INFLUENCE OF AUTOINDUCER 2 (AI-2) AND AI-2 INHIBITORS GENERATED FROM PROCESSED POULTRY ON VIRULENCE AND GROWTH OF *Salmonella enterica* SEROVAR TYPHIMURIUM

A Dissertation

by

KENNETH W. WIDMER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Food Science and Technology

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May 2007

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ABSTRACT

Influence of Autoinducer 2 (AI-2) and AI-2 Inhibitors Generated from Processed

Poultry on Virulence and Growth of *Salmonella enterica* Serovar Typhimurium.

(May 2007)

Kenneth W. Widmer, B.S., The University of Texas, El Paso;

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Bacteria produce and respond to external stimuli using molecules termed
autoinducers. Poultry meat contains inhibitors which interfere with AI-2
signaling. The primary objective of this work was to understand the effects of
AI-2 on the virulence and growth of *Salmonella* Typhimurium, and if the
introduction of AI-2 inhibiting compounds would influence these effects.

Using DNA microarray analysis, expression of 1136 virulence-related
genes in a *Salmonella* Typhimurium wild type and a *luxS* mutant strain, PJ002
(unable to produce AI-2), was monitored after exposure to treatments
containing *in vitro* synthesized AI-2 (AI-2) and poultry meat (PM) inhibitors.
Responding gene expression was unique in the presence of AI-2, with 23 genes
differentially expressed at least 1.5-fold ($p < 0.05$). The combined AI-2 + PM
treatment resulted in 22 genes being differentially expressed. Identification of
inhibitory compounds was attempted using GC analysis on a hexane solvent
extract obtained from a PM wash. From this analysis, chemical standards of

linoleic, oleic, palmitic, and stearic acid were tested for inhibition using *V. harveyi* BB170. Combined fatty acids (FA) demonstrated inhibition against AI-2 at 60 % while 10-fold and 100-fold concentrations had inhibition of 84 % and 70 %, respectively. Growth of PJ002, was studied using M-9 minimal medium with FA of varying concentrations, supplemented with either AI-2, or 1X phosphate buffered saline (PBS). Comparative analysis was done calculating the growth constants based on OD 600 values for each treatment. No significant difference in the combined FA + AI-2 treatments was observed against the AI-2 treatment. A significant increase in the growth rate constants of the AI-2 treatments was observed, however, compared to the PBS control (P = 0.01). Bacterial invasiveness, using a murine macrophage cell line, RAW 264.7, was also studied. AI-2 decreased cell invasiveness (P = 0.02), while the addition of combined FA improved invasiveness to normal levels. The results of these studies indicate that AI-2 does have an effect on the growth and virulence of *Salmonella*, but this is not uniformly modulated by the introduction of fatty acids, that inhibit AI-2 activity, suggesting that inhibition may be based on species specific transport systems.

DEDICATION

To my family.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Quorum sensing is a term that describes the overall processes of the production, secretion, and uptake of chemical signal molecules that bacteria may utilize for interspecies communication (94, 133, 148). There has been an indication that these quorum sensing molecules have some influence on virulence gene expression (22, 31, 34, 98, 105, 127). Additionally, because of the potential role quorum sensing may have on bacterial virulence, some species may utilize these signals for not only inter-species, but also intra-species, communication (38). Hence, the interaction of eukaryotic cells with bacterial pathogens, primarily by interfering with quorum sensing processes, may provide some understanding to host-pathogen interactions (25, 95).

The role of quorum sensing in of foods has not been well established. Bacteria can produce quorum sensing signals in certain food matrixes (17), but the direct role of these signals for survival, or ability to colonize, on foods is not well understood (14). A particular characteristic of some food matrixes is the ability to impede these quorum sensing signals (85). The identification of these compounds which impede quorum sensing may help illuminate how certain foods may promote or retard bacterial proliferation, and potentially enhance pathogen survival.

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This dissertation follows the style of Applied and Environmental Microbiology.

Relevance of Research

Salmonella Typhimurium is a human pathogen that is associated with foods.

Understanding the role of quorum sensing in relation to survival and virulence

gene expression in *Salmonella* may illuminate how this pathogen interacts with

bacterial flora on certain foods, and how the organism may adopt strategies to

persist in food matrices. Additionally, as certain foods contain inhibitors to

quorum sensing signals, these inhibitors may provide a means to control

Salmonella in foods. Or conversely, if the interaction of these inhibitors with

quorum sensing molecules inadvertently promotes survival, this interaction may

indicate why *Salmonella* may be a prevalent pathogen with specific foods.

Rationale

Autoinducer 2 (AI-2) is a molecule that plays a role in bacterial communication

in Gram negative bacteria. AI-2 and similar quorum sensing signals appear to

have a role in gene expression for different species of bacteria including

Enterobacteriaceae (22, 31, 34, 96, 103, 125). Poultry meat (and various other

food matrices) apparently possess inhibitors of these signals and by interfering

with quorum sensing, can modulate the effects of AI-2 (83). The organism that

was employed for this study was *Salmonella enterica* serovar Typhimurium.

This organism is a common foodborne pathogen and is associated with poultry

products (20, 21, 43, 97).

Because this organism can produce AI-2, the construction of a mutant

incapable of producing this signaling compound was needed, as it would have

been difficult to ascertain the role AI-2 has on survival and virulence. A mutant with a deletion of the *luxS* gene was employed. Previous studies have shown the deletion of this gene would not seriously affect cellular functions such as growth (128, 130, 140, 141, 146).

To measure AI-2 activity, the reporter strain *Vibrio harveyi* BB170 was employed. The *V. harveyi* wild-type strain is capable of up responding to both autoinducer-1 (AI-1) and autoinducer 2 (AI-2) in the environment. *V. harveyi* BB170 (a constructed strain that has a *luxN* deletion) was used for the AI-2 activity experiments, as this reporter strain is incapable of detecting autoinducer-1 (128, 130). Additionally since, AI-2 induces bioluminescence in *V. harveyi* AI-2 or AI-2-like activity was easily quantifiable and inhibition could readily be determined (83).

Experiments to determine the influence of AI-2 on *Salmonella* virulence were performed using cDNA microarrays. Previous research indicated that AI-2 had some impact on virulence gene expression in *E. coli* (34, 125) and, more importantly, the introduction of furanone inhibitors could modulate the expression of AI-2-induced genes (112). By using microarray analysis, it would be expected that potential targets for further analysis could quickly be determined if sufficient differences in expression could be observed. To understand the impact of inhibitors on AI-2 gene expression, washes from poultry meat were used (PM). The inhibition of these compounds had consistently demonstrated inhibition and served as a model for determining

their influence on *Salmonella* within in a poultry meat matrix. Additionally, to date the effects of AI-2 on the gene expression in *Salmonella* using microarray techniques has not been studied.

Although poultry meat washes were initially employed for the microarray experiments, it was necessary to better characterize these inhibitors. Since *in vitro* synthesized AI-2 was available, controlled experiments could be designed to understand the interaction of poultry meat inhibitors on cell signaling using purified inhibitor compounds. Although the poultry meat washes consistently expressed inhibition, there were likely also several chemical compounds that were present in these preparations that could have prevented accurate interpretation of the results. Hence it was important to purify the poultry meat derived inhibitor compounds.

Although the microarray work presented some insight to the effects of quorum sensing on *Salmonella* gene expression additional experiments were designed to determine the actual impact these signaling compounds had on virulence and survival. Two primary characteristics were investigated for *Salmonella*, namely growth and infection. The growth experiments were conducted using M-9 minimal medium supplemented with glucose. By using a defined minimal medium, it was theorized that the influence of AI-2 (with and without purified inhibitor) on the growth of *Salmonella* could be more easily discernable.

Salmonella infection assays were employed using a murine macrophage cell line, RAW 264.7 (previous published studies have used this cell line for determining changes in virulence for *Salmonella* (23, 118)). The assay employed in this work was very similar to other studies investigating *Salmonella* infection of eukaryotic cell lines (save for using different cell lines) (78, 106). Another advantage was that the assay was relatively simple and cost-effective to conduct and interpret (by enumerating plate counts based pre and post-infection numbers). Since the infection of macrophages is considered to be a critical element in the pathogenicity of *Salmonella,* this cell line served as an effective indicator that changes in virulence would be a function of the applied experimental treatments (13, 47, 56, 62, 63).

The hypothesis of this work was that AI-2 affects the virulence and survival of *Salmonella* Typhimurium, and the introduction of AI-2 inhibitory factors result in altered virulence and survival profiles.

Primary Objective

The primary objective of this work was to understand the effects of AI-2 on the virulence and survival of *Salmonella* Typhimurium, and determine if these effects can be varied by the introduction of AI-2 inhibiting compounds obtained from processed poultry meat.

Specific Objectives

1.   Determine the effect AI-2 and AI-2 inhibitors have on virulence gene expression in *Salmonella enterica* serotype Typhimurium.

2.   Characterize the compounds present in the poultry meat which

demonstrate inhibition to AI-2 activity.

3.   Determine the influence AI-2 and AI-2 inhibitors have on the growth of *S.*

Typhimurium.

4.   Determine the effects AI-2 and AI-2 inhibitors have on the virulence of *S.*

Typhimurium by using macrophage cell lines.

CHAPTER II

LITERATURE REVIEW

Morphology and Pathogenicity of *Salmonella enterica* serovar

Typhimurium

Salmonella is a Gram negative, flagellated rod and a facultative intracellular bacterial pathogen. The current classification scheme of *Salmonella* is based on two species, *bongori* and *enterica*, where *enterica* is further classified by six subspecies, I-VI (15). The Centers for Disease Control and Prevention further designate subspecies *enterica* I by serotypes, one of which is serovar Typhimurium (15). *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) has a wide variety of mammalian and avian hosts (107) and can survive in non-host environmental sources for prolonged periods (3, 145).

The primary route of infection of *Salmonella* is through the fecal oral route, where the host ingests the bacteria. Virulence of this organism is based on three primary functions, the ability to invade intestinal epithelial and macrophage cells, initiate an inflammatory response resulting in fluid secretion leading to diarrhea, and the ability to replicate and survive within host cells (62, 115, 152). Pathogenicity of Salmonella is primarily due to the function of several clusters of genes, defined as *Salmonella* Pathogenicity Islands (SPI). Two of these clusters have particular importance in *Salmonella*, defined as pathogenicity islands 1 and 2 (SPI1 and SPI2, respectively) (78, 115, 152). SPI1 is responsible primarily for encoding a type III secretion system (type III SS)

responsible for delivering effector proteins into host enterocytes and also aids in initiating an inflammatory response by the host cell. SPI-2 encodes another type III SS, which appears responsible for secreting intracellular effector proteins required for survival and replication once within the host (47, 48, 56, 63, 78, 82, 153).

Salmonella Typhimurium is able to enter the intestinal epithelial cells by an amazing ability to rearrange the host cytoskeleton. A 40-kb cluster of genes within SPI-1 encodes proteins that forms the type III secretion system, and is generally referred to as a needle structure. The structure itself is a complex arrangement of more than 20 proteins, and is comprised of three main components. The first component forms the needle base, comprised of an inner membrane and outer membrane structure. The inner membrane structure is comprised of two proteins, PrgH and PrgK, while the outer membrane is comprised of InvG (29, 47, 49, 74, 76). The second major component is a set of proteins that adhere to PrgH and serve as a complex to facilitate transport of invasion proteins through the needle complex. These proteins include of InvA (likely an ATPase transport protein), SpaP, SpaQ, SpaR, and SpaS; however, the precise conformational interaction with the needle complex is unknown (29, 47, 49, 74, 76). The final component is the rather interesting protein, PrgI, which forms the 'needle' of the system used for delivering virulence proteins within the host. This basic protein is suspected to have a structural orientation like flagellar

proteins and form a series of coils that fold over into a larger coiled confirmation forming an hollow channel (74).

The main function of this needle complex is to deliver a series of effector proteins to the host cytoplasm after bacterial attachment. These proteins induce a remarkable rearrangement of the host's cytoskeleton, which initiates the engulfing of the bacterium within the host. The rearrangement of the actin cytoskeleton is done through a series of at least 19 proteins by direct and indirect means (29, 82, 153). Indirectly, the cytoskeleton is altered utilizing GTPases within the host cell, primarily Cdc42 and Rac1. These proteins have regions that interact with GTP and, once bound, initiate other signaling molecules to begin sequences for altering the actin cytoskeleton (49, 74, 76, 81). SopE and SopE2 are *Salmonella* proteins which directly interact with Cdc42 initiating a conformational change making the GTP binding site more readily accessible. Another *Salmonella* protein that aids in cytoskeleton rearrangement is SopB, which modulates metabolism of phosphoinositide, indirectly altering Cdc42 activity (49, 74, 76, 81). The rearrangement of the actin cytoskeleton is done directly by a series of proteins which interact with the cytoskeleton, mainly proteins, SipC and SipA. SipA is directly involved with binding to actin enhancing bundling. The other protein, SipC is a protein that binds to the host membrane and functions primarily to assist in the transfer of invasion proteins within the host (49, 74, 76, 81). The result of this alteration in the host cytoskeleton is a 'ruffling' of the host cell wall and enveloping the *Salmonella*

bacterium in a form of phagocytosis (49, 81, 82, 115, 153).   Once enveloped, the host's cell wall is altered again to its original conformation using the same type III secretion system. SptP is secreted by the bacteria into the host cell cytosol, reversing the altered cytoskeleton to its normal state by primarily interacting with Rac1 (76, 81, 115). The infection of host epithelial tissue can be very rapid, and some studies have found the infection of mouse epithelial tissue by *Salmonella* Typhimurium can occur in as little as 40 seconds (115).

Once the bacteria enters the host cell, the vacuole will migrate to the basal section of the epithelial tissue. Additionally, the type III SS encoded in SPI1 also induces a cascade response resulting in an inflammatory response from the host cell and fluid accumulation (29, 47, 56, 82, 153). A primary protein from *Salmonella* responsible for initiating the inflammatory response is SipB. This protein activates caspase 1 in the host, initiating activity of interleukin-1β (IL-1β) (28, 30, 39, 152). Additionally, the host cell responds to invasion by producing a chemokine, interleukin-8 (IL-8) however the precise series of events initiating this is not currently known. These chemokines initiate an inflammatory response in the host, which attracts neutrophils and macrophages to the infected host tissue (28, 30, 39, 152).   Fluid accumulation and inflammatory response in the host is also mediated to a lesser degree by SipA, SopA, SopB, SopD, and SopE2. As some of these proteins are responsible for altering the cytoskeleton of the host, it has been suggested that the initiation of the inflammatory response

may be also attributed to the increased activity of host GTPases, Cdc42 and Rac1 (29, 47, 56, 82, 152, 153).

One response of the host, induced by IL-1β and IL-8, results in macrophage phagocytosis of *Salmonella*. Survival within macrophages is a key feature of *Salmonella* infection as the organism must be able to survive in a low pH, nutrient limited environment and be able to replicate within these phagosomes (13, 47, 56, 62, 63). The type III SS encoded in SPI-2 is theorized to be primarily responsible for encoding these genes required for intracellular survival, however the precise functions of these genes and their roles are not well known as of yet (47, 56, 62, 63). Some proteins have had their function determined, such as SSeB, SSeC, and SSeD which act as trans-locational proteins responsible for trafficking other pathogenic effector proteins (47, 56, 62). There have been some proteins identified outside of SPI-2 that are required for macrophage survival. One such protein is MgtC, whose function is to aid in *Salmonella*'s ability to replicate and survive in low Mg^{2+} conditions (13, 101). Another is SodA, a superoxide dismutase that is able to reduce the effectiveness of oxidated compounds released in phagosomes and improve survival of *Salmonella* under these conditions (137).

Once inside the macrophage, apoptosis of the macrophage occurs primarily through the production of the protein, SipB, by *Salmonella*. This protein binds, and activates, capase-1, which initiates macrophage apoptosis (47, 115, 152). As macrophage apoptosis occurs, an inflammatory response is

heightened. Neutrophils pass through the epithelial layer, and eventually (as the inflammatory response continues) the epithelial monolayer detaches causing fluid to enter the intestinal lumen resulting in diarrhea (47, 115, 152). In this nutrient rich environment, *Salmonella* can effectively propagate its numbers and shed in feces from the host (56, 115). *S.* Typhimurium may also initiate a more severe systemic infection in the host. As the bacteria are able to survive in macrophages, it is possible that the bacteria can spread to the liver and spleen (13, 86). Additionally, some strains of *Salmonella* Typhimurium are able to produce hemolytic exotoxins, and can initiate inflammatory responses in these host tissues by the exotoxin, Lipid A, causing further tissue damage (86, 138).

The regulation of these invasion genes is modulated by several regulatory factors closely associated with SPI1 and 2 and some global regulatory circuits. For SPI1, a common hyperinvasion locus (*hil*) was determined to be required for effective *Salmonella* infection in epithelial cells (78). A major transcriptional regulator of several genes required for successful invasion is encoded in this region. The protein, HilA has a transcriptional activation domain similar to a family of OmpR/ToxR transcriptional regulators and is required for the expression of three genes required for successful invasion, *invF*, *sipC*, and *prg*, where both *invF* and *prg* appear to be transcriptional promoters for other genes within SPI1 (7, 8, 47). There are other genes associated with *hilA* that work in concert, and independently, to promote gene expression of SPI1, *hilC* and *hilD* (2, 56). What is of particular interest with the activity of these genes is that their

expression can be affected by a variety of environmental conditions, including pH, osmotic potential, and oxygen availability (4, 8, 72, 86). Additionally, other signal systems appear to influence the expression of many of these transcriptional factors. One such system is the PhoP-PhoQ system that can influence *hilA* expression. PhoQ acts a sensor kinase sensitive to magnesium levels and can selectively phosphorylate PhoP, which in turn, serves as a DNA-binding protein that actively promotes several genes (up to 40) (58, 120, 121). Another global regulator that can influence type III SS expression is a DNA-binding factor for inversion stimulation (fis) and this appears to have an impact on *hilA* expression (73). It is unusual that several systems and environmental conditions appear to promote *Salmonella* virulence in an intricate, almost convoluted manner. However it has been suggested that the combination of these events provide subtle clues to how *Salmonella* can gauge if the environment presents optimal conditions for invasiveness (4, 72, 86).

Salmonella Typhimurium also possesses several other characteristics which enhance virulence. *S.* Typhimurium expresses resistance to low pH, mediated by an interesting two stage system where a mild exposure (pH 5.5-6) can initiate a much higher acid resistance to pH values as low as 3-4 (45, 46, 75, 135). The bacteria also exhibit swarming motility, and this behavior has been suggested to aid in pathogenicity along with biofilm formation (144). Aside from Mg^{2+} transport systems that increase the likelihood of the bacteria to survive in low magnesium environments such as within macrophages (13, 101), *Salmonella*

Typhimurium also possesses genes which impart resistance to high copper environments where copper is either bound (CutF) or exported out (CutC) of the cell (55). Resistance to antibiotics is another important characteristic *Salmonella* possesses which can improve virulence. A survey of clinical isolates collected from 10 European counties found that 40% of those isolates demonstrated multidrug resistance, of which *S*. Typhimurium accounted for 51% of the serotypes detected (126, 136).   In the United Kingdom in 1996, 98% of 4006 clinical isolates of *S*. Typhimurium expressed resistance to multiple antibiotics (62), while in a similar UK survey from 1998-1999, 92% of clinical isolates surveyed expressed multidrug resistance (59).

Salmonella **Typhimurium and Food-Borne Illnesses**

In a survey conducted from 1992 to 1997, non-Typhi Salmonella infections accounted, annually, for 9.7% of all food-borne illnesses in the United States, resulting in over 1.4 million cases annually (97). *Salmonella* Typhimurium as a source of food-borne illness is primarily associated with animal products, particularly poultry. In the United Kingdom, a 2002 comprehensive annual survey of commercially available raw chicken product (frozen and fresh meats) consistently detected *Salmonella* in 8 to 9.7% of the samples (98). A 1998 survey of laboratory confirmed foodborne illnesses in the United States demonstrated that *Salmonella* was the second most common bacterial agent detected, with serotype Typhimurium accounting for the highest (30%) among salmonellosis infections (20). In another 2002 survey conducted in the United

States, *S.* Typhimurium accounted for 12.3% of all the *Salmonella* serotypes identified from a variety of animal products including swine and poultry (21). Another annual survey of large broiler plants, conducted by the United States Department of Agriculture in 1998 to 1999, found that *S.* Typhimurium was the third most common serotype (accounting for 14.2% of the total isolates). In the same study although, *Salmonella* isolates were found in swine (8.4%), ground turkey (23.5%), and ground beef products (4.6%), with most of the isolates found in broilers (63.5%) (43). A Korean survey conducted on a wide variety of food products, including broiler carcasses (1334 samples total), found that 2.2% of those products were contaminated with *Salmonella* including the serotype Typhimurium (26). A comprehensive survey conducted from 2000 to 2004 in Lithuania investigated the prevalence of *Salmonella* in chicken broilers to be between 1% and 3%, with Typhimurium to be the second highest serotype detected (second to Enteriditis) (104). An additional survey in Alberta, Canada, of approximately 1,000 portions of ground beef obtained from commercial sources was completed in 1999, in which *Salmonella* was detected in 1.3% of the samples (122). A more recent survey within the United States conducted by the USDA found *Salmonella* was detected in at least 10.5%, and upwards of 14 %, of broiler carcasses surveyed nationally from October 2005 through December 2006, and *Salmonella* was present in at least 38.6% of ground chicken products surveyed in the same study (44). *Salmonella* Typhimurium has also been detected in other non-animal food products such as sesame seed products (16)

and associated with an outbreak in Ohio due to the consumption of raw milk

(94). Because *Salmonella sp.* have the ability to overcome environmental

stresses such as osmotic pressure and extreme acidity, it has been theorized that

these stresses may induce the adaptability of *Salmonella* allowing it to remain

virulent in a wide variety of uncooked food products (68).

Quorum Sensing and Autoinducer 2

Bacteria posses the ability to produce, export, and take up small chemical

molecules that can serve as a form of bacterial communication. These systems,

and the type of chemical signals, are broadly classified on the transport systems

used to take up these signals in *Vibrio harveyi*, a bioluminescent marine

bacterium.   One system is designed for the uptake of one class of signal

molecules, termed Autoinducer 1 (AI-1). These chemical signals are generally

homoserine lactones employed by Gram negative bacteria (Fig 2.1), while Gram

positive bacteria utilize oligopeptides (9, 80, 92, 105, 131, 147). The second

quorum sensing system in *V. harveyi* is receptive to a furanosyl borate diester,

Autoinducer 2 (AI-2), and this chemical signal is primarily produced by Gram

negative bacteria (Fig 2.1) (1, 105, 116, 128, 130, 146).

Autoinducer 2 is produced as a byproduct of the Activated Methyl Cycle,

where S-adenosyl-methionine is created to serve as a methyl donor in cellular

metabolism (Fig. 2.2) (18, 33, 127, 140, 146). This cycle is important in

providing methyl groups for the methylation of DNA, RNA, and proteins. The

precursor to AI-2 is formed as the hydrolysis of S-ribosyl-homocysteine is

converted into homocysteine by the enzyme, LuxS, creating 4,5-dihydroxyl-2,3-pentanedione (DPD) as a byproduct. DPD can spontaneously undergo several conformations by the removal or introduction of water, but the formation of 2-methyl-2,3,3,4-tetrahydroxytetrathydrofuran (S-THMF) acts as a precursor to AI-2. As free boron interacts with S-THMF, a furanosyl borate diester is formed, AI-2 (18, 33, 100, 127, 140).

Production of AI-2 is achieved by the enzyme, LuxS and cell culture suspensions of *E. coli* and *Salmonella* have demonstrated the ability to produce compounds that induce a response in AI-2 reporter strains (*V. harveyi* BB170, a *luxN* mutant) (57, 116, 128-130, 142). Typically, maximal production of AI-2 is observed in the mid-late log phase and is promoted by the presence of glucose in liquid medium (128-130). It is interesting that culture conditions appear to have some influence on the production of AI-2, despite no apparent differences in cell numbers as *Salmonella* (after initial growth in LB supplemented with 0.5% glucose) demonstrated increased AI-2 production once shifted into medium of 0.1 M NaCl (high osmolarity) or lower pH (pH 5.0) (129). By testing *E. coli* suspensions from a chemostat culture, DeLisa et al. (35) also observed spikes in AI-2 production after introducing glucose into the continuous culture system (35). There is some indication that AI-2 production may be influenced not by *luxS* expression, but due to signaling activity from potential metabolites (12, 57). A study from Beeston and Surette (12) showed that AI-2 production appeared independent of *luxS* expression, but was potentially tied to *pfs* expression and

the authors theorized that control of AI-2 production may be linked to methionine production.
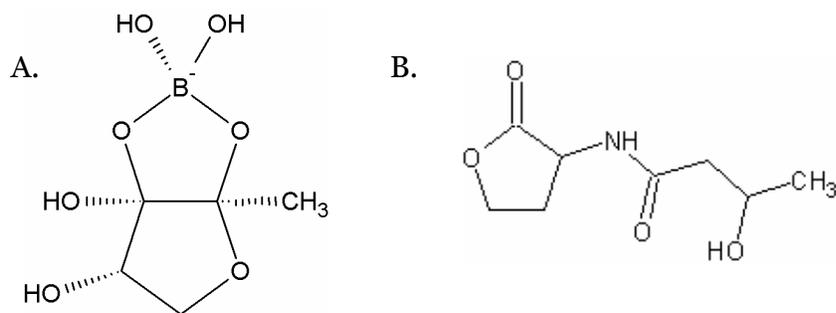
Figure 2.1. Chemical Structures of Autoinducer Signals Recognized
by *V. harveyi*

A. – Autoinducer 2 (AI-2). B. – Autoinducer 1 (AI-1)
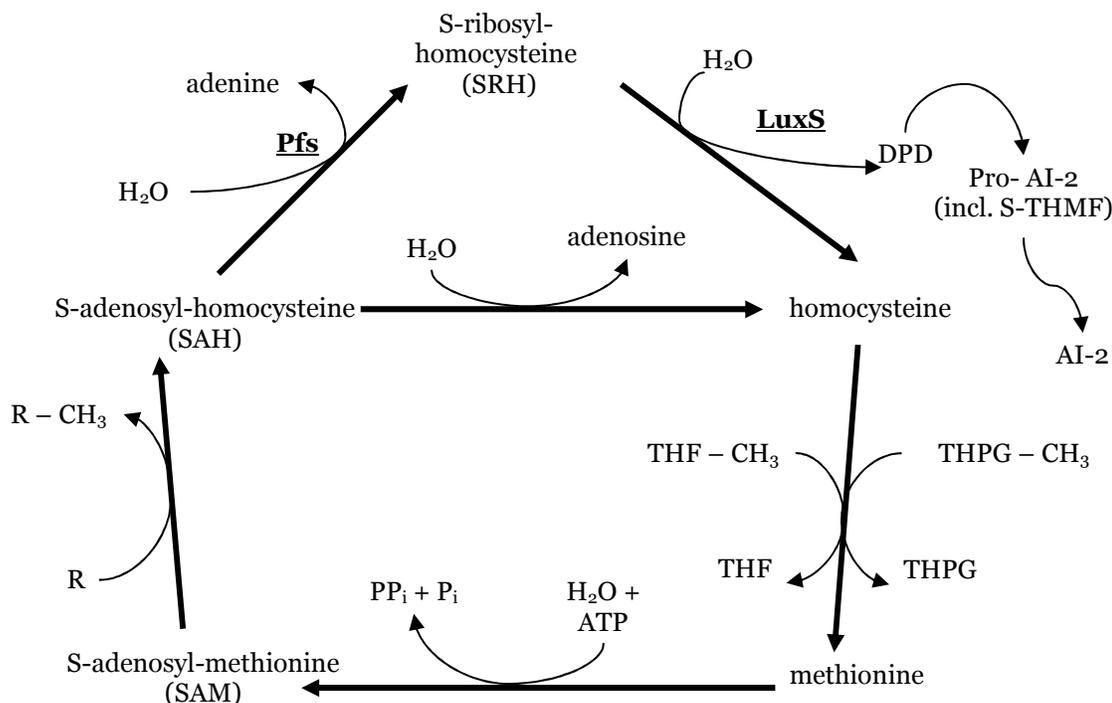
Figure 2.2. Activated Methyl Cycle

The quorum sensing system in *V. harveyi* is modulated through a series of key proteins within the system and their respective phosphorylated states (11, 60, 80, 102). When the signaling compounds are either absent or in low extra-cellular concentrations, phosphate is transferred from LuxU to LuxO (which normally is in a phosphorylated state) and in turn, LuxO down-regulates other *lux* genes. When the signaling compounds are present in high concentrations phosphate flow is reversed through the system, dephosphorylating LuxO, allowing *lux* genes to be expressed. Hence the receptor proteins that are receptors to the autoinducer compounds can act both as kinases (in the absence

of signals), and as phosphatases (in the presence of signals) (11, 60, 80, 102). A

general schematic of the signaling pathways in *V. harveyi* is presented in Figure

2.3. Chemical signals bind to the receptor proteins and either allow LuxN (AI-1),

or a combined complex of LuxP and LuxQ (AI-2), to dephosphorylate LuxU,

which in turn, will dephosphorylate LuxO (11, 60, 80, 102). The

dephosporylated LuxO, in concert with σ^{54}, helps destabilize another protein

which acts as a small RNA (sRNA) chaperone (responsible for enhancing

transcription), Hfq (80, 131, 140). As the destabilized small RNA/Hfq complex

occurs, this allows the Hfr protein to form a complex with existing LuxR mRNA

transcripts, increasing its stability. With increased transcription of LuxR, the

protein further promotes the expression of the bioluminescence genes (60, 80,

102). In short, at lower concentrations of autoinducer, LuxR mRNA/Hfr

complex is destabilized by the introduction of sRNAs and, in turn, reduces the

expression of the bioluminescent genes (80, 102).
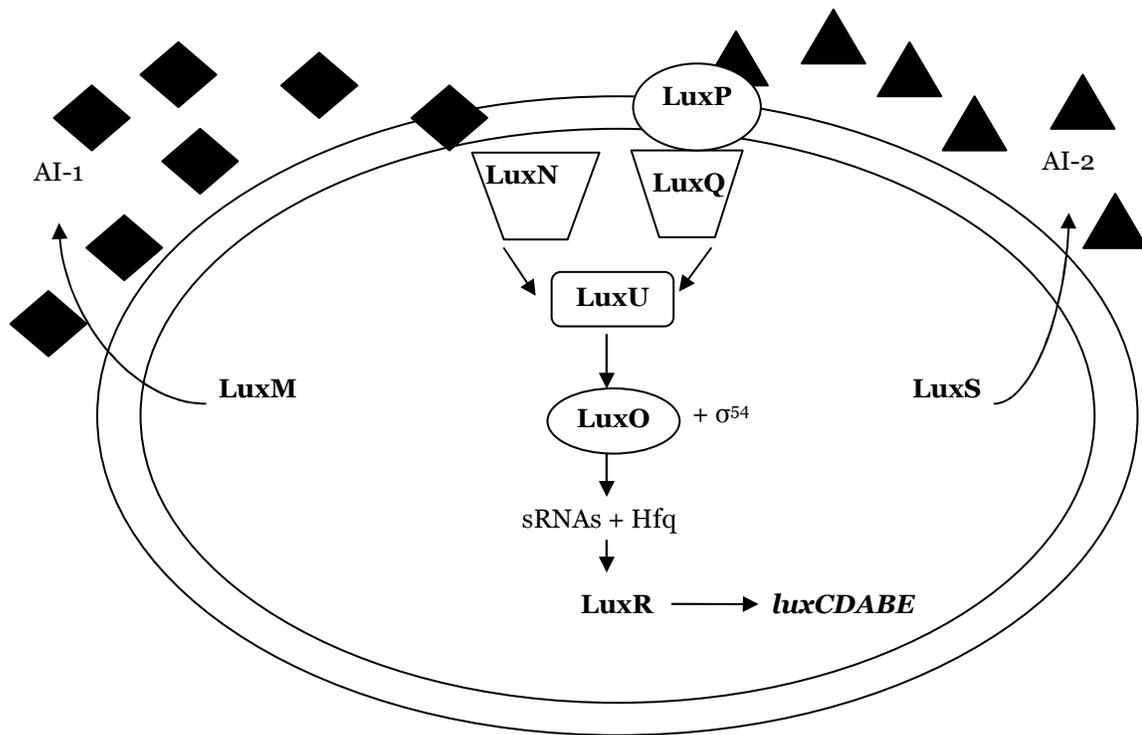
Figure 2.3. General Schematic of Quorum Sensing
Systems in *Vibrio harveyi*

In *Salmonella* and *E. coli*, the uptake of AI-2 is mediated by another set of proteins, the lsrABC complex (1, 129, 132, 133, 148). What is unique about this system is that it appears AI-2 is imported directly and modified by this complex (Fig 2.4). AI-2 is bound and transported into the cell by the lsrABC complex (which shows similar function to a ribose transport system), by initially binding to LsrB, a periplasmic binding protein.
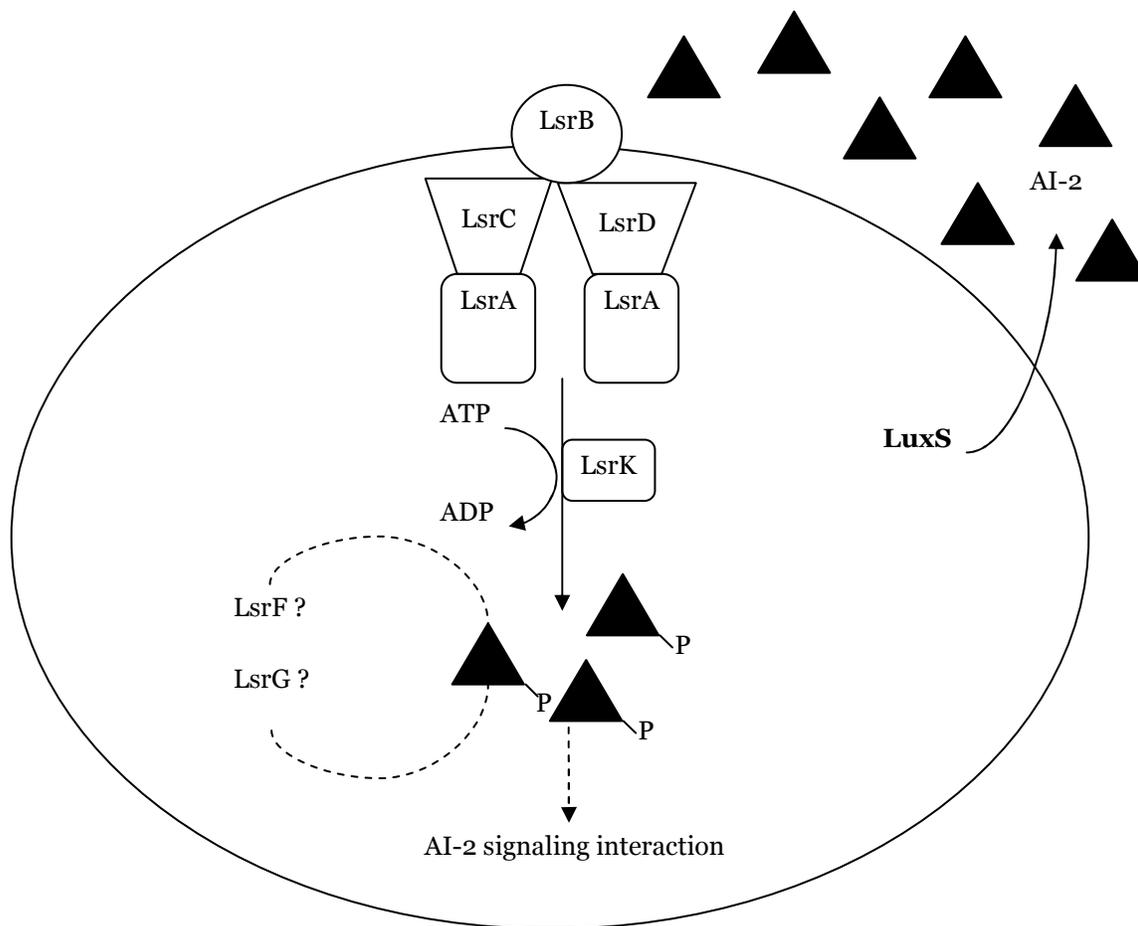
Figure 2.4. General Schematic of AI-2 Internalization
and Processing in *Salmonella enterica*

Solid triangles and solid triangles with the letter P represent, AI-2 and phosphorylated
AI-2, respectively. Dotted lines represent theoretical interaction with AI-2

The remaining proteins either form a channel structure (LsrC and LsrD)

or serve as an ATP-mediated transport protein to shuttle AI-2 across the cellular

membrane (LsrA) (132, 133, 148). Once internalized, AI-2 is phosporylated by

lsrK. There appears to be other genes involved in further processing of AI-2,

LsrF and LsrG, but their functions are yet unknown (132, 133, 148). There is

evidence that AI-2 influences the regulation of this transport system encoded by

the *lsr* operon (133, 140, 148). Under normal conditions the operon is repressed

by a DNA-binding regulatory protein, LsrR (encoded upstream from *lsr* operon)

which binds to *lsrA*. Internalized AI-2 (phosphorylated by the gene product of

lsrK) is responsible for inactivating LsrR, allowing the expression of the *lsr*

operon (Fig. 2.5) (132, 133, 140, 148).

No gene expression

LsrR

lsrK lsrR lsrA lsrC lsrD lsrB lsrF lsrG lsrE

LsrR

phosphorylated AI-2

Gene expression

P

lsrK lsrR lsrA lsrC lsrD lsrB lsrF lsrG lsrE
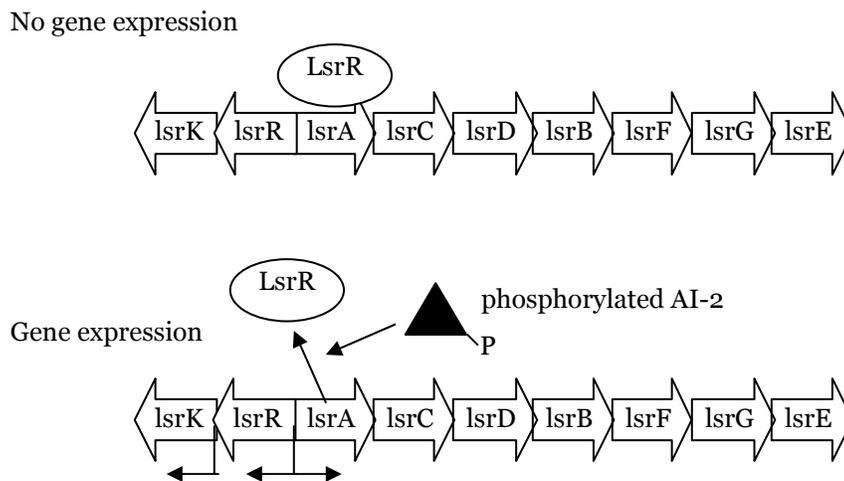
Figure 2.5. *lsr* Operon in *Salmonella enterica*

Because the lsrABC complex has functional similarity to the ribose

transport system, some researchers have investigated similar transport systems

to determine if they could be also used for the internalization of AI-2. James et

al. (71) demonstrated that the addition of purified His-tagged RbsB (a

periplasmic ribose binding protein) selectively competed with LuxP for AI-2

when cultured with *V. harveyi* BB170. Additionally, the authors found that a *rbsB* mutation in a constructed *Actinobacillus sp.* strain reduced the ability for removal of AI-2 in culture suspensions compared to the wild-type, suggesting RbsB may serve as transport system for internalization of AI-2 (71).

It has been suggested that bacteria may only be able to detect certain structural forms of AI-2. AI-2 is formed initially as an unstable product, DPD, and undergoes various chemical configurations with the addition of water and free boron to make a final, stable configuration (18, 117). A study by Miller et al. (100) found that when synthetic AI-2 was prepared under boron-free conditions, it was unable to stimulate a bioluminescent response in *V. harveyi* BB170, while the same AI-2 was detected using a *Salmonella lsr-lacZ* fusion reporter strain. The authors suggested that in the absence of boron, DPD may undergo an alternate configuration forming (2R, 4S)-2-methyl-2,3,4,- tetrahydroxytetrahydrofuran, or R-THMF, as opposed to the boronated form of AI-2 (S-THMF-borate) (18, 100, 117). Alternately, there may be other signaling molecules that are utilized for cellular communication as another compound, AI-3, has been proposed (124, 141, 142). This compound does not induce a typical response from the AI-2 reporter strain, *V. harveyi* BB170. But utilizing a lacZ fusion to *LEE1* (a promoter for a type III SS), the constructed *E. coli* reporter strain was able to detect this compound. The chemical structure of AI-3 has yet to be determined, but it is suggested to be an aromatic compound and non-polar (124, 141, 142). Despite varying opinions on whether unique signaling

compounds are indeed novel structures or merely variant forms of AI-2 (100, 124, 141), and that the transport of AI-2 may be mediated by other systems aside from the lsrABC system (71), it is important to note that both *Salmonella* and *E. coli* have demonstrated the ability to produce AI-2 and have the ability to sense this chemical signal in their environment (128, 148).

Quorum Sensing and Bacterial Virulence

A particularly interesting facet to quorum sensing is the apparent influence these chemical signals may have on bacterial virulence (42, 60, 99, 103, 119, 125).   In a study conducted by Déziel et al. (36), the authors discovered that 3,4 hydroxy-heptylquinoline (PQS) in *Pseudomonas aeruginosa* functioned in regulating quorum sensing genes as well as the production of hydroxy-alkyquinolines (HAQs). HAQs in *Pseudomonas* are potent antibiotics. The authors theorized that the diffused chemical signal, PQS, increased the expression of typical quorum sensing machinery (AI-1) and aided the organism through inhibiting competitive flora by inducing antibiotic production. Other studies found that *luxS* mutants of *Clostridium perfringens* recovered from reduced production of alpha, kappa, and theta toxins when cell free supernatants of wild type cultures were introduced (103). A particular interesting study was done by McNab et al. (96), where LuxS mutants of *Streptococcus gordonii* and *Porphyromonas gingivalis* as a mixed population were unable to produce a normal biofilm. *S. gordonii luxS* mutant bacteria were unable to accumulate on saliva coated glass slides, leaving a poor foundation for *P. gingivalis luxS* mutants to form

expansive biolfilm structures (96). Another study using a *luxS* deficient mutants of *Serratia marcescnes* were able to utilize AI-2 from wild type cell free supernatants, restoring production of prodigiosin. Prodigiosin is a tripyrrole antibiotic which is theorized to allow *Serratia sp.* to overcome competitive bacterial and fungal flora, and may have immunosuppressant properties increasing the virulence of this opportunistic pathogen (31).   Using microarray analysis, when compared to a *luxS* mutant, Sperandio *et al.* (125) reported that 404 genes were up-regulated at least five-fold in an enterohemorrhagic *Escherichia coli* wild type.   Another study found diminished transcriptional activity in a chromosomal region responsible for several pathogenic genes in enteropathogenic *E. coli*. LuxS mutants were unable to effectively transcribe the locus of enterocyte effacement (LEE), a series of promoters responsible for the type III secretion system, in *E. coli* (119).   Another published report found 242 genes in *E. coli* had a response to the introduction of AI-2 treatments (being either up regulated or down regulated) by over 2.3 fold (34). There is some indication that quorum sensing may not induce virulence factors in bacteria, but rather repress virulence as Henke and Bassler (60) demonstrated that a type III SS in *V. parahaemolyticus* was repressed in the presence of elevated AI-2 levels. And another study of particular interest investigated the effect of a *luxS* deletion had on the virulence of Gram positive organisms. While AI-2 (produced from LuxS) is typically considered a signaling compound for Gram negative bacteria, Lyon et al. (88) demonstrated that haemolytic activity (measured by an

increased streptolysin O activity) was increased in a *Streptococcus pyogenes*

luxS mutant at stages of mid-late log growth, while demonstrating limited

presence of AI-2.

Inhibition of Quorum Sensing

If quorum sensing signals are responsible for modulating bacterial virulence,

conversely, the inhibition of these signals may reduce the efficacy of these

bacterial pathogens (110, 123).   Mechanisms for interfering with quorum

sensing systems may be varied, Rasmussen and Givskov (110) indicated in a

review that interference with AHL signaling compounds (AI-1) can range from

prevention of AHL production, blocking of AHL receptors, to the direct

inactivation of the signals themselves (110).   Dong et al. (37) demonstrated that

Erwinia carotovora were unable to initiate plant rot in plant tissue samples

exposed for 20 seconds to high titer (5×10^8) suspensions of *Bacillus*

thuringiensis. *B. thuringiensis* produces a potent acyl-homoserine lactonase.

The authors theorized this enzyme degraded acyl-homoserine lactone (AHL),

which is known to be utilized by *Erwinia* to induce virulence genes (37). It has

been suggested that *Pseudomonas aeruginosa* utilizes AHL for pathogenicity in

eukaryotic hosts. In epithelial human cell culture studies, cell lines derived from

human airway epithelia were able to inhibit bioassays used for detecting AHL

produced by *Pseudomonas aeruginosa* (25). Plant tissues are also able to

produce inhibitors to autoinducer 2 signals. After incubation of legume seeds

with cell free supernatants (from bacterial suspensions previously demonstrating

AI-2 activity) washes of the seeds were extracted with equal volumes of ethyl acetate. The aqueous phases of these extracts demonstrated upwards of 900 fold reduction in AI-2 activity utilizing the *V. harveyi* bioassay (93). Another study working with extracts from various food matrixes also demonstrated inhibition to the AI-2 bioassay. Lu et al. reported relative inhibition in a variety of processed food products such as turkey patties (99.8% relative inhibition), chicken breast (97.5%), cheeses (93.7%), beef steak (90.6%) and beef patties (84.4%) (83).

Brominated furanones have also been discovered to inhibit AI-2 compounds. A study demonstrated reduced swarming in *E. coli* when exposed to furanones derived from algae (*Delisea pulchra*). The same compounds reduced AI-2 activity in *V. harveyi* reporter strains by over 5000 fold (111). Combined with microarray analysis, Ren et al. found a 49% decrease in relative light unit production using the *V. harveyi* reporter strain when it was combined with a boronated furanone. The authors found 56 genes were repressed (at least two fold) by the addition of bromonated furanone, while these same genes were up regulated by the introduction of AI-2 (112). Unlike autoinducer I systems that are mediated primarily by AHL, the inhibition of AI-2 by bromonated furanones are not well understood.

Quorum Sensing in Relation to Foods

The interaction of microorganisms and food matrices, and potential inhibition of these signaling systems are important points to address as these interactions

may indicate importance in food spoilage and propensity of certain foods to be susceptible to foodborne pathogens (105). Some studies have been conducted to determine the presence of autoinducer signals in foods, and even the presence of compounds that could inhibit these signaling systems. Bruhn et al. (17) utilized a wild-type and AHL deficient mutant of *Hafnia alevei* to determine if AHL signaling compounds had an impact on food spoilage. The authors found that there was no difference in spoilage properties when both strains were incubated in vacuum packed meat and AHL activity was detected in the wild-type after 7 days incubation. However at that time there was a predominance of lactic acid bacteria (LAB) also present in each sample in high numbers, with both strains of *H. alevei* cultured as a minor type, and no indication was made to determine if the LAB expressed any AHL signaling compounds (17). Cloak et al. (27) also conducted a study to determine if AI-2 could be recovered in certain food matrixes after inoculation with different bacterial pathogens. In both milk and chicken broth, at a variety of temperatures (4°C, 25°C, and 37°C), AI-2 activity was observed in *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella enterica* serovar Typhimurium, and *E. coli* O157:H7 with temperature seemingly having the greatest impact on AI-2 production (27). Another study found that certain foods had compounds capable of exhibiting AI-2-like activity. Lu et al. (83) created washes of several different food products and found that some foods expressed relatively high amounts of AI-2 activity (carrots, cantaloupe, tomatoes, and fish) while other food products expressed inhibition to quorum

sensing systems (poultry and meat products).   To fully understand the interaction of quorum sensing signals and inhibitors on microbial populations in foods, the presence of these compounds simply should not be the only factor considered, but also bioavailability and diffusion of these compounds in food systems. This idea has been presented by Horswill et al. (65) in a recent review and the authors suggest that further study of the transport behavior of quorum sensing compounds under environmental conditions is needed to truly deduct their impact on microbial communities, and this point is very applicable to food matrices.

CHAPTER III

DIFFERENTIAL EXPRESSION OF VIRULENCE-RELATED GENES IN A *Salmonella enterica* SEROTYPE TYPHIMURIUM *luxS* MUTANT IN RESPONSE TO AUTOINDUCER 2 (AI-2) AND POULTRY MEAT-DERIVED AI-2 INHIBITOR

Introduction

Quorum sensing, or bacterial cell signaling describes the overall process how bacteria utilize autoinducer molecules for bacterial cell-cell communication (92, 131, 148). A key cell signaling system involves the autoinducer AI-2, a furanosyl borate diester molecule produced primarily by Gram negative bacteria (1, 116, 128, 146). AI-2 is produced as a byproduct of the Activated Methyl Cycle, where S-adenosyl-methionine is created to serve as a methyl donor in cellular metabolism (140, 146). The precursor to AI-2 is formed as the hydrolysis of S-ribosyl-homocysteine is converted into homocysteine by the enzyme, luxS, creating 4,5-dihydroxyl-2,3-pentanedione (DPD) as a byproduct. DPD can undergo several conformations spontaneously with the removal or introduction of water, but the formation of 2-methyl-2,3,3,4-tetrahydroxytetrathydrofuran (S-THMF) acting as a precursor to AI-2, and the interaction of free boron with S-THMF, results in the forming the final furanosyl borate diester structure (Sun et al. 2004). Taga et al. (132) identified a *luxS* regulated operon (*lsrACDBFGE*) which encodes an ABC transporter system with similar functionality to ribose transport systems in *E. coli*. The periplasmic binding protein, *lsrB*, is

responsible for binding with extracellular AI-2, and the compound is internalized using this transport system. Normally the *lsr* operon is repressed by a DNA-binding regulatory protein, LsrR which is upstream from *lsr* operon and immediately upstream of *lsrR* is a gene which encodes a cytoplasmic phosphokinase (*lsrK*) (132, 140). Once internalized, AI-2 is phosphorylated by the gene product of *lsrK,* and is responsible for inactivating LsrR, allowing the expression of the *lsr* operon (132). There is increasing evidence that autoinducer molecules can influence virulence gene expression in bacteria (31, 34, 96, 103, 125). However, our knowledge about bacterial cell signaling in relation to food borne pathogens and food spoilage organisms is still in its infancy (14, 17, 105, 109).

Understanding the role of AI-2 and its function with food-borne bacterial pathogens and, in particular, understanding the interaction of food matrices with these signal molecules is vital (83-85). It has been previously shown that ground beef and poultry meat contain compounds that can interfere with AI-2 signaling (83). The underlying hypothesis of this study was that these inhibitory compounds by virtue of their interaction with AI-2 molecules would influence the expression of virulence genes in pathogenic bacteria. The specific objectives of this study were to understand the influence of autoinducer AI-2 and poultry meat-derived inhibitory factor interaction on the expression of specific pathogenicity, and virulence-related, *Salmonella* genes. *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) is a major causative agent in poultry-

related food-borne illnesses and also accounts for almost 1.4 million cases of food-borne illnesses annually in the United States (97). Understanding how poultry meat influences cell signaling in *S*. Typhimurium could provide insight into the factors controlling the pathogenicity of this organism on poultry products.

<div align="center">**Materials and Methods**</div>

Bacterial Strains

Salmonella enterica serovar Typhimurium (isolate # 87-26254) was obtained from the National Veterinary Service Laboratory (Ames, Iowa). The *E. coli* strains BW25113 and BW25141, which were used in the generation of the *luxS* mutant with a chloramphenicol resistance marker (25 μg/ml), were obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT). The *luxS* mutant, designated strain PJ002, was generated according to a protocol developed by Datsenko and Wanner (32). Briefly, Red recombinase expression plasmid, pKD46 was propagated and extracted from *E. coli* BW25113. *S*. Typhimurium wild-type was made competent by electroporation. Transformation of pKD46 into the competent *S*. Typhimurium cells was confirmed by ampicillin resistance on Luria-Bertani (LB) agar plates. The forward primer (5'-cgcagtcgatcatacccggatgcaagcgccggcggtccgggtgtaggctggagctgcttc-3') and reverse primer (5'-ctttcggcagcgccagctctttattgctgttcacgcgcacatgggaattagccatggtcc-3') encoding the FLP recognition target (FRT) of the plasmid, and portions

homologous to the internal sequence, 30 bp downstream, of *luxS* were generated

from a commercial source. The plasmid, pKD3 (derived from BW25141), was

extracted and used as a template for PCR, where the resulting product encoded a

chloramphenicol resistance gene flanked by the FRT/*luxS* homologous regions.

The PCR product was then incorporated into competent *S.* Typhimurium cells

containing the pKD46 plasmid (that would facilitate recombination) by

electroporation. The *Salmonella* cells were then incubated at 37° C on LB plates

amended with chloramphenicol, which would select for transformants and the

elevated temperature ensured the removal of the heat sensitive pKD46 plasmid.

E. coli strains containing the plasmids pVS212 and pVS214 were used for the *in*

vitro production of AI-2 (a gift from Dr. V. Sperandio, University of Texas

Southwestern Medical Center, Dallas). The wild-type strain (#87-26254) and the

mutant (PJ002) were used in the microarray experiments. *Vibrio harveyi* BB170

(a gift from Dr. B. Bassler, Princeton University) was used as the biosensor strain

to detect the presence of AI-2 activity.

Cell-Free Supernatant Preparation (CFS)

Salmonella Typhimurium (wild-type strain # 87-26254) was grown in LB broth

amended with 0.5% glucose (weight/vol) at 37° C to mid-late log phase (OD_{600}

~1). The culture was centrifuged (10,000 X g for 10 min), and the supernatant

filtered through a 0.2 μm sterile syringe filter (VWR, West Chester, PA). AI-2

like activity was confirmed in the cell-free supernatant as previously described

(83).

Synthesis of Autoinducer 2 (AI-2)

In vitro synthesized AI-2 was prepared according to a published protocol (124).
Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2)
were isolated and purified using a nickel resin column (Qiagen Inc., Valencia,
CA). The purified enzymes were incubated with 1 mM S-adenosyl-homocysteine
(Sigma-Aldrich, St. Louis, MO) for one hour at 37° C, and AI-2 was further
separated from the enzymes using a centrifuge filer column (Biomax-5,
Millipore, Billerica, MA). AI-2 activity was confirmed using *V. harveyi* BB170 as
described by Lu et al. (83).

Poultry Meat-Derived (PM) Inhibitors

Poultry meat (50 g) was obtained from a commercial source and stomached
with 40 ml AB medium (11), centrifuged (10,000 X g for 10 min), and the
supernatant filtered (0.2 μm). Inhibition of AI-2 like activity was confirmed
using *Vibrio harveyi* BB170 (83). Autoinducer (AI-2) activity was expressed as
Relative Light Units (RLU), a ratio of the bioluminescence of a sample compared
to its negative control (the reporter strain only). The bioluminescence was
measured using a Wallac 1420 plate reader (Perkin Elmer, Shelton, CT).
Inhibition was expressed as a % relative to a corresponding positive control
(containing either, AI-2 or CFS, without the PM inhibitor) defined as: 100 −
[(light unit measurement of sample/light unit measurement of positive control)
x 100]. The *V. harveyi* bioluminescence assay was performed to confirm that
the *luxS* mutant (PJ002) was unable to produce AI-2 activity.

Treatment Conditions, RNA Extraction, and cDNA Labeling

The *luxS* mutant (PJ002) was initially grown overnight in LB amended with chloramphenicol (25 µg/ml). The culture was diluted (1:20) in fresh LB broth (with no antibiotics) and incubated for 4 hours at 37º C with moderate shaking. Portions (600 µl) were removed and centrifuged (10,000 X g). The cell pellets were subsequently exposed to the different experimental conditions. The pellets were re-suspended in 6 ml volumes consisting of 5.4 ml LB broth, with the remaining 0.6 ml containing the different experimental treatments (ie., 10% of the final total volume of the culture).   The different treatments were i) 0.6 mL of pH 7.4 phosphate buffer (PB treatment), ii) 0.3 ml phosphate buffer + 0.3 mL *in vitro* synthesized AI-2 (AI-2 treatment), iii) 0.3 mL *in vitro* synthesized AI-2 + 0.3 mL poultry meat inhibitor (AI-2 + PM treatment), iv) 0.3 mL phosphate buffer + 0.3 mL poultry meat inhibitor (PM treatment). The four different treatments were incubated in LB medium for 3 hours (OD_{600} ~0.6) at 37º C.

Two milliliter aliquots were removed from each treatment, centrifuged (12,000 X g for 5 min), and the pellets were flash frozen with liquid nitrogen, and stored at - 80º C until total RNA was extracted. (Three replicate RNA extractions were performed from each treatment). The RNA was extracted using RiboPure kit (Ambion, Austin, TX), followed by DNase treatment per the manufacturer's directions. The triplicate RNA extractions were pooled and maintained at - 80º C. The wildtype *S*. Typhimurium strain was grown for 3 hours in LB at 37º C and total RNA was also extracted. The RNA samples were

concentrated using Quick-Precip Plus Solution (Edge Biosystems, Gaithersburg,

MD) by the manufacturer's instructions and concentration determined by UV

spectra. The RNA samples were labeled using the LabelStar Array Kit (Qiagen

Inc., Valencia, CA), using random nanomers according to the manufacturer's

instructions. Briefly 2.5 μg RNA were combined with (final concentration) 5 μM

random nanomer primers, 2 μl denaturation solution, and RNase free water to a

final 25 μl reaction volume. The reaction mixture was incubated for 5 minutes at

65 ºC and then placed on ice for 5 minutes. After sufficient incubation on ice, an

additional 25 μl of prepared Reverse Transcriptase reaction solution (final

concentration: 1X reaction buffer, 0.08 mM dCTP, 0.5 mM dNTPs, 0.02 mM

either Cy3 or Cy5 labeled dCTP (Amersham Bioscience, Piscataway, NJ), 20

units RNase inhibitor, and 2.5 μl RT enzyme, RNase free water to final 25 μl

volume) was added and incubated for 15 minutes at 25 ºC, followed by an

incubation at 37 ºC for 2 hours. After incubation, 2 μl of stop solution were

added, and the labeled cDNA was purified using the Label Star kit (Qiagen Inc.,

Valencia, CA) as per manufacturer instructions. The cDNA from each of the

treatments and the wild type strain were labeled in duplicate, but utilizing either

Cy3 or Cy5 as labeled dCTP for dye swap technical controls (53).

Microarray Preparation, Hybridization, and Scanning

Sequences specific to virulence and pathogenicity genes in *Salmonella* were

obtained from Genbank and used to design 45-mer oligo probes according to a

previously published method (38). Open reading frames for 1136 genes were

synthesized and normalized in concentration by Integrated DNA Technologies Inc. (Coralville, IA). Oligos were resuspended in Epoxide Slide Spotting Solution and printed onto Epoxide Coated Slides (Corning Inc., Corning, NY) using a GeneMachine Omnigrid Accent microarray printer (Genomic Solutions Ann Arbor, MI). Each spot printed in duplicate, and two copies of each array printed on each slide. Arrays were immersed in a pre-hybridization buffer (25 ml 20X SSC, 1 ml 10% SDS, 100μl 0.1 g/ml Bovine Serum Albumin solution, and de-ionized H_2O up to a final volume of 100 ml) for 45 minutes at 42°C. Arrays were then immersed in 0.1 X SSC at room temperature with gentle agitation (5 min). The immersion and incubation were repeated twice and then the arrays transferred to DI H_2O for 30 seconds. The DI H_2O rinsing was repeated once and then the arrays were spun in a swinging bucket centrifuge at roughly 500 X g for 5 minutes. Pre-hybridized arrays were stored at room temperature until hybridization.

The labeled cDNA samples were concentrated and dried in a speedvac system and resuspended in 20 μl pre-warmed 1X hybridization buffer (Universal Hybridization Solution, Corning Inc., Corning, NY) which was then added directly to the arrays after a 95°C incubation for 5 minutes. The arrays were hybridized overnight (16 hours) at 42°C with a sufficient volume of hybridization buffer added to the cassette chambers (Universal Hybridization Solution, Corning Inc., Corning, NY). After hybridization, the arrays were then transferred to a post-hybridization solution (2X SSC, 0.1% SDS) and incubated

for 10 minutes at 42ºC. The arrays were then washed twice with 1X SSC by immersion for 10 minutes. This was then followed by two washings with 0.1X SSC by immersion for 5 minutes. The arrays were given a final rinse in 0.01X SSC for 30 seconds and then spun in a swinging bucket centrifuge at 1200 RPM for 5 minutes. Arrays were scanned using a GenePix 4000B image scanner (Molecular Devices Corporation, Union City, CA) and saved as TIFF images.

Data Analysis

Array image analysis was done using GenePix Pro 5.1 (Molecular Devices Corporation, Union City, CA) and Acuity 4.0 (Molecular Devices, Sunnyvale. CA). All images were normalized based on a Lowess ratio for the features (150). Additionally, features were selected as ideal spot images if they had a signal to noise ratio (SNR) of at least 3 for either channel (Cy3 or Cy5) (149). By selecting features that met this SNR criterion, this would reduce error in data analysis taking into account potential variation due to dye labeling, and hybridization, efficiency. The normalized $\log_2$ Cy5/Cy3 ratios were compiled and evaluated (t test, $p < 0.05$) with ratios expressing differences of at least 1.5-fold induction (or repression) being considered biologically significant (5, 67). The following treatments were compared, namely, AI-2 vs PB, PM vs PB, and AI-2 + PM vs PB.

Results

Influence of Poultry Meat Inhibitors on AI-2 Related

Bioluminescence

The CFS from the *S.* Typhimurium mutant strain, PJ002, was unable to elicit the

characteristic bioluminescence signal with the AI-2 biosensor strain, *V. harveyi*

BB170, while the CFS from the wild type strain (*S.* Typhimurium 87-26254)

produced a mean 360-fold increase in bioluminescence (RLU) as compared to

the corresponding negative control (Table 3.1). The *in vitro* synthesized AI-2

molecules exhibited a mean 139-fold increase in bioluminescence. The poultry

inhibitor treatment (PM) resulted in 87.5 % mean reduction in bioluminescence

when compared to the CFS sample, and almost a 60% mean reduction when

Table 3.1. Autoinducer Activity, and Inhibition from Poultry Meat Inhibitor (PM), of *In Vitro* Synthesized Autoinducer 2 (AI-2) and Cell Free Supernatants (CFS) Derived from *S.* Typhimurium Wild-type and *S.* Typhimurium *luxS* Mutant (PJ002)

Sample	Relative AI-2-like Activity (RLU)[1]	% Inhibition[2]
Purified AI-2	139.6 ± 39.6	-
S. Typhimurium CFS	360.6 ± 9.9	-
S. Typhimurium *luxS* Mutant PJ002 CFS	1.6 ± 0.6	-
AI-2 + PM	57.1 ± 4	59.0% ± 2.8
S. Typhimurium CFS + PM	31.0 ± 3.5	87.5% ± 8.9

[1] Mean relative AI-2-like Activity (Relative Light Units) ± standard error (n = 3), based on negative control values.
[2] Mean percentage ± standard error (n = 3), based on positive control values.

compared to the *in vitro* synthesized AI-2 (Table 3.1).

AI-2 and Poultry Meat Inhibitors Influence Virulence-Related Gene Expression

Twenty three (23) genes had a significant difference (p<0.05) in the gene expression patterns for the AI-2 treatment compared to the control PB treatment, with roughly half the genes being up-regulated (Table 3.2). Of the 13 genes that were up-regulated, 6 of them were up-regulated 2-fold, or greater. Significant differences in gene expression were noted in the presence of the PM treatment, with 36 genes being either up-regulated, or down-regulated (Table 3.3). Out of these 36 genes, 19 genes were up-regulated 2-fold or greater, while most of the down-regulated genes expressed differences between 1.5 to 1.9-fold. The combined AI-2 + PM treatment showed that only 22 genes were being differentially expressed compared to the PB control (Table 3.4). However, unlike the other treatment most of the genes were up-regulated (15 of the 22 genes). Of these, 6 were up-regulated with a difference of 2-fold or greater, and of the 7 genes that were down-regulated, 5 were 2-fold or greater (Table 3.4).

Discussion

The *S*. Typhimurium *luxS* mutant, PJ002, generated in this study was (as expected) unable to synthesize AI-2 which was confirmed in the bioluminescence assay (Table 3.1). Taga et al. (132) had reported that even though AI-2 production may be lacking, *luxS* mutants are fully capable of responding to AI-2 activity. Therefore, it was fully expected that the mutant

strain (PJ002) would respond to AI-2 molecules, if present. The poultry meat samples inhibited the AI-2 based response in *V. harveyi* BB170 (Table 3.1) which confirmed earlier published results demonstrating the ability of PM samples to interfere with AI-2 signaling (83).

Twenty three genes exhibited significant induction or repression in the presence of AI-2 molecules. Several genes that were up-regulated were putative cytoplasmic or outer membrane proteins, while the genes being down-regulated in the presence of AI-2 appear to be directly associated with bacterial virulence (*rpoS, prgH*). The PM treatment appeared to have the greatest impact on gene expression with 36 genes being differentially expressed. The PM matrix contains a variety of compounds in contrast to just AI-2 molecules that are probably influencing the gene expression. Previous reports suggesting the modulation of gene expression by environmental conditions and food matrices provide supporting evidence for this observation (4, 8, 68). What is interesting in these results is that we are demonstrating how a matrix that is known to interfere with AI-2 signaling is having a significant impact on gene expression. The up-regulation of only 1 gene (*tolR*) was common between the AI-2 treatment effect and the PM treatments. In fact, *tolR* was the only gene that was common across all treatments. When the cells were exposed to AI-2 + PM, *Salmonella* showed a unique response in its virulence gene expression (Table 3.4). A total of 22 genes were differentially expressed in the presence of the autoinducer molecule and its putative inhibitor. A majority of the genes that were

Table 3.2. *S.* Typhimurium *luxS* Mutant (PJ002) Genes Influenced by *In Vitro* Synthesized Autoinducer 2 (AI-2)

Fold Change[1]	Gene	Gene Function and/or Product	P Value
-10.8	*sitC*	fur regulated Salmonella iron transporter	0.050
-2.3	*prgH*	cell invasion protein	0.027
-2.1	*htpG*	chaperone Hsp90, heat shock protein C 62.5	0.002
-2.0	*yhdV*	putative outer membrane lipoprotein	0.003
-1.9	*prlC*	oligopeptidase A	0.030
-1.9	*wzxE*	O-antigen translocase in LPS biosynthesis	0.020
-1.8	*stiH*	putative fimbriae	0.025
-1.7	*tldD*	suppresses inhibitory activity of CsrA	0.045
-1.6	*stbE*	putative fimbriae; chaperone	0.031
-1.5	*rpoS*	sigma S (sigma 38) factor of RNA polymerase	0.026
1.5	*cobT*	nicotinate-nucleotide dimethylbenzimidazole-P phophoribosyl transferase	0.039
1.7	STM1131	putative outer membrane protein	0.045
1.7	STM0305	putative outer membrane protein	0.033
1.8	*ybdQ*	putative universal stress protein UspA and related nucleotide-binding protein	0.022
1.8	*tolR*	tol protein, role in outer membrane integrity	0.039
1.8	*yicJ*	putative GPH family transport protein	0.023
1.8	*hslJ*	heat shock protein hslJ	0.028
2.1	STM2868	putative cytoplasmic protein	0.009
2.3	*yjfR*	putative Zn-dependent hydrolases of the beta-lactamase fold	0.039
2.3	*yecA*	putative metal-binding protein	0.039
2.5	*malZ*	maltodextrin glucosidase	0.020
3.1	STM4255	putative cytoplasmic protein	0.007
3.3	*yjiS*	putative cytoplasmic protein	0.019

[1] Calculation based on the difference of the mean ratio (treatment to reference strain) for the AI-2 treatment verses the PB negative control.

Table 3.3. *S*. Typhimurium *luxS* Mutant (PJ002) Genes Influenced by Poultry Meat (PM)

Fold Change[1]	Gene	Gene Function and/or Product	P Value
-9.4	*res*	DNA restriction (DNA helicase)	0.031
-2.6	*lpxA*	UDP-N-acetylglucosamine acetyltransferase	0.028
-2.2	*alkB*	DNA repair system specific for alkylated DNA	0.036
-2.0	*dcm*	DNA cytosine methylase	0.041
-1.9	*invI*	surface presentation of antigens	0.039
-1.7	*glnP*	glutamine high-affinity transporter	0.004
-1.7	*nmpC*	new outer membrane protein	0.022
-1.7	*stiH*	putative fimbriae	0.015
-1.7	*yhgF*	putative RNase R	0.040
-1.6	*stbE*	putative fimbriae; chaperone	0.049
-1.6	*ybiO*	putative transport protein	0.037
-1.5	*sipD*	cell invasion protein	0.030
-1.5	*holC*	DNA polymerase III, chi subunit	0.038
-1.5	*gabP*	RpoS dependent gamma-aminobutyrate transport protein	0.009
-1.5	*mfd*	transcription-repair coupling factor	0.046
-1.5	*cydD*	cytochrome-related transporter	0.016
-1.5	*nagC*	transcriptional repressor of nag (N-acetylglucosamine) operon (NagC/XylR family)	0.023
1.5	*sthA*	putative fimbrial chaperone protein	0.048
1.5	*sseA*	secretion system effector	0.026
1.5	*prpA*	serine/threonine protein phosphatase	0.041
1.5	*pqiA*	paraquat-inducible protein A	0.032
1.6	*bcfH*	putative thiol-disulfide isomerase	0.030
1.7	*pqaA*	PhoPQ-regulated protein	0.000
1.7	*hslJ*	heat shock protein hslJ	0.035
1.7	*fdx*	[2FE-2S] ferredoxin	0.043

[1] Calculation based on the difference of the mean ratios (treatment to reference strain) for the PM treatment versus the PB control.

Table 3.3. Continued.

Fold Change[1]	Gene	Gene Function and/or Product	P Value
1.7	*yojI*	putative ABC-type multidrug/protein/lipid transport system	0.015
1.7	*yiaD*	putative outer membrane lipoprotein	0.032
2.2	*speG*	spermidine N1-acetyltransferase	0.013
2.3	*rpoE*	sigma E (sigma 24) factor of RNA polymerase	0.040
2.3	*valS*	valine tRNA synthetase	0.050
2.4	*pagP*	PhoPQ-activated gene	0.005
2.5	*cutA*	putative periplasmic divalent cation tolerance protein	0.032
2.8	*cheM*	methyl accepting chemotaxis protein II	0.024
3.5	*tolR*	tol protein, role in outer membrane integrity	0.029
4.0	*ydgE*	putative membrane transporter of cations and cationic drugs	0.047
5.2	*uvrB*	UvrB with UvrAC is a DNA excision repair enzyme	0.016

[1] Calculation based on the difference of the mean ratios (treatment to reference strain) for the PM treatment versus the PB control.

Table 3.4. *S.* Typhimurium *luxS* Mutant (PJ002) Genes Influenced by the Interaction of Autoinducer 2 with Poultry Meat Inhibitor (AI-2 + PM)

Fold Change[1]	Gene	Gene Function and/or Product	P Value
-3.3	*alkB*	DNA repair system specific for alkylated DNA	0.024
-2.2	*invI*	surface presentation of antigens	0.019
-2.1	*yhhA*	putative outer membrane protein	0.043
-2.0	*yohG*	putative outer membrane efflux protein	0.015
-1.9	*cyoC*	cytochrome o ubiquinol oxidase subunit III	0.037
-1.6	*envR*	transcriptional repressor for envCD (acrEF)	0.031
-1.6	*sseG*	secretion system effector	0.016
1.5	*pphB*	serine/threonine specific protein phosphatase 2	0.016
1.5	*sopB*	Pathogenicity island encoded protein: SPI5	0.020
1.6	*tolA*	tol protein, membrane spanning protein	0.049
1.6	*pagP*	PhoPQ-activated gene	0.009
1.6	*yifK*	putative APC family amino-acid transport protein	0.032
1.6	*nusB*	transcription termination; L factor	0.036
1.7	*tatC*	component of sec-independent protein export	0.047
1.7	*ssaR*	secretion system apparatus protein	0.030
1.8	*oxyR*	regulatory protein sensor for oxidative stress	0.017
2.0	*cutA*	putative periplasmic divalent cation tolerance protein	0.046
2.2	*tolR*	tol protein, role in outer membrane integrity	0.030
2.4	*cheM*	methyl accepting chemotaxis protein II	0.014
2.6	*valS*	valine tRNA synthetase	0.033
2.6	*recG*	DNA helicase	0.016
5.9	*uvrB*	UvrB with UvrAC is a DNA excision repair enzyme	0.047

[1]Calculation based on the difference of the mean ratios (treatment to reference strain) for the AI-2 + PM treatment verses to the PB control.

differentially expressed were different from those that were expressed in the presence of PM alone. Only 7 genes (i.e. 1/3 of the targeted genes, namely, *alkb*, *cheM*, *cutA*, *invI*, *pagP*, *uvrB*, and *valS*) showed a common response between the PM and AI-2+PM treatments (Table 3.3 and Table 3.4). This supports our assertion that the interaction of AI-2+ PM elicits a unique gene expression response that is distinct from the response observed in the presence of either PM or AI-2 molecules. Of the 23 genes that were differentially expressed in the presence of AI-2 molecules, only *tolR*, was observed in the presence of both AI-2+PM or PM alone, with *hsjI* having only a similar response in the PM treatment. These results suggest that some genes in the PM and AI-2+PM treatments may have been suppressed or induced due to the lack of AI-2 or the interaction of AI-2 with the poultry meat derived inhibitors.

When the gene expression among the different treatments is compared, only one gene, *tolR* was found to be represented in all of the treatments. This gene which plays a role in the outer membrane integrity was up-regulated significantly across all treatments (Tables 3.2, 3.3 and 3.4). TolR is a component of the Tol-Pal system and the function of this complex is to provide outer membrane support, and possibly for the transport of macromolecules into the cell (52). The Tol-Pal system is suggested to also be responsible for production of outer membrane vesicles which may have a function in pathogenticity by transporting virulence factors outside the cell (61). It is possible that not only is this gene enhanced by the presence of AI-2 (1.6-fold), but it may also be

independently influenced by particular environmental conditions present in a food matrix, as the PM treatment produced over 3-fold induction of the gene (Table 3.3). The arrays used in this study represented only a portion of the genes comprising the entire *Salmonella* genome. Thus it would be difficult to derive complete functional expression pathways from this data set. Delineating the response of an organism to environmental stimuli based solely on transcriptional activity can be a daunting task, since it is likely that different regulatory circuits may be processing a variety of signaling compounds under different conditions to produce an *in vivo* response (19). Nevertheless this work does provide some insight to the response of *Salmonella* due to AI-2 and the completely different gene expression response observed when the AI-2 molecules are in the presence of a poultry matrix.

Bacterial responses to AI-2 using microarrays have been investigated in the past. Sperandio et al. (125) reported that out of 4,290 genes in *E. coli*, 404 genes were regulated > 5-fold due to quorum sensing. Under less stringent data analysis conditions, 736 genes (17%) were either up-regulated or down-regulated at least two-fold. In another study roughly 5.6% of the genes were modulated ≥ 2.3-fold when the responses of a luxS mutant and wild-type *E. coli* were compared (34). A study by Ren et al. (112), involved treatments of a brominated furanone derived from the alga, *Delisea pulchra*, which was capable of interfering with quorum sensing. The authors found 166 genes were regulated at least 2.5-fold using the AI-2 treatments (roughly 4% of the 4228 genes on the

array), and interestingly 79% of similar genes were differentially expressed in the quorum sensing inhibitor treatment (112). Yuan et al (151), found 17 genes being differentially expressed (at least 1.5 fold compared to the wild type) in a *luxS* mutant of *P. gingivalis*. When comparing the AI-2 treatment to the PB control, 23 genes exhibited a significant difference in their expression. Similar numbers were also seen in the AI-2 + PM treatment, with the highest number of genes having statistically significant differences in expression levels for the PM treatment. These significant changes in gene expression ranged roughly between 2% to 3% across all of the treatments (Tables 3.2, 3.3 and 3.4).

However, the array utilized in this study contained approximately a quarter of the entire *Salmonella* genome (95) and there may be a larger portion of genes which may respond to AI-2, or AI-2 combined with the poultry meat-derived inhibitors, that were not present on the designed array. Additionally, these experiments monitored the late-log transcriptional activity of *S.* Typhimurium as total RNA was extracted after 3h incubation (OD_{600} ~0.6). Potentially, there may be a greater modulation of gene expression due to AI-2, and in combination with poultry meat-derived inhibitors, if RNA were extracted at earlier time points. Yet other studies have employed similar approaches to what is presented here (34, 112, 125).

In conclusion, this study suggests that AI-2 does have an impact on the expression of certain virulence genes in *S.* Typhimurium. Interestingly, the interaction of the AI-2 molecules with cell-signaling inhibitors found in poultry

meat influences the expression of a completely different set of genes. The responses from these two treatments are so distinct that there is no overlap except for just one gene (*tolR*). Overall, these results suggest that virulence-related genes in *Salmonella* appear to be differentially expressed depending on whether the organism is in contact with AI-2 molecules in the presence or absence of poultry meat matrix. These findings support our original hypothesis that poultry meat based inhibitory compounds by virtue of their interaction with AI-2 molecules would influence the expression of virulence genes in *Salmonella*. Proteomic analysis is needed to elucidate the pathways in which these poultry meat inhibitors may be controlling global protein expression in this pathogen. Understanding the influence food matrices have on bacterial cell signaling and their ultimate virulence or growth is critical in the development of new food products and types that are not only safe, but are also capable of having extended shelf lives.

CHAPTER IV

IDENTIFICATION OF POULTRY MEAT-DERIVED FATTY ACIDS

THAT EXHIBIT INHIBITION OF AUTOINDUCER (AI-2) ACTIVITY

Introduction

A system of bacterial cell signaling utilizing autoinducer molecules, is referred to

as quorum sensing (92, 131, 147). One particular cell signaling system involves

autoinducer 2 (AI-2), a furanosyl borate diester molecule produced primarily by

Gram negative bacteria (1, 116, 128, 146). AI-2 is produced as a by-product of

the Activated Methyl Cycle, where S-adenosyl-methionine is created to serve as a

methyl donor in cellular metabolism (140, 146). Several studies have proposed

that autoinducer molecules can influence virulence gene expression in bacteria

(31, 34, 96, 103, 125). However, the understanding of bacterial cell signaling in

relation to food borne pathogens and food spoilage organisms is still limited (14,

17, 105, 109).

Previous research has identified compounds derived from a variety of

sources that can inhibit bacterial cell signaling systems (50, 54, 90, 91).

Additionally compounds derived from food matrixes can also demonstrate

inhibition to these autoinducer signaling systems, in particular, matrixes derived

from poultry meat (83, 84). The primary focus of this study was to further

define, and identify, compounds derived from a poultry meat wash that

exhibited AI-2 inhibition. By identifying these inhibitory compounds, it may be

possible to gain a better understanding on the interaction, of autoinducer signals with bacterial pathogens in food matrixes, particularly poultry meats.

Materials and Methods

Bacterial Strains and Cell-Free Supernatant Preparation (CFS)

Reporter strain, *Vibrio harveyi* BB170 (generously donated from Dr. B. Bassler, Princeton University) was used as the biosensor strain to detect the presence of AI-2 activity. In order to produce high yields of compounds that expressed AI-2-like activity, an environmental E. coli strain #5, obtained from a groundwater source, was used to produce cell-free supernatant (CFS). *E. coli* #5 was grown in LB broth media amended with 0.5% glucose (weight/vol) at 37° C to mid-late log phase (OD_{600} ~1). The culture was centrifuged (10,000 X g for 10 min), and the supernatant filtered through a 0.2 μm sterile syringe filter (VWR, West Chester, PA). AI-2-like activity was confirmed in the CFS as described by Lu et al. (83). Briefly described, 90 μl of a 1:5000 dilution from an overnight culture of *V. harveyi* BB170 grown in AB media was combined with 10 μl of CFS. The reporter strain was incubated at 30° C, with moderate shaking, and bioluminescence measurements were taken at periodic intervals using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT).

Synthesis of Autoinducer 2 (AI-2)

In vitro synthesized AI-2 was prepared according to a published protocol (124). Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia,

CA). The purified enzymes were incubated with 1 mM S-adenosylhomocysteine (Sigma-Aldrich, St. Louis, MO) for one hour at 37° C, and AI-2 was further separated from the enzymes using a centrifuge filter column (Biomax-5, Millipore, Billerica, MA). AI-2 activity was confirmed using *V. harveyi* BB170 as previously described.

Poultry Wash (PW) Derived Inhibitors

Poultry meat (50 g) obtained from a commercial source was stomached with 40 ml water, centrifuged (10,000 X g) for 10 min, and the supernatant filtered (0.2 μm). Inhibition of AI-2 like activity was confirmed using *Vibrio harveyi* BB170. Inhibition of AI-2 was expressed as a % relative to a corresponding positive control (100 – [(light unit measurement of sample/light unit measurement of positive control) x 100]).

Molecular Size Exclusion and Reverse Phase Column Chromatography

All column work was conducted on an ÄKTAFPLC system fitted with a UV detection system (Amersham Biosciences, Piscataway, NJ). For molecular size exclusion chromatography, PW inhibitor was loaded into a pre-packed resin column with the matrix consisting of cross-linked copolymer of allyl dextran and N,N-methylenbisacrylamide (HiPrep 16/60 Sephacryl S-300, Amersham Biosciences, Piscataway, NJ). Using a running buffer of 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2, and at a flow rate of 0.5 ml/minute, 1 ml fractions were collected after the void volume, and further concentrated to a final

0.1 ml volume under speed-vac. To correlate collected fraction volumes with approximate molecular weight, protein standards (molecular weights ranging from 13,700 – 67,000 Daltons) were previously run through the column and peaks viewed by UV detection were associated with specific fractions (Low Molecular Weight Gel Filtration Calibration Kit, Amersham Biosciences, Piscataway, NJ). Reverse phase chromatography was conducted using hand-packed C18 resin column (octadecyl-functionalized silica gel, Sigma-Aldrich, St. Louis, MO). PW inhibitor was loaded into the column under water-saturated conditions. Water was used for the initial column conditions in order to reduce potential inhibition from residual chemicals that would be present in a running buffer. Using an increasing linear gradient of methanol at 1 ml/minute up to a final concentration 100%, 5 ml fractions were collected, evaporated under nitrogen gas with heating block and resuspended in 0.5 ml water.   Selected fractions collected under both chromatography systems were tested for AI-2 inhibition (described earlier in the text).

Solvent Extraction and Fatty Acid Analysis of Poultry Meat-Derived Inhibitors

Poultry meat obtained from a commercial source was stomached with an equivalent volume weight of DI water for 2 minutes. The liquid from the stomached material was transferred to sterile polypropylene bottles and centrifuged (10,000 X g) for 10 min. The supernatant was then removed and pooled. Multiple preparations were made until one liter of wash was generated.

The poultry meat wash was mixed with three sequential 500 ml volumes of hexane. After the addition of a volume of solvent and mixing, the solution was allowed to separate, the solvent phase removed, and an additional solvent volume was added to the PW sample. The pooled solvents were evaporated resulting in approximately 200, and 400, mg material (obtained from two preparations). From the solvent extracted material, fatty acid composition was determined using gas chromatography (GC) as described in Hossen and Hernandez (66). Briefly, methyl esters of the fatty acids were generated and further extracted in hexane. Using hydrogen as a carrier gas, the extracted fatty acid esters were injected into a GC system with a flame ionization detector and passed through a fused silica capillary column. Fatty acid concentrations for the second solvent extract were determined using a standard method, AOCS official method Ce 1h-05. Briefly, methyl esters of the extracted fatty acids were prepared and then passed through a GC system with reference fatty acid methyl esters used to aid in quantifying the fatty acids present in the sample.

Evaluation of AI-2 Inhibition by Selected Fatty Acids

Four fatty acids were selected for further analysis based on percentage of composition in the extracted solvent. Linoleic acid, oleic acid, palmitic acid, and stearic acid were obtained from a commercial source (Sigma-Aldrich, St. Louis, MO), and stock concentrations of 10 mM, 1 mM, and 0.1 mM were prepared in water. AI-2 inhibition for these solutions were determined using CFS. Additionally, solutions were prepared in which linoleic acid, oleic acid, palmitic

acid, and stearic acid were combined in water at concentrations (0.014 mM,

0.033 mM, 0.02 mM, and 0.0046 mM, respectively) similar to those determined

in the chemical analysis (1X) and at concentrations of 10-fold (10X) and 100-fold

(100X) to determine if these would demonstrate a corresponding increase in

inhibition. AI-2 inhibition was determined with these combined solutions using

both CFS and *in vitro* prepared AI-2.

To determine if the fatty acids had a cytotoxic effect on the reporter

strain, another AI-2 bioassay was run with the 100X fatty acid solution and a PW

control. After sufficient incubation to demonstrate inhibition, portions of the

bioassay cultures were spread plated on LM media (10), incubated at 30° C, and

plate counts of *V. harveyi* colonies determined after 24 hours.

Results

Molecular Size Exclusion and Reverse Phase Column Chromatography

Initial experiments using centrifuge molecular weight cut-off filters indicated

that inhibitory compounds in the poultry wash were greater than 50,000

Daltons (data not shown). From the molecular size exclusion chromatography

experiments, several concentrated fractions demonstrated inhibition with the *V.*

harveyi autoinducer assay (Fig. 4.1).   Fractions greater than the 67 kD (kilo

Daltons) standard did express some activity, but the highest mean response was,

at most, 61 % (fractions 30-79). The highest number of fractions that

consistently gave a 90 % or greater inhibition were 67 kD, or smaller, and most

of the collected fractions (fractions 91-119) were of sizes less than 13.7 kD (Fig.

2.1) before a noticeable decrease in inhibition (mean 24 %) was seen in the later
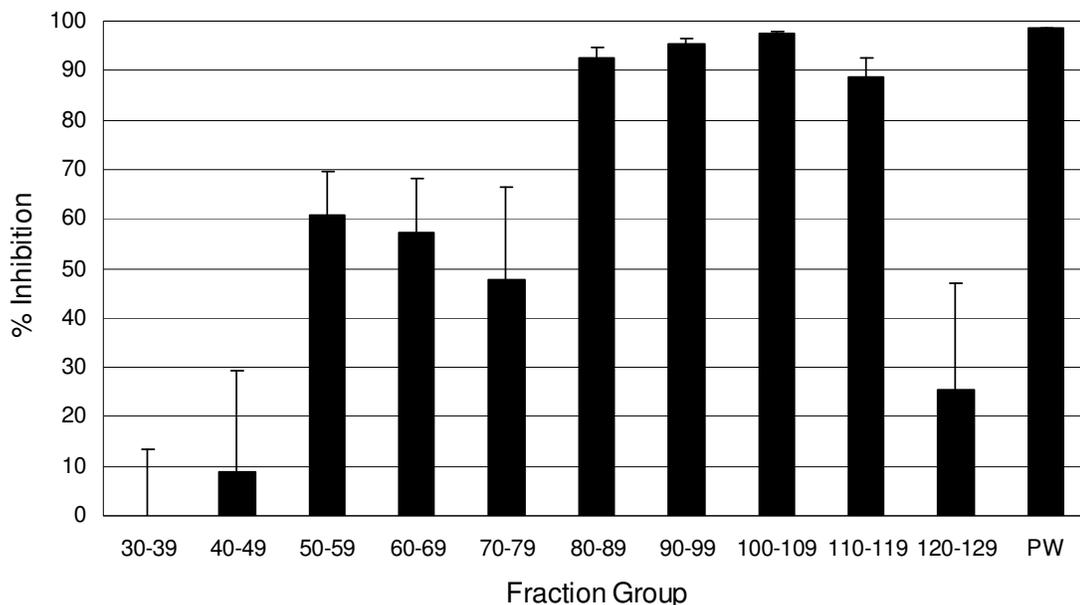
Figure 4.1. Mean AI-2-like Inhibition in Fractions Generated by Molecular Size Exclusion Liquid Chromatography

% Inhibition - mean percentage inhibition of AI-2 bioassay (n = 10) in relation to a positive control (CFS) for each fraction group (bars represent ± one standard error). Fraction Group - fractions (1 ml) collected after passing through a gel filtration molecular size exclusion column. PW - untreated poultry wash as a comparative inhibition control for the AI-2 bioassay. Molecular weight standards corresponded to specific fraction groups as follows: 67 and 43 kD standards - fractions 70-79, 25 kD standard - fractions 80-89, and 13.7 kD standard – fractions 90-99.

collected fractions (fractions 120-129). As data indicated that the size of the compounds was less than the resolution of the column, additional experiments were conducted to further separate the compounds based on hydrophobic interaction using the C18 resin column.

The average UV spectra from the collected fractions indicated that most of the compounds were released from the column when the mobile solvent reached greater than 80 % (data not shown). Based on UV absorbance, fractions were selected for concentration and further testing for inhibition. Although the inhibition of the later selected fractions varied (roughly 19-83 %), the majority of fractions that demonstrated the most inhibition (over 70 %) were collected when the mobile solvent phase was at a concentration of 90%, or greater (fractions 21 to 29), and none of the earlier fractions demonstrated any appreciable inhibition (Fig. 4.2). From these results, it was expected that the inhibitory compounds had hydrophobic properties and might be extracted from the poultry meat wash using a non-polar solvent.
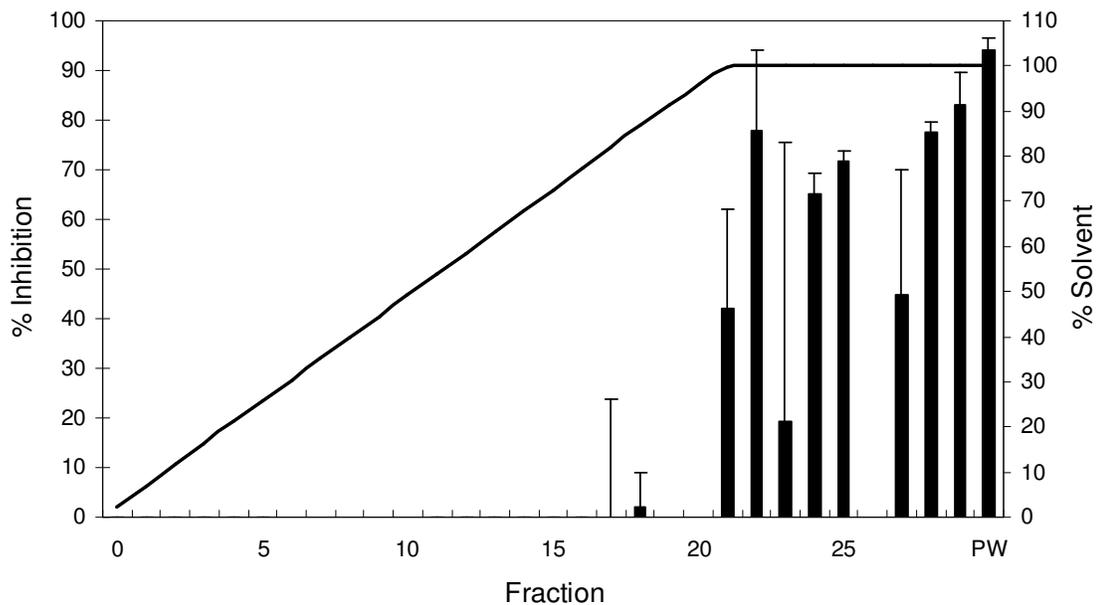
Figure 4.2. Mean AI-2-like Inhibition in Fractions Generated by Reverse Phase Liquid Chromatography

% Inhibition - bars represent the mean percentage inhibition of AI-2 bioassay (n = 3) in relation to a positive control (CFS) for each concentrated fraction (bars represent ± one standard error). % Solvent - curve represents the percentage of methanol used as the moving solvent phase. Fraction: Fractions (5 ml) collected after passing through a C18 column. PW - untreated poultry wash provided as a comparative % inhibition control for the AI-2 bioassay.

Fatty Acid Analysis Utilizing Gas Chromatography

Analysis of the first solvent extracted material indicated that oleic (38.3 %),

palmitic (29.7 %), and linoleic acid (12.3 %) were the three most common fatty

acids present (Table 4.1). Further analysis from the second solvent extracted

material indicated these fatty acids were present in the highest concentrations

ranging from 9.19, 5.13, and 3.95 µg/g, respectively (Table 4.1).

Table 4.1. Composition and Concentration of Fatty Acids Derived from Hexane
Solvent Extract of Poultry Meat

Fatty Acid	%	µg/g
Linoleic	12.3	3.95
Myristic	3.6	0.13
Oleic	38.3	9.19
Palmitic	29.7	5.13
Palmitoleic	9.9	1.40
Stearic	6.2	1.31

Percentage (%) and concentration (µg/g) of fatty acids based on gas chromatographic analysis.

AI-2 Inhibition of Selected Fatty Acids

Chemical standards of fatty acids were chosen based on those in a sufficiently

high concentration within the solvent extracts.   From the 0.1, 1, and 10 mM

preparation of fatty acid solutions all samples demonstrated some AI-2

inhibition (Fig. 4.3). Interestingly, as the concentrations of stearic acid and palmitic acid increased there was some decline in inhibition. While oleic acid and linoleic acid inhibition increased as higher concentrations were tested, with linoleic acid having the highest inhibition of over 99 % at 10 mM (Fig. 4.3).

However, these higher concentrations were far greater than those observed in the solvent extracted PW, and could be construed as a higher artificial sample compared to actual concentrations found in a food matrix. Rather than just observe the inhibition of specific fatty acids separately, preparations were made with combinations of all the selected fatty acids (1X) in concentrations similar to those determined by GC analysis and, at higher concentrations (10X and 100X). Inhibition was present in all the fatty acid solutions with the 1X, 10X, and 100X solutions averaging 65 %, 89 %, and 83 %, respectively, when CFS was used as the autoinducer signal (Table 4.2).
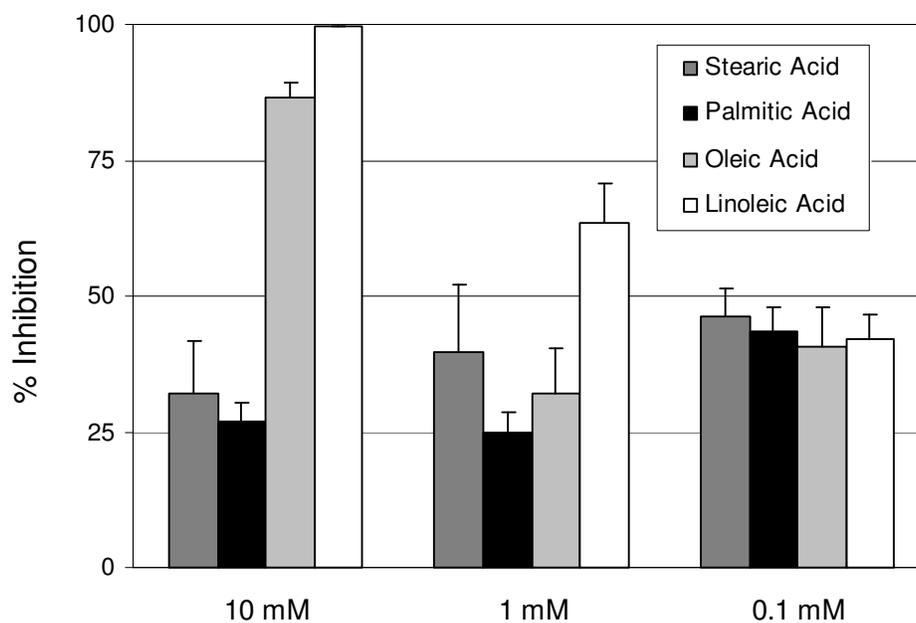
Figure 4.3. Mean AI-2-like Inhibition for Selected Fatty Acids

Bars represent mean percentage inhibition of AI-2 bioassay (n = 6) in relation to a positive control (CFS) for each fatty acid solution (± one standard error). Solutions of fatty acids were stearic acid, palmitic acid, oleic acid, and linoleic acid in, 10 mM, 1 mM, or 0.1 mM concentrations.

Table 4.2. AI-2 Inhibition for Combined Fatty Acid Treatments

Sample	% Inhibition CFS	% Inhibition AI-2
1 X Solution	64.5 (± 5.0)	59.5 (± 8.0)
10X Solution	89.2 (± 3.6)	84.4 (± 3.0)
100X Solution	83.2 (± 1.0)	69.5 (± 3.7)
PW	97.6 (± 0.3)	99.0 (± 0.2)

Sample - combined solutions of stearic acid, palmitic acid , oleic acid, and linoleic acid in various concentrations (1X, 10X, or 100X), or poultry meat wash (PW). % Inhibition CFS - mean percentage inhibition of AI-2 bioassay (n = 3) in relation to a positive control (CFS). Values in parenthesis represent the standard error in percentage. % Inhibition AI-2 - mean percentage inhibition of AI-2 bioassay (n = 3) in relation to a positive control (CFS). Values in parenthesis represent the standard error in percentage.

When *in vitro* synthesized AI-2 was used as the autoinducer signal for the *V. harveyi* bioassay, the 1X, 10X, and 100X solutions demonstrated inhibition of 59 %, 84 %, and 69 %, respectively (Table 4.2). Plate counts from the 100X combined fatty acid solution averaged 7.5 log CFU/ml, while the PW treatment and positive control (AI-2) averaged 7.8 and 7.2 log CFU/ml, respectively, indicating there was no significant decrease in viability of the reporter strain despite increased autoinducer inhibition in the 100X treatment (Fig. 4.4).

Discussion

Previously published studies have described a variety of isolated compounds which can interfere with quorum sensing systems, and many of these compounds were derived from plant and algal species. Gao et al. described the
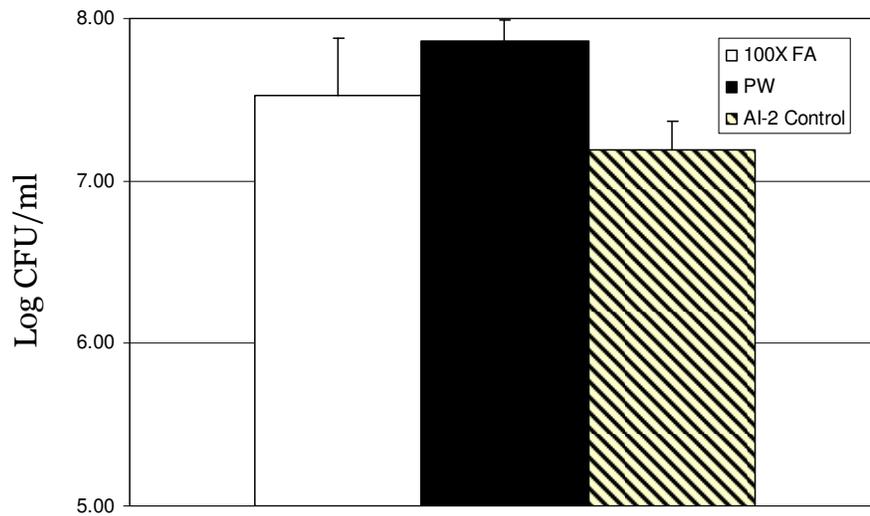
Figure 4.4. Mean Plate Counts for Combined Fatty Acid Treatment (100X), Poultry Wash Inhibitor (PW) and *In Vitro* Synthesized AI-2 (AI-2)

Mean log colony forming units per ml (n = 3) of *V. harveyi* after 24 hour incubation on LM plates (bars represent ± one standard error). Treatments were a combined solution of stearic, palmitic, oleic, and linoleic acid (100X FA), poultry wash inhibitor (PW), and *in vitro* synthesized autoinducer 2 positive control (AI-2 Control). The mean AI-2 inhibition for the 100X FA sample and PW sample were, 85.0 % (± 1.7 %) and 90.4 % (± 1.2 %), respectively.

isolation of several compounds obtained from solvent extracts of *M. truncatula* seedlings which could impede homoserine lactone signaling compounds (50). Halogenated furanones derived from the alga, *Delisea pulchra*, expressed similar characteristics of autoinducer inhibition (91) and appear to influence many bacterial processes from swarming motility (54, 64, 111) to production of intracellular cellulases and proteases (90). Although these compounds interact primarily with acyl homoserine lactones (a class of quorum sensing compounds), there has also been published studies describing the ability of furanones to act as inhibitors for AI-2, as Ren et al. showed that furanones were capable of inhibiting response of the reporter strain, *V. harveyi* BB170, by roughly 49 % (112). Compounds, other than furanones, have demonstrated inhibitory properties to AI-2 cell signaling. DeAnna et al. have described the influence of RbsB, a periplasmic ribose binding protein found in *Actinobacillus sp.*, on AI-2 activity where 50 % inhibition was seen with as little as 40 ng/ml of the protein (71).

Rasmussen and Givskov have proposed that inhibition to quorum sensing systems likely employ different strategies: i) impede the production of the signaling compound ii) inactivate the signaling compound by direct interaction, or iii) competitively bind to (or interfere with) the quorum sensing receptors in the bacteria (110). Although there has been substantial evidence of compounds which possess inhibitory properties, these compounds do not have a similar structure to the fatty acids employed in this study, particularly those isolated

compounds having a furanone structure. Interestingly, the fatty acids employed in this study were similar in structure, with only small differences in the length of, or number of double bonds present in (oleic and linoleic acid), the CH side chains, indicating that fatty acids as a class of compounds may have inhibitory properties. Also, the fatty acids employed in this study do not have a similar structure to AI-2 suggesting that inhibition may not be based on the fatty acids acting as a mimic compound, competitively binding to bacterial receptors (Fig. 4.5). Inhibition in the combined fatty acid samples did not equal that observed in the PW control samples, but there was appreciable amount of inhibition seen. However, this may be expected as only a select number of fatty acids were evaluated for inhibition. As all of the selected fatty acids demonstrated some inhibition singly and have similar chemical structures, it may be expected that other fatty acids within the poultry meat wash are present but below the detection limits of chemical analysis, and these compounds could further enhance inhibition seen in poultry wash. Additionally, it must be noted that there may be other compounds that were not retained in the initial hexane solvent extraction, which are present in the poultry wash and also contribute to AI-2 inhibtion.

There have been published studies showing that mixed fatty acids in milk have antimicrobial properties (143). Additionally, linoleic acid has also been show to inhibit the growth of food-borne bacterial pathogens (79) and it could be suggested that the inhibition seen is merely the reduction of the reporter strain
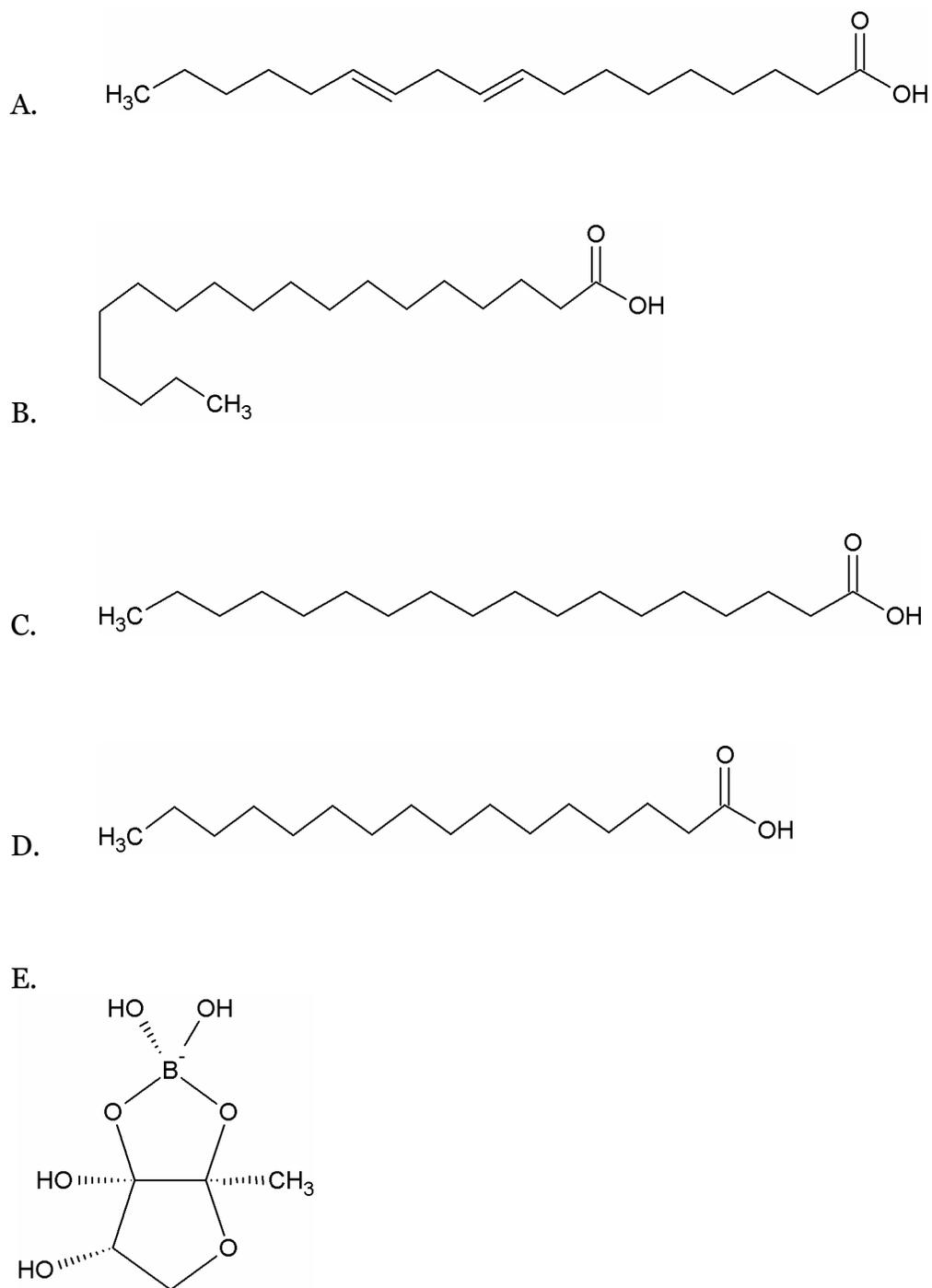
Figure 4.5. Chemical Structures of AI-2 and Selected Fatty Acids

linoleic acid (A), oleic acid (B), stearic acid (C), palmitic acid (D), and autoinducer 2 (E).

due to antimicrobial effects. However the work conducted in this study does not indicate that the reduction of AI-2 signal in the reporter strain is a result of antimicrobial activity, as there was no significant reduction in plate counts for the inhibitor treatments compared to the untreated control despite the fatty acid treatments expressing over 80 % inhibition (Fig. 4.4).

This study presents several long-chain fatty acids identified from poultry meat tissue which interfere with autoinducer 2 activity. As these different fatty acids have similar structures, they may serve as a class of compounds that possess inhibitory characteristics. Further research will be needed to better understand the mode of inhibition utilized by these fatty acids. However as studies indicate quorum sensing plays a role in the virulence of bacterial pathogens, developing strategies to modulate such signaling systems could lead to novel means to control food spoilage bacteria or food-borne pathogens.

CHAPTER V

INFLUENCE OF AUTOINDUCER 2 (AI-2) ON THE GROWTH AND INFECTION OF *Salmonella enterica* SEROVAR TYPHIMURIUM AND THE MODULATION OF THESE EFFECTS USING POULTRY MEAT-DERIVED FATTY ACIDS CHARACTERIZED TO HAVE AI-2 INHIBITORY PROPERTIES

Introduction

Salmonella Typhimurium is an enteric pathogen which can act as an agent of food-borne illness and is primarily associated with animal products, particularly poultry. A survey conducted from 1992 to 1997 showed that, annually, non-Typhi *Salmonella* infections accounted for 9.7% of all food-borne illnesses in the United States and resulted in over 1.4 million cases annually (97). An annual survey of commercial raw chicken products (frozen and fresh meats) in the United Kingdom, *Salmonella* was detected in 8 to 9.7% of the samples (98). Another annual survey of large broiler plants conducted by the USDA in 1998 to 1999, found that *S.* Typhimurium was the third most common serotype, accounting for 14.2% of the total isolates and most of the isolates were found in broilers (63.5%) (43). Because *Salmonella sp.* have the ability to overcome environmental stresses such as osmotic pressure and acid tolerance, it has been theorized that these stresses may induce the adaptability of *Salmonella* allowing it to remain virulent in a wide variety of uncooked food products (68).

Bacteria possess the ability to produce, export, and take up small chemical molecules that can serve as a form of bacterial communication. One common signaling compound is a furanosyl borate diester, Autoinducer 2 (AI-2), which is primarily produced by Gram negative bacteria (1, 116, 128, 130, 146). AI-2 is produced as a byproduct of the Activated Methyl Cycle, where S-adenosyl-methionine is created to serve as a methyl donor in cellular metabolism (127, 140, 146). The precursor to AI-2 is formed as the hydrolysis of S-ribosyl-homocystiene is converted into homocysteine by the enzyme, LuxS, creating 4,5-dihydroxyl-2,3-pentanedione (DPD) as a byproduct. DPD can undergo several conformations spontaneously with the removal or introduction of water, but the formation of 2-methyl-2,3,3,4-tetrahydroxytetrathydrofuran (S-THMF) acts as a the precursor to AI-2. As free boron interacts with S-THMF, a furanosyl borate diester is formed, AI-2 (100, 127, 140).

Studies have indicated that AI-2 has an impact on bacterial virulence. *Clostridium perfringens luxS* mutants recovered from limited production of alpha, kappa, and theta toxins when cell free supernatants of wild type cultures were introduced (103). LuxS mutants of the opportunistic pathogen, *Serratia marcescnes* were able to utilize AI-2 from wild type cell free supernatants, restoring production of prodigiosin, a compound suggested to have immunosuppresant properties (31). Other studies using microarray analysis have shown that an enterohemorrhagic *Escherichia coli* wild type, when compared to a *luxS* mutant, had 404 genes were up-regulated at least five-fold

(125).   Also an enteropathogenic *E. coli*. *luxS* mutant were unable to effectively

transcribe the locus of enterocyte effacement (LEE), a series of promoters

responsible for the type III secretion system, in *E. coli* (119).

Compounds derived from plant tissues and brominated furanones have

properties that inhibit AI-2 (93, 111, 112). Additionally, extracts from various

food matrices have been reported to inhibit AI-2 signaling system (83). Previous

experiments have indicated that fatty acids derived from a poultry meat wash

also have inhibitory properties (Chapter IV). The primary focus of this study was

to determine the impact of AI-2 on the growth and virulence of *Salmonella*

enterica serovar Typhimurium, and whether fatty acids could modulate any

effects AI-2 had on these processes.

Materials and Methods

Bacterial Strains and Cell Culture Lines

Vibrio harveyi BB170 (a gift from Dr. B. Bassler, Princeton University) was used

as the biosensor strain to detect the presence of AI-2 activity. *Salmonella*

enterica serovar Typhimurium (isolate # 87-26254) was obtained from the

National Veterinary Service Laboratory (Ames, Iowa).   A *luxS* mutant of this

strain, designated PJ002, was subsequently generated as detailed previously

(Widmer et al. 2006). Briefly, Red recombinase expression plasmid, pKD46 was

used to transform the *S*. Typhimurium wild-type by eletroporation. An

additional plasmid, pKD3, was used as a template for PCR with the resulting

product encoded a chloramphenicol resistance gene flanked by the FLP

recognition target, and *luxS* homologous, regions. The PCR product was then incorporated into competent *S.* Typhimurium cells containing the pKD46 plasmid (that would facilitate recombination) by further electroporation. The *Salmonella* cells were incubated at 37° C on LB plates amended with chloramphenicol, which would select for transformants and the elevated temperature ensured the removal of the heat sensitive pKD46 plasmid. The murine macrophage cell line, RAW264.7, was obtained from a commercial source and was used for the infection assay experiments (ATCC: Number TIB-71).

Synthesis of Autoinducer 2 (AI-2)

In vitro synthesized AI-2 was prepared according to a published protocol (REF). Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia, CA). The purified enzymes were incubated with 1 mM S-adenosylhomocysteine (Sigma-Aldrich, St. Louis, MO) for one hour at 37° C, and AI-2 was further separated from the enzymes using a centrifuge filter column (Biomax-5, Millipore, Billerica, MA). AI-2 activity was confirmed using *V. harveyi* BB170 as described by Lu et al. (REF). Briefly, 90 μl of a 1:5000 dilution from an overnight culture of *V. harveyi* BB170 grown in AB media was combined with 10 μl of CFS. The reporter strain was incubated at 30° C, with moderate shaking, and bioluminescence measurements were taken at periodic intervals using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT).

Fatty Acid Growth Experiments

Linoleic acid, oleic acid, palmitic acid, and stearic acid were obtained from a

commercial source (Sigma-Aldrich, St. Louis, MO). As previously described in

an earlier chapter (Chapter IV), it was determined that these fatty acids were

present in a solvent fraction after extraction from washes of poultry meat

samples at a particular concentration. Stock solutions of linoleic acid, oleic acid ,

palmitic acid, and stearic acid were combined in water at concentrations (0.014

mM, 0.033 mM, 0.02 mM, and 0.0046 mM, respectively) similar to those

determined by previous chemical analysis (1X) and at concentrations of 10-fold

(10X) and 100-fold (100X). Additionally solutions of individual fatty acids were

prepared in similar concentrations to those of the combined solutions to

determine effects of a particular fatty acid singly.

A portion of an overnight culture of *Salmonella* PJ002 grown in LB was

centrifuged and the cell pellet washed three times in 1X PBS. One microliter of

the washed cells was added to 80 μl M9 medium (supplemented with 0.2%

glucose as the carbon source) in a 96 well clear flat bottom plate (Corning Inc,

Corning, NY). Then either (i) 20 μl 1X PBS (PBS control), (ii) 10 μl AI-2 + 10 μl

of a fatty acid (FA) at a defined concentration (either 1X, 10X, or 100X), 10 μl 1X

PBS + 10 μl FA (either 1X, 10X, or 100X), or (iv) 10 μl AI-2 + 10 μl 1X PBS (AI-2

control) was added to bring the final concentration in each well to 100 μl. The

combinations of solutions were prepared as 8 replicates. Additionally, blank

controls were prepared (20 μl 1X PBS + 80 μl M9 medium) to provide a baseline

for OD600 readings. The plates were incubated at 37° C for 12 hours with moderate shaking in a Tecan Spectrafluor Plus plate reader (Tecan Systems Inc., San Jose, CA). The optical density of each well was measured at a wavelength of 600 nm and at 15 minute intervals the values recorded automatically using Magellan software 4.0.

OD600 values were used to estimate cell numbers (69, 70, 134). All OD600 values were corrected against the un-inoculated control, and then log transformed and graphed. Time points were selected at the extreme endpoints, encompassing the entire the linear portion of the graphed data. These points were then used to calculate the growth rate constant defined as: $k = (\ln(OD_2) - \ln(OD_1))/(T_2 - T_1)$, where OD_1 and OD_2 were the OD600 values at time 1 (T_1) and time 2 (T_2), respectively. The resulting growth rate constants (k) were then used for comparison analysis. To also determine if there was any difference in the growth rates of the *luxS* mutant versus the wild-type, an additional experiment was conducted. Cultures were prepared from each strain as previously described, inoculated in 80 μl M9 medium + 20 μl 1X PBS and grown under similar conditions.

Macrophage Infection Assay

The RAW264.7 cells were maintained on modified Eagle medium with Earle's modified salts (MEME) supplemented with 2 mM Glutamine and 10% fetal bovine serum. Six well plates were seeded with RAW cells and allowed to grow to confluence at 37° C in 5% CO_2. From an overnight culture of *Salmonella*

PJ002 was grown in LB broth, a portion was removed, centrifuged and the cell pellet resuspended in 400 μl MEME media (without FBS). Either (i) 100 μl 1X PBS (PBS treatment), (ii) 50 μl 1X PBS + 50 μl AI-2 (AI-2 treatment), or (iii) 50 ul AI-2 + 50 μl 100x of the combined fatty acids (100X FA + AI-2) were added to produce a final cell suspension volume of 500 μl. The cell invasion assays were conducted in a manner similar to previously published studies were used with some modifications (23, 118). In brief, the 500 μl cell suspensions (containing roughly 10^7 bacterial cells) were added to a single well and allowed to incubate for 1 hour at 37° C in 5% CO_2. Cells that had grown to confluence in selected wells were removed and counted using a hemocytometer. Based on these counts of RAW cells, the ratio of macrophage cells to bacteria (MOI) was approximately 1:100. The wells were washed twice with 1X PBS and 300 ul MEM supplemented with ceftriaxone (100 μg/ml) and 10% FBS were added to each well. The plates were then incubated for 2 hours at 37° C in 5% CO_2. After incubation the wells were washed 3 times with 1X PBS and 400 μl 1% Triton X-100 was added. The plates were incubated at room temperature for 10 minutes, and then 600 μl LB broth was added to each well. Portions were removed, serially diluted in 1X PBS, and plated onto LB agar plates. All LB agar plates were incubated at 37° C overnight. Dilutions of the treatments and inoculum were maintained overnight at 4° C for additional confirmation of plating results, if needed. Colonies were counted and infection efficiency calculated as the number of recovered cells (as CFU/ml) post-infection divided by the initial

inoculum multiplied by 100 (41, 108). The assays were performed twice, with 3 replications of each treatment. Additional treatments prepared as negative controls (uninoculated wells seeded with RAW cells), and controls to demonstrate the bactericidal effectiveness of ceftriaxone (*Salmonella* cell suspension in MEME), were also plated onto LB agar. To determine if the mutant strain PJ002 had a different efficacy of infection with the RAW cell line, both the mutant strain and the wild-type were used in infection assays in triplicates, where the cell suspensions were prepared in 400 μl MEM media with 100 μl 1X PBS, as described previously.

Statistical Analysis

All statistical analysis was done using a commercially available statistical software program (SPSS 11.0). Analysis included either an independent t test or, when applicable, by ANOVA with Dunnitt's test used for post hoc analysis. Results were deemed statistically significant at a calculated P value of 0.05.

<div align="center">

Results

</div>

Comparison of Growth Constants from Fatty Acid Growth Experiments

AI-2 activity was confirmed using the *V. harveryi* reporter strain and showed typical relative activity of ≈ 200-fold relative light units (data not shown). The growth constants for the different treatments were aranged into two sets for comparative analysis. The first series were treatments of the fatty acids combined with AI-2 were compared directly to the AI-2 control. As the

underlying assumption was that the AI-2 control would have a higher growth

rate constant than the combined fatty acid-AI-2 treatments, a one-sided

Dunnett's test was used for statistical analysis (Table 5.1). For the second set,

the fatty acid treatments were compared to the blank control to determine if the

fatty acids alone had an impact on *Salmonella* growth (Table 5.2).

Table 5.1. Mean Growth Constant, k, of *Salmonella* in M9 Media Supplemented with Fatty Acids in Combination with AI-2

Fatty Acid(s)	Treatment	k
Combined (All Fatty Acids)	1X + AI-2	0.0054 (± 0.0003)
	10X + AI-2	0.0051 (± 0.0004)
	100X + AI-2	0.0059 (± 0.0002)
	AI-2	0.0051 (± 0.0003)
Linoleic Acid	1X + AI-2	0.0038 (± 0.0003)
	10X + AI-2	0.0038 (± 0.0005)
	100X + AI-2	0.0043 (± 0.0003)
	AI-2	0.0039 (± 0.0004)
Oleic Acid	1X + AI-2	0.0026 (± 0.0006)
	10X + AI-2	0.0028 (± 0.0002)
	100X + AI-2	0.0037 (± 0.0005)
	AI-2	0.0025 (± 0.0006)
Palmitic Acid	1X + AI-2	0.0020 (± 0.0002)
	10X + AI-2	0.0021 (± 0.0002)
	100X + AI-2	0.0032 (± 0.0002)
	AI-2	0.0023 (± 0.0003)
Stearic Acid	1X + AI-2	0.0027 (± 0.0002)
	10X + AI-2	0.0026 (± 0.0003)
	100X + AI-2	0.0031 (± 0.0002)
	AI-2	0.0024 (± 0.0002)

Treatment - fatty acids singly, or combined, at a nominal (1X + AI-2), 10-fold (10X + AI-2), or 100-fold (100X + AI-2) concentration. AI-2 comprises of M9 medium supplemented with Autoinducer 2.   k - mean growth constant (at least 7 replicates) with standard error in parenthesis.

Table 5.2. Mean Growth Constant, k, of *Salmonella* in M9 Media
Supplemented with Fatty Acids

Fatty Acid(s)	Treatment	k
Combined (All Fatty Acids)	1X	0.0045 (± 0.0003)
	10X	0.0050 (± 0.0002)
	100X	0.0040 (± 0.0002)
	PBS	0.0044 (± 0.0003)
Linoleic Acid	1X	0.0009 (± 0.0002)
	10X	0.0012 (± 0.0002)
	100X	0.0018 (± 0.0003) [a]
	PBS	0.0008 (± 0.0002)
Oleic Acid	1X	0.0017 (± 0.0001)
	10X	0.0016 (± 0.0001)
	100X	0.0021 (± 0.0002)
	PBS	0.0017 (± 0.0003)
Palmitic Acid	1X	0.0010 (± 0.0003)
	10X	0.0016 (± 0.0002)
	100X	0.0011 (± 0.0003)
	PBS	0.0015 (± 0.0001)
Stearic Acid	1X	0.0013 (± 0.0001)
	10X	0.0019 (± 0.0001)
	100X	0.0016 (± 0.0001)
	PBS	0.0019 (± 0.0001)

Treatment - fatty acids singly, or combined, at a nominal (1X), 10-fold (10X), or 100-fold (100X) concentration. PBS comprises of M9 medium supplemented with 1X PBS. k - mean growth constant (at least 7 replicates) with standard error in parenthesis. [a] - significant difference in the mean values of k compared to the PBS control (P=0.05)

None of the calculated k values expressed any statistical significant differences
(P > 0.05) for the combined FA + AI-2 treatments compared to the AI-2 control,
indicating that the fatty acids had no effect on AI-2 which would impact the
growth rate of *Salmonella* (Table 5.1). A similar result was observed in most of
the FA treatments when compared to the PBS control, with only linoleic acid at a
100-fold concentration (100X) demonstrating a significant difference (Table
5.2).

When the mean values of the OD600 values were plotted, an overall
pattern was observed in each series of treatments. Consistently, treatments with
AI-2 (with and without fatty acids) had a more pronounced increase in OD600
absorbance readings compared to treatments that had did not have AI-2 (Fig.
5.1-5.5). From this apparent difference, further statistical analysis was done
directly comparing the growth rates of the AI-2 treatments against the PBS
control. There was a significant difference in the growth rates, where mean k
values for the AI-2 and PBS controls were 0.0033 and 0.0021, respectively
(Table 5.3). Neither the wild-type, nor the mutant strain, demonstrated a
significant difference in mean k values, indicating no difference in growth rates
in M9 media (combined with PBS) (Table 5.4).

Figure 5.1. Mean OD 600 Values of Combined FA Treatments
with, and without, AI-2 in M9 Minimal Medium

Points represent the mean OD600 values. Treatments are 1x PBS (PBS), Autoinducer 2 (AI-2) and combined fatty acids at a nominal concentration (1X), 10-fold (10X) or 100-fold (100X) without AI-2, or with AI-2 (1X + AI-2, 10x + AI-2, 100X + AI-2).

Figure 5.2. Mean OD 600 Values of Linoleic Acid Treatments
with, and without, AI-2 in M9 Minimal Medium

Points represent the mean OD600 values. Treatments are 1x PBS (PBS), Autoinducer 2
(AI-2) and linoleic acid at a nominal concentration (1X), 10-fold (10X) or 100-fold (100X)
without AI-2, or with AI-2 (1X + AI-2, 10x + AI-2, 100X + AI-2).

Figure 5.3. Mean OD 600 Values of Oleic Acid Treatments
with, and without, AI-2 in M9 Minimal Medium

Points represent the mean OD600 values. Treatments are 1x PBS (PBS), Autoinducer 2
(AI-2) and oleic acid at a nominal concentration (1X), 10-fold (10X) or 100-fold (100X)
without AI-2, or with AI-2 (1X + AI-2, 10x + AI-2, 100X + AI-2).
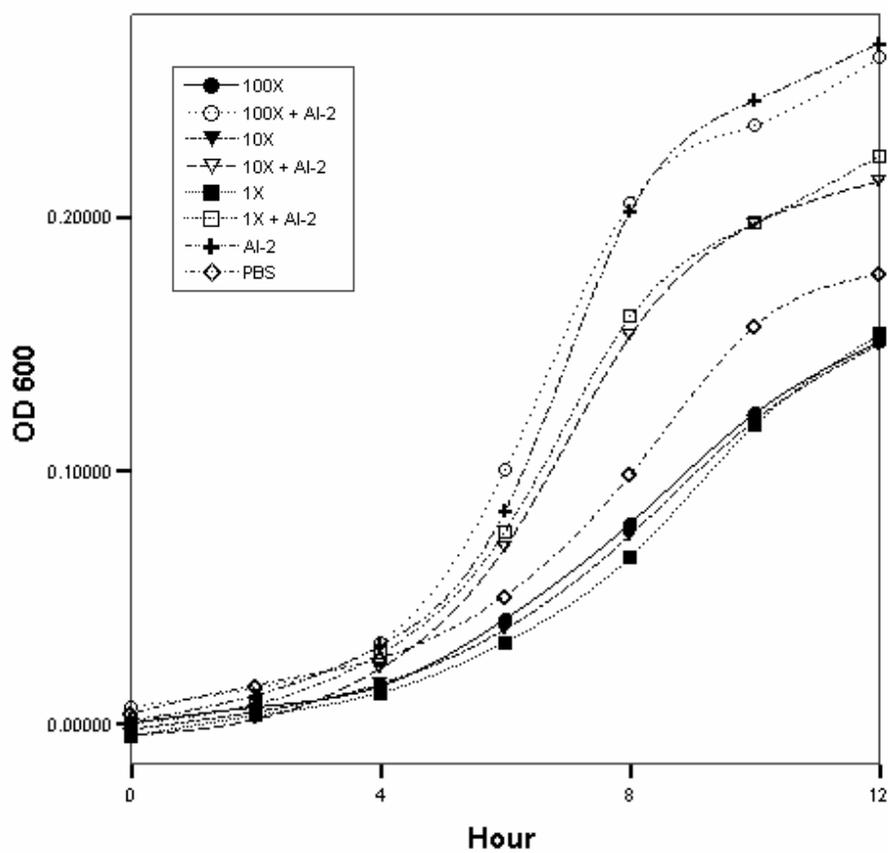
Figure 5.4. Mean OD 600 Values of Palmitic Acid Treatments
with, and without, AI-2 in M9 Minimal Medium

Points represent the mean OD600 values. Treatments are 1x PBS (PBS), Autoinducer 2
(AI-2) and palmitic acid at a nominal concentration (1X), 10-fold (10X) or 100-fold (100X)
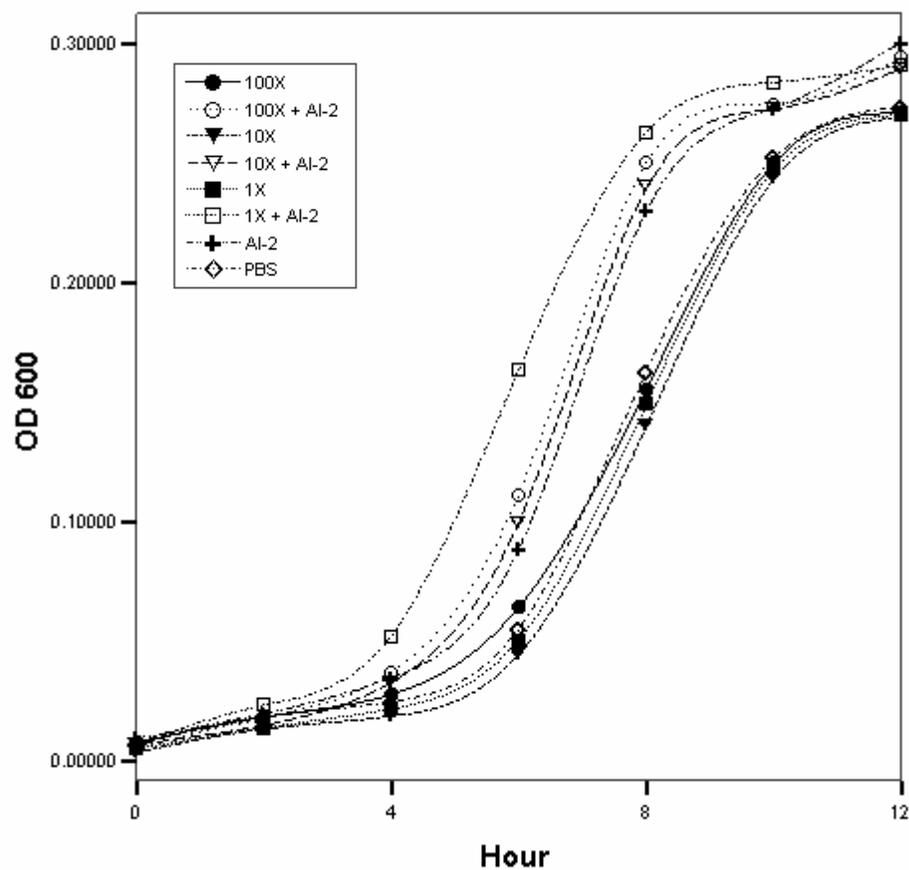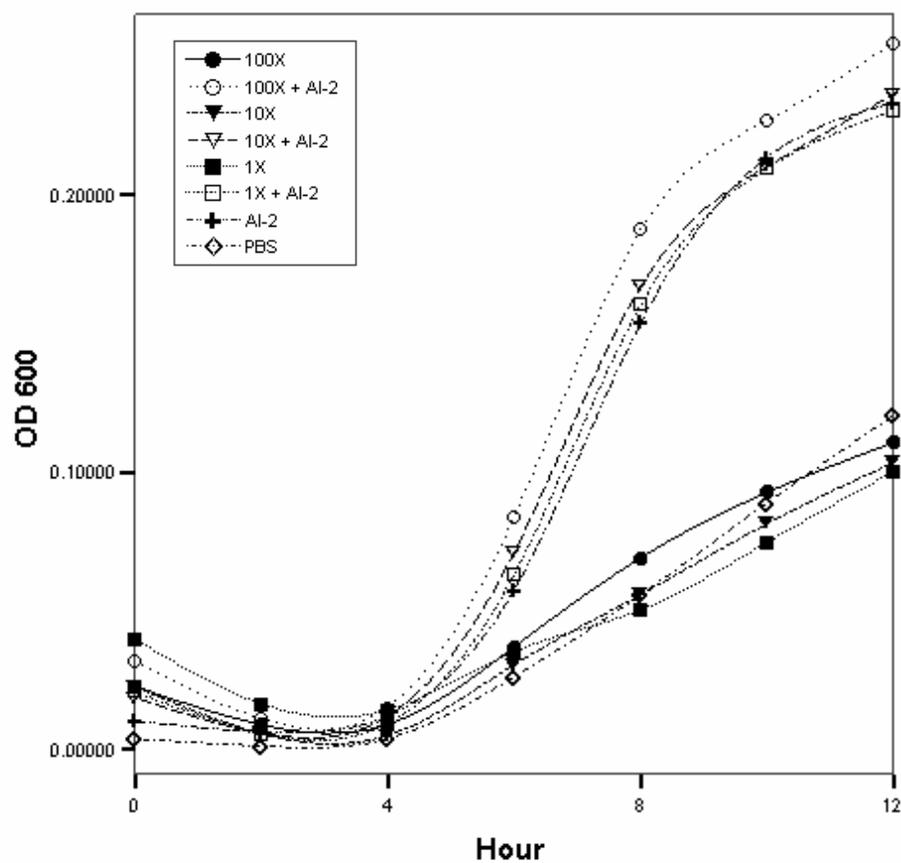without AI-2, or with AI-2 (1X + AI-2, 10x + AI-2, 100X + AI-2).
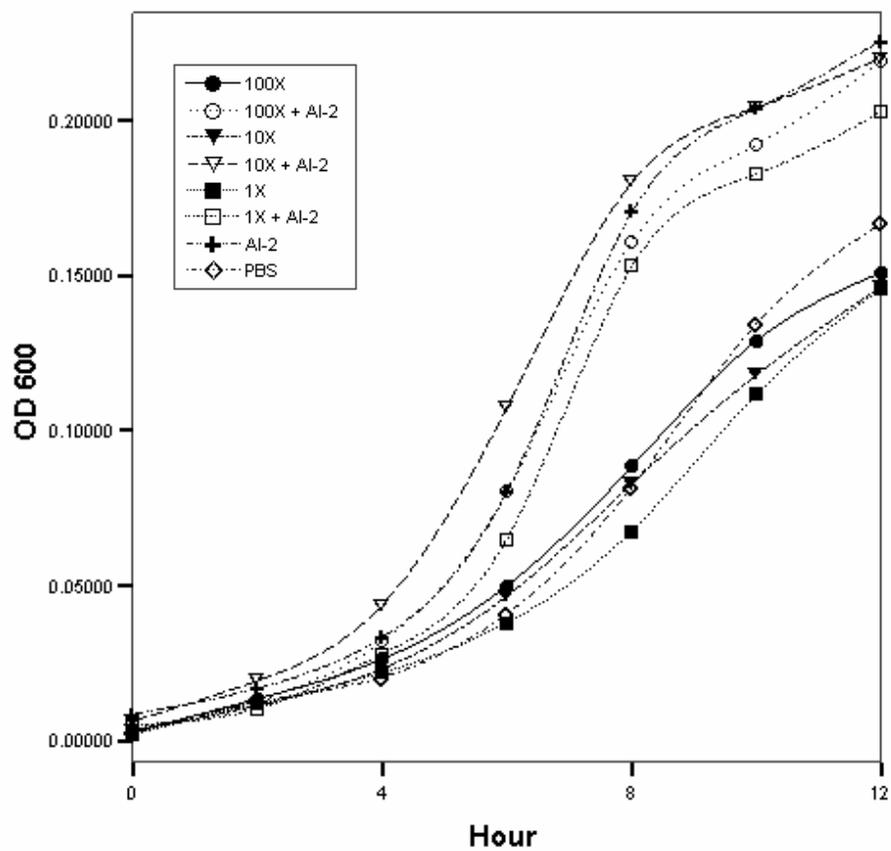
Figure 5.5. Mean OD 600 Values of Stearic Acid Treatments
with, and without, AI-2 in M9 Minimal Medium

Points represent the mean OD600 values. Treatments are 1x PBS (PBS), Autoinducer 2
(AI-2) and stearic acid at a nominal concentration (1X), 10-fold (10X) or 100-fold (100X)
without AI-2, or with AI-2 (1X + AI-2, 10x + AI-2, 100X + AI-2).

Table 5.3. Comparative Mean Growth Constant, k, of *Salmonella* in M9 Media Supplemented with AI-2 against PBS Control

Treatment	k
AI-2	0.0033 (± 0.0002)[a]
PBS	0.0021 (± 0.0002)

Treatment – Medium supplemented with either AI-2 or PBS, respectively. k - mean growth constant (at least 39 replicates) with standard error in parenthesis. [a] - significant difference in the mean values of k compared to the PBS control (P=0.01).

Table 5.4. Comparative Mean Growth Constant, k, of *Salmonella* Wild-Type and *luxS* Mutant in M9 Media

Strain	k
Wild-Type	0.0047 (± 0.0005)
Mutant	0.0051 (± 0.0002)

k - mean growth constant (n = 8) with standard error in parenthesis.   No significant difference in the mean values of k observed (P=0.49).

Impact of AI-2 and Combined Fatty Acids on Macrophage Infection

The removal of the *luxS* gene had no significant impact on the infectivity of

Salmonella Typhimurium mutant compared to the wild-type. Although the *luxS*

mutant did have a slight increase in infective efficiency compared to the wild-

type (0.74% and 0.34%, respectively), this was not statistically significant (Fig.

5.6). There were some differences in infection efficiency that were observed to be

statistically significant. The AI-2 treatment demonstrated a reduced infection

efficiency of 0.28% compared to the untreated PBS control of 0.67%, while the
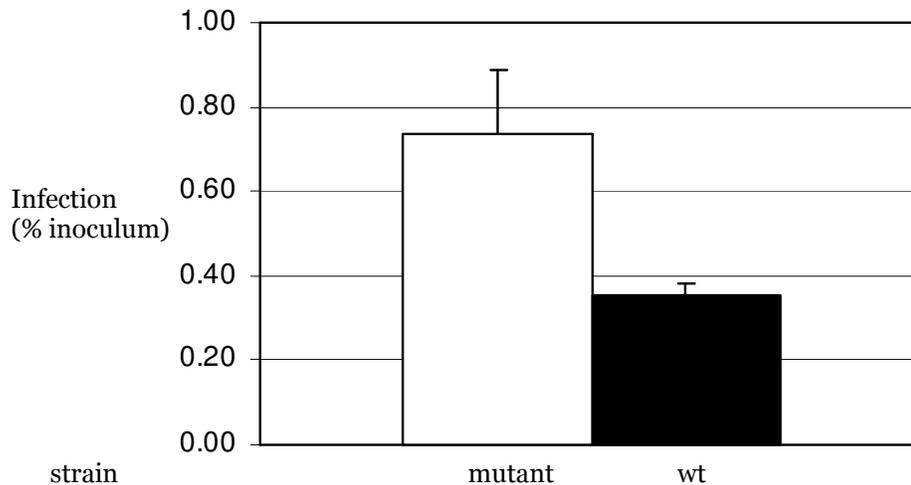
Figure 5.6. Infection Efficiency of *Salmonella* Mutant
and Wild-Type in RAW Macrophage Cells

Bars represent means for each treatment (n = 3) ± standard error.

combined FA + AI-2 treatment had an infectivity of 0.43% (Fig. 5.7). Directly

compared to the PBS control, the AI-2 treatment did express a statistically

significant difference (P = 0.02). However, the combined FA + AI-2 treatment

did not have a significant difference to either the PBS control treatment, or the

AI-2 treatment, when directly compared (P > 0.05). Because of these results,

further analysis was done to determine if the combined fatty acid treatment

alone improved infection efficiency. The 100X fatty acid treatment did have

improved infection (1.4%) compared to the PBS control (0.67%), but this was not

statistically significant (P > 0.05). However the differences in mean % infection

efficiency were significant compared to the AI-2 treatment (P = 0.02) (Fig 5.8).
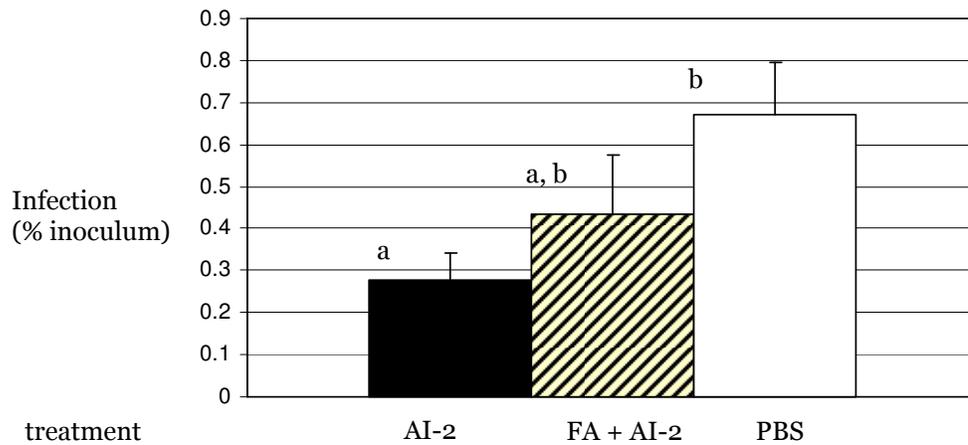
Figure 5.7. Infection Efficiency of *Salmonella* Mutant
with AI-2, Fatty Acids + AI-2, and PBS in RAW
Macrophage Cells

Bars represent the means for each treatment (n = 6) ± standard error.   Treatments were
Autoinducer 2 (AI-2), 100X combined fatty acids and AI-2 (FA + AI-2), and phosphate
buffered saline control (PBS). Letters (a, b) represent groups of statistically significant
differences in the mean % infection compared to the PBS control (P = 0.02).
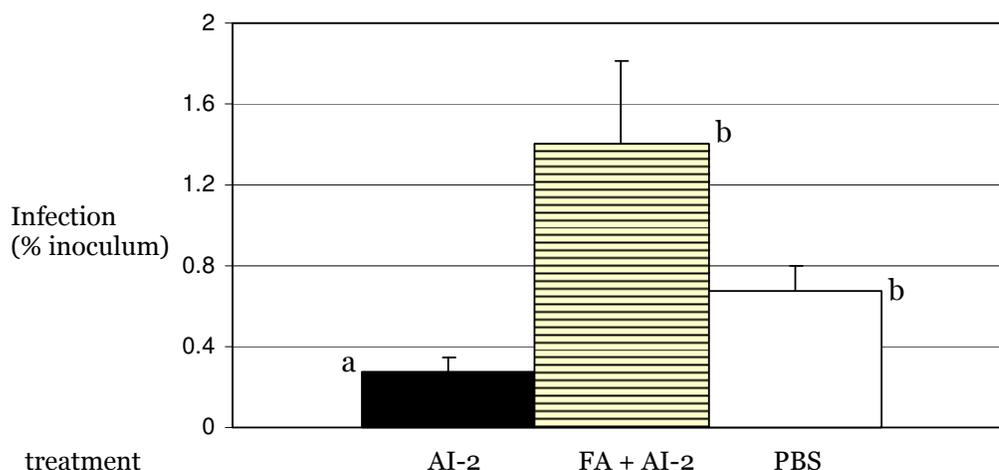
Figure 5.8. Infection Efficiency of *Salmonella* Mutant with
AI-2, Fatty Acids, and PBS in RAW Macrophage Cells

Bars represent the means for each treatment (n = 6) ± standard error. Treatments were
Autoinducer 2 (AI-2), 100X combined fatty acids (FA), and phosphate buffered saline
control (PBS). Letters (a, b) represent groups of statistically significant differences in the
mean % invasion compared to the PBS control (P = 0.02).

Discussion

AI-2 has an impact on the growth of *Salmonella*, in such that, under minimal

medium culture conditions growth during the exponential phase is increased

(Table 5.2.). Although it is a simplistic model and there are relatively better

methods to estimate the growth constant, k, calculating the growth rate for the

linear portion of the exponential growth phase can be a useful tool for

understanding the growth kinetics of bacteria (24, 154). These parameters have

been used to compare the growth and survival of bacteria in certain food

matrixes under varying environmental conditions (6, 89). What is of particular

interest is that the addition of varying fatty acids, solely and in combination, at

different concentrations to the AI-2 treatments did nothing to impede the growth of *Salmonella* (Fig. 5.1-5.5). Previous experiments indicated that these compounds possess characteristics that interfere with the AI-2 quorum sensing system (as discussed in the previous chapter). It was expected that the introduction of these compounds would interfere with the influence of AI-2, in such that the growth would be impeded to mimic that of the PBS treatment. Furthermore, the addition of the fatty acids alone appeared to have little influence on the growth of *Salmonella*, as these treatments caused little difference in mean OD 600 values compared to the PBS treatment (Fig. 5.1-5.5). This point is further reinforced by the analysis of the growth constants, as only one treatment (linoleic acid at 100-fold concentration) had any significant difference in growth rates compared to the PBS control (Table 5.1). It may not be surprising that AI-2 has influence on the exponential growth of *Salmonella*, as typical maximal production in enriched medium is at the mid-to-late log phase and the compound is slowly removed over time (128, 132, 133). It is interesting to note that there were no differences in the growth rates of the mutant compared to the wild type, supporting the notion that the removal of the *luxS* gene does not significantly impact growth of *Salmonella* (Table 5.3). Although one may be surprised that there is no apparent difference in the growth rates for the mutant and wild-type, whereas, in the case of the *luxS* mutant, the addition of AI-2 does improve the growth rate. Other studies have demonstrated that substantial production of AI-2 were in Luria Bertaini broth, a relatively

enriched medium, (133) and in some cases further supplemented with 0.5%

glucose (128, 130). It is likely that the levels of AI-2 produced by the wild-type in

M9 medium were less that those seen in the AI-2 supplemented treatments and

these elevated levels induced an improved growth rate in the *luxS* mutant (when

compared to the PBS control) (Table 5.3).

How well *Salmonella* responds to host defenses, in particular survival in

macrophages, is a key aspect of determining the efficacy and progression of

Salmonella infection (114). The ability to not only survive phagocytosis, but also

replicate within macrophage vacuoles is a key aspect for the pathogenicity of

Salmonella (51, 113). This study demonstrates that AI-2 impedes infection of

Salmonella in murine macrophages and such reduction in infectivity can be

reversed with the addition of fatty acids (Fig. 5.7). However, it is unlikely that

improved infection is solely due to fatty acids interfering with AI-2 on the basis

of cell signaling. Rather, it is likely that fatty acids have an influence on

virulence directly, and in turn counter-balance the effects AI-2. This point of a

lack of interaction with AI-2 on the basis of cell signaling is also supported by the

growth experiments, where the addition of fatty acids had no impact on the

response of the organism to AI-2 (Table 5.1).

There are several published studies indicating that fatty acids influence

virulence of *Salmonella*, but these effects vary by inducing, or repressing,

virulence depending on the compounds in question. Lucas et al. (87) found that

genes responsible for the metabolism and uptake of fatty acids also affected the

expression of *hilA*, which acts as a direct promoter (or a transcriptional effector for other promoters) of genes encoded in the *Salmonella* Pathogeniticy Island 1 (SPI1). The authors found that a mutation for FadD (responsible for the uptake and degradation of long chained fatty acids) resulted in reduced expression of *hilA* (87). Using lacZ fusions, El-Gedaily et al. (40) also showed that short-chained fatty acids induced gene expression of the *spvABCD* operon, which is utilized for initiating plasmid-mediated virulence. Interestingly, the authors found that there was improved expression of *spvB* for fatty acids that had carbon side chains of C3 to C6, but higher carbon side chains (C8, C10) resulted in a severe reduction of *spvB* expression (40). Lawhon et al. (77) suggested that different short chained fatty acids have a varying effect on *Salmonella* virulence gene expression. The authors suggested that fatty acids may provide a unique environmental signal for *Salmonella* infection depending on the composition of the fatty acids present. The authors found that propionate and butyrate reduced virulence gene expression, while another short chained fatty acid, acetate, improved expression. They further commented that the presence of these fatty acids could be indicative of their location in a host, where the distal ileum (an effective site for *Salmonella* invasion) had typically higher concentrations of acetate, while other sites within the host (such as the colon), would contain higher concentrations of other fatty acids like butyrate (77). This varying response of infection with different fatty acids was also seen by Van Immerseel et al. (139). The authors demonstrated that proprionate and butyrate reduced

Salmonella enteritidis infection of an avian intestinal epithelial cell line. However when combined with other fatty acids that mimicked the contents and concentrations of an adult chicken caecum, infection was normalized (139). The fatty acids employed in this study were long chained fatty acids (greater than C10) and their impact on *Salmonella* virulence has not been fully studied compared to previous work done with short chained fatty acids. It may be likely that the metabolism of these long chained fatty acids, and their resulting products, may have some influence on *Salmonella* similar to that seen with particular short chained fatty acids.

As AI-2 is produced by Gram negative bacteria, it has been considered to be a 'universal' signal capable of being detected by different bacterial species (1, 9, 124, 140). Although the production of the signaling compound appears to be conserved, different bacterial species appear to utilize varying routes and transport systems for the uptake of AI-2. For the internal transport of AI-2, *Vibrio harveyi* utilizes two proteins, LuxP and LuxQ. These two proteins form a combined receptor complex which extra-cellular AI-2 binds to. The bound signaling compound then initiates the dephosphorilization of LuxU, and LuxU, in turn, dephosphorylates LuxO. The dephosporylated LuxO, in concert with σ^{54}, help destabilize another protein which acts as a small RNA (sRNA) chaperone, responsible for enchaining transcription, Hfq (80, 131, 140). As the destabilized small RNA/Hfq complex occurs, this allows the Hfr protein to form a complex with existing LuxR mRNA transcripts, increasing stability and further

transcription of LuxR which, further promotes the expression of the bioluminescence genes (80). In *Salmonella* and *E. coli*, the uptake of AI-2 is mediated by another set of proteins, the *lsr*ABC complex (1, 129, 132, 133, 148). Here, AI-2 is bound to LsrB, a periplasmic binding protein. The compound is then taken up by LsrC and LsrD, both channel proteins, and finally taken inside the cell using an ATPase energy transport system protein, LsrA. Internalized AI-2 is then phosphorylated by LsrK, and it is theorized this form of AI-2 may then have some regulatory function for various quorum sensing genes (116, 133, 148). Recent studies have also indicated a ribose-binding transport system may have similar function to the *lsr*ABC complex, where RbsB (a periplasmic ribose binding protein) has demonstrated the ability to also bind to AI-2 (71). As more research is conducted on quorum sensing, it is becoming indicative that different species of bacteria may utilize varying binding, and transport systems, for the internalization of AI-2. Sun et al. (127) has suggested this, as their research found that in a comparative study among 138 genomes, *luxP* was found to be commonly present in only three species, all of them *Vibrio* species.

Compounds have been identified to be inhibitors to the AI-2 quorum sensing system, specifically furanones derived from algae (*Delisea pulchra*) which reduced AI-2 activity in *V. harveyi* reporter strains by over 5000 fold (111). Ren et al. (112) also demonstrated a decrease of 49% in relative light units using a *V. harveyi* reporter strain when combined with a boronated furanone. Based on the inhibitory properties of the brominated furanones, the authors

wished to determine if varying gene expression could be observed in an *E. coli* K12 strain cultured under conditions that would promote AI-2 production, and also in combination of furanone inhibitors. The authors found that out of the 56 genes down-regulated in the quorum sensing inhibitor treatment, 44 were up-regulated in conditions promoting AI-2 (112). Initially, this idea of a direct impact on virulence due to the modulation of gene expression in *Salmonella* could be supported from the infection assay experiments presented in this study. Previous research indicates that fatty acids interfere with quorum sensing (as discussed in Chapter 4), and there was a statistical difference observed in the infection efficiency of the AI-2 treatment when compared to the PBS control while none was observed when the combined fatty acid and AI-2 treatment was similarly compared (Fig. 5.7). However, cell signaling inhibition was based on the response of a *Vibrio harveyi* reporter strain. *Salmonella*, as previously discussed, utilizes a different transport system for the importation of AI-2. Hence, it may be important to account for how AI-2 is taken up by a particular bacterial species when discussing quorum sensing inhibition, particularly if this inhibition is seen using the *Vibrio harveyi* reporter strain. This may indicate the limitation of extrapolating the effects of inhibitory compounds when applied to other (non-*Vibrio*) bacterial species. Thus, while the fatty acids employed in this study have characteristics of AI-2 inhibition, the response in *Salmonella* from these compounds may not be due to impeding the AI-2 signaling system.

This study establishes that AI-2 does have varying effects on *Salmonella*, improving the growth of *Salmonella* in minimal medium, while decreasing the infectivity of macrophages. The addition of fatty acids had a limited effect on the growth of *Salmonella*, but did improve infection normalizing it to control levels. Although in the case of infection, there was an opposite effect in activity compared to the AI-2 treatments, it is likely that the increase in infection could be due to promotion of specific virulence genes rather than interfering with AI-2 as a quorum sensing system. To better understand inhibition of AI-2 in specific bacterial species, utilization of reporter strains that employ similar transport systems for the uptake of AI-2 may be needed to truly understand the impact these inhibitor compounds have on bacterial functions and processes mediated by AI-2.

CHAPTER VI

SUMMARY

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an intracellular pathogen and major causative agent in food-borne illnesses. Bacteria are capable of producing, secreting, and utilizing chemical compounds for cell-to-cell communication, generally termed as quorum sensing. A key cell signaling system involves autoinducer AI-2, a furanosyl borate diester molecule produced primarily by Gram negative bacteria. A constructed reporter strain, *Vibrio harveyii* BB170, is typically used to detect AI-2 activity by measuring bioluminescence of the organism. Autoinducer signals bind to the receptor protein, LuxP and forms a complex with another membrane protein, LuxQ. This protein complex then will dephosphorylate LuxU, and in turn, dephosphorylate LuxO. The dephosporylated LuxO, in concert with σ^{54}, help destabilize another protein which acts as a small RNA (sRNA) chaperone, responsible for enchaining transcription, Hfq. As the destabilized small RNA/Hfq complex occurs, this allows the Hfr protein to form a complex with existing LuxR mRNA transcripts, increasing its stability, and in turn promoting luciferase genes. In *Salmonella* there has been identified a *luxS* regulated operon (*lsrACDBFGE*) which encodes an ABC transporter system with similar functionality to ribose transport systems in *E. coli*. The periplasmic binding protein, *lsrB*, is responsible for binding with extracellular AI-2, and the compound is internalized using this transport system. Once internalized, AI-2 is phosphorylated by the gene product of *lsrK,* and is

responsible for inactivating LsrR, allowing the expression of the *lsr* operon. However other functions are proposed to be affected by the phosphorylated AI-2 complex and there is increasing evidence that AI-2 has a role in influencing gene expression and bacterial processes, including those processes involved with virulence.

Previous research studies have demonstrated that food matrices, particularly poultry meat extracts, have compounds which can interfere with AI-2 activity. This inhibition is primarily based on observing a reduction of light production in the *V. harveyii* reporter strain when comparing treatments containing inhibitors combined with autoinducer signals verses positive controls. The objective of this work was to determine if AI-2 influenced virulence gene expression, and if combined with inhibitors in poultry meat, could those effects be modulated. Experiments were also performed to define the compounds in the poultry meat matrix that had AI-2 inhibitory properties. *Salmonella* growth and virulence were studied directly to determine the impact AI-2 had on these processes, and if the implementation of inhibitory compounds could modulate these effects.

AI-2 has limited influence on virulence gene expression, but this expression is modulated by poultry meat derived inhibitors. Experiments were conducted to determine if there was varying expression of *Salmonella* Typhimurium genes (using spotted microarrays) in response to AI-2 in the presence, and absence, of poultry meat (PM) derived AI-2 inhibitors.

Expression of 1136 virulence-related genes in *Salmonella* Typhimurium wild type and its isogenic *luxS* mutant strain (unable to produce AI-2) was monitored when the cells were exposed (3 hours) to different treatments containing *in vitro* synthesized AI-2 and the PM inhibitor (AI-2, AI-2 + PM, or PM alone). The responses of the genes were unique in the presence of *in vitro* synthesized AI-2. Out of 1136 genes on the array, 23 genes were differentially expressed (either up-regulated or down-regulated) at least 1.5-fold ($p < 0.05$) in the presence of AI-2. Exposure to the PM inhibitor resulted in 36 genes being differentially expressed, while the combined AI-2 + PM treatment resulted in 22 genes being differentially expressed out of which only 7 genes showed overlap with the PM treatment suggesting a unique response when AI-2 interacts with the inhibitor molecules. The results suggest that *Salmonella* gene expression can vary depending on the presence or absence of the poultry meat matrix and/or AI-2 molecules.

Fatty acids are key components in poultry meat extracts which demonstrate AI-2 inhibition. Previous research has shown that certain food matrixes have the properties to inhibit this signaling compound. Using the reporter strain, *V. harveyi* BB170, poultry meat wash (PW) samples were characterized by their molecular weight and hydrophobic properties using liquid chromatography systems and the resulting fractions tested for AI-2 inhibition. Based on a molecular size exclusion column, the majority of collected fractions that possessed inhibition to AI-2 were 13.7 k Daltons, or less, in size. Using

reverse phase liquid chromatography, the majority of fractions which expressed inhibition were obtained when methanol solvent, acting as a mobile phase in a water-saturated C18 resin column, reached 100% in concentration. Based on these initial experiments, hexane was used to extract the inhibitory compounds from a PW preparation. With gas chromatography (GC) analysis, several fatty acids were identified and quantified. Chemical standards of linoleic acid, oleic acid, palmitic acid, and stearic acid were obtained and singly tested for inhibition at 0.1 mM, 1 mM, and 10 mM concentrations. All samples expressed inhibition (ranging from approximately 25-99 % inhibition). When combined in concentrations determined by GC analysis, inhibition of AI-2 was approximately 60 %. At higher concentrations (10 and 100 fold), the combined fatty acids produced an inhibition of 84.4 % and 69.5 %, respectively. At concentrations of 100 fold, the samples of combined fatty acids did not demonstrate a significant decrease in plate counts, despite presenting high AI-2 inhibition, indicating that the reduction in AI-2 activity was due to interferance with cell signaling, rather than antimicrobial effects against the *V. harveyi* reporter strain.

AI-2 promotes growth, but reduces infection in murine macrophages. When AI-2 is combined with fatty acids, there is no modulation of growth, but normalized infection was restored. Fatty acids have demonstrated characteristics of AI-2 inhibition using the reporter strain, *Vibrio harveyii*, BB170. The primary focus of this study was to determine the impact of AI-2 on the growth and virulence of *Salmonella enterica* serovar Typhimurium, and

whether fatty acids could modulate any effects of AI-2 observed. A luxS mutant, PJ002, was grown in M-9 minimal medium supplemented with glucose and long-chained fatty acids (FA) of varying concentrations. In addition, treatments were supplemented with *in vitro* synthesized AI-2 or 1X PBS. After a 12 h incubation recording OD600 values, comparative analysis was done calculating the growth constants for each treatment. No significant difference was seen in the combined FA + AI-2 treatments compared to the AI-2 treatment. Additionally, the majority of FA treatments had no impact on the growth of *Salmonella*. However, there was a significant increase in the growth rate constants of the AI-2 treatments when compared to the PBS control (P = 0.01). Bacterial invasiveness using a murine macrophage cell line, RAW 264.7 was also investigated. AI-2 decreased cell invasiveness (P = 0.02), while the addition of combined FA restored invasiveness to normalized function. Because literature suggests varying medium and short-chained fatty acids have an impact on virulence for *Salmonella*, it is likely the modulation of invasion is due to the direct promotion of virulence genes, rather than interfering with AI-2 signaling. The results of this study show that although AI-2 does have an effect on the growth and virulence of *Salmonella*, this cannot be modulated by fatty acids, although these compounds have AI-2 inhibitory characteristics. This indicates that the validity of inhibition based on *V. harveyi* may have limited applicability when observing quorum sensing systems in other bacterial species.

Conclusions

The results of the research conducted in these studies can be summarized as follows:

1.   AI-2 has some influence on virulence gene expression, and this expression is modulated by poultry meat-derived inhibitors.

2.   Some genes were commonly expressed in treatments of poultry meat extracts singly, and in combination with AI-2, indicating that the food matrix itself has some influence on *Salmonella* virulence gene expression.

3.   Fatty acids are components in poultry meat extracts which demonstrate AI-2 inhibition, and may serve as a class of compounds that are quorum sensing inhbitors.

4.   AI-2 promotes growth of Salmonella in minimal medium. The addition of fatty acid inhibitors does not affect the growth rate of Salmonella under similar conditions, even when combined with AI-2.

5.   AI-2 reduces the infection efficiency in murine macrophages. When combined with fatty acids, infection is normalized to control levels.

6.   Because the *V. harveyi* reporter strain, BB170, uses an AI-2 transport system that may be unique to that species, it is likely responses observed in the combined AI-2 + fatty acid treatments from these experiments may be due to other interactions other than those affiliated with quorum sensing.

7.   To better understand the impact of AI-2 inhibitors in enterobacteriaceae, there can be limitations to using *V. harveryi* BB170, and it may be more applicable to utilize other reporter strains that use similar internalization systems for these quorum sensing signals.

REFERENCES

1. **Ahmer, B. M. M.** 2004. Cell to cell signalling in *Escherichia coli* and *Salmonella enterica*. Mol. Microbiol. **52:**933-945.

2. **Akbar, S., L.M. Schechter, C.P. Lostroh, and C.A. Lee.** 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in Salmonella Typhimurium. Mol. Microbiol. **47:**715-728.

3. **Allan, J. T., Z. Yan, and J.L. Kornacki.** 2004. Surface material, temperature, and soil effect on the survival of selected foodborne pathogens in the presence of condensate. J. Food. Prot. **67:**2666-2670.

4. **Altier, C.** 2005. Genetic and environmental control of *Salmonella* invasion. J. Microbiol. **43:**85-92.

5. **Arfin, S. M., A.D. Long E.T. Ito, L. Tolleri, M.M. Riehle, E.S. Paegle, and G.W. Hatfield.** 2000. Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factor. J. Biol. Chem. **275:**29672-29684.

6. **Bachrouri, M., E.J. Quinto, and M.T. Mora.** 2002. Survival of *Escherichia coli* O157:H7 during storage of yogurt at different temperatures. J. Food Sci. **67:**1899-1903.

7. **Bajaj, V., C. Hwang, and C.A. Lee.** 1995. *hilA* is a novel *opmR/toxR* familiy member that activates the expression of *Salmonella* Typhimurium invasion genes. Mol. Microbiol. **18:**715-727.

8. **Bajaj, V., R.L. Lucas, C. Hwang, and C.A. Lee.** 1996. Co-ordinate regulation of *Salmonella* Typhimurium invasion genes by environmental and regulatory factors is mediates by control of hilA expression. Mol. Microbiol. **22:**703-714.

9. **Bassler, B. L.** 2002. Small talk cell-to-cell communication in bacteria. Cell **109:**421-424.

10. **Bassler, B. L., M. Wright, and M.R. Silverman.** 1994. Multiple signaling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273-286.

11. **Bassler, B. L., M. Wright, and M.R. Silverman.** 1994. Multiple signaling systems controlling expression of luminescence in *Vibrio harveyi* sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:** 273-286.

12. **Beeston, A. L., and M.G. Surrette.** 2002. pfs-dependant regulation of autoinducer 2 production in *Salmonella enterica* serovar Typhimurium. J. Bact. **184:**3450-3456.

13. **Blanc-Potard, A. B., and E.A. Groisman.** 1997. The *Salmonella* selC locus contains a pathogenicity island mediating intramacrophage survival. EMBO J. **16:**5376-5385.

14. **Brandl, M. T., W.G. Miller, A.H. Bates, and R.E. Mandrell.** 2005. Production of autoinducer 2 in *Salmonella enterica* serovar Thompson contributes to its fitness in chicken but not on cilantro leaf surfaces. Appl. Environ. Microbiol. **71:**2653-2662.

15. **Brenner, F. W., R.G. Villar, F.J. Angulo, R. Tauxe, and B. Swaminathan.** 2000. *Salmonella* nomenclature. J. Clin. Microbiol. **38:**2465-2467.

16. **Brockmann, S. O., I. Piechotowski, and P. Kimmig.** 2004. *Salmonella* in sesame seed products. J. Food. Prot. **67:**178-180.

17. **Bruhn, J. B., A.B. Christensen, L.R. Flodgaard, K.F. Nielsen, T.O. Larsen, M.L. Givskov, and L. Gram.** 2004. Presence of acylated homoserine lactones (AHLs) and AHL-producing bacteria in meat and potential role of AHL in spoilage of meat. Appl. Envir. Microbiol. **70:**4293-4302.

18. **Camilli, A., and B.L. Bassler.** 2006. Bacterial small-molecule signalling pathways. Sci. **311:**1113-1116.

19. **Cases, I., and V. de Lorenzo.** 2005. Promoters in the environment transcriptional regulation in its natural context. Nat. Rev. Microbiol. **3:**105-118.

20. **Centers for Disease Control and Prevention.** 1999. Incidence of foodborne illnesses: preliminary data from the foodborne disease active surveillance network (FoodNet) - United States, 1998. M.M.W.R. **48:**189-194.

21. **Centers for Disease Control and Prevention.** 2003. *Salmonella* surveillance summary, 2002. United Stated Department of Health and Human Services, Atlanta, GA.

22. **Chen, S., S. Zhao, P.F. McDermott, C.M. Schroeder, D.G. White, J. Meng.** 2005. A DNA microarray for identification of virulence and antimicrobial resistance genes in *Salmonella* serovars and *Escherichia coli*. Mol. Cell Probe **19:**195-201.

23. **Chopra, V., A.A. Fadl, J. Sha, S. Chopra, C.L. Galindo, and A.K. Chopra.** 2006. Alterations in the virulence potential of enteric pathogens and bacterial-host cell interactions under simulated microgravity conditions. J. Tox. Environ. Health Pt. A **69:**1345-1370.

24. **Chrzanowski, T. H., K. Šmek, R.H. Sada, and S. Williams** 1993. Estimates of bacterial growth rate constants from thymidine incorporation and variable conversion factors. Microb. Ecol. **25:**121-130.

25. **Chun, C. K., E.O. Ozer, M.J. Welsh, J. Zabner, and E.P. Greenberg.** 2004. Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proc. Nat. Acad. Sci. **101:**3587-90.

26. **Chung, Y. H., S.Y. Kim, and Y.H. Chang.** 2003. Prevalence and antibiotic susceptibility of *Salmonella* isolated from foods in Korea from 1993 to 2001. J. Food. Prot. **66:**1154-57.

27. **Cloak, O. M., B.T. Solow, C.E. Briggs, C. Chen, and P.M. Fratamico.** 2002. Quorum sensing and production of autoinducer-2 in *Campylobacter spp., Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium in foods. Appl. Envir. Microbiol. **68:**4666-71.

28. **Coburn, B., Y. Li, D. Owen, B.A. Vallance, and B.B. Finlay.** 2005. *Salmonella enterica* serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. Infect. Immun. **73:**3219-27.

29. **Collazo, C. M., and J.E. Galán.** 1997. The invasion-associated type-III protein secretion system in *Salmonella* - a review. Gene **192:**51-59.

30. **Cossart, P., and P.J. Sansonetti.** 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. Sci. **304:**242-248.

31. **Coulthurst, S. J., C.L. Kurz, and G.P.C. Salmond.** 2004. LuxS mutants of *Serratia* defective in autoinducer-2-dependant 'quorum sensing' show strain-dependant impacts on virulence and production of carbapenem and prodigiosin. Microbiol. **150:**1901-1910.

32. **Datsenko, K. A., and B.L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. **97:**6640-6645.

33. **De Keersmaecker, S. C. J., K. Sonck, and J. Vanderleyden.** 2006. Let luxS speak up in AI-2 signaling. Trend Microbiol. **14:**114-119.

34. **Delisa, M. P., C. Wu, L. Wang, J.J. Valdes, and W.E. Bentley.** 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. J. Bact. **183:**5239-5247.

35. **Delisa, M. P., J.J. Valdes, and W.E. Bentley.** 2001. Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. J. Bact. **183:**2918-2928.

36. **Dézel, E. F. L., S. Milot, J. He, M.N. Mindrinos, R.G. Tompkins, and L.G. Rahme.** 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkyquinolines (HAQs) reveals arole for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc. Nat. Acad. Sci. **101:**1339-1344.

37. **Dong, Y. H., X.F. Zhang, J.L. Xu, and L.H. Zhang.** 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia cartovora* virulence by a new form of microbial antagonism, signal interference. Appl. Environ. Microbiol. **70:**954-60.

38. **Dowd, S. E., and H. Ishizaki.** 2006. Microarray based comparison of two *Escherichia coli* O157:H7 lineages. BMC Microbiol. **6:**30.

39. **Eckmann, L., M.F. Kagnoff, and J. Fierer.** 1993. Epithelial cells secrete the chemokine interleukin-8 in the response to bacterial entry. Infect. Immun. **61:**4569-4574.

40. **El-Gedaily, A., G. Paesold, C.Y. Chen, D.G. Guineyn, and M. Krause.** 1997. Plasmid virulence gene expression induced by short-chain fatty acids in *Salmonella dublin*: identification of *rpoS*-dependant and *rpo-S*-independant mechanisms. J. Bact. **179:**1409-1412.

41. **Fahlen, T. F., N. Mathur, and B.D. Jones.** 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella* Typhimurium. FEMS Imm. Med. Microbiol. **28:**25-35.

42. **Falcão, J. P., F. Sharp, amd V. Sperandio.** 2004. Cell-to-cell signaling in intestinal pathogens. Curr. Iss. Intestin. Microbiol. **5:**9-18.

43. **Food Safety and Inspection Service.** 1999. *Salmonella* serotypes isolated from raw meat and poultry, January 26, 1998 to January 25, 1999, United States. Department of Agriculture, Washington, DC

44. **Food Safety and Inspection Service.** 2007. Quarterly progress report on *Salmonella* testing of selected raw meat and poultry products: preliminary results, October - December, 2006. United States Department of Agriculture, Washington, DC

45. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bact. **173:**6896-6902.

46. **Foster, J. W., Y.K. Park, I.S. Bang, K. Karem, H. Betts, H.K. Hall, and E. Shaw.** 1994. Regulatory circuits involved with pH-regulated gene expression in *Salmonella* Typhimurium. Microbiol. **140:**341-352.

47. **Galán, J. E.** 2001. *Salmonella* interactions with host cells: typle III secretion at work. Annu. Rev. Cell Dev. Biol. **17:**53-86.

48. **Galán, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella Typhimurium to penetrate tissue culture cells. Proc. Nat. Acad. Sci. **86:**6383-6387.

49. **Galán, J. E., and D. Zhou.** 2000. Striking a balance modulation of the actin cytoskeleton by *Salmonella*. Proc. Nat. Acad. Sci. **97:**8754-8761.

50. **Gao, M., M. Teplitski, J.B. Robinson, and W.D. Bauer.** 2003. Production of substances by *Meducago truncatula* that affect bacterial quorum sensing. Mol. Plant-Microbe Interact. **16:**827-834.

51. **Garvis, S. G., C.R. Beuzón, and D.W. Holden.** 2001. A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and *Salmonella*-containing vacuoles in macrophages. Cell. Microbiol. **3:**731-744.

52. **Germon, P., M.C. Ray, A. Vianney, and J.C. Lazzaroni.** 2001. Energy-dependant conformational change in the TolA protein of *Escherichia coli* involves its n-terminal domain, TolQ and TolR. J. Bact. **183:**4110-4114.

53. **Goryachev, A. B., P.F. MacGregor, and A.M. Edwards.** 2001. Unfolding of microarray data. J. Comp. Biol. **8:**443-461.

54. **Gram, L., R. de Dys, R. Maximilien, M. Givskov, P. Steinberg, and S. Kjelleberg.** 1996. Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming and motility of *Proteus mirabilis*. Appl. Environ. Microbiol. **62:**4284-4287.

55. **Gupta, S. D., H.C. Wu, P.D. Rick.** 1997. A *Salmonella* Typhimurium genetic locus which confers copper tolerance on copper-sensitive mutants of *Escherichia coli*. J. Bact. **179:**4977-4984.

56. **Hansen-Wester, I., and M. Hensel.** 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microb. Inf. **3:**549-559.

57. **Hardie, K. R., C. Cooksley, A.D. Green, and K. Winzer.** 2003. Autoinducer 2 activity in *Escherichia coli* culture supernatants can be actively reduced despite maintenance of an active synthase, LuxS. Microbiol. **149:**715-728.

58. **Heithoff, D. M., C.P. Conner, U. Hentschel, F. Govantes, P.C. Hanna, and M.J. Mahan.** 1999. Coordinate intracellular expression of Salmonella genes induced during infection. J. Bact. **181:**779-807.

59. **Helms, M., S. Ethelberg, and K. Mølbak.** 2005. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emer. Inf. Dis. **11:**859-867.

60. **Henke, J. M., and B.L. Bassler.** 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bact. **186:**3794-3805.

61. **Henry, T., S. Pommier, L. Journet, A. Bernardac, J.P. Gorvel, and R. Lloubès.** 2004. Improved methods for producing outer membrane vesicles in gram-negative bacteria. Res. Microbiol. **155:**437-446.

62. **Hensel, M.** 2000. *Salmonella* pathogenicity island 2. Mol. Microbiol. **36:**1015-1023.

63. **Hensel, M., J.E. Shea, B. Raupach, D. Monack, S. Falkow, C. Gleeson, T. Kubo, and D.W. Holden.** 1997. Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. Mol. Microbiol. **24:**155-167.

64. **Hjelmgaard, T., T. Persson, T.B. Rasmussen, M. Givskov, and J. Nielsen.** 2003. Synthesis of furanone-based natural product analogues with quorum sensing antagonist activity. Bioorg. Med. Chem. **11:**3261-3271.

65. **Horswill, A. R., P. Stoodley, P.S. Stewart, M.R. Parsek.** 2007. The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. Anal. Bioanal Chem. **387:**371-380.

66. **Hossen, M., and E. Hernandez.** 2005. Enzyme-catalyzed synthesis of structured phopholipids with conjugated linoleic acid. Eur. J Lipid. Sci. Technol. **107:**730-736.

67. **Hughes, T. R., M.J. Marton, A.R. Jones, C.J. Roberts, R. Stoughton, C.D. Armour, H.A. Bennett, E. Coffey, H. Dai, Y.D. He, M.J. Kidd, A.M. King, M.R. Meyer, D. Slade, P.Y. Lum, S.B. Stepaniants, D.D. Shoemaker, D. Gachotte, K. Chakraburtty, J. Simon, M. Bard, and S.H. Friend.** 2000. Functional discovery via a compendium of expression profiles. Cell **102:**109-126.

68. **Humphrey, T.** 2004. *Salmonella*, stress responses and food safety. Nat. Rev. Mirobiol. **2:**504-509.

69. **Hung, D. T., and J.J. Mekalanos.** 2005. Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independant manner. Proc. Nat. Acad. Sci. **102:**3028-3033.

70. **Isaac, D. D., J.S. Pinkner, S.J. Hultgren, and T.J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc. Nat. Acad. Sci. **102:**17775-17779.

71. **James, D., J., H. Shao, R.J. Lamont, and D.R. Demuth.** 2006. The *Actinobacillus actinomycetemcomitans* ribose binding protien RbsB interacts with cognate and heterologous autoinducer 2 signals. Inf. Immun. **74:**4021-4029.

72. **Jones, B. D.** 2005. *Salmonella* invasion gene regulation: a story of environmental awareness. J. Microbiol. **43:**110-117.

73. **Kelly, A., M.D. Goldberg, R.K. Carrol, V. Danino, J.C.D. Hinton, and C.J. Dorman.** 2004. A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. Microbiol. **150:**2037-2053.

74. **Kimbrough, T. G., and S.I. Miller.** 2002. Assembly of the type III secretion needle complex of *Salmonella* Typhimurium. Microb. Inf. **4:**75-82.

75. **Koutsoumanis, K. P., and J.N. Sofos.** 2004. Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habitation at different pH conditions. Let. Appl. Microbiol. **38:**321-326.

76. **Kubori, T., A. Sukhan, S.I. Aizawa, and J.E. Galán.** 2000. Molecular characterization and assembly of the needle complex of the *Salmonella* Typhimurium type III protein secretion system. Proc. Nat. Acad. Sci. **97:**10225-10230.

77. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter *Salmonella* Typhimurium invasion gene expression and virulence through BarA/SirA. Mol. Microbiol. **46:**1451-1464.

78. **Lee, C. A. B. D. J., and S. Falkow.** 1992. Identification of a *Salmonella* Typhimurium invasion locus by selection for hyperinvasive mutants. Proc. Nat. Acad. Sci. **89:**1847-1851.

79. **Lee, J. Y., Y.K. Kim, and D.H. Shin.** 2002. Antimicrobial synergistic effect of linoleic acid and monoglyceride against *Bacillus cereus* and *Staphylococcus aureus*. J. Agric. Food Chem. **50:**2193-2199.

80. **Lenz, D. H., K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen, and B.L. Bassler.** 2004. The small RNA chaperone hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. Cell **118:**69-82.

81. **Lilić, M., and C.E. Stebbins.** 2004. Re-structuring the host cell up close with *Salmonella*'s molecular machinery. Microb. Inf. **6:**1205-1211.

82. **Lostroh, C. P., and C.A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microb. Inf. **3:**1281-1291.

83. **Lu, L., M.E. Hume, and S.D. Pillai.** 2004. Autoinducer-2-like activity associated with foods and its interaction with food additives. J. Food Prot. **67:**1457-1462.

84. **Lu, L., M.E. Hume, and S.D. Pillai.** 2005. Autoinducer 2-like activity in poultry-associated enteric bacteria in response to subtherapeutic antibiotic exposure. Avian Dis. **49:**74-80.

85. **Lu, L., M.E. Hume, S.D. Pillai.** 2005. Autoinducer-2-like activity on vegetable produce and its potential involvement in bacterial biofilm formation on tomatoes. Foodborne Pathog. Dis. **2:**242-249.

86. **Lucas, R. L., and C.A. Lee.** 2000. Unraveling the mysteries of virulence gene regulation in *Salmonella* Typhimurium. Mol. Microbiol. **36:**1024-1033.

87. **Lucas, R. L., C.P. Lostroh, C.C. DiRusso, M.P. Spector, B.L. Wanner, and C.A. Lee.** 2000. Multiple factors independently regulate hilA and invasion gene expression in *Salmonella enterica* serovar Typhimurium. J. Bact. **2000:**1872-1882.

88. **Lyon, W. R., J.C. Madden, J.C. Levin, J.L. Stein, and M.G. Caparon.** 2001. Mutation of luxS affects growth and virulence factor expression in *Streptococcus pyogenes*. Mol. Microbiol. **42:**145-157.

89. **Mamani, Y., E.J. Quinto, J. Simal-Gandara, and M.T. Mora.** 2003. Growth and survival of *Escherichia coli* O157:H7 in different types of milk stored at 4ºC or 20ºC. J. Food Sci. **68:**2558-2563.

90. **Manefield, M., M. Welch, M. Givskov, G.P. Salmond, and S. Kjelleberg.** 2001. Halogenated furanones from red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. FEMS Microbiol. Let. **205:**131-138.

91. **Manefield, M., T.B. Rasmussen, M. Henzter, J.B. Anderson, P. Steinberg, S. Kjelleberg, and M. Givskov.** 2002. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiol. **148:**1119-1127.

92. **March, J. C., and W.E. Bentley.** 2004. Quorum sensing and bacterial cross talk in biotechnology. Cur. Opinion Biotech. **15:**495-502.

93. **Mathesius, U., S. Mulders, M. Gao, M. Teplitski, G. Caetano-Anollés, B.G. Rolfe, and W.D. Bauer.** 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proc. Nat. Acad. Sci. **100:**1444-1449.

94. **Mazurek, J., E. Salehi, D. Propes, J. Holt, T. Bannerman, L.M. Nicholson, M. Bundesen, R. Duffy, and R.L. Moolenaar.** 2004. A multistate outbreak of *Salmonella enterica* serotype Typhimurium infection linked to raw milk consumption-Ohio, 2003. J. Food Prot. **67:**2165-2170.

95. **McClelland, M., K.E. Sanderson, J. Spieth, S.W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R.K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852-856.

96. **McNab, R. S. K. F., A. El-Sabaeny, B. Barbieri, G.S. Cook, and R.J. Lamont.** 2003. Luxs-based signaling in *Streptococcus gordonii* autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J. Bacteriol. **185:**274-284.

97. **Mead, P. S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe.** 1999. Food-related illness and death in the United States. Emer. Inf. Dis. **5:**607-625.

98. **Meldrum, R. J., D. Tucker, and C. Edwards.** 2004. Baseline rates of *Campylobacter* and *Salmonella* in raw chicken in Wales, United Kingdom, in 2002. J. Food Prot. **67:**1226-1228.

99. **Miller, M. B., K. Skorupski, D.H. Lenz, R.K. Taylor, and B.J. Bassler.** 2002. Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. Cell **110:**303-314.

100. **Miller, S. T., K.B. Xavier, S.R. Campagna, M.E. Taga, M.F. Semmelhack, B.L. Bassler, and F.M. Hughson.** 2004. *Salmonella* Typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol. Cell **15:**677-687.

101. **Moncrief, M. B., and M.E. Maguire.** 1998. Magnesium and the role of mgtC in growth of *Salmonella* Typhimurium. Infect. Immun. **66:**3802-3809.

102. **Neiditch, M. B., M.J. Fererle, S.T. Miller, B.L. Bassler, and F.M. Hughson.** 2005. Regulation of *luxpq* receptor activity by the quorum-sensing signal autoinducer-2. Mol. Cell **18:**507-518.

103. **Ohtani, K., H. Hayashi, and T. Shimizu.** 2002. The luxS gene is involved in cell-cell signaling for toxin production in *Clostridium perfringens*. Mol. Microbiol. **44:**171-179.

104. **Pieskus, J., J. Milius, I. Michalskiene, and G. Zagrebneviene.** 2006. The distribution of *Salmonella* serovars in chicken and humans in Lithuania. J. Vet. Med. **53:**12-16.

105. **Pillai, S. D., and P.R. Jesudhasan.** 2006. Quorum sensing: how bacteria communicate. Food Technol. **60:**42-50.

106. **Pizarro-Cerdá, J., and K. Tedrin.** 2004. The bacterial signal molecule, ppGpp, regulates *Salmonella* virulence gene expression. Mol. Microbiol. **52:**1827-1844.

107. **Rabsch, W., H.L. Andrews, R.A. Kingsley, R. Prager, H. Tschäpe, L.G. Adams, and A.J. Bäumler.** 2002. *Salmonella enterica* serotype Typhimurium and its host-adapted variants. Infect. Immun. **70:**2249-2255.

108. **Raffatellu, M., R.P. Wilson, D. Chessa, H. Anrews-Polymenis, Q.T. Tran, S. Lawhon, S. Khare, L.G. Adams, and A.J. Bäumler.** 2005. SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella enterica* serotype Typhimurium invasion of epithelial cells. Inf. Immun. **73:**146-154.

109. **Rasch, M., J.B. Andersen, K.F. Nielsen, L.R. Flodgaard, H. Christensen, M. Givskov, and L. Gram.** 2005. Involvement of bacterial quorum sensing signals in spoilage of bean sprouts. Appl. Environ. Microbiol. **71:**3321-3330.

110. **Rasmussen, T. B., and M. Givskov.** 2006. Quorum sensing inhibitors: a bargain of effects. Microbiol. **152:**895-904.

111. **Ren, D., J.J. Sims, and T.K. Wood.** 2001. Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)-4-bromo-5-

(bromomethylene)-3-butyl-2(5H)-furanone. Eviron. Microbiol. **3:**731-736.

112. **Ren, D., L.A. Bedzyk, R.W. Ye, S.M. Thomas, and T.K. Wood.** 2004. Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. Biotech. Bioeng. **88:**630-642.

113. **Richter-Dahlfors, A., A.M. Buchan, and B.B. Finlay.** 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella* Typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J. Exp. Med. **186:**569-580.

114. **Rosenberger, C. M., M.G. Scott, M.R. Gold, R.E.W. Hancock, and B.B. Finlay.** 2000. *Salmonella* Typhimurium infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression. J. Immun. **164:**5894-5904.

115. **Santos, R. L., R.M. Tsolis, A.J., Bäumler, and L.G. Adams.** 2003. Pathogenesis of *Salmonella*-induced enteritis. Braz. J. Med. Biolog. Rea. **36:**3-12.

116. **Schauder, S., K. Shokat, M.G. Surette, and B.L. Bassler.** 2001. The luxs family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. **41:**463-476.

117. **Semmelhack, M. F., S.R. Campagna, C. Hwa, M.J. Federle, and B.L. Bassler.** 004. Boron binding with the quorum sensing signal AI-2 and analogues. Org. Let. **6:**2635-2637.

118. **Sha, J., A.A. Fadl, G.R. Klimpel, D.W. Niesel, and V.L. Popov.** 2004. The two murine lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. Inf. Immun. **72:**3987-4003.

119. **Sircili, M. P., M. Walters, L.R. Trabulsi, and V. Sperandio.** 2004. Modulation of enterophathogenic *Escherichia coli* virulence by quorum sensing. Infec. Immun. **72:**2329-2337.

120. **Soncini, F. C., E. García Véscovi, and E.A. Groisman.** 1995. Transcriptional autoregulation of the *Salmonella* Typhimirium *phoPQ* operon. J. Bact. **177:**4364-4371.

121. **Soncini, F. C., E. García Véscovi, F. Solomon, and E.A. Groisman.** 1996. Molecular basis of the magnesium deprivation response in *Salmonella* Typhimurium: identification of PhoP-regulated genes. J. Bact. **178:**5092-5099.

122. **Sorensen, O., J. Van Donkersgoed, M. McFall, K. Manninen, G. Gensler, and G. Ollis.** 2002. *Salmonella spp.* shedding by Alberta beef cattle and the detection of *Salmonella spp.* in ground beef. J. Food Prot. **65:**484-491.

123. **Sperandio, V.** 2004. Striking a balance inter-kingdom cell-to-cell signaling, friendship or war? Trend. Immunol. **25:**505-507.

124. **Sperandio, V., A.G. Torres, B. Jarvis, J.P. Nataro, and J.B. Kaper.** 2003. Bacteria-host communication the language of hormones. Proc. Nat. Acad. Sci. **100:**8951-8956.

125. **Sperandio, V., A.G. Torres, J.A. Girón, and J.B. Kaper.** 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J. Bact. **183:**5187-5197.

126. **Su, L. H., C.H. Chiu, C. Chu, and J.T. Ou.** 2004. Antimicrobial resistance in nontyphoid *Salmonella* serotypes a global challenge. Antimicrob. Res. **39:**546-551.

127. **Sun, J., R. Daniel, I. Wagner-Döbler, and A. Zeng.** 2004. Is autoinducer-2 a universal signal for interspecies communication a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. BMC Evol Biol, vol. 4.

128. **Surette, M. G., and B.L. Bassler.** 1998. Quorum sensing in *Escherichia coli* and *Salmonella* Typhimurium. Proc. Nat. Acad. Sci. **95:**7046-7050.

129. **Surette, M. G., and B.L. Bassler.** 1999. Regulation of autoinducer production in *Salmonella* Typhimurium. Mol. Microbiol. **31:**585-595.

130. **Surette, M. G., M.B. Miller, and B.L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella* Typhimurium, and *Vibrio harveyi* a new family of gene responsible for autoinducer production. Proc. Nat. Acad. Sci. **96:**1639-1644.

131. **Taga, M. E., and B.L. Bassler.** 2003. Chemical communication among bacteria. Proc. Nat. Acad. Sci. **100 Suppl2:**14549-14554.

132. **Taga, M. E., J.L. Semmelhack, and B.L. Bassler.** 2001. The luxS-dependant autoinducer AI-2 controls the expression of an ABC transported that functions in AI-2 uptake in *Salmonella* Typhimurium. Mol. Microbiol. **42:**777-793.

133. **Taga, M. E., S.T. Miller, and B.L. Bassler.** 2003. Lsr-mediated transport and processing of AI-2 in *Salmonella* Typhimurium. Mol. Microbiol. **50:**1411-1427.

134. **Thibessard, A., A. Fernandez, B. Gintz, N. Leblond-Bourget, and B. Decaris.** 2002. Effects of *rodA* and *pbp2b* disruption on cell morphology and oxidative stress response of *Streptococcus thermophilus* CNRZ368. J. Bact. **184:**2821-2826.

135. **Tiwari, R. P., N. Sachdeva, G.S. Hoondal, and J.S. Grewal.** 2004. Adaptive acid tolerance response in *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Typhi. J. Basic. Microbiol. **44:**137-146.

136. **Trelfall, E. J., I. Fisher, C. Berghold, P. Gerner-Smidt, H. Tschäpe, M. Cormican, I. Luzzi, F. Schnieder, W. wannet, J. Machado, and G. Edwards.** 2003. Antimicrobial drug resistance in isolates of *Salmonella enterica* from cases of salmonellosis in humans in Europe in 2000 results of international multi-centre surveillance. Eurosurveil. **8:**41-45.

137. **Tsolis, R. M., A.J. Bäumler, and F. Heffron.** 1995. Role of Salmonella Typhimurium Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Inf. Immm. **63:**1739-1744.

138. **Van Asten, A., and J.E. Van Dijk.** 2005. Distribution of 'classic' virulence factors among *Salmonella spp.*. FEMS Immun. Med. Microbiol. **44:**251-259.

139. **Van Immerseel, F., J. De Buck, F. Pasmans, P. Velge, E. Bottreau, V. Fievez, F. Haesebrouck, and R. Ducatelle.** 2003. Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells *in vitro* is influenced by short-chain fatty acids. Int. J. Food Microbiol. **85:**237-248.

140. **Vendeville, A., K. Winzer, K. Heurlier, C.M. Tang, and K.M. Hardie.** 2005. Making 'sense' of metabolism autoinducer-2, LuxS, and pathogenic bacteria. Nat. Rev. Microbiol. **3:**383-396.

141. **Walters, M., and V.E. Sperandio.** 2006. Quorum sensing in *Escherichia coli* and *Salmonella*. Int. J. Med. Microbiol. **296:**125-131.

142. **Walters, M., M.P. Sircili, and V. Sperandio.** 2006. AI-3 synthesis is not dependant on *luxS* in *Escherrichia coli*. J. Bact. **188:**5668-5681.

143. **Wang, L. L., and E.A. Johnson.** 1992. Inhibition of *Listeria monocytogenes* by fatty acids and monoglycerides. Appl. Envir. Microbiol. **58:**624-629.

144. **Wang, Q., J.G. Frye, M. McClelland, and R.M. Harshey.** 2004. Gene expression patterns during swarming in *Salmonella* Typhimurium genes specific to surface growth and putative new motility and pathogenicity. Mol. Microbiol. **52:**169-187.

145. **Winfield, M. D., and E.A. Groisman.** 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Appl. Env. Microbiol. **69:**3687-3694.

146. **Winzer, K., K.R. Hardie, and P. Williams.** 2003. LuxS and autoinducer-2 their contribution to quorum sensing and metabolism in bacteria. Adv. Appl. Microbiol. **53:**291-396.

147. **Xavier, K. B., and B.L. Bassler.** 2003. LuxS quorum sensing more than just a numbers game. Curr. Opin. Microbiol. **6:**191-197.

148. **Xavier, K. B., and B.L. Bassler.** 2005. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. J. Bacteriol. **187:**238-248.

149. **Yang, M. C., Q.G. Ruan, J.J. Yang, S. Eckenrode, S. Wu, R.A. McIndoe, and J.X. She.** 2001. A statistical method for flagging weak spots improves normalization and ratio estimates in microarrays. Physiol. Genomics **7:**45-53.

150. **Yang, Y. H., S. Dudoit, P. Luu, D.M. Lin, V. Peng, J. Ngal, and T.P. Speed.** 2002. Normalization for cDNA microarray data: a robust method addressing single and multiple slide systematic variation. Nuc. Acids. Res. **30:**e15.

151. **Yuan, L., J.D. Hillman, and A. Progulske-Fox.** 2005. Microarray analysis of quorum-sensing-regulated genes in *Porphyromonas gingivalis*. Inf. Immun. **73:**4146-4154.

152. **Zhang, S., R.A. Kingsley, R.L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueirdo, J. Nunes, R.M. Tsolis, L.G. Adams, and A.J. Bäumler.** 2003. Molecular pathogenesis of *Salmonella enterica* serotype Typhimurium-induced diarrhea. Inf. Immun **71:**1-12.

153. **Zhou, D., and J. Galán.** 2001. *Salmonella* entry into host cells the work in concert of type III secreted effector proteins. Microb. Inf. **3:**1293-1298.

154. **Zweitering, M. H., I. Jongenburger, F.M., Rombouts, and K. Van 'T Riet.** 1990. Modeling of the bacterial growth curve. Appl. Env. Microbiol. **56:**1875-1881.

APPENDIX A

PROTOCOLS AND SUPPORTING EXPERIMENTS

AI-2 Bioluminescence Assay

Vibrio harveyi BB170 was grown overnight in AB medium at 30 °C (with moderate shaking). Two 2 µl was transferred into 10 ml fresh AB medium (1:5000 dilution). AB (Autoinducer Bioassay) medium was prepared as follows: (per L) 17.5 g NaCl, 12.3 g MgSO4, 2 g Casamino acid, pH adjusted to 7.5 (1 N NaOH) and autoclaved at 121 °C for 15 min. After cooling to room temperature the following was added: 10 ml 1M KH2PO4 pH adjusted to 7.0 (autoclaved), 10 ml 0.1M L-arginine (fresh prepared and 0.2 µm filtered), and 20 ml 50% glycerol (autoclaved). Ninty 90 µl of diluted reporter cells was added into a 96 well plate for each sample and the controls, all samples and controls were prepared as triplicates. Inhibition assay samples were prepared as 5 µl sample, with 5 µl Preformed AI-2 CFS added to the 90 µl of diluted reporter cells for a total volume of 100 µl for each well. For negative controls, 10 µl fresh AB medium was added to the wells. For the positive controls, 5 µl fresh AB medium, and 5 µl Preformed AI-2 CFS were added.

The well plate was incubated with a plate cover at 30 °C, 100 RPM. After approximately 3 hours of incubation, the plate was removed for luminescence readings and at 30 minute intervals thereafter. Readings were taken in a Perkin Elmer-Wallac Victor 2 plate reader set to measure luminescence, live display settings set to a logarithmic reading, high scale 0-100000. At these settings the

plate reader measured light emitted (with no excitation) from each sample within the visible spectrum. Measurements were taken until the average luminescence values of the negative controls approximately doubled from the previous 30 minute reading, at that point the assay was stopped and those values were used for determining inhibition. Inhibition was determined as a percentage based on the positive control using the following equitation: Relative Percentage Activity: 100 – [(Average Negative Control/Average Sample Value) x 100].

Macrophage Cell Infection Assay

The RAW264.7 cell line was maintained on modified Eagle medium with Earle's modified salts (MEME) supplemented with 2 mM Glutamine, 10 mg/ml streptomycin, 25 µg/ml amphotericin B, and 10% fetal bovine serum (FBS) in a T25 flask (a T75 flask was used for cell passage preceeding seeding experiments) at 37° C in 5% CO_2.   Six well plates were seeded with RAW cells and allowed to grow to confluence at 37° C in 5% CO_2. *Salmonella* PJ002, was grown overnight at 35 °C in LB broth amended with 25 µg/ml choramphenicol. One ml of the overnight culture was centrifuged at 12,000 x g for 5 minutes, the supernatant removed, and resuspended in 1 ml of MEME, with sodium bicarbonate (no serum).   Treatments were prepared as 400 µl cell suspension with 100 µl sample (example 100 µl 1x PBS, 50 µl 1 X PBS + 50 µl AI-2, 50 µl fatty acid mixture + 50 µl AI-2, etc). Media from the 6 well plates were removed and discarded, 500 µl of the *Salmonella* cell treatments were added to each well and incubated for 1

hour at 37° C in 5% CO2.   Two additional controls were prepared. A blank

control prepared from one well in each 6 well plate by adding 500 µl MEME

media, without bacteria. And an antibiotic (AB) control, where 500 µl of the

Salmonella cell suspension that was prepared in a microcentrifuge tube

(incubated under similar conditions as the infection assay well plates).

To determine the original inoculum, a portion of the cell suspension was serially

diluted in 1 X PBS and plated onto Luria Bertani agar plates (LBA).

After incubation each well was washed with 300ul 1X PBS, 3 times. For the AB

control, the microcentrifuge tube was spun down at 12,000 x g for 5 min, the

supernatant removed and discarded. Three hundred µl MEME medium (with

sodium bicarbonate and 10% FBS) amended with 100 µg/ml Ceftriaxone was

added to each well (or the cell pellet resuspended in the case of the AB control).

The well plates (and AB control) were incubated at 37° C in 5% CO_2 for 2 hours.

Following the incubation period, the media was removed from plate wells and

each well was rinsed twice with 300 µl 1X PBS. The AB control at this point was

be directly plated onto LB plates. Four hundred µl 1% Triton X-100 (in 1X PBS)

was added to each well and incubated at room temperature for 10 minutes,

followed by the addition of 600 µl LB broth. The LB solution was drawn up

dispersed several times to further remove the mono-cell layer and portions were

removed for dilution (in 1X PBS) and plating onto LBA plates.   All LBA plates

were incubated for 24H at 35 °C. Colonies were counted on the plate the

following day, converted to CFU/ml, and infection efficiency calculated as

follows: % infection = [cells in inoculum (CFU/ml) / cells recovered post-infection (CFU/ml)] X 100. The assay was considered successful if both the blank control samples and the AB control expressed no colonies after plating on LBA.

Confirmation of Cell Infection by Microscopy

To determine if *Salmonella* was capable infecting the RAW cells, despite the washing steps employed during the cell infection assay, an experiment was run to confirm the presence of *Salmonella* by bright-field microscopy. RAW cells were seeded in 6 well plates, with the addition of 25 mm plastic coverslips (Thermanox coverslips, Nunc, Rochester, NY). A cell infection assay was run as previously described except that after the final washing steps post-incubation with MEME medium amended with chloramphenicol, the RAW cells were not lysed with Triton-X 100. Instead, the coverslips were removed for Giemsa staining.

The cover slips were air dried and then dehydrated with methanol (dipped twice in a methanol solution). The cover slips were then submerged in a diluted Giemsa stain, modified, solution (1:20 dilution in water) for 20 minutes. After sufficient staining, the cover slips were removed and briefly washed with water (dipped in solution 2-3 times), and air dried in a near vertical position. The stained cover slips were mounted on glass slides and viewed at 1000 X total magnification under bright field using an oil immersion lens. The Giemsa stain is typically used to differentiate nuclei from cytoplasmic morphology in

eukaryotic cells, and can be used to indicate if a cell line is contaminated with a *Mycoplasma* infection due to the presence of dark blue-purple nuclei within the cytoplasm (typically a pinkish stain). The infected samples demonstrated several dark stained rod shaped bacteria, within the cytoplasm of the RAW host cells, and these were noticeably absent in the blank controls (Fig. A.1). This indicated that despite several washings after incubation in the MEME supplemented antibiotic medium, *Salmonella* cells were still present and closely associated with the RAW host cells.
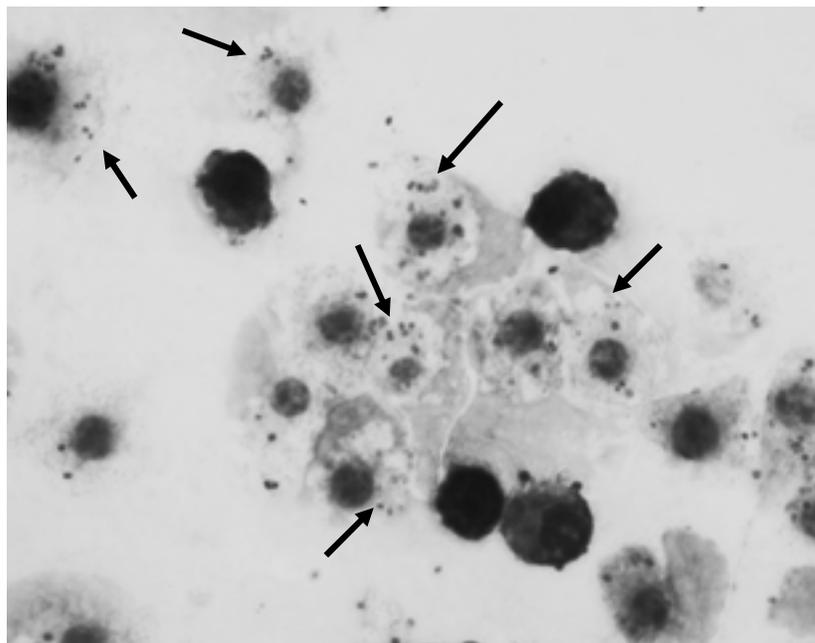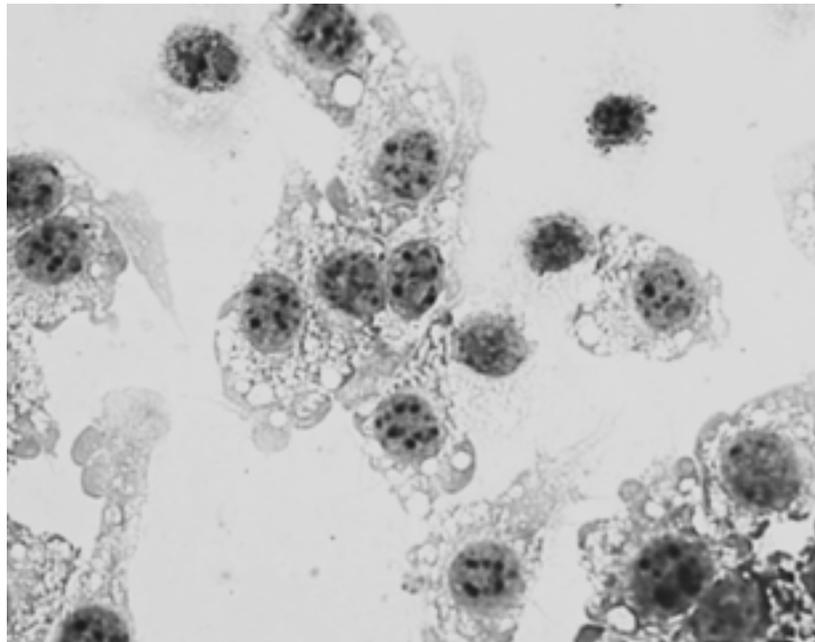
Figure A.1 Giemsa Stained RAW Cells after *Salmonella enterica* Infection Assay

Top panel - negative control, Bottom panel – infected cells (selected sites demonstrating typical infection indicated by arrows).

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Widmer, K.W., S. Deepak, and S.D. Pillai. 2005. Use of artificial neural networks to accurately identify *Cryptosporidium* oocyst and *Giardia* cyst images. Appl. Environ. Microbiol. 71:80-84
Widmer, K.W. 2003. Development of Artificial Neural Networks (ANN) Capable of identifying *Cryptosporidium parvum* oocysts stained with 4', 6 diamidino-2-phenylindole (DAPI). J. Rap. Meth. Auto. Microbiol. 11:97-110
Widmer, K.W., K.H. Oshima, and S.D. Pillai. 2002. The identification of *C. parvum* oocsts using an artificial neural network approach. Appl. Environ. Microbiol. 68:1115-1121.

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