. Employing QS, bacteria regulate various functions such as sporulation, swarming motility, biofilm formation, and production of virulence factors and antibiotics. Among these phenomena, QS regulation of virulence in human pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and enterohaemorrhagic *Escherichia coli*, has been well documented¹⁷⁸.

Bacterial infections are generally treated with antibiotics; however abuse of use of antibiotics in humans and animals that humans consume has caused the emergence of a number of antibiotic resistant bacteria¹⁷⁹. This has created the need for the identification of classes of new antibacterial compounds that are efficient in attacking the aforementioned resistant bacteria. To that end, the discovery of compounds that may interfere with bacterial communication may provide with a potential alternative to current classes of antibacterial drugs^{52,180}. Molecules that can interfere with QS should be able to mislead the bacteria about their surrounding environmental conditions, and therefore, affect their growth and behavior¹⁸¹. In the past few years, a number of such molecules have been identified. For instance, some synthetic analogs of AI-2 were found to act as agonists in *Vibrio harveyi*, while others were antagonists in *P. aeruginosa*¹⁸². More recently, modified imidazolines have been reported to inhibit QS³³. The list of such interfering compounds is extensive and there are several possible mechanisms of interference as pointed out in a recent review by Ni *et al.*⁵² One of the most significant advantages of using QS interfering molecules as a means to limit bacterial growth and pathogenicity is that QS interfering molecules, unlike antibiotics, do not directly kill the

bacteria and, therefore, do not add evolutionary pressure to develop resistance against interfering molecules^{183,184}.

To date several molecules that interfere with QS have been identified, but their use in practical applications is limited because of potential side-effects on human health. An approach to address this issue is to test compounds that are safe to consume, or known to improve the quality of food products, as QS interfering agents¹⁸⁵⁻¹⁸⁷. The United States Department of Food and Drug Administration maintains a list of food additives that are considered safe and approved suitable for consumption. These substances are classified as Generally Recognized As Safe (GRAS). Sources of GRAS substances include angelica, basil, bergamot, chamomile roman, chamomile german, cinnamon bark, citrus rind (all), clary sage, clove, coriander, dill, eucalyptus, frankincense, galbanum, geranium, ginger, grapefruit, hyssop, juniper, jasmine, bay laurel, lavender, lemon, lemongrass, lime, melissa (lemon balm), marjoram, myrrh, myrtle, nutmeg, orange, oregano, patchouly, pepper, peppermint, petitgrain, pine, rosemary, rose, savory, sage, sandalwood, spearmint, spruce, tarragon, tangerine, thyme, valerian, vetiver, and ylang ylang, among others.

Phenolic compounds, which are known to protect the plants from pathogens¹⁸⁸, constitute a key ingredient of a number of GRAS substances. The three major classes of phenolic compounds are tannins, flavonoids, and lignins. Among them, flavonoids have a variety of beneficial biological properties and, therefore, are the most studied among all¹⁸⁹. To date, a limited number of compounds originating from GRAS substances have

been shown to affect QS. For example, Niu *et al.* have reported that cinnamaldehyde, an ingredient of cinnamon, inhibited the bacterial growth in sub-millimolar ranges³⁶. In addition, tannic acid, which is abundant in oak bark, antagonized QS in *Chromobacterium violaceum* as well as reduced biofilm formation in *Proteus mirabilis*¹⁹⁰. In another study, garlic extract and *p*-coumaric acid were proven to act as antagonists in the QS systems of various bacteria such as *Escherichia coli*, *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, *Pseudomonas putida*, and *Pseudomonas chlororaphis*¹⁹¹.

Phenolic compounds originating from GRAS substances are under investigation at the College of Agriculture of the University of Kentucky; some of them have been shown to possess antimicrobial properties¹⁹² and are being considered for use as food preservatives. However, the mechanism of action of these compounds is not known. We postulated that they may act by interfering with bacterial QS. There are a number of ways in which a molecule can interfere with QS; Figure 2 represents some of them. Specifically, we hypothesized that test GRAS compounds 1) may affect the concentration of QSMs produced by food contaminating bacteria or 2) may act as agonists or antagonists in the QS systems of bacteria. The former may occur if QSM synthesis is inhibited. In order to test this hypothesis, we selected 12 compounds that had shown antimicrobial effect, and incubated each of them with cultures of QSM producing bacteria. QSM production was then monitored in the culture media at different time intervals using a panel of bacterial whole-cell sensing systems designed to quantitatively respond to AI-2, short chain AHLs, and long chain AHLs, respectively. These experiments allowed us to detect the amount of QSMs that the cultured bacteria

produced and verify if GRAS compounds affected the QSM synthesis. To test the latter, we performed experiments to determine if the GRAS compounds had an effect on the QS response of the above sensing bacteria, both, in the presence and absence of QSMs. These studies provided insight into the mode of action of GRAS compounds based on their effects on the QS sensing/regulatory elements of the whole-cell biosensing systems.

Materials and methods

Materials. AHLs, *N*-hexanoyl-DL-homoserine lactone (C-6 HSL) and *N*-dodecanoyl-DL-homoserine lactone (C-12 HSL), ampicillin, kanamycin, and compounds originating from GRAS substances, namely curcumin, capsaicin, thymol, thymoquinone, coumarin, ascorbic acid, *p*-coumaric acid, *t*-cinnamic acid, tannic acid, eugenol, quercetin, and *o*-dianisidine, were purchased from Sigma (St. Louis, MO). AI-2 was purchased from Omm Scientific (Dallas, TX). Luria Bertani (LB) broth, nutrient broth, and vitamin free casamino acids were purchased from Difco (Sparks, MD). Acetonitrile used in the experiments was of HPLC grade and was purchased from VWR Scientific (Pittsburgh, PA). The 96-well microtiter plates were purchased from Costar (Corning, NY). The microcentrifuge was purchased from Eppendorf (Westbury, NY). The orbital shaker incubator was purchased from Fisher Scientific (Fair Lawn, NJ). Bioluminescent measurements were performed using the FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC).

Plasmids, bacterial strains, and culture conditions. The plasmids pSB406 and pSB1075 were originally provided by Dr. Paul Williams (University of Nottingham, Nottingham, UK) and previously transformed in *E. coli* JM109 cells. The transformed AHL-sensing cells were then stored at -80 °C as glycerol stocks. Fresh cell cultures were obtained from the glycerol stocks, grown in LB media (100 µg/mL ampicillin) overnight, in the orbital shaker at 37 °C, 250 rpm, and then refreshed and allowed to grow until an optical density (OD) at 600 nm of 0.45-0.50 was reached. *V. harveyi* MM32 (AI-2-sensing strain), *E. coli* AB1157 (AI-2-producing strain), and *Hafnia alvei* 718 (AHL-producing strain) were purchased from American Type Culture Collection (Manassas, VA). Cultures of *V. harveyi* MM32 cells were setup in autoinducer bioassay (AB) media containing 30 µg/mL kanamycin and grown overnight in the orbital shaker at 30 °C, 250 rpm. Overnight cultures were then diluted 1:100 to obtain an OD (600 nm) of 0.01-0.02. The procedure to prepare AB media has been described elsewhere¹²⁹. *E. coli* AB1157 cells were grown in LB media (no antibiotic) at 37 °C, 250 rpm, while *H. alvei* 718 cells were grown in nutrient media (no antibiotic) at 30 °C, 250 rpm, in the orbital shaker.

Dose-response curves. Commercially available *N*-acyl homoserine lactones were dissolved in acetonitrile to obtain 1×10^{-2} M stock solutions, which were serially diluted with RO filtered water to obtain standard solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. A 1% solution of acetonitrile in RO filtered water was used as blank (upon addition to the bacterial culture, the acetonitrile final concentration of 0.1% did not result toxic for the sensing cells). A volume of 10 µL of each of these solutions was added in triplicate to a 96-well white polystyrene microtiter plate containing 90 µL/well

of cell culture grown to an OD (600 nm) of 0.45-0.50. C-6 HSL solutions were employed for the whole-cell sensor with pSB406, while C-12 HSL solutions were employed for the whole-cell sensor with pSB1075. The microtiter plate was then incubated in the orbital shaker at 37 °C, 175 rpm for two hours. The produced bioluminescence was then measured using the microplate reader. The light intensity was expressed in relative light units (RLU).

AI-2 aqueous stock solution (3.7×10^{-3} M) was serially diluted with RO filtered water to prepare AI-2 standard solutions at concentrations ranging from 1×10^{-4} M to 1×10^{-8} M. RO filtered water was used as blank. A volume of 10 μ L of each of these standard solutions and blank was added in triplicate to a 96-well black polystyrene microtiter plate containing 90 μ L/well of *V. harveyi* MM32 cell culture grown to an OD (600 nm) of 0.01-0.02. The microtiter plate was then incubated in the orbital shaker at 30 °C, 175 rpm for three hours. The produced bioluminescence was then measured using the microplate reader. The light intensity was expressed in relative light units (RLU).

Monitoring of QSM production by *E. coli* AB1157 and *H. alvei* 718. *E. coli* AB1157 was grown in LB media (no antibiotic) overnight, in the incubator shaker at 37 °C, 250 rpm. *H. alvei* 718 was grown in nutrient media (no antibiotic) overnight, in the incubator shaker at 30 °C, 250 rpm. Overnight cultures were diluted 1:100 and 1:15, respectively, with fresh media and allowed to grow in the above conditions. One-milliliter fractions of culture media were collected at each hour for 8 hours, followed by collection of an

overnight fraction. The collected fractions were centrifuged at 13,000 rpm for 5 minutes at room temperature and the supernatants were stored at -20 °C until analyzed. To measure the QSMs produced, 10 µL of each supernatant sample was added in triplicate to the wells of a microtiter plate followed by addition of 90 µL of the proper sensing cell suspension, prepared as described above. For *E. coli* AB1157 culture media fractions, *V. harveyi* MM32 sensing cells were used, while for *H. alvei* 718 culture media fractions, the whole-cell sensing systems containing pSB406 and pSB1075 were employed. The assays were performed as described above.

Solubility study and preparation of GRAS compounds solutions. While the selected GRAS compounds are soluble in several solvents, the cell based biosensing systems employed in this work have been characterized in terms of behavior in the presence of water and acetonitrile. Therefore, when solubility properties allowed, we prepared solutions of GRAS compounds in either of the two solvents. Tannic acid, eugenol, and ascorbic acid were dissolved in RO filtered water to obtain 10 mg/mL stock solutions. A set of 10 mg/mL stock solutions of coumarin, *p*-coumaric acid, *t*-cinnamic acid, capsaicin, thymoquinone, and thymol were prepared in acetonitrile. Curcumin was not completely soluble either in water or acetonitrile at 10 mg/mL; therefore, a 1 mg/mL stock solution in acetonitrile was prepared. A 10 mg/mL stock solution of *o*-dianisidine was prepared in dimethyl sulfoxide (DMSO). Quercetin was dissolved in 0.1 N NH₄OH to obtain a 10 mg/mL stock solution. The GRAS compound stock solutions were serially diluted with RO filtered water to obtain the desired range of concentrations. Table 1 lists the GRAS compounds, their structures, solvents used to dissolve them, and the

concentration ranges that were tested. All of the GRAS compound solutions were evaluated in the experiments described below along with the respective controls. For each compound, the control was comprised of a RO filtered water solution containing an amount of solvent equivalent to that present in the solution of GRAS compound at the highest concentration tested.

Effect of GRAS compounds on the response of the bacterial sensing systems in the absence of QSMs. A 10 μL volume of the GRAS compound solutions at various concentrations and of the respective controls was added in triplicate to 90 μL of cell culture of each of the sensing systems, prepared as described above, in a microtiter plate. The assays were performed as described above for standard QSM solutions. A matched standard dose-response curve was included in each analytical run.

Effect of GRAS compounds on the response of the bacterial sensing systems in the presence of QSMs. A fixed concentration of QSM was added to each of the GRAS compound solutions at various concentrations and to the respective controls. Specifically, 2×10^{-6} g/mL C-6 HSL, 2.8×10^{-7} g/mL C-12 HSL, or 1.9×10^{-6} g/mL AI-2 was added to each sample. A 10 μL volume of the obtained GRAS compound solutions at various concentrations and of the respective controls, containing a fixed concentration of QSM, was added in triplicate to 90 μL of cell culture of the corresponding sensing system, prepared as described above, in a microtiter plate. The assays were performed as described above for standard QSM solutions. A matched standard dose-response curve was included in each analytical run.

Effect of GRAS compounds on the production of QSMs by *E. coli* AB1157 and *H. alvei*

718. *E. coli* AB1157 produces AI-2 molecules, while *H. alvei* 718 produces AHL molecules. The two bacterial strains were grown overnight, and the overnight cultures were then diluted 1:100 with fresh LB media (*E. coli* AB1157) and 1:15 with fresh nutrient media (*H. alvei* 718), respectively, as described above. Volumes of 100 µL of GRAS compound solutions at various concentrations and of the respective controls were added in triplicate to 1-mL aliquots of diluted cell cultures in 14 mL culture tubes; cells were then allowed to grow in proper conditions, as described above, for a period of six hours (time needed for maximum production of QSMs by bacteria). At the end of this time period, the cultures were centrifuged at 12,000 rpm for 5 minutes at room temperature. The supernatants were stored at -20 °C until analyzed. The QSMs produced were measured in the supernatant samples following the protocol described above for monitoring of QSM production by bacteria.

Results and discussion

Bacterial QS plays a major role in the survival of bacteria and their success in colonizing various environments. Since QS is utilized by a number of bacteria, interference with QS could be employed as a general tool to mitigate bacterial growth and pathogenicity. To that end, a variety of natural and synthetic compounds have been investigated and shown to be able to disrupt the bacterial chatter⁵². The compounds that we selected in our study were initially evaluated for antimicrobial properties at the College of Agriculture of the University of Kentucky. Specifically, phenolic compounds of GRAS origin were tested against some of the most common Gram-positive and Gram-negative foodborne pathogens to determine their minimum inhibitory concentration (MIC) values¹⁹². Compounds that exhibited antimicrobial properties were chosen to be tested as QS interferants (Table 1). For that, we utilized whole-cell based biosensing systems that can quantitatively detect QS signaling molecules, namely AHLs and AI-2. *E. coli* based whole-cell biosensing systems either containing plasmid pSB406 or plasmid pSB1075 detect short-chain AHLs and long-chain AHLs, respectively. These bioluminescent cell-based sensors have been previously optimized, characterized, and employed as analytical tools in our lab to detect AHLs in physiological samples²⁷ and to assess QS-interfering characteristics of antibiotics¹⁹³. *V. harveyi* MM32 is derived from spontaneously bioluminescent marine bacterium *V. harveyi*, which controls light emission by means of various QS systems; in strain MM32, bioluminescence is only triggered by AI-2 since the other QS regulatory systems have been silenced¹⁵. Recently, we developed a whole-cell biosensing system based on *V. harveyi* MM32 for

quantitative detection of AI-2 (this dissertation, Chapter 5). All three biosensing systems contain genetic modifications so that the sensing cells cannot produce their own QSMs and, therefore, emit bioluminescence only in response to the QSMs present in the environment. Examples of QSMs detected by these biosensing systems are shown in Figure 1(b).

Initial solubility tests were performed to select a suitable solvent for each of the GRAS compounds, while making sure that it did not have harmful effects on the bacterial sensing cells. A range of solutions of GRAS compounds were prepared after dissolving each compound in appropriate solvent by serially diluting the stock solution with RO filtered water. In order to test the effect of GRAS compounds on the response of the whole-cell biosensing systems, these solutions were then incubated with the biosensing cells in the absence as well as in the presence of a fixed concentration of the respective QSM.

GRAS compounds, curcumin, capsaicin, eugenol, quercetin, *p*-coumaric acid, thymol, and *o*-dianisidine did not alter the bioluminescence emitted by all of the sensing cells, both, in the absence and presence of the QSM. Namely, the compounds alone did not trigger a response in the sensing systems and, when incubated along a fixed concentration of QSM, they did not affect the sensing systems' responses to the respective QSMs. As an example, Figure 3 shows the response of the whole-cell sensing system that harbors pSB406 to various concentrations of *p*-coumaric acid (from 1×10^{-4} g/mL to 1×10^{-9} g/mL), in the absence and presence of 2.0×10^{-7} g/mL C-6 HSL.

Decreased signal intensities with 1×10^{-4} g/mL *p*-coumaric acid were likely due to the cell toxicity of the compound. In addition, when the above mentioned GRAS compounds were incubated with AHL producing *H. alvei* 718 cells and AI-2 producing *E. coli* AB1157 cells, no change in the QSM production profile of these bacteria was observed. Hence, we concluded that these compounds did not interfere with QS, at least through the investigated mechanisms, that is, interaction with QS recognition/regulatory proteins and effect on QSM synthesis.

Thymoquinone is known to inhibit the formation of bacterial biofilms¹⁹⁴; however, its mechanism of action is not known. A potential mechanism may involve interference with bacterial QS. Therefore, we employed whole-cell biosensing systems to investigate if thymoquinone interferes with QS. In our study, we found that thymoquinone affected the bioluminescence response of the biosensing system harboring plasmid pSB1075, both, in the absence and presence of C-12 HSL (Figure 4). The increase in bioluminescence observed with 1×10^{-5} g/mL of thymoquinone alone (Figure 4a) indicates that the compound induces a response in the sensor and may be recognized by the LasR QS recognition/regulatory protein present in the sensing cells, thus suggesting potential for interference with QS. The observed decrease in bioluminescence, when both 1×10^{-5} g/mL thymoquinone and C-12 HSL are present (Figure 4b), suggests that thymoquinone may interfere with the binding between C-12 HSL and the receptor protein LasR. Based on the overall results, one could speculate that thymoquinone may bind to LasR, although causing a less effective response as compared to C-12 HSL. The hydrophobic binding pocket of LasR, being more

hydrophobic than that of RhlR, may interact with thymoquinone. This might help explain the effect on the pSB1075 based whole-cell biosensing system. Further experiments and molecular modeling studies are underway to investigate and elucidate the observed effects. Thymoquinone did not alter the bioluminescence emitted by the *E. coli* pSB406-based and *V. harveyi* MM32-based whole-cell biosensing systems, both, in the absence and presence of the respective QSMs. Additionally, when thymoquinone was incubated with AHL and AI-2 producing bacteria, no effect was observed on the QSM production.

Ascorbic acid has been previously reported to reduce bioluminescence of the AI-2 reporter strain *V. harveyi* BB170¹⁹⁵. Therefore, we studied ascorbic acid with the *V. harveyi* MM32-based whole-cell biosensing system. In our study, ascorbic acid solutions were incubated with *V. harveyi* MM32 sensing cells in the presence and absence of AI-2. We observed that, in both conditions, ascorbic acid at concentrations from 1×10^{-3} g/mL to 1×10^{-8} g/mL did not influence the bioluminescence response of the biosensing cells. Similarly, ascorbic acid did not induce a response in the developed FRET biosensing system for AI-2 detection (this dissertation, Chapter 6). Therefore, we may conclude that ascorbic acid does not bind to the LuxP protein. On the other hand, in both experiments, with and without AI-2, a sharp decrease in bioluminescence signal was observed with 1×10^{-2} g/mL of ascorbic acid, which may be attributed to general toxicity of ascorbic acid to the sensing cells (data not shown). Next, ascorbic acid solutions (1×10^{-2} g/mL to 1×10^{-8} g/mL) were incubated with AI-2 producing *E. coli* AB1157 cells, and cell culture supernatants were analyzed with *V. harveyi* MM32 sensing cells to measure the levels of AI-2 produced. Increased levels of bioluminescence, corresponding to

increased AI-2 concentrations, were detected in supernatant samples derived from *E. coli* AB1157 cultures incubated with ascorbic acid at concentrations from 1×10^{-5} g/mL to 1×10^{-3} g/mL (Figure 5). These data suggest that ascorbic acid seems to enhance the production of AI-2 by *E. coli* AB1157 cells, although there are currently no clear indications that the ascorbic acid metabolic pathway is linked to the AI-2 synthetic pathway. Further studies are required to clarify if ascorbic acid acts through a specific effect on the QS regulatory system or a general effect on the cell metabolism. Ascorbic acid at the concentrations tested did not affect the response of the whole-cell biosensing systems containing plasmids pSB406 or pSB1075, both, in the absence and presence of the respective AHLs. In addition, ascorbic acid did not influence the AHL production by *H. alvei* 718.

When *t*-cinnamic acid at concentrations of 1×10^{-3} g/mL to 1×10^{-9} g/mL was incubated with the whole-cell biosensing systems containing pSB406 or pSB1075, in the absence of AHLs, a significant increase in bioluminescence was observed at 1×10^{-4} g/mL and 1×10^{-5} g/mL of *t*-cinnamic acid (Figure 6). The effect was dose-dependent, at least for the sensor bearing pSB1075. These data indicate that *t*-cinnamic acid may be able to bind to the AHL receptors. No data are available at this time to confirm whether *t*-cinnamic acid binds to the AHL-binding site or to other binding pockets on the AHL receptors. If the binding was to occur in the AHL-binding site, the fact that the receptors have higher affinity for their cognate AHLs would explain why a much higher concentration of *t*-cinnamic acid (1×10^{-5} g/mL) is needed to obtain a detectable response, as compared to the cognate AHLs that induce a response at nanomolar levels.

t-Cinnamic acid at the concentrations tested did not affect the response of the whole-cell biosensing systems containing plasmids pSB406 or pSB1075 in the presence of the respective AHLs, as well as the response of *V. harveyi* MM32 sensing system, both, in the presence and absence of AI-2. In addition, *t*-cinnamic acid did not influence either the AHL production by *H. alvei* 718 or AI-2 production by *E. coli* AB1157.

Recently, the anti QS activity of tannic acid was reported by Taganna *et al.*¹⁹⁶, who demonstrated that tannin-rich methanolic leaf extract of *Terminalia catappa* (tropical almond) inhibited AHL-dependent QS regulated violacein production in *Chromobacterium violaceum*. The same extract also inhibited AHL-dependent biofilm maturation in *P. aeruginosa*. Further, tannic acid and its derivatives are known to inhibit biofilm formation in *E. coli*¹³⁹ and *Proteus mirabilis*¹⁹⁰. Our studies have confirmed that tannic acid interferes with AHL based quorum sensing; however, we observed that the sensors bioluminescence response increased as the concentration of tannic acid increased. This trend was observed when tannic acid solutions were incubated with whole-cell biosensing systems containing plasmid pSB406 or pSB1075 (Figure 7), both, in the presence and absence of C-6 HSL or C-12 HSL, respectively. In addition, tannic acid also interfered with the AI-2 based QS system as evident by increased bioluminescence signals obtained when tannic acid was incubated with *V. harveyi* MM32-based whole-cell biosensing system, both, in the presence and absence of AI-2 (data not shown). Tannic acid does not have any structure similarities with either AHLs or AI-2. However, it has been shown that certain compounds can interfere with QS by binding to the QSM recognition proteins in a non-agonistic manner^{52,140}. Therefore, we speculate that tannic

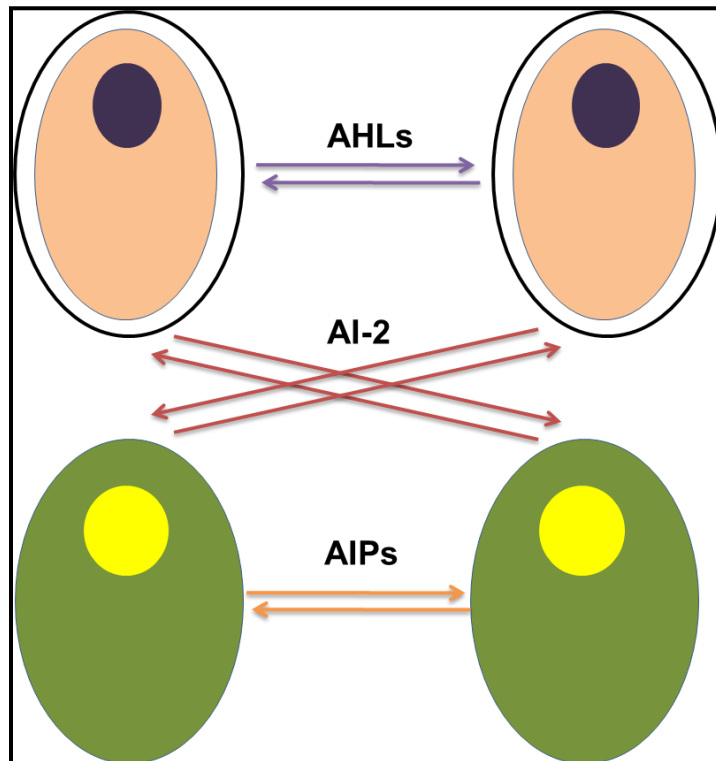
acid may interact with the AHL or AI-2 recognition proteins by binding to a site other than the ligand binding pocket. No effect was observed on the AHL production by *H. alvei* 718 or AI-2 production by *E. coli* AB1157. Since tannic acid interferes with QS systems of both Gram-negative and Gram-positive bacteria, it may potentially be used as a universal molecule to fight bacterial infections.

Although coumarin is not a GRAS compound, we analyzed it due to its known antimicrobial properties. In a study by Ojana *et al.*, coumarin exhibited antimicrobial activity against *E. coli*, *P. aeruginosa*, and *S. aureus*¹⁹⁷. In addition, coumarin, alone and in combination with antibiotics, significantly reduced incidence of local peritoneal infections in animal models¹⁹⁸. In our study, coumarin did not show any effect on the biosensing system with plasmid pSB406, both, in the presence and absence of C-6 HSL, as well as on the biosensing system with plasmid pSB1075, both, in the presence and absence of C-12 HSL. However, when coumarin solutions were incubated with *V. harveyi* MM32-based whole-cell biosensing system, both, in the presence and absence of AI-2, bioluminescence signals higher than those of the controls (no coumarin) were obtained between 1×10^{-4} g/mL and 1×10^{-5} g/mL of coumarin (Figure 8). Additionally, when coumarin was incubated with *E. coli* AB1157, higher levels of bioluminescence, corresponding to higher AI-2 concentrations, were measured in supernatant samples from cultures of *E. coli* AB1157 exposed to 1×10^{-8} g/mL to 1×10^{-6} g/mL of coumarin. No effect on AHL production by *H. alvei* 718 was observed. The data obtained supports that coumarin is capable of interfering with AI-2 dependent bacterial QS, both, by interacting with the QS regulatory system and affecting the synthesis of AI-2.

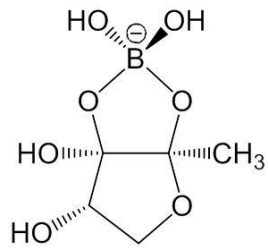
Conclusion

We evaluated twelve compounds for their effect on bacterial QS. Although some of them did not exert an effect that we could measure using our whole-cell biosensing systems, other compounds, such as, thymoquinone, *t*-cinnamic acid, ascorbic acid, tannic acid, and coumarin affected the bioluminescence response of the bacterial biosensing systems. In these biosensing systems, bioluminescence depends on activation of QS regulated circuits; therefore, a molecule that causes a change in bioluminescence in the sensors may have an effect on the QS systems of naturally occurring bacteria. While some of the compounds that influenced bioluminescence are structurally related to QSMs, for instance, ascorbic acid and AI-2 are both furanones, others are not. Therefore, there must be various ways by which these compounds interfere with QS. Further investigation is essential to elaborate on the mechanisms of action of the tested compounds. Moreover, animal studies using these GRAS compounds should provide further insight into the feasibility of employing them as alternatives to antibiotic treatment and as food preservatives.

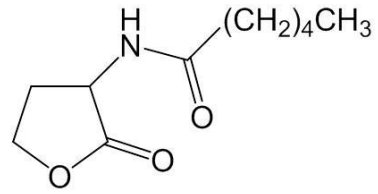
Figure 1. (a) General schematic of quorum sensing in bacteria. For intra-species communication, Gram-negative bacteria utilize AHLs and Gram-positive bacteria utilize AIPs. For inter-species communication, AI-2 molecules are utilized by both Gram-negative and Gram-positive bacteria. **(b)** Chemical structures of AI-2, C-6 HSL, and C-12 HSL.



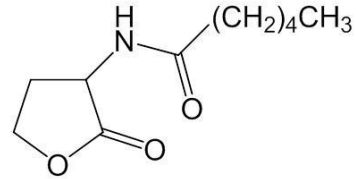
(b)



Borate form of autoinducer-2



N-Hexanoyl-DL-homoserine lactone



N-Dodecanoyl-DL-homoserine lactone

Figure 2. Interference with QS. Possible interference mechanisms include (1) interference with the binding between QSM and its cognate recognition protein, (2) interference with activation of gene expression, including genes encoding QSM synthases, and (3) degradation of QSM.

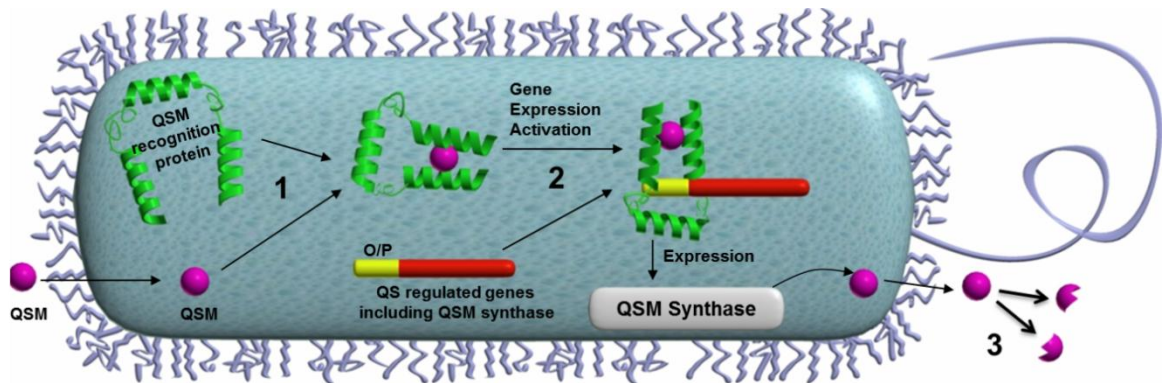
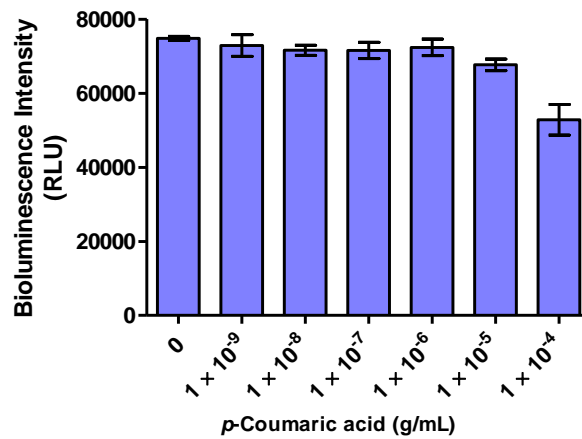


Figure 3. (a) *p*-Coumaric acid solutions were incubated with the whole-cell biosensing system containing plasmid pSB406 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL *p*-coumaric acid) contains 1% acetonitrile (v/v). **(b)** *p*-Coumaric acid solutions containing 2.0×10^{-6} g/mL C-6 HSL were incubated with the whole-cell biosensing system containing plasmid pSB406 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL *p*-coumaric acid) contains 1% acetonitrile (v/v), along with 2.0×10^{-6} g/mL C-6 HSL. Upon addition to the biosensing cells, the final concentration of C-6 HSL is 2.0×10^{-7} g/mL.

(a)



(b)

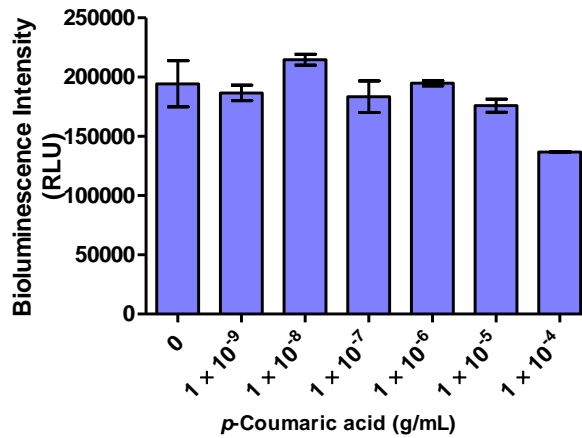
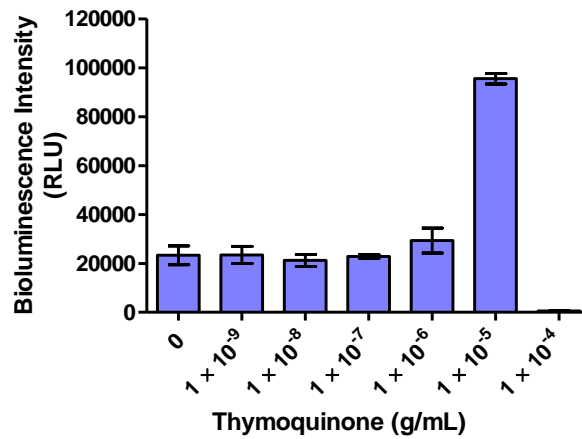


Figure 4. (a) Thymoquinone solutions were incubated with the whole-cell biosensing system containing plasmid pSB1075 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL thymoquinone) contains 1% acetonitrile (v/v). **(b)** Thymoquinone solutions containing 2.8×10^{-7} g/mL C-12 HSL were incubated with the whole-cell biosensing system containing plasmid pSB1075 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL thymoquinone) contains 1% acetonitrile (v/v), along with 2.8×10^{-7} g/mL C-12 HSL. Upon addition to the biosensing cells, the final concentration of C-12 HSL is 2.8×10^{-8} g/mL.

(a)



(b)

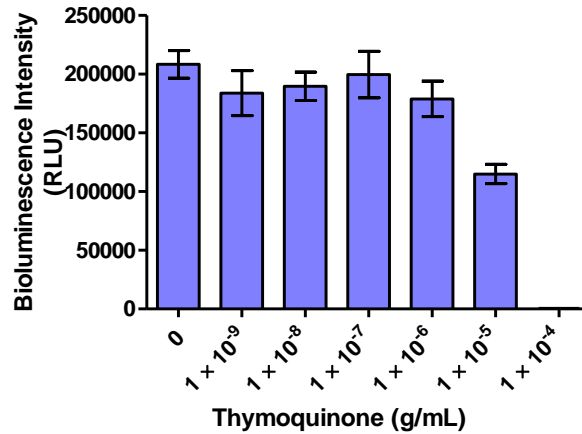


Figure 5. Ascorbic acid solutions, from 1×10^{-2} g/mL to 1×10^{-8} g/mL, were added to cultures of AI-2 producing *E. coli* AB1157 cells. After 6 hours (time required for maximum AI-2 production) the cell cultures were centrifuged, and the supernatants were analyzed for AI-2 content using the *V. harveyi* MM32 based whole-cell biosensing system. Blank (0 mg/mL ascorbic acid) contained RO filtered water.

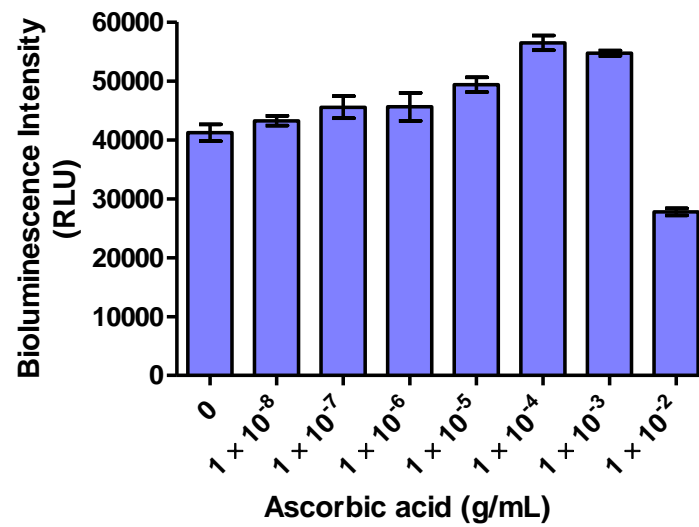
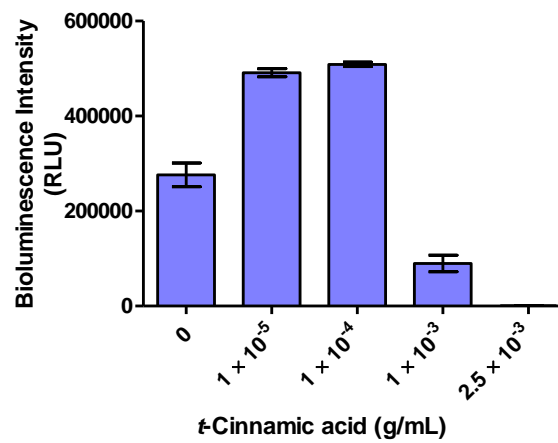


Figure 6. (a) *t*-Cinnamic acid solutions were incubated with the whole-cell biosensing system containing plasmid pSB406 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL *t*-cinnamic acid) contains 1% acetonitrile (v/v). **(b)** *t*-Cinnamic acid solutions were incubated with the whole-cell biosensing system containing plasmid pSB1075 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL *t*-cinnamic acid) contains 1% acetonitrile (v/v).

(a)



(b)

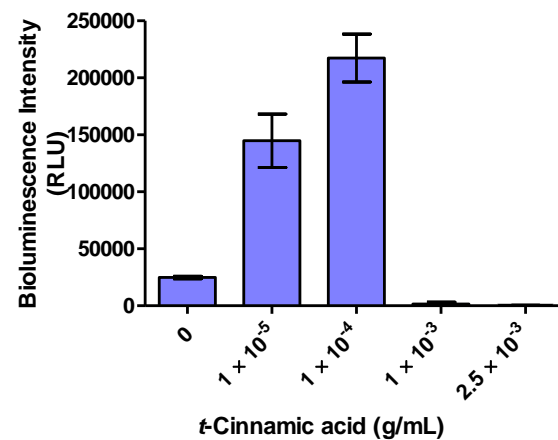
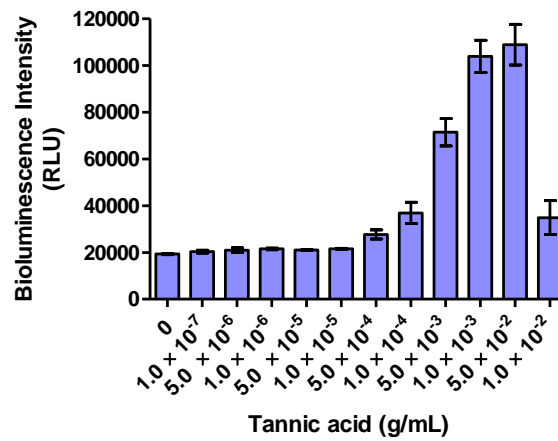


Figure 7. (a) Tannic acid solutions were incubated with the whole-cell biosensing system containing plasmid pSB1075 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL tannic acid) contains RO filtered water. (b) Tannic acid solutions containing 2.8×10^{-7} g/mL C-12 HSL were incubated with the whole-cell biosensing system containing plasmid pSB1075 for 2 h at 37 °C, 175 rpm. Blank (0 mg/mL tannic acid) contains RO filtered water, along with 2.8×10^{-7} g/mL C-12 HSL. Upon addition to the biosensing cells, the final concentration of C-12 HSL is 2.8×10^{-8} g/mL.

(a)



(b)

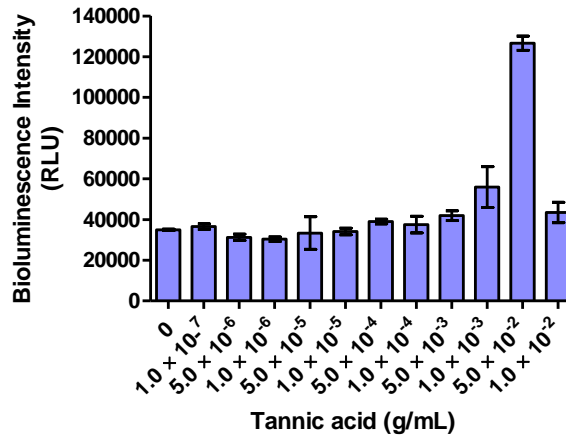
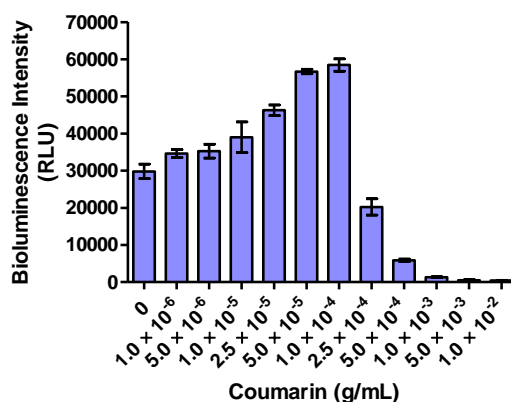


Figure 8. (a) Coumarin solutions, from 1×10^{-6} g/mL to 1×10^{-2} g/mL, were incubated with the *V. harveyi* MM32 based whole-cell biosensing system for 3 h at 30 °C, 175 rpm. Blank (0 mg/mL *t*-cinnamic acid) contains 1 % acetonitrile (v/v). **(b)** Coumarin solutions, from 1×10^{-6} g/mL to 1×10^{-2} g/mL, containing 2.0×10^{-6} g/mL AI-2 were incubated with the *V. harveyi* MM32 based whole-cell biosensing system for 3 h at 30 °C, 175 rpm. Blank (0 mg/mL *t*-cinnamic acid) contains 1 % acetonitrile (v/v), along with 2.0×10^{-6} g/mL AI-2. Upon addition to the biosensing cells, the final concentration of AI-2 is 2.0×10^{-7} g/mL.

(a)



(b)

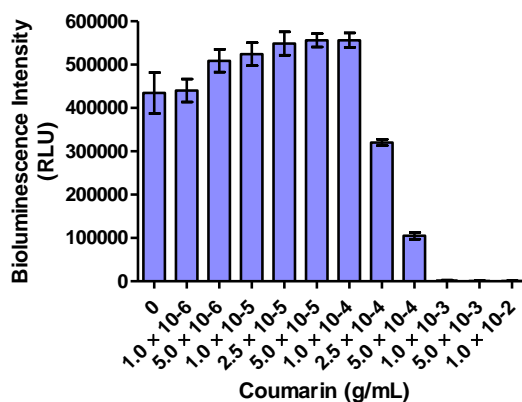


Table 1. GRAS compounds and their properties.

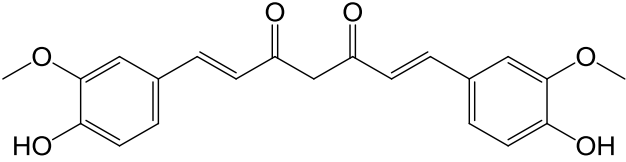
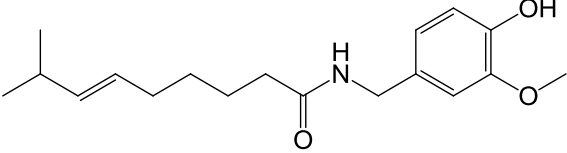
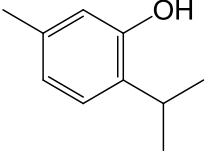
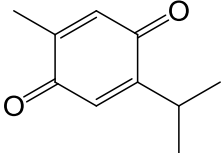
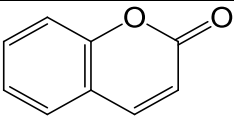
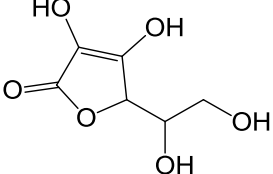
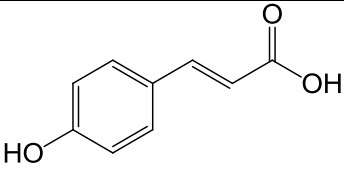
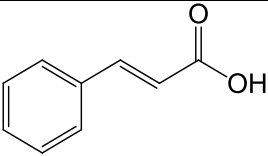
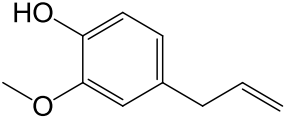
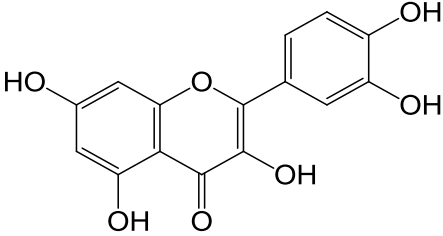
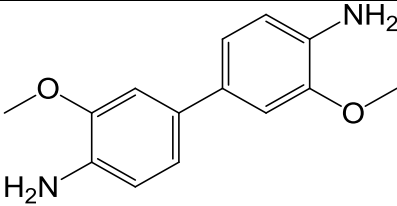
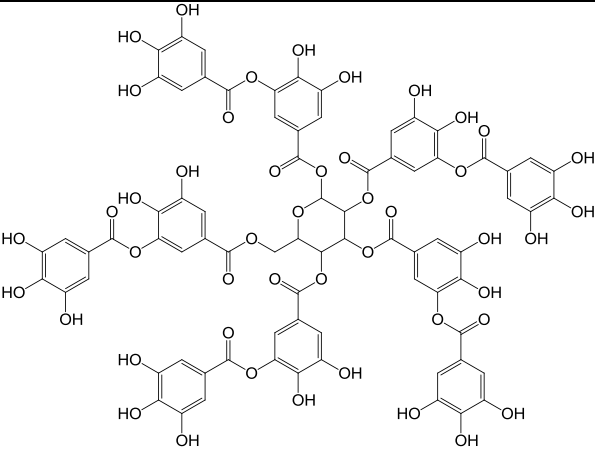
GRAS compounds	Structure	Solubility
Curcumin		Acetonitrile
Capsaicin		Acetonitrile
Thymol		Water
Thymoquinone		Acetonitrile
Coumarin		Acetonitrile
Ascorbic acid		Water
<i>p</i> -Coumaric acid		Acetonitrile

Table 1 (continued)

<p><i>t</i>-Cinnamic acid</p>		<p>Acetonitrile</p>
<p>Eugenol</p>		<p>Water</p>
<p>Quercetin</p>		<p>0.1 N ammonium hydroxide</p>
<p><i>o</i>-Dianisidine</p>		<p>DMSO</p>
<p>Tannic acid</p>		<p>Water</p>

CHAPTER SIX

BREAKING INTO THE BACTERIAL QUORUM SENSING CIRCUITS: DETECTION OF THE AUTOINDUCER-2 VIA A FLUORESCENCE BIOSENSING SYSTEM

Introduction

Bacteria, both Gram-positive and Gram-negative modulate the expression of certain genes in a cell-density dependent manner via the production, release, and sensing of small signaling molecules known as quorum sensing molecules (QSMs). This phenomenon, termed quorum sensing (QS), is known to regulate a diverse array of functions including the production of virulence factors^{9,199,200} and antibiotics^{142,201}, biofilm formation²⁰², and motility²⁰³. Specifically, specialized genes involved in the above functions are expressed when a QSM threshold concentration representative of a certain population size is reached. The most common types of QSMs are *N*-acyl homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2). Gram-negative and Gram-positive bacteria employ AHLs and AIPs, respectively, for communicating with members of the same species. AI-2 molecules are used by both types of bacteria and also involved in communication between species²⁰⁴. Bacteria are not limited to the use of just one type of QSM or QS circuit, and many bacteria utilize multiple QS circuits to regulate their various functions²⁰⁵⁻²⁰⁷. In that regard, *Vibrio harveyi*, a marine bacterium, employs multiple QS systems to regulate multiple functions, such as siderophores²⁰⁸ and metalloproteases production²⁰⁹, type III secretion²¹⁰, biofilm formation²¹¹, and bioluminescence²⁰⁵. These QS circuits are based

on the use of different QSMs (Figure 1A), namely, *N*-(3-hydroxybutanoyl) homoserine lactone (HAI-1), (*Z*)-3-aminoundec-2-en-4-one (CAI-1), (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (BAI-2), and nitric oxide (NO), which was recently shown to participate in QS regulation of bioluminescence through a newly discovered QS pathway²¹². HAI-1 is found only in *V. harveyi* and its close relative *Vibrio parahaemolyticus*, CAI-1 is mostly present in the *Vibrio* species, while the class of AI-2 molecules is found in a wide variety of bacterial species and genera²¹¹. This suggests that HAI-1 is involved in intra-species communication, CAI-1 in intra-genera communication, and AI-2 in inter-bacterial communication amongst different species and genera.

The class of AI-2 molecules, including borated autoinducer-2 (BAI-2), is composed by a family of compounds that originate from the precursor 4,5-dihydroxy-2,3-pentanedione (DPD). In *V. harveyi*, DPD is formed as a by-product of an enzymatic reaction part of a bacterial metabolic pathway. Specifically, the essential coenzyme *S*-adenosyl methionine (SAM) is converted into *S*-adenosyl homocysteine (SAH) upon transfer of methyl groups to DNA, RNA, and proteins⁴⁸. SAH is toxic to cells as it inhibits SAM-dependent methyltransferases²¹³ and is metabolized to *S*-ribosyl homocysteine (SRH) by 5'-methylthioadenosine-SAH nucleosidase as a first step to dampen its toxic effects. SRH is subsequently converted into homocysteine by the enzyme LuxS, with DPD being formed as a by-product of this enzymatic reaction. DPP is then employed by the bacterial quorum sensing circuit to produce AI-2. The biosynthesis of AI-2 from DPD is illustrated in Figure 1B. Under aqueous conditions, DPD undergoes spontaneous

cyclization to generate various interconverting hydrated forms, (2*R*,4*S*)- and (2*S*,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*R*- and *S*-DHMF, respectively), as well as (2*R*,4*S*)- and (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*- and *S*-THMF, respectively). In the presence of borate, *S*-THMF is converted into (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate), i.e., BAI-2¹⁰⁴. Two of these AI-2 molecules, BAI-2 and *R*-THMF, are known to be utilized as QSMs by *Vibrio harveyi* and *Salmonella typhimurium*, respectively, while other forms of AI-2 are used by other bacterial species¹⁵.

AI-2 serves a vital role in QS-regulated pathways in a number of human and foodborne pathogens, including *S. typhimurium* and various pathogenic *E. coli* strains^{15,214}. Thus, detection of AI-2 in physiological samples may be critical in the early detection of bacterial infections and monitoring of the efficacy of antibacterial therapy. To that end, we have developed a rapid, highly sensitive, and selective protein-based biosensing system aimed at the detection of AI-2 molecules in physiological samples.

In *V. harveyi*, the periplasmic binding protein LuxP is responsible for recognition and binding of the BAI-2 molecule. Similar to other periplasmic binding proteins^{94,215}, LuxP undergoes a change in conformation when it binds BAI-2, a property that has been employed by researchers to create protein biosensing systems for AI-2 detection. For example, Rajamani *et al.* developed a Förster Resonance Energy Transfer (FRET)-based system¹²⁸ in which the gene sequence of the LuxP protein was inserted between that of two different variants of the Green Fluorescent Protein, Cyan Fluorescent Protein (CFP)

and Yellow Fluorescent Protein (YFP) to create a hybrid CFP-LuxP-YFP fusion protein. Upon excitation at 440 nm, CFP emits at 485 nm, while YFP excitation and emission wavelengths are 485 nm and 527 nm, respectively. Thus, excitation of the CFP-LuxP-YFP fusion protein at 440 nm causes emission of CFP at 485 nm, which, in turn leads to fluorescence resonance energy transfer (FRET) from CFP to YFP, resulting in YFP fluorescence emission at 527 nm. When BAI-2 is present in the environment, it binds to the LuxP part of the fusion protein, changes its conformation, and alters the fluorescence emission at 527 resulting from the FRET between CFP and YFP. Specifically, addition of BAI-2 caused a dose-dependent decrease in FRET of the sensing CFP-LuxP-YFP fusion protein that was used to detect the levels of the QSM. This biosensing system responded selectively to BAI-2 with an apparent K_d of 270 nM. In another approach, Zhu *et al.* modified the LuxP protein with environmentally sensitive fluorophores²¹⁶, such as, 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), 6-bromoacetyl-2-(dimethylamino) naphthalene (badan), N,N'-didansyl-L-cystine (DDC), and Dapoxyl (2-bromoacetamidoethyl)sulfonamide (Dapoxyl). The fluorophores were chemically conjugated to Cysteines near the ligand binding site so that upon analyte binding, change in the microenvironment surrounding the fluorophore would lead to a change in the fluorescence emission. These systems were able to detect BAI-2 at micromolar levels.

The biosensing systems developed by Rajamani *et al.* and Zhu *et al.* exhibited a selective response to BAI-2, but the detection limits were not sufficient for their use in clinical samples, such as saliva and stool. Data obtained by us (this dissertation, chapter

3) and others^{119,217}, suggests that AI-2 is present in a sub-micromolar to low nanomolar range. Physicochemical methods, such as GC-MS¹²⁴ and LC-MS-MS¹¹⁹, have also been employed to detect DPD with limits of detection of 5.6 nM and 0.23 nM, respectively. However, DPD must be derivatized with the proper chemical moiety before analysis can be performed, negating the usefulness of these methods for direct quantitative detection without prior pre-treatment of any physiological sample.

In order to address the current need for systems that can detect QSMs in physiological samples in a quantitative manner, we designed and developed a fluorescence-based sensing system that takes advantage of the exquisite molecular recognition properties of the LuxP protein to its ligand, AI-2, coupled with the sensitive detection afforded by fluorescence resonance energy transfer methods. The design of our system is based on a combination of genetic fusion methods with site-selective incorporation of a fluorophore into selected the amino acids of LuxP. Specifically, we prepared a fusion protein between LuxP and enhanced green fluorescent protein (EGFP). This LuxP-EGFP fusion protein was capable of emitting fluorescent light upon excitation at 485 nm, and the fluorescence produced was not significantly altered by the presence of AI-2. This characteristic of the system inspired us to design a FRET biosensing system for AI-2 detection that involved labeling the LuxP part of the LuxP-EGFP fusion protein with a fluorophore. In particular we utilized the fluorophore 7-diethylamino-3-[*N*-(2-maleimidoethyl)carbamoyl]coumarin (MDCC) and the fusion of the LuxP protein with EGFP as the donor-acceptor pair for FRET. MDCC, when excited at 425 nm, transferred energy to EGFP in the LuxP-EGFP fusion protein, causing EGFP

excitation and consequent emission with a peak at 507 nm. Addition of BAI-2 caused a dose-dependent decrease in FRET, which translated in a lower fluorescence emission signal at 507 nm. The biosensing system developed was selective to AI-2 molecules, had detection limits in the sub-micromolar range, and its utility was validated by determining the concentration of AI-2 molecules in human saliva and blood serum.

Materials and Methods

Materials

Plasmid pGEX-4T-1-LuxP was kindly provided by Dr. Bonnie Bassler (Princeton University). Plasmid pEGFP and ExTaq DNA polymerase were purchased from Clontech (Mountain View, CA). Plasmid pET28a(+) was purchased from EMD Millipore (Billerica, MA). Chemically competent *Escherichia coli* cells BL21 (DE3) were purchased from American Type Culture Collection (Manassas, VA). Pfu DNA polymerase was purchased from Promega (Madison, WI). The TOPO TA cloning kit, MDCC, and Microcon YM-10 centrifugal device were purchased from Invitrogen (Carlsbad, CA). Restriction endonuclease enzymes, NheI and BamHI, Calf Intestinal Alkaline Phosphatase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). The QIAprep Miniprep DNA extraction kit and Nickel-NitriloTriAcetic acid (Ni-NTA) agarose were purchased from Qiagen (Valencia, CA). Primers were purchased from Operon (Huntsville, AL). AI-2 was purchased from Omm Scientific (Dallas, TX). Isopropyl- β -D-thiogalactoside (IPTG) was purchased from Gold Biotechnology (Houston, TX). The

Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Polystyrene petri dishes and culture tubes were purchased from BD Biosciences (Sparks, MD). Boric acid was purchased from Fisher Scientific (Pittsburgh, PA). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Matheson Scientific (Houston, TX). Luria Bertani (LB) media and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid (HEPES) were purchased from VWR International (Bridgeport, NJ). Ampicillin, kanamycin, and sodium chloride were purchased from Sigma (St. Louis, MO). All solutions were prepared using deionized reverse osmosis filtered water (Milli-Q Water Purification System, Millipore, Bedford, MA).

Apparatus

Cell cultures were grown using an orbital shaker incubator purchased from Forma Scientific (Marietta, OH). Cells were harvested using a Beckman J2-MI centrifuge (Palo Alto, CA). DNA amplification and overlap Polymerase Chain Reaction (PCR) reactions were performed using an Eppendorf Mastercycler personal thermal cycler (Hauppauge, NY). Bacterial colonies were grown on agar plates using an Isotemp incubator from Fisher Scientific (Pittsburgh, PA). Fluorescence studies were performed using a PTI Laserstrobe™ Fluorescence Lifetime Spectrofluorometer (Birmingham, NJ). The optical density of bacterial cultures was measured using a Spectronic 21D spectrophotometer (Artisan Scientific, Champaign, IL).

Isolation and cloning of DNA

The plasmids pGEX-4T-1-LuxP and pEGFP, respectively, were transformed and maintained in *E. coli* BL21 (DE3) cells. Cultures of pGEX-4T-1-LuxP and pEGFP containing cells were grown overnight in LB media supplemented with 100 µg/mL of ampicillin using an orbital shaker incubator set at 37 °C and 250 rpm. The DNA from each culture was harvested using the Qiagen DNA extraction kit. LuxP DNA was amplified from pGEX-4T-1-LuxP by PCR using primers 5'-GCTAGCACACAAGTTTTGAATGGGTACTGG-3' and 5'-TATTCAGATAATTCAGGTGGAGGTGGATCA-3'. EGFP DNA was amplified from pEGFP by PCR using primers 5'-TCAGGTGGAGGTGGATCAGTGAGCAAGGGC-3' and 5'-CTCGGCATGGACGAGCTGTACAAGGGATCC-3'. By using these primers, a site for the nuclease NheI was introduced at the beginning of the LuxP DNA sequence and a site for the nuclease BamHI was introduced at the end of the EGFP DNA sequence. An overlap PCR reaction was then carried out using the primers 5'-TATTCAGATAATTCAGGTGGAGGTGGATCA-3' and 5'-TCAGGTGGAGGTGGATCAGTGAGCAAGGGC-3'. A high fidelity Pfu polymerase was used for the above PCR reactions. The overlap PCR product was further incubated with ExTaq polymerase to introduce A (adenine) overhangs at the 3' terminus of the amplified DNA. The obtained DNA product was then cloned into the TA vector using a TA cloning kit. The plasmid ligation product was transformed and maintained in the TOP10F' cells supplied with the TA cloning kit. The primers used enabled creation of a fusion between the DNAs of LuxP and EGFP connected by a linker corresponding to the peptide Ser-Hys-Hys-Hys-Ser. Using the restriction enzymes, NheI and BamHI, the LuxP-EGFP DNA

was cloned into vector pET28a(+) to obtain plasmid pET28a(+)-LuxP-EGFP, which was transformed and maintained in *E. coli* BL21 (DE3) cells.

Purification of LuxP-EGFP fusion protein

A culture of *E. coli* BL21 (DE3) cells containing plasmid pET28a(+)-LuxP-EGFP was grown overnight in LB media supplemented with 30 µg/mL kanamycin using an orbital shaker incubator set at 37 °C and 250 rpm. A 4 mL volume of overnight grown culture was added to 500 mL of fresh LB media (30 µg/mL kanamycin) and allowed to grow at 37 °C, 250 rpm until an optical density (600 nm) of 0.40 to 0.50 was reached. The culture was moved to an orbital shaker incubator that was set at 16 °C, 250 rpm, and protein expression was induced by adding IPTG at a final concentration of 10 mM. The following day, the fusion protein was harvested and purified using a Ni-NTA resin drip column. The purified protein was analyzed using SDS-PAGE and dialyzed extensively (16 buffer changes over 2 days) using a buffer containing 50 mM HEPES and 150 mM NaCl (pH 7.0). The concentration of the purified protein was determined using the Bradford protein assay according to the manufacturer's instructions.

DNA and protein sequence analysis

DNA was isolated from a culture of *E. coli* BL21 (DE3) cells containing plasmid pET28a(+)-LuxP-EGFP using QIAprep miniprep kit and sent for sequencing at the University of Kentucky Advanced Genetic Technologies Center. The fusion protein was expressed, purified, and analyzed by SDS-PAGE, as reported above. The gel band of the protein was digested with trypsin, and LC-ESI-MS-MS analysis was performed using a

ThermoFinnigan LTQ linear ion trap instrument at the University of Kentucky Mass Spectrometry Facility. The resulting MS-MS spectra were searched in the Swiss-Prot protein sequence database using the Mascot search engine to confirm the presence of both proteins, LuxP and EGFP.

Method optimization for FRET signal detection

The fluorophore MDCC was dissolved in anhydrous dimethyl sulfoxide (DMSO) to obtain a 1×10^{-4} g/mL stock solution, which was serially diluted with 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) to obtain 1×10^{-5} to 1×10^{-9} g/mL MDCC solutions. LuxP-EGFP solutions, ranging from 1×10^{-6} M to 1×10^{-10} M, were prepared by serial dilution of the purified fusion protein solution using 50 mM HEPES, 150 mM NaCl buffer (pH 7.0). The excitation and emission peak wavelengths of MDCC were 425 nm and 475 nm, respectively. The excitation and emission peak wavelengths of EGFP in the fusion protein were 488 nm and 507 nm, respectively. The overlap between the emission spectrum of MDCC and absorption spectrum of the fusion protein, which contained EGFP, was determined by using a Varian Cary Eclipse Spectrophotometer. A 900 μ L volume of MDCC solution, at a fixed concentration, was incubated with 100 μ L of fusion protein solutions at various concentrations for 15 minutes. The obtained solutions were excited at 425 nm and the emission spectra were recorded from 450 nm to 600 nm. The optimal FRET, i.e., the maximum transfer of energy from MDCC to the fusion protein, as demonstrated by the emission intensity at 507 nm, was observed when 2.5×10^{-7} M

MDCC was used in combination with 5×10^{-9} M fusion protein. The resulting FRET donor-acceptor solution was referred to as FRET complex.

Dose-response curve using BAI-2

The FRET complex contained 90% (v/v) MDCC at 2.5×10^{-7} M concentration and 10% (v/v) fusion protein at 5×10^{-9} M concentration. A 1×10^{-3} M solution of boric acid was prepared in 50 mM HEPES, 150 mM NaCl buffer (pH 7.0). The commercially available AI-2 aqueous solution was serially diluted using the 1×10^{-3} M boric acid solution to obtain standards of the borate isomeric form (BAI-2) ranging in concentration from 1×10^{-4} M to 1×10^{-10} M. A 180 μ L volume of the FRET complex was incubated with 1.8 μ L of BAI-2 solutions at various concentrations in triplicate for a period of 3 minutes. The incubation was performed in a 300 μ L quartz microcuvette. The samples were then excited at 425 nm and the emission spectra were recorded from 450 nm to 600 nm. A 180 μ L volume of the FRET complex with 1.8 μ L volume of 1×10^{-3} M boric acid solution in 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) served as blank.

Selectivity studies

The compounds tested included AI-2, ascorbic acid, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (DMHF). AI-2 (3.7×10^{-3} M) and ascorbic acid (1×10^{-3} M) stock solutions were prepared in water, while DMHF stock solution (1×10^{-3} M) was prepared in ethanol. These stock solutions were serially diluted with 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) to obtain 1×10^{-4} M to 1×10^{-10} M solutions. A 1.8 μ L aliquot of each solution in triplicate was incubated with 180 μ L of FRET complex and fluorescence

measurements were performed as described above. A 1.8 μL volume of 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) served as blank.

Dose-response curves in saliva and blood serum

Saliva samples were obtained from healthy volunteers. Each saliva sample was centrifuged to remove debris and the supernatant was stored at $-80\text{ }^{\circ}\text{C}$ until needed. A pool of saliva was obtained by mixing equal volumes of individual saliva sample supernatants followed by 5 min of vortexing. BAI-2 standard solutions, ranging in concentration from $1 \times 10^{-3}\text{ M}$ to $1 \times 10^{-9}\text{ M}$, were prepared as described above. A 10 μL aliquot of each BAI-2 solution was added to 90 μL of pooled saliva to obtain spiked saliva samples ranging from $1 \times 10^{-4}\text{ M}$ to $1 \times 10^{-10}\text{ M}$ BAI-2 concentration. A 1.8 μL volume of the spiked saliva samples was incubated in triplicate with the FRET complex as described above. A 10 μL volume of $1 \times 10^{-3}\text{ M}$ boric acid solution in 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) was added to 90 μL of pooled saliva to serve as a blank.

Blood serum samples were obtained from healthy volunteers. A pool of serum was prepared by mixing equal volumes of individual serum samples followed by vigorous vortexing. A 10 μL aliquot of each BAI-2 solution, ranging in concentration from $1 \times 10^{-3}\text{ M}$ to $1 \times 10^{-9}\text{ M}$, was added to 90 μL of pooled serum to obtain spiked serum samples ranging from $1 \times 10^{-4}\text{ M}$ to $1 \times 10^{-9}\text{ M}$ BAI-2 concentration. A 1.8 μL volume of the spiked serum samples was incubated in triplicate with the FRET complex as described above. Because a serum matrix effect was observed, similar spiking studies were repeated by diluting the pooled serum 1:10, 1:25, 1:50, 1:75, and 1:100 with 50

mM HEPES, 150 mM NaCl buffer (pH 7.0). A 10 μL volume of 1×10^{-3} M boric acid solution in 50 mM HEPES, 150 mM NaCl buffer (pH 7.0) was added to 90 μL of undiluted and diluted pooled serum to serve as a blank.

Detection of AI-2 in saliva and blood serum samples

Saliva samples were obtained from healthy volunteers and processed as described above. A 1.8 μL volume of each saliva sample was added in triplicate to the FRET complex and fluorescence measurements were performed as described above. Serum samples were obtained from patients with inflammatory bowel disease (IBD) and diluted 1:100 using 50 mM HEPES, 150 mM NaCl buffer (pH 7.0). A 1.8 μL volume of each 1:100 diluted serum sample was added in triplicate to the FRET complex and fluorescence measurements were performed as described above. A total of 10 saliva and 10 serum samples were analyzed. A dose-response curve was included in each analytical run and data were plotted using GraphPad Prism 5. AI-2 concentrations in samples were calculated after interpolating the FRET ratio values and multiplying by the dilution factor.

Results and discussion

In this work, we designed and developed a biosensing system to detect the quorum sensing autoinducer-2, AI-2, class of molecules. The biosensing system was based on the principles of fluorescence resonance energy transfer, FRET, by employing a fluorophore attached to a recognition protein as the donor of a donor-acceptor pair where the acceptor is a fluorescent protein part of a LuxP-EGFP fusion protein. Specifically, MDCC is the donor and EGFP the acceptor. LuxP undergoes a conformational change upon binding BAI-2^{128,218}. The X-ray crystal structure analysis of LuxP without BAI-2, i.e., apoLuxP and with bound BAI-2, i.e., holoLuxP revealed differences in the protein conformation (Figure 2). Hence, we postulated that we could detect BAI-2 with high sensitivity and selectivity by employing an appropriate reporter system to monitor this conformational change.

EGFP and its variants are traditionally used as reporter elements in fusion protein constructs^{219,220}. Employing overlap PCR, the two genes, *luxP* and *egfp*, were joined together at the 3'-terminus of *luxP*. The two genes were separated by 18 bases that encoded for a six-amino acid peptide linker, to allow for enough flexibility so that BAI-2 binding to LuxP was not hindered by EGFP. In addition, to ease the purification process, nucleotides encoding for six-His residues to be employed as an affinity chromatography tag to bind a Ni-NTA resin column were incorporated at the N-terminus of the protein. The *luxP-egfp* construct was cloned into the pET28a(+) vector and placed under the control of a *lac* operon, which enabled overexpression of the fusion protein.

The use of the fluorophore MDCC, a coumarin derivative, as donor in the FRET system was based on our observation that coumarin caused an increase in the bioluminescence response of a *V. harveyi* MM32-based whole-cell biosensing system for AI-2 detection (Thesis Chapter 5). In this sensing system, the binding of AI-2 to the recognition element, LuxP, triggers a cascade of phosphorylation and dephosphorylation events that lead to the expression of the *luxCDABE* cassette and production of bioluminescence. One possible reason for the observed effect of coumarin is that it may act as an agonist and bind to the LuxP protein at the BAI-2 binding site or bind to another binding pocket on the protein. Therefore, we hypothesized that MDCC, which is structurally related to coumarin, may also bind to the LuxP protein. Additionally, since EGFP is connected to LuxP, it may be in close enough proximity to MDCC so that FRET between MDCC and EGFP may occur. Furthermore, MDCC was excited at 425 nm, and the emission spectrum was recorded. An absorption spectrum for the fusion protein LuxP-EGFP was also obtained. We confirmed that the emission of MDCC overlaps with the absorption of the fusion protein, thus deeming it suitable as a donor for use in our proposed FRET system (Figure 3).

In order to optimize the experimental conditions, various molar ratios of MDCC and fusion protein were tested; upon exciting at 425 nm, the emission intensities of MDCC at 475 nm and LuxP-EGFP at 507 nm were measured. The LuxP-EGFP construct alone exhibited background emission at 507 nm when excited at 425 nm; however, in the presence of MDCC, the emission intensity of the construct increased. Figure 4 shows the emission spectra from three different concentrations of the fusion protein, with and

without a fixed concentration of MDCC. An increase in emission intensity of the fusion protein was observed with increases in its concentration, both, in the presence and absence of MDCC. Protein concentrations greater than 5×10^{-8} M did not show any improvement in the FRET ratio, i.e., the ratio of the emission intensities of acceptor and donor. Further, we found that a solution of MDCC and fusion protein, at final concentrations of 2.5×10^{-7} M and 5×10^{-9} M, respectively, resulted in the highest FRET ratio. This solution of LuxP-EGFP and MDCC, which was used in all subsequent experiments, is referred to as “FRET complex”. Incubation times longer than 15 min did not improve the FRET ratio from the fusion protein-MDCC solution. Therefore, all experiments were performed by incubating the FRET complex for a period of 15 min.

The FRET complex was then incubated with solutions of BAI-2 at concentrations ranging from 1×10^{-5} M to 1×10^{-12} M. The BAI-2 standard solutions were prepared using 1×10^{-3} M boric acid in 50 mM HEPES, 150 mM NaCl buffer, pH 7.0. It has been previously reported that in the biosynthesis of BAI-2, a 4 equivalent amount of borate, with respect to the starting material *S*-ribosylhomocysteine, was sufficient to obtain a borate isomer of AI-2¹⁰⁴. Hence, we ensured that boric acid was in sufficient excess while preparing our BAI-2 standards. The presence of boric acid did not affect the pH of the solution²²¹, thus not affecting the properties of the fluorophore and the conformation of the LuxP-EGFP fusion. As shown in Figure 5, as the concentration of BAI-2 increased, the acceptor emission peak at 507 nm decreased, while the donor emission peak at 475 nm increased; this caused a dose-dependent decrease in the FRET ratio. The biosensing system developed herein allowed us to detect BAI-2 at sub-

nanomolar levels with a limit of detection (LOD) of 1×10^{-10} M, which is the lowest limit of detection reported for a LuxP protein-based biosensing system^{128,216}. The limit of detection was defined as the analyte concentration that produced a signal lower than the average of the blank minus three standard deviations of the blank. In addition, a dynamic range of five orders of magnitude and linear response over three orders of magnitude was observed (Figure 6).

To test our hypothesis, which postulates direct binding of MDCC to the LuxP protein in the FRET process followed by an observed FRET signal and not to a direct interaction of MDCC with EGFP, we performed experiments using MDCC and commercially available EGFP. While maintaining all the experimental conditions described above, various concentrations of EGFP were incubated with 2.5×10^{-7} M MDCC. When the solutions were excited at 425 nm, no changes in the emission intensity of EGFP were observed as compared to EGFP alone, thus showing that the EGFP emission intensity was independent of the presence of MDCC. This clearly indicates that there are no interactions taking place between MDCC and EGFP that could result in a FRET signal. Further, when standard solutions of BAI-2 were added to the solution containing MDCC and EGFP, no changes in the emission intensities of MDCC and EGFP were observed (data not shown). These results point to the requirement for MDCC to bind to LuxP in order to be in close proximity of EGFP for the energy transfer to occur.

One of the limitations of the previously reported LuxP protein-based sensors is their inability to respond to non-borate forms of AI-2^{128,216}. As noted in the introduction,

the BAI-2 precursor, DPD, generates various hydrated non-containing boron isomeric forms that exist in equilibrium. Rather recently, Globisch *et al.* reported the existence of an additional isomer of DPD²²², although currently, only two forms of AI-2 are known to take part in QS, namely BAI-2 in *V. harveyi* and R-THMF in *S. typhimurium*. Nevertheless, it is speculated that other isomeric forms might also be involved in the quorum sensing regulatory pathways of certain bacteria²²². To that end, we tested the ability of our sensing system to respond to the hydrated forms of DPD. A dose-response curve for DPD was obtained by incubating various concentrations of DPD with the sensing system. The dose-response curve had a dynamic range of over three orders of magnitude with a limit of detection of 1×10^{-9} M (Figure 6). The ability to detect both forms of AI-2 is significant as the sensor can be utilized in applications where samples might contain borate free as well as borated AI-2.

The selectivity of our protein biosensing system was evaluated by exposing it to (5R)-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (ascorbic acid) and 5-methyl-4-hydroxy-3(2H)-furanone (MHF). The reason for choosing furanone derivatives was because of literature reports claiming that furanones affect the AI-2 bacterial quorum sensing circuitry, although the mechanisms of action have not been elucidated^{195,223,224}. Solutions of these compounds at concentrations ranging from 1×10^{-5} M to 1×10^{-12} M, were incubated with the protein biosensing system and the potential emission due to FRET was observed. Ascorbic acid did not elicit a change in the concentration range tested. On the other hand, MHF induced a response in the protein biosensing system at concentrations of 1×10^{-6} M and 1×10^{-5} M. Due to solubility

constraints, higher concentrations of MHF could not be tested. Based on these observations, we conclude that the biosensing system is able to respond to the AI-2 class of compounds and to structurally related furanones. However, it is important to point out that the biosensing system responded to MHF at concentrations at least three orders of magnitude higher than that of AI-2 molecules. The response of the sensing system to various concentrations of BAI-2, DPD, MHF, and ascorbic acid is shown in Figure 6.

Next we explored the feasibility of employing our sensing system for the detection of AI-2 in saliva and blood serum. For that, we investigated potential matrix effects by creating pools of saliva and serum samples, respectively, and spiking them with various concentrations of BAI-2 to obtain dose-response curves in these matrices. As shown in Figure 7, the slope of the dose-response curve obtained in saliva was nearly identical to the slope of the dose-response curve obtained in buffer and both curves can be superimposed. Serum, on the other hand, caused a shift of over 25 nm in the MDCC emission peak. Therefore, serum dilution studies were performed that showed that 1:100 dilution was necessary in order to effectively eliminate the matrix effect mentioned above. As it can be seen from Figure 7, the dose-response curve in 1:100 diluted serum overlapped with the dose-response curve obtained in buffer.

We then employed the biosensing system to detect AI-2 molecules in individual saliva and serum samples. AI-2 was not detected in any of the 10 blood serum samples tested. These results conform to those of our previous study where, using a *V. harveyi*

MM32-based whole-cell biosensing system, we were not able to detect AI-2 in serum samples (this dissertation, chapter three). While it is possible that serum does not contain levels of AI-2 that are detectable by protein or whole-cell biosensing systems, physical-chemical methods¹¹⁹ may be employed to confirm the presence or absence of AI-2 in serum. On the other hand, three out of ten saliva samples showed distinct levels of AI-2, varying between approximately 1×10^{-8} M and 1×10^{-10} M (Table 1). The presence of AI-2 in saliva was also demonstrated in another study using a whole-cell biosensing system (this dissertation, Chapter 2). Analysis of additional samples is necessary to potentially establish the method developed herein as an analytical tool for detection of AI-2 to be employed in the diagnosis and monitoring of bacterial-related conditions.

Conclusion

We designed and developed a FRET protein-based sensing system for AI-2 detection, which employed the LuxP protein as the recognition element along with the fluorophore MDCC and the photoprotein EGFP, genetically fused to LuxP, as the FRET donor-acceptor pair. The biosensing system proved to be rapid, in that it required an incubation time with the analyte of only 3 min to obtain a dose-dependent response; this represents a significant improvement as compared to conventional physico-chemical methods. Furthermore, the protein biosensing system described in this work has a lower limit of detection for BAI-2 (1×10^{-10} M) than previously reported protein biosensing systems employing LuxP combined with synthetic fluorophores²¹⁶ (1×10^{-6}

M) or yellow and cyan fluorescent proteins as reporters¹²⁸ ($> 1 \times 10^{-8}$ M BAI-2). One of the main advantages of our biosensing system lies in its ability to respond to non-borate forms of AI-2. This broadens the scope of the biosensing system for use in applications where detection of non-borate forms of AI-2 is required; for instance, human pathogens, such as *S. typhimurium*, are known to employ non-borate forms of AI-2 for QS regulation. Detection of QSMs in clinical samples has attracted attention, especially in bacteria related conditions such as cystic fibrosis²⁵ and Crohn's disease¹⁷⁰. We demonstrated that the developed biosensing system can be applied to the detection of QS AI-2 molecules in saliva and blood serum. In conclusion, our FRET-based protein biosensing system offers rapid, sensitive, selective, and quantitative detection of AI-2 molecules, and has potential to be used in clinical applications.

Figure 1(A). Quorum sensing signaling molecules of *V. harveyi*. (a) HAI-1, (b) CAI-1, (c) BAI-2, and (d) NO. **(B)** Biosynthesis of BAI-2.

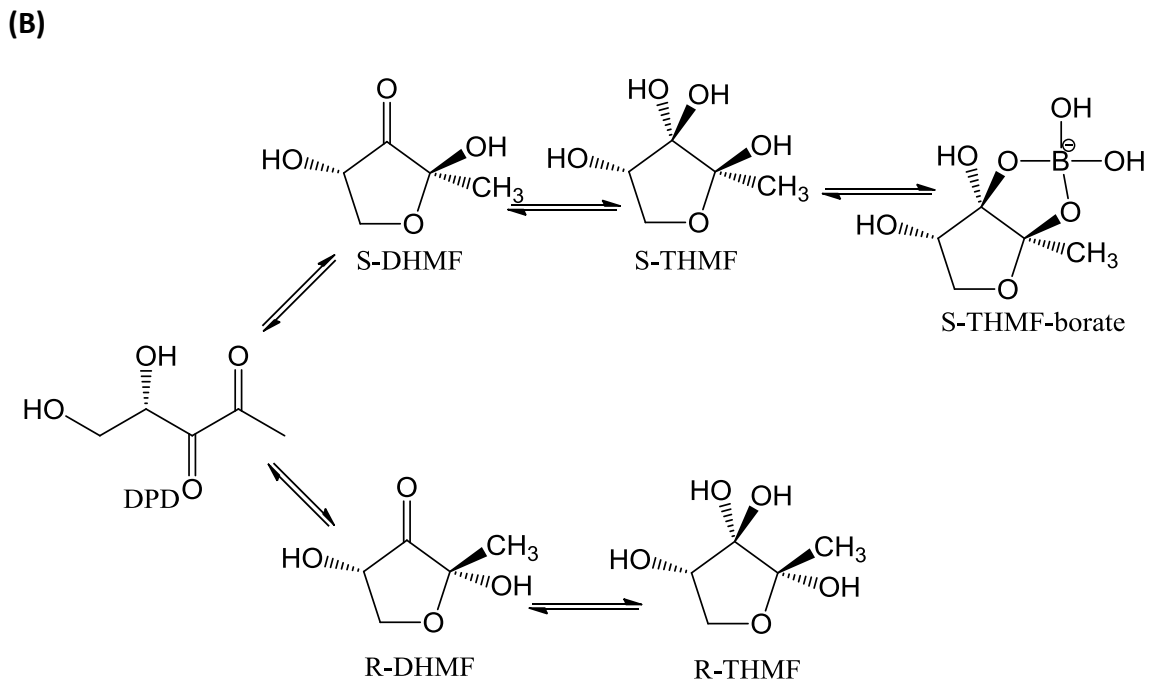
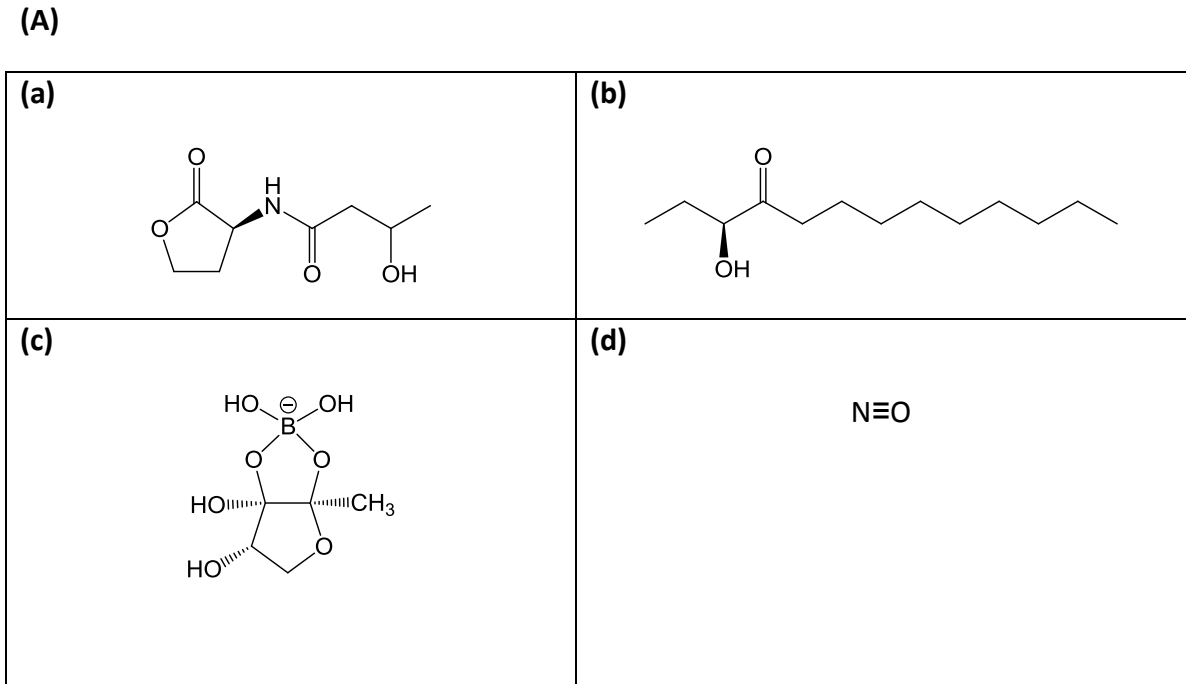


Figure 2. The 3-D crystal structures of apo-LuxP, without bound AI-2, (left) and holo-LuxP, with bound AI-2, (right). Protein data bank structures 1ZHH (apo-LuxP) and 2HJ9 (holo-LuxP).

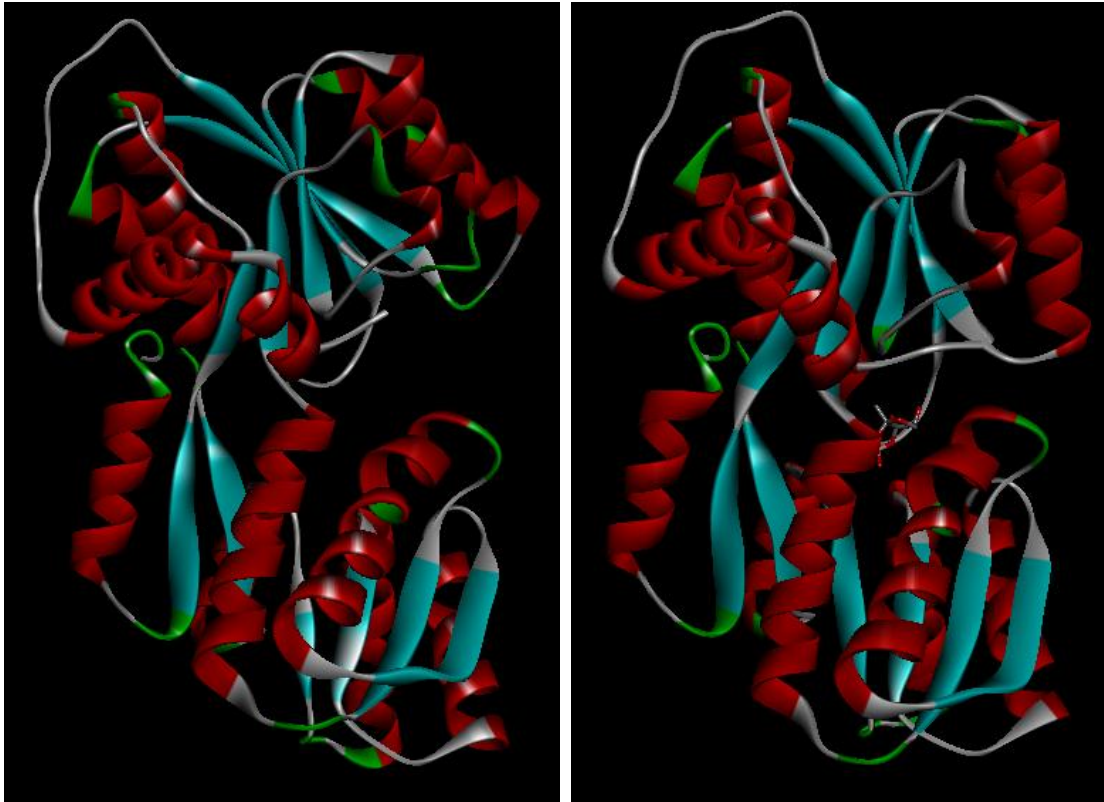


Figure 3. The emission spectrum of MDCC (blue) overlaps with the absorption spectrum of LuxP-EGFP (red).

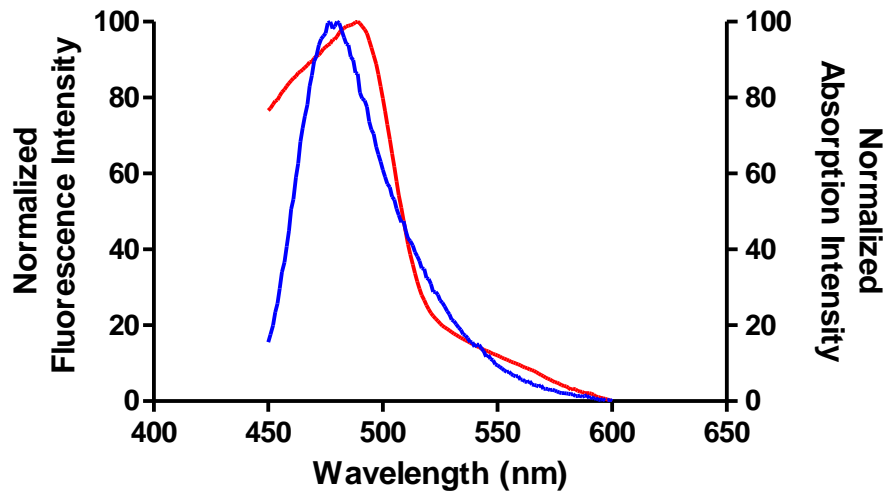


Figure 4. Emission spectra of the LuxP-EGFP fusion protein. LuxP-EGFP at 1×10^{-10} M (orange), 1×10^{-9} M (green), and 5×10^{-8} M (blue) concentrations in the presence of 2.5×10^{-7} M MDCC. LuxP-EGFP at 1×10^{-10} M (pink), 1×10^{-9} M (red), and 5×10^{-8} M (white) concentrations in the absence of MDCC. Samples were excited at 425 nm.

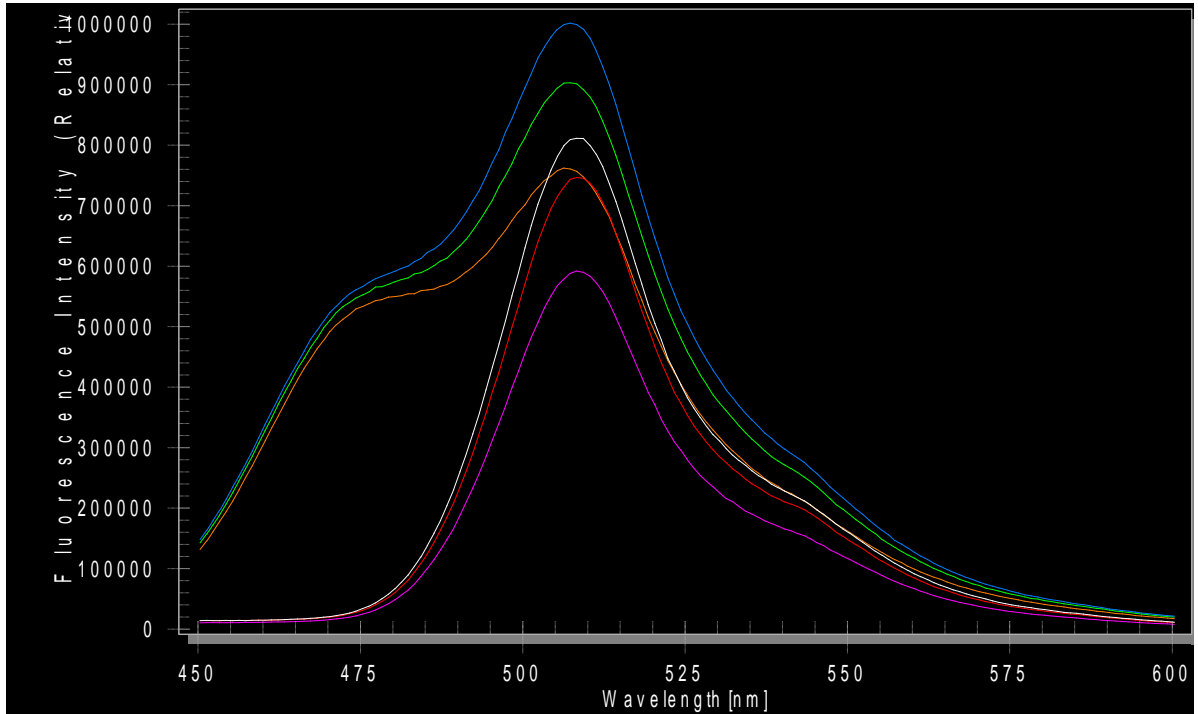


Figure 5. FRET response of construct to BAI-2. A decrease in FRET signal with increase in concentration of BAI-2 was observed. The solutions were excited at 425 nm; the emission peak wavelengths of MDCC and LuxP-EGFP are 475 nm and 507 nm, respectively. The FRET complex contained 2.5×10^{-7} M MDCC and 5×10^{-8} M LuxP-EGFP. BAI-2 concentrations were 1×10^{-5} M (red), 1×10^{-6} M (violet), 1×10^{-7} M (green), 1×10^{-8} M (yellow), 1×10^{-9} M (orange), 1×10^{-10} M (pink), and 1×10^{-11} M (blue). The FRET complex with 1×10^{-3} M boric acid (BAI-2 diluent) served as control (white).

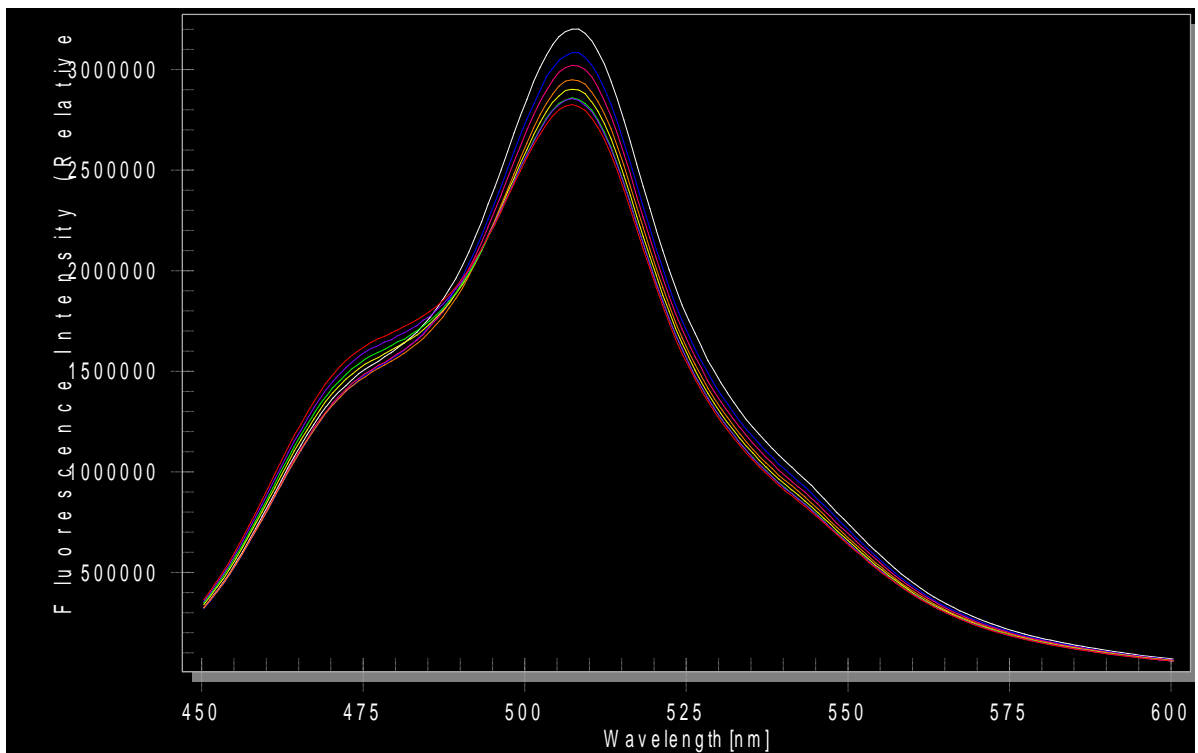


Figure 6. Dose-dependent response of the protein biosensing system to BAI-2 (red), DPD (blue), MHF (black), and ascorbic acid (green).

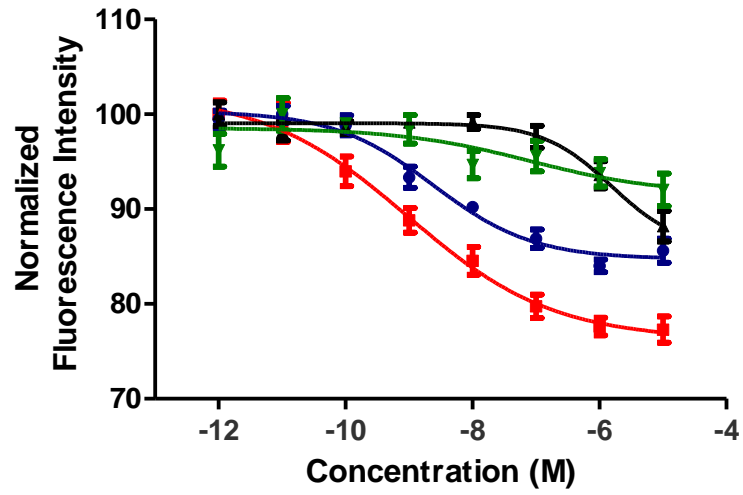


Figure 7. Comparison of dose-response curves obtained in buffer (red), undiluted saliva (blue), and 1: 100 diluted blood serum (black).

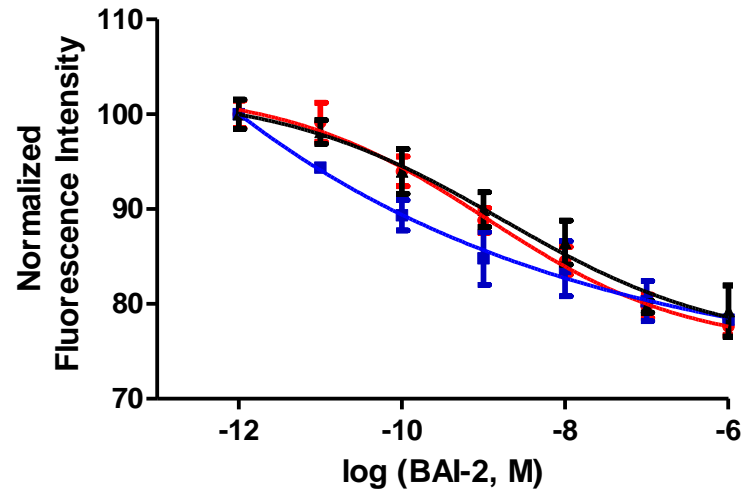


Table 1. Analysis of clinical samples using FRET based biosensing system.

Sample	AI-2 concentration (M)	%RSD
Saliva 1	2.5×10^{-10}	2
Saliva 2	4.2×10^{-10}	6
Saliva 3	6.9×10^{-9}	3

CHAPTER SEVEN

CONCLUSION AND FUTURE STUDIES

Similar to higher organisms, bacteria have the ability to communicate with each other. This phenomenon, termed quorum sensing (QS), enables bacteria to coordinate their behavior in a cell-density dependent manner. Bacteria synthesize small molecules, known as quorum sensing molecules (QSMs), which are transported out of the cell and can enter other bacteria. By monitoring these molecules, bacteria can monitor the cell-population density since the QSM concentration in the surrounding environment is proportional to the number of bacterial cells. At a certain population size, several genes that are under the control of QS regulation are expressed. In many species of bacteria, QS controls behaviors such as biofilm formation, antibiotic and virulence factor production, swarming motility, and sporulation.

Inflammatory Bowel Disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic gastrointestinal inflammatory condition with unknown etiology. The involvement of bacteria in IBD is supported by the identification of serological markers against microbial antigens and improvement in patient health after treatment by antibiotics. In addition, the bacterial flora has been shown to be different in healthy individuals and patients with IBD, with less diversity and modified relative abundance of species in IBD. Given that QSMs are bacterial products involved in the regulation of functions involved in bacterial pathogenicity, we hypothesized that monitoring of the levels of QSMs in physiological samples might provide information on

the bacterial load and on the status of the disease, thus offering a non-invasive diagnostic tool that can be used for management of IBD as well as other bacteria related conditions.

The first step towards achieving this goal is to develop tools and methods that can detect QSMs in physiological samples in a sensitive and selective manner. Physiological samples such as saliva, stool, and bowel secretions might contain QSMs at concentrations that represent their systemic level. To that end, we developed a method to detect QS autoinducer-2 (AI-2) molecules in these physiological samples by employing a *Vibrio harveyi* based biosensing system. Whole-cell biosensing systems offer several advantages; for instance, they can withstand a variety of environmental conditions (ionic strength of media, wide range of pH) and provide information about the analyte bioavailability. Due to their sensitivity and selectivity, whole-cell biosensing systems have been used in a variety of environmental and biomedical applications. The *V. harveyi* based whole-cell biosensing system developed in this work encompasses a QS regulatory system as recognition element and the bioluminescent *luxCDABE* gene cassette as reporter element. The biosensing system produces bioluminescence in response to the presence of AI-2 molecules. Most importantly, the bioluminescence produced is proportional to the amount of AI-2 present in the environment of the sensing cells. Therefore, by measuring bioluminescence we can quantitate the amount of AI-2 present in a sample. By employing the developed method, we obtained limits of detection of 25 nM and 50 nM in saliva and stool matrices, respectively. In addition, analysis of physiological samples obtained from IBD patients revealed the presence of

AI-2. Specifically, different levels of AI-2 were observed in stool samples from different individuals; this observation supports the hypothesis that changes in the microflora of the inflamed intestine may result in changes in the levels of AI-2. To our knowledge, this is the first attempt to quantify AI-2 levels in saliva, stool, and bowel secretion samples from IBD patients.

Furthermore, we investigated blood serum for the presence of QSMs. Saliva and stool samples are advantageous to use since they are collected non-invasively; however, blood samples only require minimally invasive collection and are commonly employed to detect a variety of biomarkers pertaining to several diseases. Additionally, blood is also routinely collected from IBD patients to determine various parameters, including inflammation status and presence of specific antibodies, and to differentiate between CD and UC. Since we were able to detect QSMs, both, *N*-acyl homoserine lactones (AHLs) and AI-2 in saliva and stool samples, we hypothesized that these molecules may also enter the blood stream. To that end, we developed analytical methods for the detection of QSMs in blood serum by utilizing whole-cell biosensing systems for AHL and AI-2 molecules, respectively. *E. coli* based whole-cell biosensing systems containing plasmid pSB406 or pSB1075 were employed to detect short and long chain AHL molecules, respectively, while a *V. harveyi* based biosensing system was used to detect AI-2 molecules. The developed methods allowed us to achieve nanomolar limits of detection in serum with all three sensors. Most importantly, the analysis of blood serum samples from IBD patients and controls revealed differing levels of short-chain AHLs. Serum samples of healthy individuals exhibited undetectable levels of short chain AHLs,

while IBD samples showed the presence of short chain AHLs with slightly higher levels in UC patients as compared to CD patients. Since no previous reports existed that could support the presence of AHLs in serum, we deemed it necessary to employ a conventional analytical method to validate our findings. To that end, we confirmed the presence of AHLs in blood serum by analyzing pooled serum samples using high performance liquid chromatography coupled with tandem mass spectrometry. Furthermore, when we employed a mice model of colitis, which is representative of human IBD, we detected a difference between the levels of AHLs in, both, serum and stool samples of colitic mice and control mice. Although a limited number of mice were used, this observation supports our hypothesis that microflora perturbations caused by acute inflammation can lead to changes in the levels of QSMs. These findings can serve as a basis for further studies aimed at proving the use of QSM detection as a diagnostic and monitoring tool for IBD as well as other bacteria-related conditions.

In addition to whole-cell based biosensing systems for QSM detection, we also developed a protein based biosensing system to detect AI-2 molecules. In wild type *V. harveyi*, the borated form of AI-2 (BAI-2) binds to the periplasmic binding protein LuxP, which undergoes a change in conformation upon binding. By using techniques of molecular cloning, we fused *egfp*, coding for the enhanced green fluorescent protein (EGFP), to the C-terminus of *luxP*. The LuxP in the LuxP-EGFP fusion protein is able to bind to 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC), an environmentally sensitive fluorophore. Based on the emission and absorption spectra of MDCC and EGFP, we developed a protein biosensing system, LuxP-EGFP-MDCC,

exploiting the principle of Förster Resonance Energy Transfer (FRET). In this construct, energy is transferred from MDCC to EGFP. The presence of BAI-2, which is the cognate molecule of LuxP, causes a decrease in the FRET ratio. Using this system, we achieved a limit of detection of 0.1 nM, the lowest obtained using LuxP protein based sensing systems and lower than that afforded by the developed whole-cell based biosensing system. Additionally, the sensor is selective to the AI-2 class of molecules and is able to respond to, both, BAI-2 and non-borated forms of AI-2, thus widening the sensor's utility for applications where AI-2 as well as BAI-2 molecules might be present. Furthermore, the protein biosensing system was characterized and optimized for detecting AI-2 molecules in physiological samples such as saliva and blood serum.

In addition to disease detection, monitoring of QSMs can also prove useful for early detection of food contamination and, thus, prevention of foodborne illnesses. Bacteria are known to thrive on a variety of food surfaces. Moreover, certain foodborne pathogens are known to employ QS regulation and QSMs have been found in various foods, including milk and meat products. Therefore, detecting QSMs in food samples can provide a convenient method for early detection of food spoilage. To that end, we developed methods to detect QSMs in food matrices by employing whole-cell biosensing systems that can detect AHLs and AI-2 molecules. We selected ground beef and milk as model foods. We showed that QSMs can be detected in these foods with minimal sample processing, while maintaining the analytical parameters of the sensing systems. In addition, we applied the sensing systems to detect QSMs produced in

artificially spoiled food samples. This further corroborates that whole-cell biosensing systems can be applied to detect food spoilage.

Finally, we tested a number of compounds originating from Generally Recognized As Safe (GRAS) substances for their effect on QS. These compounds were previously found to act as antibacterials, although their mechanisms of action have not been elucidated. For that, we tested these compounds to evaluate whether they may act through interference with bacterial quorum sensing. Such compounds were incubated with whole-cell sensing systems alone and in the presence of QSMs. This allowed us to assess their ability to induce a response in the sensing systems, as well as to interfere with the bacterial sensing systems response to QSMs. Selected compounds were also incubated with bacteria that produce QSMs to evaluate their effect on QSM synthesis. Our study identified four such compounds, namely ascorbic acid, t-cinnamic acid, tannic acid, and coumarin that affected bacterial QS.

As research at the molecular level in the fields of biology and microbiology continues, novel features of bacteria are revealed. QS is one such feature of bacteria that has been discovered and studied extensively only over the past two decades. Given the close association between bacteria and humans, it is imperative that we enhance our understanding of QS. We believe that the ability to detect QSMs represents a step towards understanding the intricate workings of bacteria in the human body. While we lay the foundation, additional work is necessary to illustrate that QSM detection can be utilized as a diagnostic tool. This would involve analysis of large numbers of clinical

samples, such as saliva, stool, bowel secretions, and blood serum, as well as tissue specimens to ascertain that the levels of QSMs correlate with the status of the disease.

While in the present work we mainly focused on IBD, in principle, these biosensing systems could also be used to detect QSMs in other bacteria-related conditions. Furthermore, the developed cell and protein sensing systems could be immobilized on optical fibers or incorporated into microfluidic platforms to generate portable analytical devices. This would enable the sensing systems to be used in on-site applications, such as, in the physician's office and at the patient's bedside in hospitals.

Further enhancements in the protein-based sensing system can be brought about by employing a variety of approaches. For instance, the length of the linker between the two proteins in the fusion can be changed. A linker length of six amino acids was employed in the protein construct described in this work; varying the linker length may further improve the sensor's limit of detection for AI-2 molecules. Additionally, the selectivity of the sensor could be tailored in order to enable the recognition of individual forms of AI-2. In that regard, it is possible to alter the protein construct by employing other variants of the GFP protein, which would facilitate multiplex detection.

QS biosensing systems are ideal for the screening of synthetic as well as natural compounds. The list of such compounds is extensive; while we tested a few compounds originating from GRAS substances, many other compounds have shown antibacterial properties and should be evaluated for their effect on QS. The compounds found to

affect QS could then be studied in animal models to investigate their effects *in vivo*. This could be achieved by feeding the animals with a known amount of the QS interfering compound for a period of time and then comparing the QSMs levels found in various specimens of the treated animals with those of control animals. Bacterial involvement in conditions such as IBD is supported by improvement in patient health due to antibiotic treatment. It would be interesting to study QS interfering compounds, alone and in combination with antibiotics, to determine if they are effective in treating bacterial conditions. This hypothesis could also be tested using animal models and evaluating a number of circulating molecules and tissue parameters.

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Patent:

1. Daunert, Sylvia; Deo, Sapna K.; Pasini, Patrizia; Kumari, Anjali; Shashidhar, Harohalli; Auer Flomenhof, Deborah R; **Raut, Nilesh**. *Systems and methods for diagnosis and monitoring of bacteria-related conditions*. WO/2009/036081

Publications:

1. **Raut, N. G.**; Pasini, P; Joel, Smita; Kumar, Manoj, Daunert, S. "Breaking into the bacterial quorum sensing circuits: detection of the autoinducer-2 via a fluorescence biosensing system" To be submitted 2012.

2. **Raut, N. G.**; Pasini, P; Daunert, S. "Whole-cell sensing system for detection of the quorum sensing universal signal autoinducer-2 in physiological samples." To be submitted 2012.
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6. Struss, A.; Pasini, P.; Ensor, M., C.; **Raut, N.**; Daunert, S. "Paper Strip Whole Cell Biosensors: A Portable Test for the Semiquantitative Detection of Bacterial Quorum Signaling Molecules." *Analytical Chemistry* (2010), 82(11), 4457-4463.
7. Turner, K; **Raut, N.**; Pasini, P.; Daunert, S; Michelini, E.; Cevenini, L.; Mezzanotte, L.; Roda, A. "Cell-based bioluminescent biosensors." *Chemiluminescence and Bioluminescence: Past, Present and Future*. Royal Society of Chemistry. Chapter 15, 514-545.
8. Flomenhoft, D. A.; Ballard H. O.; Hardin, G.; Shashidhar, H.; Paranjape, S.; Struss, A.; **Raut, N.G.**; Subbaswami, A.; Pasini, P.; Daunert, S. "Evaluation of acyl

homoserine lactone activity in the stool of low birthweight infants at risk for necrotizing enterocolitis.”

Notable presentations:

1. Poster presentation at 240th national meeting of American Chemical Society, August 22-26, 2010, Boston, MA, United States. “Biosensing systems for the detection of quorum sensing signaling molecules in clinical samples.” **Raut, Nilesh G.**; Pasini, Patrizia; Harohalli, Shashidhar; Auer Flomenhof, Deborah R.; Daunert, Sylvia.
2. Poster presentation at 238th national meeting of American Chemical Society, August 16-20, 2009, Washington, DC, United States. “Detection of bacterial quorum sensing signaling molecules on a compact disk microfluidic platform employing whole-cell-based biosensing system.” **Raut, Nilesh G.**; Pasini, Patrizia; Daunert, Sylvia.
3. Poster presentation at 39th Central Regional Meeting of the American Chemical Society, May 20-23, 2007, Covington, KY, United States. “Non-Invasive Quorum-Based Biosensors for the Evaluation of Bacterial Behavior in GI disorders.” Kumari, Anjali; Pasini, Patrizia; Flomenhoft, Deborah A.; Shashidhar, Harohadli; **Raut, Nilesh**; Daunert, Sylvia.

4. Poster presentation at PITTCON 2009 conference and expo, March 8-13, Chicago, IL, United States. "Whole Cell Based Biosensing System for the Detection of Autoinducer-2 in IBD Patient Samples." **Raut, Nilesh**; Pasini, Patrizia; Deborah A. Flomenhoft; H. Shashidhar; Daunert, Sylvia.