EVALUATION OF INTERCELLULAR SIGNALING IN

LEGIONELLA PNEUMOPHILA

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EVALUATION OF INTERCELLULAR SIGNALING IN *LEGIONELLA PNEUMOPHILA*

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DISSERTATION ABSTRACT

EVALUATION OF INTERCELLULAR SIGNALING IN

LEGIONELLA PNEUMOPHILA

Stephanie Denise Zeigler-Ballerstein

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The number of prokaryotes known to participate in intercellular signaling is growing. The objective of this research was to determine if *Legionella* species are able to communicate through the use of intercellular signals with other bacteria and with eukaryotic hosts. The evaluation of intercellular signaling in *L. pneumophila* was conducted in three phases: i) determine if *Legionella* species produce intercellular signals; ii) determine if *L. pneumophila* responds to signals produced by unrelated microorganisms, and iii) evaluate if *L. pneumophila* can respond to signals or other metabolites produced by its natural, protozoan hosts.

Among bacteria, known intercellular signals include N-acyl homoserine lactones (AHSL), autoinducer-2 (AI-2), and quinolone autoinducers. In the evaluation of *Legionella* species' ability to produce signaling metabolites, biological sensors that detect

AHSL, AI-2, and quinolone autoinducers were used. The data from each of the trials demonstrated conclusively that *Legionella* species do not produce AHSL, AI-2, or quinolone signals.

In the evaluation of the ability of *L. pneumophila* to respond to interspecies or inter-kingdom signals, the pathogen was exposed to supernatants of signal producing microorganisms and that of protozoa known to support their intracellular growth. An evaluation of the protein profile of *L. pneumophila* following its exposure to these supernatants by two-dimensional electrophoresis (2-DE) revealed the expression of multiple proteins. Subsequent analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) aided in the presumptive identification of many of these proteins.

Of the 40 proteins that were identified, 36 were differentially expressed in *L. pneumophila* following exposure to the supernatants of signal producing bacteria. These proteins included global stress protein GspA and a 24kDa macrophage induced protein. Following exposure to protozoa, 36 proteins were identified as a part of the *L. pneumophila* response and include a *Legionella* long chain fatty acid transporter and cold shock domain protein CspA. In terms of its protein expression, the reaction demonstrated by *L. pneumophila* in the presence of unrelated prokaryotes and potential host protozoa represented a stress response and suggested that *L. pneumophila* can respond to these organisms via intercellular signaling.

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I. INTRODUCTION

Legionella pneumophila is an aquatic bacterium associated with a variety of aqueous habitats. This bacterium is also the causative agent of Legionnaire's Disease in human hosts. Common to both of these environments is the organisms requirement for intracellular growth. Multiplication is supported by numerous species of environmental protozoa; whereas, in humans this function is accommodated by alveolar macrophages during the progression of Legionnaire's Disease (LD). LD is a form of community acquired pneumonia that causes the hospitalization of an estimated 18,000 diseased persons annually (Fields et al., 2002). Infection typically results from exposure to aerosols laden with infectious bacteria. Person-to-person transmission of LD does not occur, but environmental reservoirs are frequently implicated in the genesis of LD outbreaks. Given the importance of the environmental reservoir in such circumstances, determining the mechanisms by which *L. pneumophila* survives and subsequently maintains its growth prior to the incidence of human infection undoubtedly should reveal measures that will assist in developing efficient control measures. Available data suggest a possible contribution of cohabiting microorganisms and protozoa in the persistence of *L. pneumophila*, which is crucial to the ability of this organism to establish a successful respiratory infection (reviewed by Fields et al., 2002; Molmeret et al., 2004). Given these facts, the review that follows will focus on the ecology of *Legionella* species and their association with both prokaryotes and protozoan hosts. Furthermore, the concept of

intercellular signaling among prokaryotes will be explored, as these signals are crucial to the development of the *Legionella* habitat, the aquatic biofilm.

II. REVIEW OF LITERATURE

The Ecology of Legionella

A common denominator among *Legionella* species is their persistence in aquatic environments, both natural and man-made. Legionellae have been isolated from numerous aqueous environments including lakes, ponds, effluent from wastewater treatment plants, showerheads, spas, decorative fountains, misters, and cooling towers (Barbaree et al., 1986; Stout et al., 1985; Fliermans et al., 1981; Atlas, 1999). In naturally untreated waters, *Legionella* concentrations have been enumerated at 10^3 cfu/mL; however, their population densities are substantially higher within man-made systems (Tison and Seidler, 1983). Within these systems, where solid surfaces are continously bathed with water, the development of biofilms are inevitable. Biofilm genesis requires the cooperation of a complex and densely populated array of microorganisms, with microbial concentrations substantially higher than in the aqueous phase. Thus, legionellae densities within these matrices are much higher than that of their free-living counterparts.

Of the artificial environments, cooling towers are prone to have biofilms heavily contaminated with legionellae. Cooling towers are known to disperse contaminated aerosols over wide distances and thus, are frequently linked to large scale outbreaks of legionellosis. With legionellae densities of 10^5 to 10^6 cfu/ml of water in these systems,

water cooling towers are capable of generating aerosol volumes equal to 4,300 liters per day that easily covers a dispersion radius of 6 km ((Negron-Alvira et al., 1988; Nguyen, 2006). Anecdotal evidence of the relationship between cooling towers and outbreaks of legionellosis also exists where the absence of LD was noted following the discontinued use of contaminated systems (Sabria et al., 2006). Although the majority of outbreaks are the result of transmission from artificial aquatic environments, some exceptions do exist. *L. longbeachae*, a prominent cause of legionellosis in Australia, is frequently isolated from moist potting soil mixtures (Steele et al., 1990a; Steele et al., 1990b). Although approximately one-half of *Legionella* species have been associated with legionellosis, humans appear to be incidental, rather than primary hosts.

Legionella species are virtually ubiquitous in the aquatic environment. The isolation of legionellae from aqueous sources following outbreaks of legionellosis has demonstrated the extent to which aqueous reservoirs facilitate transmission of these pathogens, thereby establishing their epidemiological significance. With the first documented incident of the Legionnaire's disease attributed to contaminated air conditioning units during the historic outbreak in 1976, the majority of subsequent exposures have been directly linked to aqueous niches (Fraser et al., 1977; Fields et al., 2002). Virtually any man-made equipment or facility that is bathed in, or frequently in contact with water is susceptible to colonization by legionellae. Natural aqueous habitats, although infrequently implicated as a dissemination source in legionellosis outbreaks, are an expected niche for these microbes. Freshwater lakes, rivers, ponds, springs, and creeks have been documented as natural sources of various *Legionella* species (Fliermans et al., 1981). A study by Sheehan et al (2005) purported the isolation of four *Legionella*

species, including *L. micdadei* and *L. sainthelensi*, from an acidic (pH 2.7), geothermal (60 ºC) creek in Yellowstone National Park.

The environmental phase of the legionellae life cycle is multi-faceted, characterized by a period of intracellular growth within protozoa, after which these organisms exist as both biofilm and planktonic forms. The latter corresponds to the period that transmission is most likely to occur. Biofilms are known for their microbial diversity and for the cooperation required amongst microorganisms for their creation. Under experimental conditions, the ability of *L. pneumohpila's* to produce biofilms in the absence of pioneer microorganisms has been demonstrated (Hu et al., 2003, Stoodley et al., 2002). *tatB*, *tatC*, and *fliA* have been linked to this activity. *L. pneumophila* Tat (for twin arginine translocation) mutants, like *Pseudomonas aeruginosa tat* mutants, form biofilm matrices that are less dense than their wildtype counterparts (De Buck et al., 2005). After evaluating multiple *L. pneumophila* mutants for their ability to manufacture biofilms, Mampel et al (2006) noted that *fliA* mutants lacking the flagellar sigma factor FliA (σ^{28}) produced matrices that lacked 30% of the biomass of that produced by the wild type. Contrary to other progenitor microorganisms, mutations affecting pili and flagellar biogenesis as well as the synthesis of the stationary phase sigma factor *rpoS*, produced no observable deficiencies in *L. pneumophila*'s ability to establish biofilms (Mampel et al., 2006).

Although legionellae are isolated amongst numerous microorganisms from biofilms, the natural consortia associated with this habitat has rarely been characterized. With respect to *L. pneumophila*, the majority of biofilm analysis has focused on simply documenting the presence of this pathogen. Identification is typically accomplished

through culture or molecular detection methods and in the aftermath of large-scale outbreaks of legionellosis. Therefore, the identification of other microbial flora within *Legionella*-positive specimens has received little attention. However, understanding the ecology of these microorganisms requires knowledge of the diversity of the consortium in which they reside. Of the studies that have identified the microbial composition of biofilms harboring *Legionella*, multiple *Pseudomonas* species (*P. aeruginosa, P. fluorescens, P. testosteroini, P. paucimobilis, P. versicularis,* and *P. maltophila*), actinomycetes, *Methylobacterium* sp., *Flavobacterium* sp., *Alcaligines* sp., *Acinetobacter* sp., *Vibrio* sp., *Chromobacterium* sp., *Klebsiella* sp., and *Achromobacter* sp have been noted (Rogers et al., 1994; Surman et al., 1994; Walker et al., 1994; Rogers, 1993). The association of *L. pneumophila* with cyanobacteria in algal-bacterial mat communties, namely *Fischerella, Phomidium*, and *Oscillatoria* spp., has also been documented (Tison et al., 1980). Algae, fungi, and more importantly, protozoa are also frequently isolated. Given the fact that these organisms are isolated synchronously with *Legionella* and that growth of *Legionella* species proceeds only with the aid of an intracellular, eukaryotic host it is likely that the interactions of legionellae with non-*Legionellaceae* bacteria results in the maintenance of metabolic activity and their persistence within biofilms.

The importance of legionellae association with other prokaryotes was underscored by various studies of the interactions between *L. pneumophila* and unrelated microorganisms. Collectively, the results demonstrated that intracellular growth is enhanced and survivability is extended under laboratory conditions (Tison et al., 1980; Surman et al., 1994; Wadowsky and Yee, 1985). The significance of this data is derived from the pathogen's sharp decline in culturability over extended periods. In the absence

of a suitable intracellular host (or other heterotrophic microorganisms), *L. pneumophila* can assume a non-culturable state over an undefined period of time. The data regarding the period over which viability is completely diminished are conflicting, but they do suggest that *Legionella* can be maintained as a free-living entity for up to 2.4 years. At one end of the spectrum, culturability is lost in as little as 50 days in autoclaved, ultra-pure water (Yamamoto et al., 1996). On the contrary, a complete loss of culturability after 125 days for bacilli stored in distilled water and up to 600 days in filter-sterilized, tap water has been reported (Steinert et al., 1997; James et al., 1999). Paszko-Kolva *et al* (1992) reported the recovery of viable microorganisms following a 2.4 year incubation period in various aquatic microcosms, i.e. those supplied with non-sterile drinking water, creek water, or estuarine water. In the research reviewed here, the anomalies in nutrient conditions employed during the evaluation of the long term survival of *L. pneumophila*'s influenced the extent to which viability was sustained.

One mechanism through which viability is retained involves the use of poly-3-hydroxybutyrate (PHB). The presence of intracellular PHB is enhanced during periods of low iron or nutrient availability. However, in the case of *L. pneumophila*, PHB consumption does not appear to be related to culturability (James *et al* 1999). While PHB provides a plausible explanation for survival, it fails to account for the enhanced survival of cultures maintained in tap water cultures compared to those maintained in distilled water. One explaination, however, is the influence of cohabiting microorganisms and the influence of metabolic products secreted by these organisms on survival of legionellae. As will be discussed at a later point in this review, loss of culturability has little effect on the pathogens ability to parasitize protozoa. However, a

"non-culturable" status does correlate with attenuated pathogenicity because non-culturable organisms are unable to parasitize human alveolar macrophages (Steinart et al., 1997).

Among the first reports suggesting the importance of cohabiting microflora in the long term survival of legionellae were two studies involving cyanobacteria. The first of these demonstrated the extension of culturability to a minimum of 12 weeks when legionellae were maintained in a suspension of *Fischerella* supernatants (Tison et al., 1980). A subsequent investigation revealed the pathogens extended viability upon aerosolization following suspension in *Fischerella* conditioned medium. In contrast, a loss of viability was demonstrated following its resuspension in dialyzed supernatants. This indicated the production of low molecular weight compounds as a potential source of *Legionella*'s sustainment (Berendt, 1981). These data suggest the dependence of *Legionella* on other organisms for growth supporting activity. Best et al (1985) defined "syntrophy" as an interaction during which a "donor" microorganism contributes to the maintenance of a "recipient" as a result of its ability to produce a factor required for growth in the latter. Under laboratory conditions, syntrophic interactions can be identified by the evolution of satellite colonies within close proximity to the donor strain. This method has also been used to demonstrate the effects of cohabiting microbes on the survival of legionellae. Surman et al (1994) substantiated this concept by demonstrating *L. pneumophila* satellites on R2A minimal medium in the presence of *P. vesicularis*, *P. paucimoilis*, *Flavobacterium* sp. and *Methylobacterium* sp. The satellites remained viable for more than 6 weeks after initial culture.

The principle of syntrophy with regard to *Legionella*'s viability was also supported by the fact that viability was not observed when the same cells were exposed to either heat killed non-Legionellaceae or their cell free extracts (Surman et al., 1994). Additional data also support the concept of syntrophy with regard to the persistence of legionellae. A study conducted with environmental microflora indicated *Pseudomonas, Flavobacterium, Alcaligines,* and *Acinetobacter* stimulate satellite development of *L. pneumophila* (Stout et al., 1985). Syntrophic species were also noted for their capacity to extend the pathogens viability over time when compared to unsupported organisms. Wadowsky et al (1985) noted similar results; however, common to each of the studies mentioned is the inability of *L. pneumophila* to grow in aqueous cultures in the presence of syntrophs. As the authors suggested, the factors secreted by these organisms may be diluted to the extent that growth of legionellae is not supported (Tinson, 1980; Stout et al., 1985). However, given the small colony size and low abundance of legionellae, an equally plausible explanation is that the numbers of progeny was too low to be detected using standard plate count methods.

The ability *L. pneumophila*'s to grow syntrophically under laboratory conditions is likely a reflection of its capacity for limited growth outside of a host. The displacement of syntrophic interactions between legionellae and supportive microorganisms from the laboratory to the biofilm environment, which is known for its ability to concentrate legionellae, suggests the plausibility of extracellular growth. Tightly clustered microorganisms are distributed throughout biofilms, and the close proximity of organisms within these microcolonies facilitates their interaction. One interaction is the onset of intercellular signaling between prokaryotes (Allison et al.,

1998). Syntrophy may also be facilitated where neighboring microbes are encased within biofilm matrices. Such conditions may hinder the diffusion of potential extracellular growth factors, creating the conditions required for growth and/or survival of legionellae given the appropriate syntrophic association. Rogers et al (1992) noted the presence of legionellae microcolonies distributed throughout biofilms in their studies and suggested that these small aggregates are potentially evidence of growth within the consortia given that protozoa were absent. This suggestion was refuted in a separate study in which microcolonies were shown to be the product of continuous aggregation of planktonic microorganisms rather than accumulation of cells through clonal replication (Mampel et al., 2006). This finding is intriguing in that sessile *L. pneumophila* remained metabolically active despite the fact that they failed to grow extracellularly during biofilm genesis in a medium capable of supporting the growth of legionellae *in vitro.*

Further assessment of biofilm formation by *L. pneumophila* AA100 by Mampel et al (2006) demonstrated that this activity is stimulated by intracellular growth in host amoebae. Planktonic prokaryotes recently liberated from amoebal hosts were shown to be actively involved in biofilm establishment. As pioneering organisms during initial development, planktonic cells continuously increased biofilm density during its maturation; however, as planktonic organisms became sessile, additional growth or replication was not observed, suggesting the induction of a metabolic switch that becomame active during sessile growth. The deposition and persistence of planktonic cells on the substratum was stimulated not only by the presence of amoebae, but also by the presence of specific microorganisms. These included biofilms established by *Empedobacter brevis, Microbacterium* sp., and *Acinetobacter baumanii*. Other species

were noted for their ability to antagonize *L. pneumophila* colonization including an unnamed *Pseudomonas* species, *Corynebacteirum glutamicum*, and *Klebsiella pneumoniae* (Mampel et al., 2006).

Legionella and its association with protozoa

Protozoa are also closely associated with legionellae within biofilms. As extracellular growth of *Legionella* species in the environment has not been substantiated, protozoa are a critical component in their reproduction. Legionellae are readily ingested by and grow to high densities within protozoa, and infected protozoa are a natural source of *Legionella* isolates (Rowbotham, 1983; Barbaree et al., 1986). Following the first description of intracellular growth in protozoa in 1980, many susceptible species have been identified. Permissive hosts include *Hartmannella vermiformis*, two *Acanthamoeba* species (*A. polyphaga, A. castellanii*), *Balamuthia mandrillaris, Dictyostellium discoidium*, 2 *Naegleria* species (*N. fowleri, N. lovaniensis)*, *Vahlkampfia jugosa*, and *Echinamoeba exudans* (Holden et al., 1984; Fields et al., 1990; Tyndall and Domingue, 1982; Newsome et al, 1985; Shadrach, 2005; Fields et al., 1989; Rowbotham,1986; Solomon et al., 2000). Ciliates, namely the *Tetrahymena* species *T. pyrmiformis T. vorax*, and *T. thermophila*, are also susceptible to infection (Smith-Somerville et al., 1991; Fields et al., 1984; Kikuhara et al., 1994). Of these organisms *Hartmannella*, *Acanthamoeba, Tetrahymena*, and *Naegleria* species are frequently demonstrated in legionellae biofilms, some of which are typically infected with the bacillus. These organisms serve as important reservoirs for legionellae, and are typically isolated concurrently with *L. pneumophila* following outbreaks of legionellosis (Fields et al., 1990).

The association between intracellular growth in protozoa and the subsequent capacity for invasion of human macrophages is well documented. When compared to agar grown bacteria, amoebae derived microorganisms demonstrated enhanced potential for HEp-2 (human epidermoid carcinoma), RAW 264.7 (a mouse macrophage cell line), and THP-1 cells (human monocytic cells) (Cirillo et al., 1994). The extent to which agar grown microorganisms become attenuated is reminiscent of this fact *in situ.* In the extended absence of an environmental host, legionellae enter a dormant or viable-but-nonculturable (VBNC) state. This is characterized by an inability to grow on media under laboratory conditions, which typically impedes their detection in the absence of molecular methods, and an inability to cause respiratory infection in guinea pigs. Both of these shortcomings are fully reversed when dormant legionellae are passaged through protozoa (Steinart et al., 1997).

An obligatory association between *Legionella* and protozoa has been established; however, the factors that facilitate the initial interaction between these organisms are not well characterized. Protozoa feed on multiple bacterial species by grazing in areas heavily populated by prokaryotes. Rather than serendipity on *Legionella*'s part, however, other intervening factors are likely to ensure that the organism is ingested. Such an assumption yields the hypothesis that *Legionella* species actively pursue the feeding grounds of amoebae, and this function is mediated by chemotactic functions involving the perception of amoeboid signal compounds. This hypothesis is supported by Rowbotham (1983) in his recognition that "virulent legionellae are attracted to and attack suitable host amoebae, rather than [cause an] infection following chance ingestion". He noted in his observations that i) actively feeding *Acanthamoeba polypaga* trophozites,

rather than cysts, were attacked by *L. pneumophila;* ii) the pathogen was attracted to extended trophozites; iii) the bacilli formed aggregates at the site of the amoebae's contractile vacuole; iv) motile legionellae actively pursued healthy trophozoites within 20 minutes of exposure; and v) the gram-negative bacterium *Alcaligines* was attracted to the amoebae, but failed to stimulate ingestion. The significance in the attraction of legionellae to extended trophozites suggests that these are uninfected, and that the prokaryotes actively avoid or are repelled by rounded, i.e. infected, or encysted protozoa. Thus, *L. pneumophila* appeared not only to actively pursue its host, but it also stimulated its ingestion (Rowbotham 1983).

Not all protozoa are susceptible to intracellular infection by legionellae. In an investigation of multiple cooling towers implicated in an outbreak of legoinellosis, ciliates (*Tetrahymena* sp. and *Cyclidium* sp.), and unidentified species were collected. With the exception of *Cyclidium* species, all of the protozoa isolates were found to be susceptible to infection (Barbaree et al., 1986). Although *Tetrahymena* sp. are susceptible to legionellae, these organisms were shown to resist infection under certain experimental conditions. Previously stated, *T. vorax* can sustain a legionellae infection in the absence of a subsequent increase in intracellular bacilli and with little mortality if any (Smith-Sommerville et al., 1991). The infection of other *Tetrahymena* species appears to be a function of nutrient limitation. *In vitro*, *T. pyriformis* is resistant to infection by *L. pneumophila* and *L. longbeachae* when proteose peptone (PP) concentrations exceed 0.5g/L in a co-culture medium. While a reduced PP concentration of 0.2g/L partially induces starvation conditions and subsequently minimizes resistance to infection, the addition of 2.25g/L NaCl renders these organisms completely susceptible to invasion

(Steele and McLennan 1996). Legionellae-*Tetrahymena* interaction is facilitated in part by nutrient accessibility, and thus raises multiple questions, i.e., do the ciliates elect to feed on legionellae when other nutrients are limiting or do *Tetrahymena* emit signals that would indicate their susceptibility to infection by receptive legioenellae?

A role for intercellular signaling has been demonstrated in the interactions of microorganisms, particularly within those habitats in which legionellae species are known to reside. Thus, the concept of this type of communication will be the subject presented in the second half of this review.

Intercellular communication among prokaryotes

In a manner that is reminiscent of higher organisms, prokaryotes possess the ability to communicate between themselves via the aid of diffusible, chemical signals. These signals are as diverse in nature as the microorganisms that synthesize them and are characterized as small peptides, proteins, or carbohydrates (Holden et al., 1999; Eberhard, et al., 1981). Equally as diverse are the phenotypes that are induced in signal producing bacteria by these compounds which include activation of bacterial luminescence, antibiotic synthesis, induction of crown gall tumors in plants, twitching motility, and bacterial pigmentation (reviewed by Whitehead et al., 2001; McClean et al., 1997a). Although these events are stimulated by extracellular signals, a threshold concentration of signaling molecules must be attained. This requires a large population of microorganisms or a bacterial "quorum". Therefore, any biological functions among bacteria that are stimulated by the presence of concentrated, chemical signals are said to

be the results of "quorum sensing". The signaling compounds that induce these activities are termed "autoinducers".

The signaling molecules that provide the foundation for quorum sensing are synthesized constitutively during the exponential phase of bacterial growth. The concentration of extracellular signal increases coordinately with increasing bacterial density, eventually reaching the concentration required for induction of gene expression. Since the level of autoinducer is directly proportional to the number of cells, a direct correlation between gene function and cell density (quorum size) can be established. Multiple compounds have been associated with signaling activity. What appears to be the most prolific among Gram-negative microorganisms are *N*-acylhomoserine lactones (AHSL) (Whitehead et al., 2001). Conversely, quorum sensing among gram-positive bacteria is typically mediated by oligopeptides and amino acids (Reading and Sperandio, 2006; Waters and Bassler, 2005). An additional signal, furanosyl borate diester (AI-2), is commonly referred to as an interspecies signal due to the fact that this compound is synthesized by a multitude of gram-positive and gram-negative prokaryotes (Xavier and Bassler 2003, Sun et al., 2004). The primary focus of the review that follows will be communication mediated by AHSL, AI-2, and an additional form of intercellular signaling involving a quinolone compound that is unique to the prokaryote *P. aerugionsa* (Pesci et al., 1999).

The earliest reference to autoinducers appeared in 1970 with the description of the autoinduction of luminescence in the marine bacterium *Vibrio fischeri* by Nealson et al (1970). *V. fisheri* cultures were noted for the maximal production of light during late exponential phase and early stationary phase, which roughly corresponded to maximal

cell densities in batch culture. Interestingly, weakly bioluminenscent cultures in early exponential phase demonstrated maximum luminescence when resuspended in spent supernatants collected from stationary phase cells. This observation indicated the likely presence of a diffusible factor in culture supernatants, which was isolated by Eberhard et al in 1981. This factor, isolated by Eberhard et al in 1981, was identified as *N*-(3-oxohexanoyl)-L- homoserine lactone (oxo-C_6-HSL). As a class, homoserine lactones are characterized as carbohydrate-fatty acid derivatives. Structurally, these compounds consist of a lactone ring to which a fatty acid acyl side chain is attached. The side chain is variable in length, with a minimum of 4 carbon residues and a typical range of C_4 to C_8. It is the length of the acyl side chain, together with the oxidation state of the third carbon in the sequence that relegates specificity of the signal to the species by which it is synthesized. For example, two well known quorum sensing, gram-negative bacilli *Agrobacterium tumifaciens* and *Chromobacterium violacium*, produce *N*-(3oxooctanoyl)-L-homoserine lactone and *N*-(hexanoyl)-L-homoserine lactone, respectively (Zhang et al., 1993; McClean et al., 1997a). To date, the longest AHSL is recognized to be synthesized by the soil bacterium *Sinorhizobium meliloti*, which produces six separate autoinducers, including N-(tetrahydro-2-oxo-furanyl)-octadecanamide or C_{18}-HSL (Marketon et al., 2002).

Normal metabolic activity includes the constitutive synthesis of an autoinducer or AHSL. These molecules diffuse freely into and out of the bacterial cell, accumulating to concentrations that are commensurate with cell density. In seawater, *V. fischeri* occurs at population densities below10^2 cells/mL. Under these conditions, the autoinducers fail to accumulate and luminescence is not observed. The threshold concentration required

for the bioluminescent phenotype of *V. fischeri* is 10nM, with maximal induction at an AHSL concentration of 200nM. This corresponds to a cell density of roughly 10^{10} cfu/mL, which corresponds to the concentration of bacilli growing symbiotically within the light organ of the Japanese pinecone fish *Monocentris japonicus* (Kaplan and Greenberg, 1985). Subsequent analysis of *V. fischeri* luminescence *in vivo* has demonstrated AHSL concentrations in the light organs of sepiolid squids *Euprymna scolopes* and *Euprymna morsei* at approximately 118nM and 140nM, respectively when bacterial densities reached 10^8 cfu/mL (Boettcher and Ruby, 1995).

With regard to *V. fischeri*, the genes under the control of AHSL responsible for bioluminescence, are known collectively as the *lux* genes. The *lux* operon contains two divergently transcribed units. The first unit in the *lux* operon is *luxI*. LuxI is an autoinducer synthase that produces the AHSL signal molecule. The remaining *lux* genes, *luxICDABEG*, provide the structural components required for luminescence via the enzymatic activity of luciferase (Engebrecht and Silverman, 1984; Engebrecht et al., 1983). Directly upstream of the *luxICDABEG* transcriptional unit is the second unit, referred to as the *lux* transcriptional activator, *luxR*. The *luxR* protein, LuxR, is also required for *lux* activity where its activity is regulated by a threshold concentration of autoinducer. Specificity is required for successful interaction between LuxR and its cognate, species specific AHSL (Stevens and Greenberg, 1997).

The LuxR protein is synthesized constitutively in the presence of low concentrations of AHSL, persisting in an inactive conformation. Below threshold concentrations of autoinducer, the N terminus of LuxR physically restricts the activity of the C terminus; however, interaction by the cognate autoinducer with the N-terminus

relaxes its inhibition, resulting in a fully functional LuxR via C terminal activation (Sitnikov et al., 1996). In conjunction with RNA polymerase, active LuxR molecules bind the promoter region of the *lux* operon. This action stimulates transcription of both the autoinducer synthase (LuxI) as well as the luciferase genes, and culminates with the expression of bioluminescence in *V. fisheri* (Stevens et al., 1994; Stevens and Greenberg, 1997).

The *lux* system is the model against which subsequent discoveries of AHSL mediated quorum sensing systems in other Gram-negative bacilli have been identified. In addition to multiple *Vibrio* species which include *V. fischeri, V. harveyi*, *V. vulnificus* and *V. anguillarum* (Eberhard et al., 1981; Bassler et al., 1993; Kim et al., 2003; Milton et al., 1997), AHSL and *luxR/I* homologs have been identified in more than 40 species of Gram-negative bacilli. This group includes the *Pseudomonas* species *P. aeruginosa,* and *P. aureofaciens* (Wood and Pierson, 1996; Pearson et al., 1994), the plant pathogens *Agrobacteriu*m *tumifaciens* (Cha et al., 1998) and *Erwinia carotovora* (Jones et al., 1993), fish pathogens *Aeromonas hydrophila* and *Aeromonas salmonicida* (Swift et al., 1997), *Chromobacterium violaceum* (McLean et al., 1997), *Burkholderia cepacia* (Lewenza et al., 1999), the intracellular pathogen *Brucella melitensis* (Taminiau et al., 2002) and multiple members of the family *Enterobacteriaciae* including *Proteus mirabilis*, *Serratia marcescens* (Swift et al., 1993), *Serratia liquifaciens* (Eberl et al., 1996), *Enterobacter agglomerans* (Swift et al., 1993), the insect pathogen *Xenorhabdus nematophilus*, and *Yersenia* species *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (Throup et al., 1995; Atkinson et al., 1999; Kirwan et al., 2006).

The functions controlled by quorum sensing systems of these species are both diverse and extensive. Included are such processes as the regulation of pigmentation and chitinolytic activity in *C. violaceum* (McClean et al., 1997, Chernin et al., 1998), the conjugal transfer of the Ti (tumor inducing) plasmid in *A. tumifaciens* (Hwang et al., 1995), synthesis of carbapenem antibiotics in *E. carotovora* (Chhabra et al., 1993), swarming and proteolytic activity in *S. liquifaciens* (Swift et al., 1997), and the production of serine protease (exoprotease) by both *Aeromonas* pathogens (Swift et al., 1999).

Among bacterial species that produce AHSL, the quorum sensing system associated with *P. aeruginosa* has been elucidated with much detail. *P. aeruginosa* is a prominent opportunistic pathogen in the progression of cystic fibrosis and other diseases, demonstrating a propensity for biofilm formation *in vivo*. This organism has also been isolated from biofilms originating in man-made water systems (Hu et al., 2003). Three signal compounds have been isolated from *P. aeruginosa*, i.e. *N*-(3-oxododecanoyl)-L-homoserine lactone (C_{12}-HSL, OdDHL), *N*-butanoyl-L-homoserine lactone (C_4-HSL, BHL), and 2-heptyl-3-hydroxy-4-quinolone, also known as the *Pseudomonas* quinolone signal [PQS] (Pearson et al., 1994; Pearson et al., 1995; Pesci et al., 1999). OdDHL and BHL contribute to the *lasRI* and *rhlRI Pseudomonas* quorum sensing systems respectively, where the "R" protein functions as an autoinducer dependent transcriptional activator and "I" protein is the AHSL synthase.

The model characterizing the *lasRI* and *rhlRI* quorum sensing systems is based on a hierarchical network in which *rhlRI* is dependent on the *las* system for transcriptional activation. Several of the virulence factors that define pathogenicity for *P. aeruginosa*

are controlled by the *lasRI* system, i.e. LasA and LasB elastase, hemolysin, hydrogen cyanide, alkaline protease, pyoverdin, and exotoxin A. Although LasR-OdDHL exerts control over the expression of *rhlI* expression and the subsequent synthesis of BHL, this system is also required for the optimum biosynthesis of multiple virulence factors and enzymatic products including elastase, alkaline protease, hydrogen cyanide, and hemolysin, rhamnolipid, pyocyanin, and chitinase (reviewed by Whitehead *et al* 2001).

P. aeruginosa is a significant opportunist in the lungs of individuals with cystic fibrosis (CF). Within this environment, these organisms can grow to densities that are conducive to quorum sensing, i.e., 10^9 cfu/mL (Singh et al., 2000). Both of the autoinducers synthesized by *P. aeruginosa* have been detected in the sputum of CF patients. In this environment, C_{12}-HSL is the dominant species with concentrations as high as 21.20 nM compared to 1-5 nM BHL. The positive correlation between i) *lasI* and *lasR* transcripts and ii) the high ratio of lasA and lasB (elastase), *toxA* (exotoxin A), and *algD* (alginate) mRNA transcripts, suggests that autoinducers are indispensably linked to the pathogenesis of *P. aeruginosa* (Erickson et al., 2002).

The effects of various mutations in the genetic quorum sensing network of *P. aeruginosa* and other microorganisms have been used to establish the role of intercellular communication in pathogenicity. Rumbaugh et al (1999) demonstrated that *P. aeruginosa* quorum sensing mutants were less virulent in burned mouse models of infection. Attenuation was attributed to the inability of the mutant's to disseminate throughout the body and across damaged epithelial tissue. *P. aeruginosa* mutants deficient in the production of either signal displayed an appreciable reduction in pathogenicity in burned mouse models. Mortality rates were measured at 6.7% in mice

infected with double mutants sustaining the loss of both autoinducer synthases. Although mortality rose to 46.7% when mice were infected with either Δ*lasI* or Δ*rhlI* mutants, these data demonstrated the impact of both AI synthases on virulence in that infections mediated by the fully virulent PAO1 wild type resulted in the mortality of 94.3% of the burned mouse models (Rumbaugh et al., 1999).

Another signal, *Pseudomonas* quinilone signal (PQS), has also been detected in various CF lung specimens including sputum, bronchoalveolar lavage fluid, and mucopurulent fluid. In these specimens, a maximum concentration of 2μM PQS was detected (Collier et al., 2002). Although the role of PQS in intercellular signaling has not been completely elucidated, it has a defined role in the quorum sensing hierarchy of *P. aerugionosa*. The signal has been implicated in the up-regulation of C_4-HSL and the control of extracellular metabolite synthesis in the absence of autoinducer (Diggle et al., 2003). Interestingly, *P. aeruginosa* produces 55 quinolones and quinolines, but only 2-heptyl-3-hydroxy-4-quinolone (PQS) has been characterized as an intercellular signal (Camilli and Bassler, 2006). The concentration of PQS is greatest during late stationary phase (up to 25μM) suggesting that its synthesis is induced by starvation or as a stress response rather than the attainment of a critical cell density (McKnight et al., 2000). Furthermore, PQS appears to modulate the synthesis of *rhlI* dependent phenotypes (rhamnolipid, elastase, and pyocyanin expression, etc) during the organism's passage into stationary phase (Diggle et al., 2003)

The expression of both PQS and AHSL appears to be a requirement for both the maintenance and development of *P. aeruginosa* during both environmental and *in vivo* phases of growth. Beyond their association with virulence, however, AHSL and to a

lesser extent PQS, are also important in their regulation of biofilm genesis. AHSL has been detected in and/or isolated from biofilms occurring naturally, and evidence continues to mount as to its regulatory value to biofilm differentiation and maturation (McClean et al., 1997). Although PQS has not been demonstrated in environmental biofilms, it has been detected in the lungs of cystic fibrosis (CF) patients (Collier et al., 2002; Guina et al., 2003).

Quorum sensing and biofilm development

Studies evaluating the contribution of intercellular signaling, in particular that mediated by AHSL, to biofilm development, have collectively shown that signaling molecules contribute to biofilm genesis and sustainability. The control of biofilm formation and maturation is regulated by multiple genetic functions and phenotypes in progenitor microorganisms, including AHSLs. Autoinducer activity was first reported in naturally occurring aquatic biofilms recovered from the surface of limestone rocks (McLean et al., 1997). Since this discovery, much attention has been focused on the contribution of intercellular signaling to biofilm development. Primary stages of biofilm formation are likely not regulated by quorum sensing as the requirement for higher cell densities would negate the involvement of density-dependent signals. The initial stage of substratum colonization, which is fulfilled by microbial attachment, is recognized as an individual event. With regard to *P. aeruginosa*, evidence of AHSL activity during the second stage of biofilm development (irreversible attachment) has been cited via *lasB* reporter fusions, where *lasB* is responsive to C_{12}-HSL. Genes under the control of C_4-HSL are fully expressed during the third stage of biofilm development, where the

incidence of microcolony formations appear as the first signs of biofilm maturation (Sauer et al., 2002).

Expression of autoinducer at precise stages in biofilm formation indicates numerous control points susceptible to regulation by AHSL. Quorum sensing does not, however, regulate the initial attachment of bacteria to the substratum. This has been demonsrated by observing the sedimentation rates and cell density of both HSL mutants and wild types are nearly identical. In processes following colonization, the presence of quorum sensing signals is crucial to the development of biofilms as dynamic, yet steadfast, recalcitrant structures. During biofilm development, both of the autoinducers synthesized by *P. aeruginosa* are constituitively secreted. While *lasI* expression decreases over time, the concentrations of both autoinducers remain high at the biofilm's substratum (De Kievet, 2001). Biofilms composed of wild type organisms produce structures that are typically composed of loosely packed microcolonies in which prokaryotes are spatially separated and the colony's interstices are comprised of EPS (Davies et al., 1998; Sauer et al., 2002). AHSL mediates microcolony maturation, which contributes to the 3-dimensional "mushrooms-like" or "pillar-like" architecture of biofilms, and the organism's ability to detach from mature biofilm matrices (Sauer et al., 2002, Boles et al., 2005).

Any impairment of *P. aerugionosa*'s principle quorum sensing system (*lasRI*) is manifest as a defect in its ability to become physically differentiated from planktonic cells during substratum colonization, which is expressed as a deficiency in biofilm formation. *lasI-rhlI* double mutants for AHSL synthesis form tightly packed aggregates with a thin EPS closely associated with the cell surface rather than filling intercellular

interstices. This conformation of EPS is the same form associated with free living, planktonic cells (Davies et al., 1998; Sauer et al., 2002). The flat, undifferentiated structures formed by these organisms appear much more uniform with regard to EPS and cellular distribution with a significantly diminished density, i.e. one-fifth of the wild-type's biomass. An evaluation of individual mutations indicated that no appreciable loss in biofilm density or structure could be attributed to a deletion of *rhlI* (i.e. no C_4-HSL synthesis); however, *lasI* mutants constructed loosely organized biofilms composed of compressed, incomplete glycocalyx. This was reminiscent of the matrix constructed by the double mutants and in turn demonstrated significant susceptibility to biocidal detergents. For example, within 5 minutes of exposure to 0.2% SDS, these structures completely detached from the substratum (Davies et al., 1998). Given the remarkable stability of natural biofilms, which are notoriously recalcitrant to biocides *in situ* and antibiotic activity and immune response *in vivo*, the contribution of AHSL to biofilm fitness and persistence is substantial and presents a potential "Achilles Heel" where biofilm eradication is concerned.

In addition to regulation of *Pseudomonas* biofilms, quorum sensing has also been implicated in the regulation of biofilm formation of other quorum sensing prokaryotes, thus demonstrating the global application of this signal for biofilms genesis. *Burkholderia cepacia* produces C_6- and C_8-HSL. Aberrations in its *cep* quorum sensing system result in reduced efficiency during substratum colonization following attachment, a pronounced lack of microcolony maturation, and a severe reduction in the organism's ability to accumulate biomass through the acquisition of surface area. Following the addition of 200nM C_8-HSL, however, these defects were completely restored. These

mutations, which occurred in the *cepR* (transcriptional regulator) and *cepI* (autoinducer synthase) genes had no detectable effect on microcolony formation during the initial stages of biofilm formation (Huber et al., 2001). Likewise, *Burkholderia cenocepacia* lacking *cepRI* and an additional quorum system, *cciRI*, accumulate 50% less biomass than their wild type counterparts. A loss of both systems also contributes to an enhanced susceptibility of biofilms to the antibiotic ciprofloxacin, and is indicated by an impressive reduction in the minimum inhibitory concentration (MIC) from 1,024 μg/mL to 64μg/mL (Tomlin et al., 2005). Susceptibility of *cepRI* mutants to 0.2% SDS was also noted. The biofilms inclusive of both *Burkholderia* species represent an important element in their pathogenic ability to sustain a chronic infection in persons with cystic fibrosis (Conway et al., 2002).

AI-2 and quorum sensing

AHSL is prolific among gram-negative bacilli, and while an inherent specificity exists for its detection by the species by which it is produced, the similarity of the signaling molecule from species to species suggests a potential for interspecies communication. While this has been documented in limited form (Bassler et al., 1997; Duan et al., 2003), another prominent intercellular signal, i.e. AI-2, is noted for its universal activity among microorganisms regardless of Gram reaction or morphology. Since the discovery of AI-2 in *V. harveyi* supernatants, this signal has been demonstrated in numerous prokaryotes. The presence of AI-2 in multiple organisms, regardless of Gram reaction and cellular morphology, together with the fact that *luxS* is highly conserved among these organisms suggests that this signal serves as a global signal for intercellular communication among microorganisms. A consolidated list of the more

than 55 AI-2 secreting species would include *Salmonella enterica* serovar Typhimurium, *Helicobacter pylori*, *Borrelia burgdorferi*, *Clostridium perfringens, Porphryomonas gingivalis, Neisseria meningitides, Shigella flexneri, Campylobacter jejuni, Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria meningitides, Vibrio vulnificus, Actinobacillus actinomycetemcomitans,* and *E. coli* O157:H7 (Surette et al., 1999; Chung et al., 2001; Stevenson and Babb, 2002). The signal has been detected in biofilms and (under experimental conditions) in consumable items including milk, chicken broth, and apple juice (Wen and Burne, 2004; Cloak et al., 2002).

AI-2 is chemically characterized as a furanosyl borate diester, or more specifically (*2S, 4S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran, i.e. *S*-THMF (Miller et al., 2004, Schauder et al 2001). AI-2 was first described by Bassler et al (1994) in association with the regulatory structure of bioluminescence in the marine bacterium *Vibrio harveyi*. Like *V. fischeri*, bioluminescence in *V. harveyi* is regulated by the production of AHSL, specifically β-hydroxybutryl homoserine lactone (C_4-HSL); unlike *V. fischeri*, however, two distinct autoinducers exert a regulatory role over the expression of luminescence by *V. harveyi.* The first molecule, C_4-HSL, is referred to as AI-1 and is the product of the *luxLM* locus. The use of *luxLM* rather than *luxI* terminology is employed to reflect a lack of homology with the *luxI* family of autoinducer synthases. While LuxR is the protein required for the expression of the *luxCDABEGH* operon in *V.harveyi*, it also lacks homology with the LuxR family of transcriptional activators related to that of *V. fischeri* (Bassler et al., 1994, Schauder et al., 2001).

AI-2 is the second autoinducer synthesized by *V. harveyi*. This compound is the biosynthetic product of the LuxS autoinducer synthase produced by the *luxS* gene

(Bassler et al., 1994). *luxS*, the gene for autoinducer synthase, is located in the same biosynthetic operon as *pfs*, another gene required for AI-2 synthesis. At low cell densities, *lux* expression is repressed through the action of an unidentified repressor. Expression of the repressor is stimulated by LuxO, which requires phosphorylation for complete activation. Bioluminescence is inhibited by the repressor by providing an obstacle to LuxR's interaction with the lux operon. Detection of AI-1 (C_4-HSL) and AI-2 are perceived by transmembrane sensors LuxN and LuxPQ (Bassler et al., 1994). At densities below threshold, each of these periplasmic binding proteins function as a kinase, which in turn phosphorylate LuxU, an intermediate protein in the signaling cascade. LuxU transfers its phosphate to LuxO, which is required for the activation of the *lux* operon repressor (Mok et al., 2003).

Intercellular signaling via AI-2 influences several bacterial processes, including biofilm formation and pathogenesis (Wen and Burne, 2004; Sircili et al., 2004; Kim et al., 2003). Biofilms formed with LuxS mutants are known for aberrations in its 3-dimensional structure that are reminiscent of those composed ofAHSL mutants. These biofilms consist of homogenous matrices that lack the structural integrity required to resist environmental stress. Defects in AI-2 production by *Streptococcus mutans* result in the formation of single-species biofilms characterized by large microcolonies and an incomplete matrix. However, in contrast to AHSL deficiencies, the lack of homogenicity appears to enhance the resistance of these structures, as indicated by their resistance to disruption by 1% SDS treatment.

In contrast to the enhanced resistance in biofilms established by *S. mutans* AI-2 mutants, the absence of AI-2 signaling can negatively regulate mixed-species biofilm

formation. The formation of *Streptococcus gordonii* and *Porphyromonas gingivalis* based oral biofilms, also known as dental plaque, is enhanced by *luxS*. *In vitro*, *S. gordonii* has been identified as a prerequisite for successful biofilm colonization by *P. gingivalis*. McNab et al (2003) demonstrated that while the presence of a functional copy of *luxS* in either microorganism was sufficient for biofilm formation, a complete deficit in signal capacity is manifest as an inability to form microcolonies which are required for biofilm accumulation. In addition to biofilm formation, AI-2 regulates the expression of multiple proteins in *P. gingivalis*, among them heme acquisition protein, a hemin-regulated outer membrane protein (HemR), and a siderophore acquisition protein (Chung et al., 2001). Given the propensity of *P. gingivalis* for biofilm colonization and its role in the development of periodontitis in humans, LuxS represents a promising target in the control of periodontal disease.

The ecological existence of *Legoinella* species is defined by a complex microcosm that includes multiple species of bacteria and protozoa and interactions that are governed, in part, by intracellular signals. Interactions that include syntrophy with other prokaryotic organisms, intracellular growth within protozoa, and the influence of AHSL and AI-2 mediated quorum sensing in virulence and biofilm formation suggests that these associations contribute to the development and/or maintenance of legionellae viability and virulence during the extracellular phase of its life cycle. Given this potential, the objectives of this research are as follows: i) to determine if *Legionella* species, in particular *L. pneumophila* are capable of producing AHSL, AI-2, and PQS; ii) evaluate the ability of *L. pneumophila* to respond to the presence of unrelated, signal

producing bacteria, and iii) determine if *L. pneumophila* is able to detect and respond to the presence of potential eukaryotic hosts.

III. EVALUATION OF INTERCELLULAR SIGNALING BY ACYL HOMOSERINE LACTONES (AHSL), FURANOSYL BORATE DIESTERS (AI-2), AND QUINOLONES IN *LEGIONELLA* SPECIES

Abstract

Numerous *Legionella* virulence genes are strictly relegated to expression during stationary phase. This phase of growth can be associated with high densities of bacterial cells, which is consistent with the quorum sensing paradigm. The objective of the following set of experiments is to determine if *Legionella* specie produce the intercellular signals associated with quorum sensing, i.e. AHSL, AI-2, or quinolone autoinducers. To determine the presence of these metabolites, biosensors with broad detection ranges were employed. Each experiment incorporated the analysis of an autoinducer in *Legionella* supernatants prepared from stationary phase cultures grown planktonically *in vitro* and *in vivo*. *Legionella* species were also assayed for autoinducer production on agar plates (sessile). The data from each of the trials conclusively demonstrates that *Legionella* species do not produce AHSL, AI-2, or quinolone signals.

1. Introduction

Beyond chemical evidence of quorum sensing via the detection of chemical signals, microorganisms participating in "quorum sensing" exhibit social behaviors characterized by phenotypic expression of the group following the accumulation of a dense population of like organisms. In a manner reminiscent of signal producing

prokaryotes, *L. pneumophila* exhibits quorum sensing like behaviors, suggesting that this microorganism synthesizes the chemical signals required for intercellular communication. Rowbotham noted that *L. pneumophila* bacterial cultures achieve rapid motility in a synchronous manner during the stationary phase (Rowbotham 1980). In that the expression of flagella, and thus motility, are a positive predictor of virulence in *L. pneumophila*, if motility is regulated by quorum sensing, so is the organism's pathogenicity (Bosshardt et al., 1997). This concept is also supported by observations that *L. pneumophila* demonstrate quorum sensing "like" behaviors *in vitro*, where exponential phase cultures exposed to stationary phase, cell-free supernatants become cytotoxic to macrophages (Byrne and Swanson, 1998).

In addition to the phenotypic responses as evidence of legionellae quorum sensing, other data also suggests the influence of intercellular signals on *Legionella's* life cycle. This data rests on the current model for *L. pneumophila* development, which is subdivided into two reciprocal phases, i.e., replication and transmission. Both of these phases appear to be globally regulated, in part, by nutrient availability (Molofsky and Swanson, 2004). A deficit in nutrient stores results in the accumulation of guanosine tetraphosphate (ppGpp), a stringent response signal. ppGpp has a regulatory role in the signaling cascade that includes the stationary phase sigma factor RpoS, and influences the coordinated passage of *L. pneumophila* from the exponential to the stationary phase of growth, and the subsequent expression of traits necessary for survival during passage between hosts (Bachman and Swanson, 2001).

In their biphasic model, Molofsky and Swanson (2004) correlate the replication and transmission phases of *L. pneumophila* with exponential and stationary phases,

respectively. These periods are further defined by the induction or inhibition of virulence gene expression, which results in a distinction between growth-phase with respect to phenotype. Among the phenotypes that are distinguished during the replicative phase are microbial growth and replication, basal expression of the type IV secretion apparatus, and elevated activity of the global repressor *csrA*. CsrA (Carbon Storage Regulator A) is a small RNA-binding protein that is constitutively expressed during exponential phase, and represses the expression of the transmissive (or post exponential phase) phenotype, thereby advocating replication. Conversely, phenotypic expression of multiple virulence traits is evident during *L. pneumophila*'s transmissive phase. Many of the factors susceptible to repression by CsrA, including motility, cytotoxicity, stress resistance (UV, heat, and osmotic pressure), and the ability to prevent phagolysome genesis, are fully expressed presumably as the result of LetA (a stationary phase stress-response regulator), which relieves the repression by CsrA during the post exponential stage (Molofsky and Swanson, 2003; Molofsky and Swanson, 2004).

While the involvement of ppGpp, CsrA, and LetA are apparent in *L. pneumophila*'s transition into the transmissive phase, nutrient limitation is not the sole factor stimulating ppGpp synthesis. These gaps are identified in the model, by i) the demonstration that *letA* mutants are capable of establishing infections and inducing cytotoxicity in macrophage monolayers at rates comparable to the wild type within a period of 72 hours and ii) transmission traits (the stationary phase phenotype) are not abolished in *relA* mutants (Hammer et al., 2002). Taken together, a reasonable conclusion suggests the involvement of additional factors, possibly the influence of

exogenous autoinducers as environmental signals or of a possible quorum sensing system in *L. pneumophila* itself.

Among the alternatives used for autoinducer detection is the biosensor or indicator strain. For AI-2 detection, a single biosensor, *V. harveyi* BB170, has been used. Multiple AHSL detectors are available. Three features common to AHSL biosensors are: i) a promoter controlled by quorum sensing regulatory elements that has been linked to the *lux* operon or a *lacZ* reporter gene, ii) the disruption of its *luxI* homolog or the gene for AHSL synthase, and iii) a functional transcriptional regulator, i.e. the *luxR* homolog, which is required for detection of and response to AHSL. Collectively, the biosensors that are currently available can detect the range of known signal molecules, from a minimum acyl side chain of C_4 to a maximum of C_{18} (Llamas et al., 2004; McClean et al., 1997)

The collection of indicator strains includes a wide variety of microorganisms. *Chromobacterium violaceum* CV026, is a *cviI*::Tn5*xylE* mutant deficient in its ability to synthesize native AHSL, i.e. *N*-hexanoyl-L-homoserine lactone (C_6-HSL). The double mini-Tn5 insertion mutants are white, turning purple in response to exogenous AHSL. In reporter assays, violacein production is stimulated by short and medium chain AHSL (C_4-C_8), as well as N-acylhomocysteine thiolactone (AHT) analogues (McClean et al., 1997; Cha et al., 1998). *C. violaceum* CV017, the parent strain of CV026, is a mini Tn5 mutant that constitutively produces AHSL and is thus, strongly pigmented. This strain can be utilized in a reverse assay for the detection of AHSL in that signals with extended acyl side chains are inhibitory to violacein production by this species. Failure of *C. violaceum*

CV017 to express pigmentation in the presence of exogenous signals would provide an indication of long chain ($C_{10} - C_{14}$) AHSLs (Cherin et al., 1998; McClean et al., 1997).

The biosensor *Agrobacterium tumifaciens* pZLR4 is capable of detecting several varieties of 3-oxo-*N*-acyl homoserine lactone. This bacterium's *luxR* homolog, *traR*, requires the activity of N-(3-oxo-octanoyl)-L-homoserine lactone (C_8-HSL) for transcription of the *tra* operon, which is responsible for the conjugal transfer of Ti plasmids. pZLR4 contains the *traCDG* operon with its native promoter region. The presence of a *traG:lacZ* reporter fusion together with *traR*, and the absence of the *traI* autoinducer synthase, constitute the necessary components for the detection of exogenous autoinducer (Cha et al., 1998). This strain possesses a broad detection range for multiple AHSL derivatives (Cha et al., 1998).

Reporter strains *Escherichia coli* MG4 (pKDT17) and *Escherichai coli* XL1-Blue (pECP61.5) are characterized as *lacZ* fusions. Each biosensor possesses quorum sensing regulated genes cloned from *Pseudomonas aeruginosa* PAO1, and detects PAI-1 (C_{12}-HSL) and PAI-2 (C_4-HSL), respectively. The plasmid pKDT17 is characterized by a *lasB::lacZ* translational reporter fusion, the *lasB* promoter, and *lasR* under the control of a *lac* promotor (p*lac-lasR*) (Pearson et al., 1994). In that the autoinducer associated with the *las* system (elastase biosynthesis) of *P.aeruginosa* is N-(3-oxododecanoyl) homoserine lactone, the detection capabilities of this biosensor are relegated to metabolites with longer acyl side chains. The second reporter strains plasmid, pECP61.5, possesses a *rhlA::lacZ* reporter translational fusion with *rhlR* under the control of a *tac* promotor (*tacp-rhlR*). *E. coli* XL1-Blue (pECP61.5) is suited for the detection of short to medium chained varieties of AHSL (Pearson et al., 1997).

Other biosensors include *Pseudomonas aeruginosa* PAO-R1 (*lasR*$^-$)(pTS400) and *Vibrio harveyi* BB170, for the detection of 2-heptyl-3-hydroxy-4-quinolone (PQS) and furanosyl borate diester (AI-2), respectively (Pesci et al., 1999; Surette and Bassler, 1998). *P. aeruginosa* PAO-R1 (pTS400) carries a *lasB*'-*lacZ* translational reporter fusion. This strain is characterized by a genomic *lasR* deletion, failing to respond to 3-oxo-C_{12}- and only mildly responsive to C_4-HSL; however, the transcription of *lasB* is stimulated by exogenous PQS. 5μM PQS is required for adequate stimulation of the *lasB* promoter with an equivalent of ~150 Miller units (Pesci et al., 1999). *V. harveyi* BB170 is a *luxN*:Tn5 mutant. The *luxN* mutation renders this indicator unresponsive to N-(3-hydroxybutanoyl)-L-homoserine lactone; thus, BB170 responds solely to the presence of exogenous AI-2, which is detected by the induction of bioluminescence (Surette et al., 1999).

Other technologies have been employed to detect and isolate signaling compounds. Standard methods for AHSL distillation include acidified ethyl acetate or dichloromethane extraction from culture supernatants. For characterization, distilled extracts are typically subject to C_{18} reverse phase thin layer chromatography (TLC). This method can be used in conjunction with known AHSL standards for putative identification of AHSL variants or as a preparative method, after which spots are removed from the chromatogram's matrix and subject to mass spectrometry (MS) or nuclear magnetic resonance (NMR) for complete characterization (Shaw et al., 1997; Pearson et al., 1999).

The fact that *L. pneumophila* demonstrates behaviors that are consistent with quorum sensing suggested that this organism produces autoinducers. Therefore, the

objectives of this study were to evaluate *Legionella* species for their ability to produce AHSL, AI-2, and the *Pseudomonas* quinolone signal.

2. Materials and Methods

2.1 *Bacterial strains and cultivation methods*

Three strains of *L. pneumophila* (RI243, D4897, and F1068) and 24 additional *Legionella* species were used. These species are as follows: *L. micdadei* D-5202, *L. bozemanii* D-5064, *L. longbeachae* D-5197, *L. israelensis, L. sainthelensi, L. feeleii, L. oakridgensis, L. anisa, L. fairfieldensis, L. jordanis, L. moravica, L. brunensis, L. cherrii, L. spiritensis, L. steigerwaltii, L. cincinnatiensis, L. birminghamensis, L. quinlivanii, L. tucsonensis, L. adelaidensis, L. hackeliae, L. parisiensis, L. maceachernii*, and *L. jamestowniensis*. All *L. pneumophila* strains, as well as *L. micdadei* D-5202, *L. bozemanii* D-5064, *L. longbeachae* D-5197 were supplied by the Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia. The remaining *Legionella* species were supplied by the Barbaree laboratory, Auburn University. L*egionella* bacteria were cultured on buffered charcoal yeast extract agar (BCYE), in a 3-5% CO_2 atmosphere, for 3 days at 37 °C. When broth cultures were required, legionellae were subcultured into buffered yeast extract broth (YEB) (10g/L N-(2-Acetamido)-2-aminoethanesulfonic Acid [ACES], 10g/L yeast extract, 0.4 g/L L-cysteine, and 0.25g/L ferric pyrophosphate; pH adjusted to 6.9 with 10N KOH and filter sterilized). Broth cultures were incubated at 37 ºC, with aeration (200 rpm), for a period of 40 hours.

For detection of acyl-HSL, five biosensors were used in this study: i) *Chromobacterium violacium* CV026, ii) *Chromobacterium violacium* CV017, iii) *Escherichia coli* MG4 (pKDT17), iv) *Escherichai coli* XL1-Blue (pECP61.5), and v) *Agrobacterium tumifaciens* pZLR4. Other species utilized throughout the study included positive controls obtained from Auburn University. These were wild type strains of the following: *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa*. *E. coli* DH5a, for use as a negative AI-2 control, was also obtained from a laboratory at Auburn University.

In addition to AHSL detection, the presence additional signal compounds, i.e. *Pseudomonas* quinolone signal (PQS) and furanosyl borate diesters (AI-2), were also evaluated. To accomplish this task, *P. aeruginosa* PAO-R1 (pTS400) and *V. harveyi* BB170 and BB120, reporters with sensitivities for PQS and AI-2 respectively, were incorporated. *P. aeruguinosa* PAOR1 was provided by Everett Pesci, Ph.D. (East Carolina University School of Medicine; Greenville, N.C.) and *V. harveyi* BB170 was supplied by Bonnie Bassler, Ph.D. (Princeton University; Princeton, N.J)

Both of the *Chromobacterium* biosensors were maintained on trypticase soy agar (TSA), with 50 μg/mL kanamycin added for strain CV026. *E. coli* (pKDT17 and pECP61.5) were maintained on TSA with 100 μg/mL ampicillin. All *A. tumifaciens* strains were cultured on TSA, with the exception that 20 μg/mL gentamycin was added for cultivation of *A. tumifaciens* pZLR4. The *P. aeruginosa* PQS biosensor was maintained on TSA with 200μg/mL carbenicillin (Pesci et al 1999). TSA and/or sorbitol MacConkey agar was used to cultivate wild type strains, i.e. *E. coli* O157:H7, *S. enterica* serovar Typhimurium, and *P. aeruginosa*. *V. harveyi* BB170 and BB120 were

maintained on TSA or Good Vibrio Medium (GVB); composition per liter: 10.0 g tryptone, 5.0 g casamino acids, 25.0 g NaCl, 4g $MgCl_2$, and 1.0 g KCl (Winfrey et al., 1997).

Bioassays incorporating either of the *E. coli* or *Agrobacterium* biosensors required the use of AB Minimal Medium, with the following composition (per liter): 3.0 g K_2HPO_4, 1.0 g NaH_2PO_4 , 1.0 g NH_4Cl, 0.3g $MgSO_4 \cdot 7H_2O$, 0.15g KCl, 0.01g $CaCl_2 \cdot 2H_2O$, and 0.0025g $FeSO_4 \cdot 7H_2O$, and 0.2% glucose (Chilton et al., 1974). All biosensors required cultivation at 30 ºC for 18-24 hours. Positive control strains, other than *V. harveyi* and *Agrobacterium* strains, were cultured at 37 ºC with the latter requiring culturing at 30 ºC.

For long-term storage, all biosensors were grown in trypticase soy broth (TSB) to late exponential phase, adjusted to 15% glycerol, and frozen in 1.0 mL aliquots at -70 ºC. For *Legionella* species, the same protocol was followed with the exception that these species were cultured in buffered YEB prior to freezing.

2.2 *Eukaryotic cell culture*

For studies requiring intracellular growth, two eukaryotic cell types were used as hosts: the amoeba *Hartmannella vermiformis* and Vero cells (Green African Monkey kidney cells). The amoeba and eukaryotic cell line have been previously identified as hosts permissive to the intracellular growth of *L. pneumophila* (Oldham et al., 1985; Fields et al., 1990). *H. vermiformis* was provided by Dr. Barry Fields (CDC, Atlanta, GA); and Vero cells were obtained from Dr. Sharon Roberts (Auburn University, Auburn, AL). *H. vermiformis* was grown in ATCC medium 1034, and is prepared by

combining solution A (10.0 g peptone, 10.0 g yeast extract, 1.0 g yeast nucleic acid [SIGMA], 0.015 g folic acid, and 0.001 g hemin in 880 mL ddH_20) with 20 mL of solution 2 (per liter: 18.1 g KH_2PO_4 and 25.0 g Na_2HpO_4) (www.atcc.org). Both components are combined after autoclaving, the pH adjusted to 6.9 with 1N KOH, and the solution is filter sterilized using a 0.22 μm filter. Prior to *H. vermiformis* culture, the medium is adjusted to a 10% concentration of fetal bovine serum (FBS). The amoebae were grown in T-75 polystyrene tissue culture flasks at 37 ºC until confluence was observed, typically 3-5 days. When required for co-culture assays with *L. pneumophila*, Puck's saline is used and prepared as follows (per liter): 0.016 g $CaCl_2$, 0.285 g KCl, 0.083 g KH_2PO_4, 0.154 g $MgSO_4 \cdot 7H_2O$, and 1.10 g of glucose (Page, 1967).

Vero cell culture required the use of Modified Eagles Medium (MEM) containing Hanks balanced salts, adjusted to 5% FBS, 2mM L-glutamine, and 100 μg/ml penicillin/streptomycin. MEM, FBS, and antibiotics were purchased from Fisher Scientific (Pittsburgh, PA). Vero cells were cultivated in T-75 polystyrene tissue culture flasks, and maintained in a 5% CO_2 atmosphere at 37 ºC. Growth was allowed to proceed until confluence was observed, typically 3 days (Oldham et al., 1985).

For long term storage, *H. vermiformis* and Vero cells were frozen in their respective culture medium with the addition of dimethylsulfoxide (DMSO). For Vero storage, cells were washed 2 times with 1X PBS, after which they were inundated with 1.5 mL of a solution composed of 10% trypsin-EDTA and 1X PBS. Dislodged cells combined and pelleted at 250 x g for 5 minutes at room temperature, after which the supernatant was discarded. The remaining pellet was resuspended in DMEM with 10% DMSO prior to freezing. Preparatory procedures for *H. vermiformis* storage included the

concentration of trophozoites via centrifugation at 200 x g for 15 minutes at room temperature, after which the pellet was resuspended in ATCC medium #1034 with 20% DMSO. Both cell types were stored at -70 ºC.

2.3 *AHSL assessment in liquid culture*.

To assess AHSL, AI-2, and PQS synthesis by *Legionella* species, the microorganisms were cultured for a period of 48 hours in YEB. Following culture, cell-free supernatants were prepared by centrifuging cultures for 10 minutes at 12,000 rpm, after which the fluid was passaged through a 0.45 μm syringe filter. Supernatants were stored at 4 °C until required for use. The biosensors utilized for AHSL evaluations were *C. violaceum* CV026 and CV017, *E. coli* (pECP61.5 and pKDT17), and *A. tumifaciens* (pZLR4 and NT1).

In that *E. coli* (pECP61.5), *E. coli* (pKDT17), and *A. tumifaciens* (pZLR4) produce β-galactosidase in response to the presence of AHSL, the assay for β-galactosidase activity according to Miller was used as an indirect measure of AHSL activity. To measure enzyme activity, biosensors were grown overnight in AB Minimal medium, and subsequently combined with *Legionella* supernatant at a ratio of 1:1. Following the incubation of these cultures at 30 ºC for 3 hours with agitation (100 rpm), the number of Miller units was quantified using *o*-nitrophenyl β-D-galactoside (ONPG) hydrolysis as the substrate for the assessment of β-galactosidase activity (Miller, 1972). *A. tumifaciens* and *P. aeruginosa* supernatants were used as both standards for comparison and as positive controls. All experiments were completed twice.

2.4 *Cross-feeding assays for AHSL detection*.

To ensure that growth conditions (i.e. sessile vs. planktonic) do not impact AHSL synthesis, *Legionella* species were heavily streaked on one side of a BCYE agar plate supplemented with 40 μg/ml X-gal and cultivated for three days at 37 °C in 5% CO_2. Following this period, biosensors *E. coli* (pKDT17), *E. coli* (pECP61.5), and/or *A. tumifaciens* (pZLR4) were streaked parallel to the *Legionella* culture, and further incubated at 30 °C for two additional days. The appearance of blue colonies following incubation of the *lacZ* biosensors was interpreted as a positive result. The same format was also utilized for bioassays conducted with *C. violacium* CV026 and CV017. Assays were conducted on BCYE agar plates without X-gal. The appearance of purple *C. violaceum* CV026 colonies or colorless *C. violaceum* CV017 colonies was interpreted as a positive result (Pearson et al., 1994; Pearson et al., 1997; McClean et al., 1997; Chernin et al., 1998).

2.5 *Distillation of Legionella supernatants and assessment of AHSL activity by thin layer chromatography (TLC).*

In the detection of AHSL or other intercellular signals, low concentrations of autoinducer present a potential obstacle to their discovery by conventional bioassay of supernatants; therefore, *Legionella* supernatants were distilled via the protocol used for AHSL extraction. 300 mL of cell free supernatants were prepared from *L. pneumophila* strains RI243, F1068, and D4987; *L. bozemani;*, two positive control standards (*P. aeruginosa* and *C. violaceum*); and *L. pneumophila* RI243/*H. vermiformis* co-culture. *Legionella* species were grown for a period of 40 hours and supernatants rendered cell

free by centrifugation at 18,000 x g for 15 minutes and passage through a 0.45 um filter. Following two extractions of the aqueous phase of the supernatant with an equal volume of acidified ethyl acetate (0.1 ml glacial acetic acid/L), the remaining solution was dried with several grams of anhydrous magnesium sulfate. The pooled phases were concentrated by rotary evaporation at 40 °C until an oily residue remained (typically 3 to 4 hours) (Pearson et al., 1994). The residue was collected, combined with 1mL of acidified ethyl acetate, and stored at -70 °C until required.

For analysis by thin layer chromatography, samples were spotted onto octadecyl (C_{18}) reverse phase TLC plates (JT Baker; Phillipsburg, NJ) in volumes of 1 to 5 µL and allowed to air dry. The chromatograms were developed in 60/40 methanol:distilled water, air-dried, and overlayed with a 100 mL of a soft agar, biosensor laden suspension. The agar suspension was prepared with 1.13% (w/v) agar and a 100 µl inoculum of a *C. violaceum* CV026 or *A. tumifaciens* (pZLR4) overnight culture. For development with *A. tumifaciens* (pZLR4), 60µg/ml X-gal was added to the agar suspension. Overlayed TLC plates were incubated at 30 °C for 24-48 hours, observed for the development of spots characteristic of acyl-HSL, and photographed with a digital camera (Shaw et al., 1997).

2.6 *Assessment of AHSL production by L. pneumophila in vivo.*

H. vermiformis was cultured to confluence in 75 mm tissue culture flasks in PYNFH Medium (ATCC #1034). Confluent layers were attained by culturing the amoeba at 37 °C for a period of 3 to 5 days. Cultures were observed using an inverted microscope and when confluent, were dislodged from the flasks, and concentrated to a significantly smaller volume (~10 mL) by centrifugation at 200 x g for 15 minutes, 20

°C. Trophozoites were enumerated via hemocytometer, and dispensed into culture flasks containing an assay medium. For coculture assays, the culture medium consisted of a 1:1 suspension of FBS free ATCC medium #1034 and Puck's Saline. Amoebae were suspended in the culture medium at a density of 10^5 cells/mL. A MOI (multiplicity of infection) of 100:1 was established by inoculating the *H. vermiformis* suspension with an inoculum of an overnight culture of *L. pneumophila* RI243 adjusted to 10^3 cfu/ml. To ensure an accurate assessment of bacterial concentration, culture densities were estimated by using a previously established value, where $O.D._{595}$ of 0.9 is the equivalent of $1x10^9$ cfu/mL. Cocultures were incubated at 37 °C up to a 14 day maximum. Culture supernatants were collected at days 3, 5, 7, and 10 to assay for the presence of AHSL as previously described.

Intracellular cultures were also established by culturing *L. pneumophila* RI243 in transformed cell lines, i.e. Vero cells. For preparation of cocultures, confluent Vero cells were gently washed 2 times with 1X PBS. Following this procedure, Vero cells were resuspended in fresh MEM devoid of antibiotics. 10 µl aliquots of an overnight *L. pneumophila* culture were added to each monolayer, after which attachment was allowed to proceed for 2 hours. Monolayers were then washed three times with 1X PBS to remove unattached prokaryotes, and the culture medium was replaced. Incubation was allowed to proceed at 37 °C in 5% CO_2, and culture supernatants were removed from the cultures at discrete intervals (1, 3, 5, and 7 days) (Oldham and Rodgers, 1985). Coculture supernatants were sterilized by passage through 0.45 µM syringe filters and refrigerated until assay for ASHL activity.

2.7 *PQS evaluation.*

P. aeruginosa PAOR1 (pTS400) was used for the detection of quinolone activity in *Legionella* supernatants prepared *in vivo* (amoebae and Vero coculture) and *in vitro.* β-galactosidase activity was also quantified using the assay by Miller (Miller, 1972). *Legionella* supernatant values were measured against a positive standard (a laboratory strain of *P. aeruginosa*) and a negative control (*E. coli*).

2.8 *AI-2 evaluation*

Overnight cultures of the AI-2 biosensor, *V. harveyi* BB170 were prepared in AB minimal medium at 30 ºC with aeration (200 rpm). The cultures were diluted 1:5000 in 5 ml volumes of test medium comprised of a 1:1 ratio of fresh AB medium and *Legionella* culture supernatants prepared *in vivo* and *in vitro*. Following a 4-hour incubation period, 1 ml samples were used to measure bioluminescence using the TD-20/20 Luminometer (Turner Designs; Sunnyvale, CA). Bioluminescence activity was evaluated against a *V. harveyi* positive control (BB120), as well as *E. coli* 0157:H7 and *Salmonella enterica* serovar Typhimurium, all of which produce AI-2. In accordance with Bassler et al, culture supernatants stimulating *V. harveyi* BB170 by more than 10% of the bioluminescence values produced by *V. harveyi* BB120 were considered a positive result (Bassler et al., 1997).

2.9 *Quality control*

The growth requirements of *L. pneumophila* and the majority of legionellae preclude its growth on media lacking L-cysteine. To ensure the quality of *Legionella* cultures during the preparation of supernatants, negative growth controls consisting of

Legionella inoculated into TSB or YEB without L-cysteine were prepared with each experiment. Additionally, when broth cultures were required, each was streaked on BCYE with and without L-cysteine following all incubation periods. These measures were incorporated throughout all experiments in which any legionellae were incorporated.

3. Results

3.1 *Evaluation of AHSL activity in Legionella species.*

The presence of acyl-homoserine lactone was evaluated by various bioassays and concluded with thin layer chromatography of distilled *Legionella* supernatants. The results of all of trials demonstrated that *Legionella* supernatants failed to induce or completely inhibit violacein production in CV026 and CV017, respectively (Figure 3.4, Table 3.2); however, with a few species, namely *L. bozemanii, L. israelensis, L. feeleii, L. anisa, L.moravica,* and *L. brunensis,* a partial inhibition of the CV017 biosensor was noted.

The data from from assays using the *lacZ* reporter strains were used to substantiate the *C. violaceum* results. These included *Escherichia coli* (pKDT17), *E. coli* (pECP61.5), and *Agrobacterium tumefaciens* (pZLR4). *Legionella* supernatants were subject to quantitation of β-galactosidase activity using the assay by Miller. The average value of β-galactosidase (measured via ONPG hydrolysis) in the presence of *Legionella pneumophila* supernatants was 2.34 Miller units, and for all *Legionella* species tested was 0.99 Miller units. These values contrasted substantially from the average positive control average values of 73 Miller units (Table 3.1). This value was even greater in the

presence of distilled supernatant extracts and synthetic AHSL. For example, *A. tumifaciens* (pZLR4) β-galactosidase activity was measured at 230 Miller units in the presence of 5 μM OHHL. In accordance with the absence of *Chromobacterium* pigmentation, this data also denies the presence of AHSL in *Legionella* supernatants.

TLC of distilled *Legionella* supernatants failed to demonstrate the presence of AHSL species in the presence of either *C. violaceum* CV026 or *A. tumifaciens* pZLR4 biosensors (Figures 3.1 and 3.2). This was noted by the absence of spots following the incubation of TLC plates spotted with concentrated extracts prepared from *L. pneumophila* and *L. bozemanii* supernatants as well as coculture supernatants (*H. vermiformis* and Vero cells). As noted in Figures 3.1 and 3.2, spots developed where positive controls extracts were applied, i.e. the distilled residues from the wild type *C. violaceum* and *A. tumifaciens* or synthetic versions of C_4, C_6, and C_{12} HSL.

3.2 *Evaluation of quinolone signal production by Legionella species.*

The presence of the PQS signal was evaluated indirectly by measuring β-galactosidase activity (Miller, 1972). *Legionella* supernatants failed to yield significant results following the culture of the PQS biosensor with varying concentrations of *Legionella* supernatant. Coculture supernatants also failed to indicate the presence of quinolone signals. Average values over a three trial period were 0.80 Miller units for all *Legionella pneumophila* supernatants, regardless of culture conditions. Positive control (*P. aeruginosa*) average values were 143 Miller units (Table 3.3), and the disparity between these values suggests that *L. pneumophila* does not produce a quinolone intercellular signal.

3.3 *Evaluation of AI-2 signal expression by Legionella species*

The presence of AI-2 could not be confirmed in *L. pneumophila* supernatants prepared *in* bioluminescence was assessed *vitro* and *in vivo*. Following the culture of biosensors, *Vibrio harveyi* BB170 was incubated for a 3 hour period in *Legionella* supernatants,. The average values associated with all *Legionella* supernatants was measured at less than 0.45 units. In accordance with the parameters utilized by Bassler et al (1997), the value of 0.45 units is substantially less than 10% of the control value, which averaged at 1047 light units (Table 3.4).

4. Discussion

Taken together, the results indicate that *Legionella* does not produce the autoinducers commonly associated with Gram-negative bacteria, i.e. AHSL or AI-2. Quinolone signals are also not produced by *Legionella* species. A few species, namely *L.bozemanii, L. moravica, L. anisa, L. israelensis, L. fairfieldensis,* and *L. oakridgdensis* produced noteworthy results in the qualitative assays that required visual inspection (Figure 3.4); however, these results could not be substantiated other the same assays when β-galactosidase activity was measured (Table 3.1) or by thin layer chromatography (Figures 3.1 and 3.2).

L. pneumophila was originally hypothesized to participate in AHSL mediated signal interactions because of its display of quorum sensing "like" behaviors. Several lines of data supported this hypothesis. When observed in culture, these bacteria demonstrate a synchronized expression of flagella and rapid motility, suggesting the potential role of an extracellular signal in the expression of motility (Rowbotham, 1980).

Furthermore, phenotypic and physiological data regarding *L. pneumophila* activities *in vitro* are reminiscent of quorum sensing. When exponential phase cultures were exposed to the stationary phase, cell-free supernatants became cytotoxic to macrophages, demonstrated enhanced resistance to elevated sodium concentrations, U.V. light, and increases in osmotic potential, all of which support the possible involvement of an extracellular signal (Byrne and Swanson, 1998; Hamer et al., 2002). T

The affinity of legionellae for biofilms also suggests its dependence on autoinducers, as these metabolites are often crucial to the adequate deposition and maintenance of biofilm matrices. Multiple lines of experimental evidence demonstrate the necessity of AHSL and AI-2 in the genesis of properly manufactured biofilms where integrity and mechanical strength are directly related to the expression of autoinducer (De Kievit et al., 2001, Davies et al., 1998). Although *L. pneumophila* has proven inadequate in its ability to form robust monospecies biofilms, the fact that biofilms typically harbor legionellae suggests that these organisms contribute to its architecture, particularly through interaction with the prokaryotic consortia. An analysis of biofilms extracted from natural and man-made sources demonstrate that legionellae represent a frequent component of mixed microbial consortia, where many of the component members are known to secrete autoinducers; therefore, a reasonable inference would suggest that *Legionella* may either produce an autoinducer, become adapted to autoinducer synthesis, or be affected by the diversity of autoinducers to which it is constantly exposed under natural conditions. These observations provide sufficient support for the experimental hypothesis.

Legionellae are typically isolated from biofilms. Together with legionellae and other prokaryotic colonizers, biofilms supports a large diversity of protozoa, including amoebae, cilitates, and highly motile flagellates (Parry 2004). While varoius phenotypic data support the likelihood of quorum sensing interaction in the legionellae life cycle, other ecological data suggests the negative implications of quorum sensing regulated behaviors. Several of these would prevent or impede the ability of legionellae to facilitate intracellular infection in protozoan hosts. For example, quorum sensing facilitation of anti-predator fitness, anti-predator phenotypes would be contrary to legionellae growth requirements. The formation of large microcolonies, secretion of anti-protozoal toxins, and phenotypic conversion into form large, inedible morphotypes are all regulated or influenced by quorum sensing in some species and employed to evade ingestion by grazing protozoa (Matz, 2004; Matz, 2005).

Weitere et al (2005) demonstrated the contribution of quorum sensing to the anti-predator fitness of *Pseudomonas aeruginosa* PAO1. Mature PAO1 biofilms remained completely protected from grazing by *Acanthamoeba polyphaga* due to the AHSL mediated production of antiprotozoal toxins. This inability to feed was also associated with the flagellate *Bodo saltans*; however, *Tetrahymena* species remained adept at consuming *P. aeruginosa* microcolonies. Matz et al (2004) also demonstrated the correlation between quorum sensing function and a reduction in grazing efficiency. *P. aeruginosa lasR*/*rhlR* mutants demonstrate a reduced ability to form inedible microcolonies. As opposed to acutely toxic wild type biofilms, those comprised of *lasR*/*rhlR* mutants were rapidly consumed by the surface feeding flagellate *Rhynchomonas nasuta*, presumably due to the loss of quorum sensing regulated exotoxins

such as hydrogen cyanide, rhamnolipids, and pyocyanin. Whether *R. nasuta* is a suitable intracellular host for *L. pneumophila* has not been determined; however, the implication is clear. *A. castellanii*, which is permissive to the intracellular growth of legionellae, is a prolific biofilm grazer, preferentially attaching to and feeding on adherent communities (Huws et al., 2005). Thus, a quorum sensing system that mediated the synthesis of anti-protozoal toxins thereby denying their interaction with potential hosts, would be contrary to the survival of *Legionella* species.

In accordance with *Pseduomonas* species, impairment of the quorum sensing in *Serratia marcescens*, *Vibrio cholerae*, and *Chromobacterium violaceum* resulted in a significant increase in the vulnerability to grazing by protists (Matz, 2005). Prodigiosin and violacein, both of which are toxic pigments synthesized by *S. marcescens* and *C. violaceum*, are under the biosynthetic control of AHSL. Weekers *et al* observed a substantial decrease (0.01×10^5 cfu/mL) in *Acanthamoeba* and *Hartmannella* yields when these amoebae were cultured in the presence of pigmented microorganisms. Conversely, amoebae were noted for substantial growth (167.5×10^5) in the presence of the non-pigmented microorganisms *E. coli* and *Klebsiella aerogenes* (Weekers et al., 1993).

Although *Legionella* species appeared to be good candidates for quorum sensing, the evidence presented here supports the fact that legionellae do not secrete autoinducers. *Legionella* species maintain a strict requirement for an intracellular host with regard to bacterial replication. Within the context of this argument, a density dependent adaptation that would facilitate microcolony formation, polymicrobial aggregation, or the secretion of anti-protozoan cytotoxins would be contrary to their survival, substantially reducing legionellae fitness. In that microcolonies are resistant to predation by the protozoa in

which *Legionella* are known to replicate, any anti-grazing mechanism preventing protist ingestion of the bacilli would constitute a negative selection pressure that would undoubtedly contribute to rapid elimination of legionellae from the environment. Thus, any legionellae acquiring a grazing-resistant phenotype, such as that mediated in some prokaryotes by quorum sensing, would preclude opportunities for ingestion, and therefore intracellular replication. Such a mutation or adaptation would have likely resulted in the newly formed variants elimination via natural selection in the absence of efficient mechanisms for extracellular replication. This is also applicable to those species with an affinity for signal producing prokaryotes as this association would also interfere with their ingestion by an available host.

Despite the obvious advantages for the absence of AHSL in *Legionella* species, a reasonable expectation exists where these organisms are in possession of some type of novel, intercellular signaling system. This prediction was recently substantiated by the discovery of *Legionella* autoinducer-1 (LAI-1). The compound has been identified as 3-hydroxypentadecan-4-one and is structurally similar to the *Cholera*e autoinducer-1 (CAI-1) produced by *Vibrio cholera* (Sprig et al., 2008). While a direct role for this compound in intercellular signaling has yet to be established, mutations in the *lqs* gene cluster have been linked to virulence attenuation in *L. pneumophila*, where the pathogen demonstrated a loss of cytoxicity and an impairment in its ability to sustain intracellular growth in *A. castellanii* and macrophages (Taiden et al., 2008).

The presence of LAI-1 may also explain the partial inhibition observed in *C. violaceum* CV017 following its culture in the presence of several *Legionella* species. The lack of corroborating evidence for the existence of an AHSL substantiated its absence

among legionellae; however, the reaction also suggested the presence of a potential antagonist. Since *C. violaceum* CV017 constituitively produces AHSL, the amount of LAI-1 or other compound produced by legionellae appeared to be sufficient to produce the reactions shown in Figure 3.4. However, the metabolites produced by these legionellae failed attain the concentrations required to inhibit violacein in a manner consistent with those organisms that produce C_{10} or greater AHSL. However, an assay conducted between a wild type strain of *C. violaceum* and the *Legionella* species that caused partial inhibition may reveal an ability to profoundly inhibit violacien and potentially quorum sensing. If antagonistic effects are mediated by legionellae, the further analysis of this reaction would determine the extent of these effects on other quorum sensing organisms, for example the interruption of prodigiosin production by *Serratia marcescens*, pyocyanin by *P. aeruginosa*, or bioluminescence by *V. fischeri* (Matz and Kjelleberg, 2005). The inhibition of violacien, prodigiosin, and pyocyanin, all of which are poisonous to grazing protozoa, would prove highly advantageous to legionellae.

Given *Legionella's* inability to synthesize either AHSL or AI-2 and the fact that this organism exhibits behaviors that are prominent at higher cell densities and thus, reminiscent of quorum sensing, this organism's lack of *this type of* signaling ability is contrary to its lifestyle. It is important to note that while the presence of AHSL, AI-2, or quinolone signals could not be confirmed, this does not preclude the interaction of an unidentified signaling metabolite in legionellae development and maturation. This is supported by the suggestion by Hammer and Swanson that an "unorthodox" quorum sensing or signaling system likely facilitates *L. pneumophila*'s development and its

transition from a replicative phase to its transmission phase during its lifecycle (Hammer and Swanson 2002).

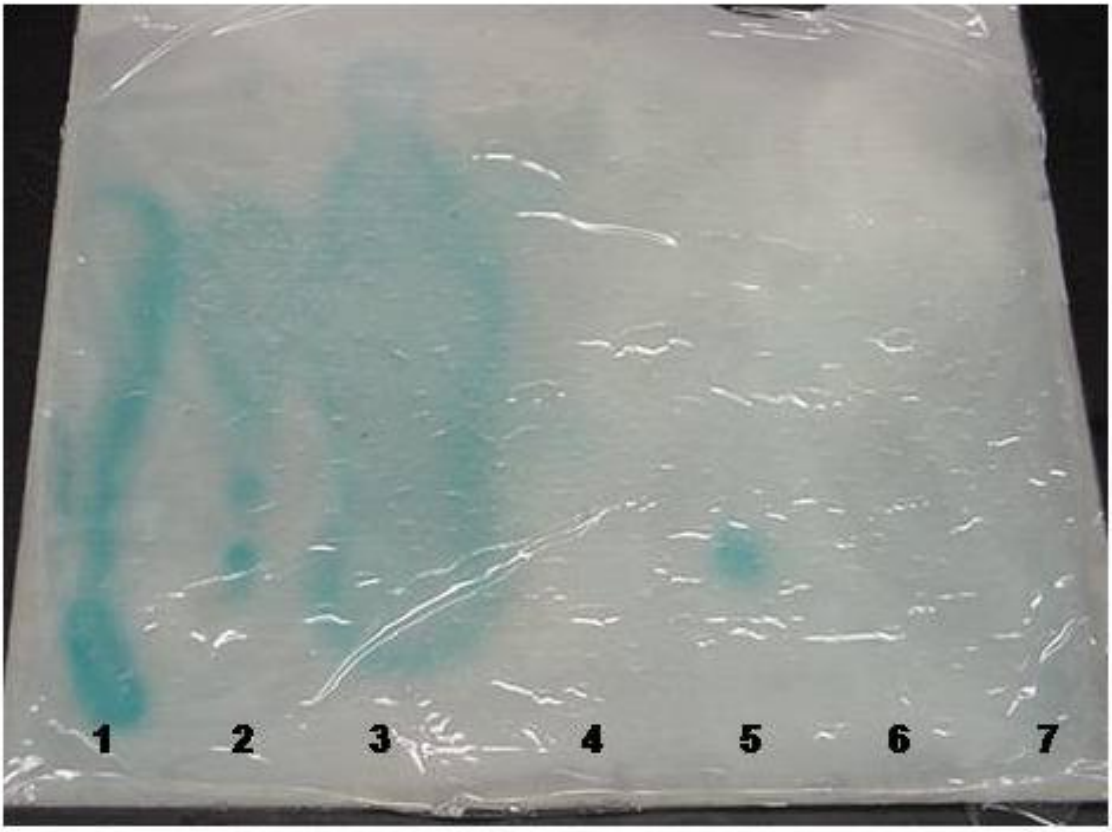

Figure 3.1. TLC plate overlayed with *A. tumifaciens* (pZLR4) for spot detection. Lane 1. 1mM synthetic C_{12}-AHSL ; Lane 2. 100 µM Cµ-HSL; Lane 3. 5 µl *A. tumifaciens* distilled extract; Lanes 4 and 6. *L. pneumophila-H. vermiformis* distilled coculture supernatants; Lanes 5 and 7. *L. pneumophila*-Vero coculture distilled supernatant extract.

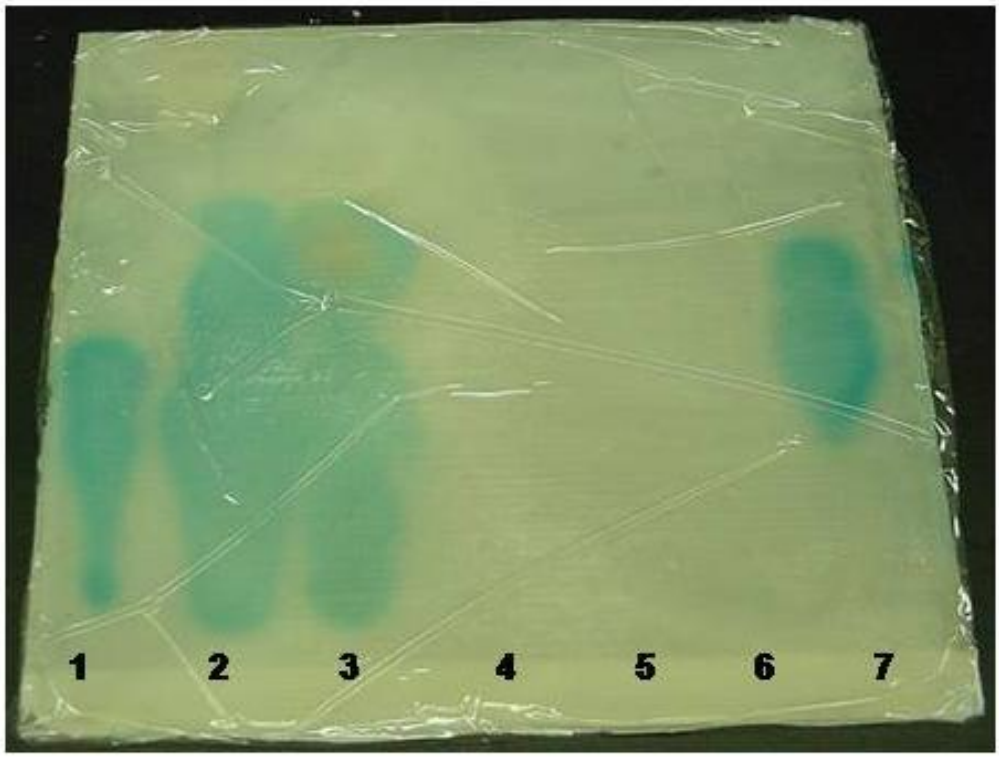

Figure 3.2. TLC plate of distilled supernatant extracts. For spot detection, the chromatograph was over-layed with *A. tumifaciens* (pZLR4). 1: 100 μM synthetic C_{12}-HSL (OdHSL); 2: *A. tumifaciens* extract; 3: *P. aeruginosa* extract; 4: *L. pneumophila* D4987 extract; 5: *L. pneumophila* F1068 extract; 6: 100 μM C_4-HSL (BHL); 7: *C. violaceum* CV017 extract.

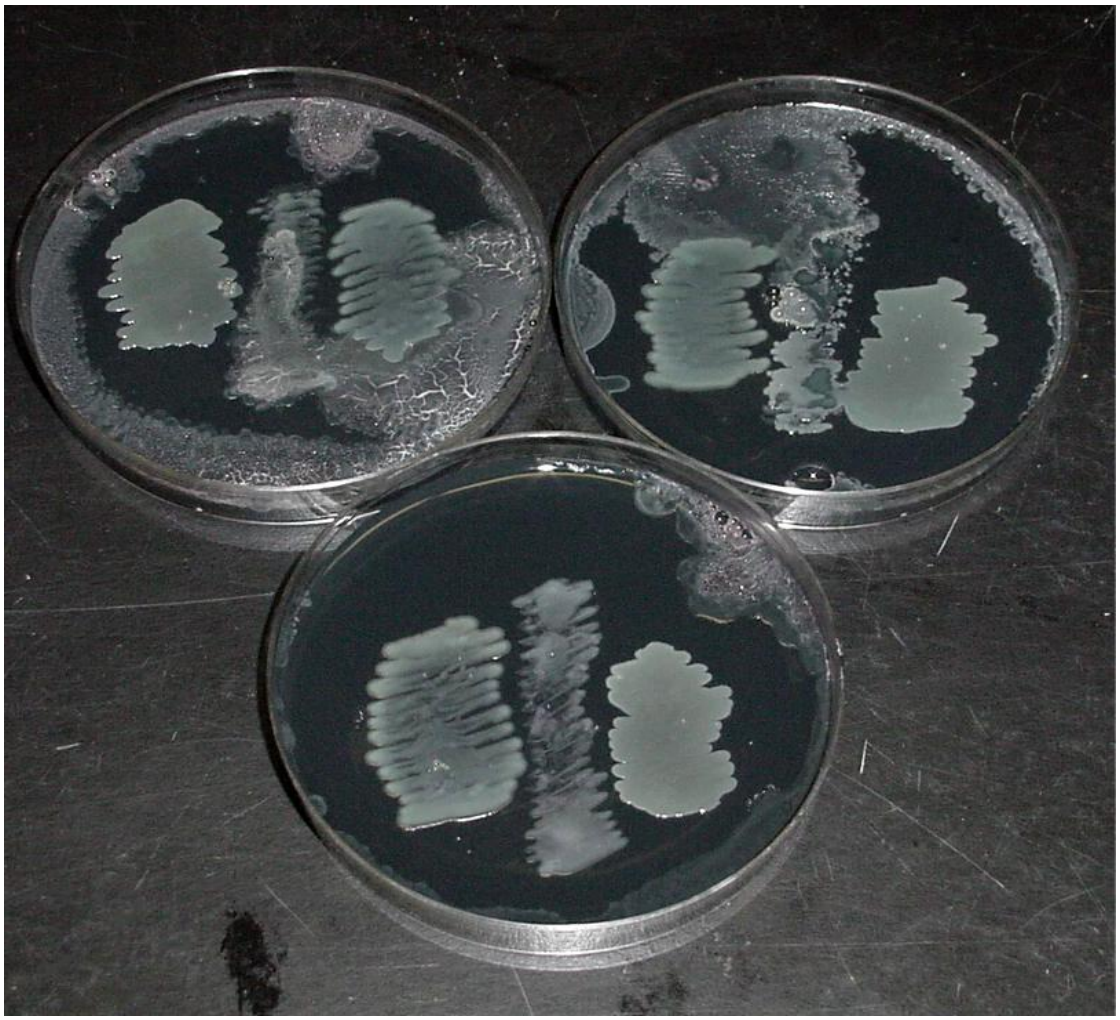

Figure 3.3. Cross feeding bioassays for the detection of AHSL on BCYE with 40μg/ml X-gal. On plates pictured, *E. coli* (pECP61.5) is streaked left, *Legionella* species center, and *E. coli* (pKDT17) is streaked on the right. Clockwise, from the top left corner: *L. pneumophila*, RI243; *L. pneumophila* F1068; *L. pneumophila* D4897. Biosensors failed to turn blue, indicating the absence of AHSL.

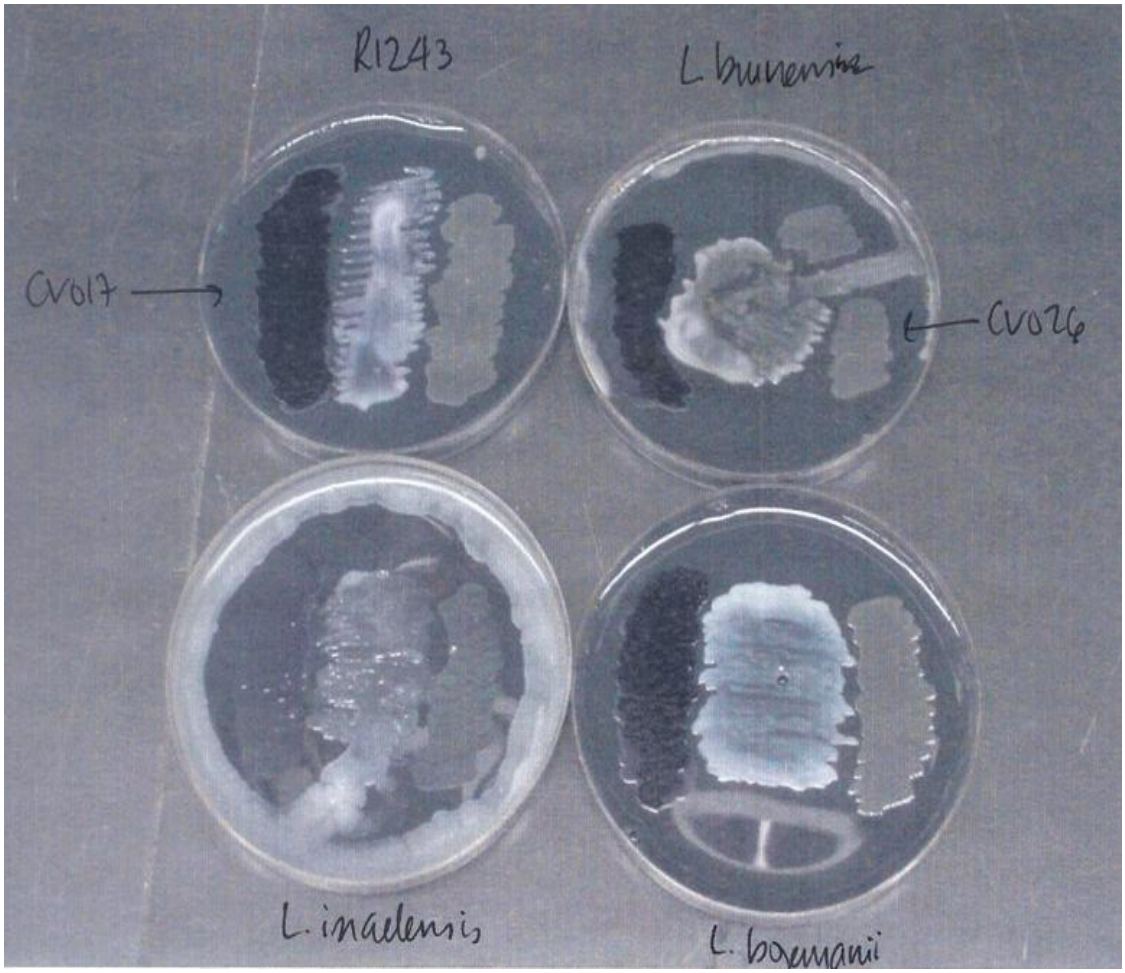

Figure 3.4. *C. violaceum* CV026 and CV017 crossfeeding bioassays. Streaking format per plate: CV017 is streaked left, *Legionella* species center, and CV026 is streaked right. Clockwise from the top left: *L. pneumophila* RI243; *L. brunensis; L. bozemanii*; and *L. israelensis*. Strain CV026 failed to turn purple in response to any of the *Legionella* species. Pigmentation was not completely inhibited by any species with regard to strain CV017; however, a slight inhibition of pigmentation was observed in CV017 when grown in close proximity to *L..israelensis.*

	pZLR4	pECP61.5 (PAI-2)	pKDT17 (PAI-1)
L. pneumophila RI243 (sup.)	8.160	0.857	0.763
L. pneumophila cells	6.891	7.739	0.233
L. pneumophila D4897	0.521	0.227	0.153
L. pneumophila F1068	0.894	1.31	0.556
L. micdadei D-5202	0.794	0.660	0.625
L. bozemanii D-5064	0.556	0.623	0.445
L. longbeachae D-5197	0.332	0.365	0.442
L. israelensis	0.556	0.488	0.763
L. sainthelensi	0.110	0.221	0.115
L. feelei	0.772	0.641	0.550
L. oakridgensis	0.339	0.452	0.441
L. anisa	0.785	0.774	0.822
L. fairfieldensis	0.440	0.529	0.448
L. jordanis	0.995	0.872	0.440
L. moravica	0.889	1.055	0.784
L. brunensis	2.055	0.899	0.477
L. cherrii	0.899	0.876	0.522
L. spiritensis	0.499	0.652	0.255
L. steigerwaltii	0.788	0.719	0.412
L. cincinnatiensis	0.301	0.225	0.284
L. birminghamensis	0.544	0.606	0.235
L. quinlivanii	0.529	0.412	0.440
L. tusconensis	0.336	0.255	0.362
L. adelaidensis	0.658	0.852	ND
L. hackeliae	ND	ND	ND
L. parisiensis	0.566	0.458	0.115
L. maceachernii	0.761	0.683	0.422
L. jamestowniensis	0.338	0.471	0.279
A. tumifaciens (positive ctrl)	60.2	72.0	2.28
P. aeruginosa (positive ctrl)	75.63	85.26	0.59
E. coli (negative ctrl)	1.33	0.650	0.215

Table 3.1. Evaluation of AHSL production by *Legionella* species**.** Consolidated list of values from AHSL bioassays conducted with biosensors maintaining *lacZ* transcriptional fusions. Values reported are for β-galactosidase activity measured in Miller units. ND = not determined.

	CV026	CV017
L. pneumophila RI243	Neg	Neg
L. pneumophila D4897	Neg	Neg
L. pneumophila F1068	Neg	Neg
L. micdadei D-5202	Neg	Neg
L. bozemanii D-5064	Neg	weak Pos.
L. longbeachae D-5197	Neg	Neg.
L. israelensis	Neg	weak Pos.
L. sainthelensi	Neg	Neg.
L. feelei	Neg	weak Pos.
L. oakridgensis	Neg	Neg.
L. anisa	Neg	weak Pos.
L. fairfieldensis	Neg	Neg.
L. jordanis	Neg	Neg.
L. moravica	Neg	weak Pos.
L. brunensis	Neg	weak Pos.
L. cherrii	Neg	Neg
L. spiritensis	Neg	Neg
L. steigerwaltii	Neg	Neg
L. cincinnatiensis	Neg	Neg
L. birminghamensis	Neg	Neg
L. quinlivanii	Neg	Neg
L. tusconensis	Neg	Neg
L. adelaidensis	Neg	Neg
L. hackeliae	ND	ND
L. parisiensis	Neg	Neg
L. maceachernii	Neg	Neg
L. jamestowniensis	Neg	Neg
C. violaceum	ND	Neg
P. aeruginosa	Pos.	Pos.

Table 3.2. Evaluation of medium and long chain AHSL production by *Legionella* species**.** Consolidated list of values from AHSL cross-feeding bioassays conducted on agar plates. Results for trials with *C. violaceum* CV026 listed as positive or negative with regard to the induction of pigmentation. With regard to CV017, positive or negative values refer to the inhibition of pigmentation, where a positive reading denotes the inhibition of pigmentation, inferring the presence of AHSL due to antagonism; CV017 demonstrating the absence of pigmentation on the periphery of its colonies were termed "weakly positive". ND = not determined

	Miller Units	Result
L. pneumophila RI243 supernatant	0.822	Neg.
L. pneumophila D897 distilled supernatant	0.609	Neg.
L. pneumophila D487 supernatant	1.202	Neg.
L. pneumophila/H. vermiformis coculture	0.660	Neg.
L.pneumophila/Vero coculture	0.703	Neg.
H. vermiformis supernatant	0.794	Neg.
5 uM BHL	0.795	Neg.
P. aeruginosa (positive control)	143.13	Pos.
P. aerugionosa pTS400 (biosensor; growth control)	0.822	Neg.

Table 3.3. Evaluation of PQS activity in *Legionella* supernatants**.** Detection of quinolone signal using *P. aeruginosa* (pTS400). Data shown are average values for β-galactosidase activity measured in Miller units.

Supernatants Evaluated	Avgerage Luminometer Reading	Result
L. pneumophila RI243	0.177	Neg.
L. pneumophila + 0.5% glucose	1.363	Neg.
H. vermiformis coculture (17 day)	0.295	Neg.
H. vermiformis coculture (3 day)	0.286	Neg.
H. vermiformis coculture (2 day) supernatant	0.243	Neg.
H. vermiformis coculture (1 day)	0.221	Neg.
Vero coculture supernatant	0.444	Neg.
Vero supernatant	0.339	Neg.
H. vermiformis supernatant (5 day)	0.479	Neg.
S. typhimurium supernatant	1161.0	Pos.
E. coli O157:H7 supernatant	856.7	Pos.
V. harveyi BB152 (positive control)	1047.0	Pos.
V. fischeri supernatant	0.030	Neg.
1uM BHL (negative control)	0.373	Neg.

Table 3.4. Evaluation of AI-2 activity in *Legionella* supernatants. Average values of AI-2 activity evaluated via the luminescence activity of *V.harvey* BB170. Units represent the amount of light activity measured by the TD-20/20 luminometer. Values exceeding 10% of the positive control (i.e. *V. harveyi* BB152) were considered positive. *All coculture assays conducted with *L. pneumophila* RI243

	pZLR4 (Miller units)	CV026 (violecein)	PQS (Miller units)	AI-2 (luminometer)
L. pneumophila/ H. vermiformis	0.06	Neg.	0.955	Neg. (0.261)
L. pneumophila/ Vero cells	1.13	Neg.	0.526	Neg. (0.444)
A. tumifaciens	65.0	Neg.	ND	ND
P. aeruginosa	63.5	Pos.	143.13	ND
E. coli O157:H7	ND	Pos.	ND	Pos. (856.7)

Table 3.5. Evaluation of autoinducer in coculture supernatants**.** Average values for autoinducer detection in cocultured supernatants. Biosensors were used to detect various species of autoinducer in cell free supernatants prepared from *L. pneumophila* cocultures established with *H. vermiformis* and Vero mononuclear cells. *A.tumifaciens, P.aeruginosa*, and *E. coli* O157:H7 served as positive controls for one or more of these assays. ND = not determined

	pZLR4	**pKDT17**	**pECP61.5**
L. pneumophila RI243	Neg.	Neg.	Neg.
L. pneumophila F1068	Neg.	Neg.	Neg.
L. pneumophila D4897	Neg.	Neg.	Neg.
L. bozemanii D-5064	Pos.	Neg.	weak Pos.
L. moravica	weak Pos.	Neg.	Neg.
L. anisa	weak Pos.	Neg.	Neg.
L. micdadei D-5202	weak Pos.	Neg.	Neg.
L. longbeachae D-5197	Neg.	Neg.	Neg.
L. fairfieldensis	ND	Neg.	Neg.
L. israelensis	weak Pos.	Neg.	Neg.
L. oakridgensis	weak Pos.	Neg.	Neg.
L. jordanis	Neg.	Neg.	weak Pos.
L. cherii	Neg.	Neg.	weak Pos.
L. sainthelensis	weak Pos.	Neg.	weak Pos.
L. spiritiensis	Neg.	Neg.	Neg.
L. feeleii	Neg.	Neg.	Neg.
L. brunensis	Neg.	Neg.	weak Pos.
C. violaceum CV017 (positive control)	Pos.	Neg.	Pos.
P. aeruginosa (positive control)	Pos.	Neg.	Pos.
A. tumifaciens (positive control)	Pos.	Neg.	Neg.
Biosensor only (negative control)	Neg.	Neg.	Neg.

Table 3.6. Evaluation of AHSL production by cross feeding bioassay. Results of cross feeding assays conducted with biosensors on BCYE with 40μg/ml X-gal. Reactions are distinguished as being positive (pos.), weakly positive (weak pos), or negative (neg.). ND = not determined.

IV. EVALUATION OF INTERCELLULAR SIGNALING BETWEEN *LEGIONELLA PNEUMOPHILA* AND MULTIPLE PROKARYOTES

Abstract

Intercellular communication between prokaryote and the subsequent cross species induction of genes under the control of intercellular communication signals has been documented. Although previous attempts at demonstrating that *L. pneumophila* participates in quorum sensing via the production of acyl-homoserine lactones or AI-2 were unsuccessful, this does not negate the probability that this species is capable of this activity. Given that intercellular communication typically results in the alteration of gene expression, changes in protein expression by *L. pneumophila's* were used as the foundation for determining if this pathogen can participate in cross-species, intercellular communication. This ability was evaluated by exposing *L. pneumophila* to supernatants produced by signal producing microorganisms. Following a brief incubation, proteins were extracted from the organisms and analyzed by 2-dimentional gel electrophoresis (2-DE). A comparison of proteomes (exposed and un-exposed to heterospecific supernatants) yielded differentially expressed proteins that were subsequently analyzed via matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) for mass spectrometry. The identification of multiple proteins, especially those associated with the stress response, suggests that *L. pneumophila* can respond to heterospecific signals (or other metabolic products) produced by unrelated microorganisms.

1. Introduction

Gene induction by heterologous autoinducers has been documented (Houdt et al., 2006; Bassler et al., 1997). This phenomenon, also referred to as signal interception, is characterized by the initiation or alteration of gene expression in response to autoinducers produced by *another* species of microorganism. The ability of one organism to detect heterologous signals produced by an unrelated microorganism is widespread among prokaryotes and has been extensively documented in *E. coli, Salmonella* enteric serovar Typhimurium, *Vibrio harveyi,* and, to a lesser extent *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and multiple AI-2 secreting microorganisms (Ahmer et al., 1998, McKenney et al., 1995; Duan et al., 2003, Bassler et al., 1997). The genes responsive to these, some of which include virulence factors, are diverse in their function. AI-2 and AHSL have been detected in mixed populations of naturally occurring prokaryotes known to include *L. pneumophia;* therefore, legionellae appear to be viable candidates for heterologous signal interception, and cross-species communication. While limited data exists in support of this hypothesis, some observations do support a role for interspecies communication in *Legionella* development.

According to Mampel et al, *L. pneumophila* demonstrates a predilection for biofilms formed by *Empedobacter brevis* and *Microbacterium* sp. With regard to the latter, the pathogen exhibited an apparent tropism toward *Microbacterium* microcolonies, suggesting an interaction that may be supported by intercellular signaling or species-specific, metabolic interactions (Mampel et al., 2006). The latter is corroborated by observations of *Microbacterium* in an unrelated investigation. *Microbacterium phyllosphaerae* is not known to produce AHSL. The substitution of *M. phyllosphaerae*

with its culture supernatants during the formation of a multi-species biofilm resulted in a moderate reduction (11.3%) in biomass. When the bacterium contributed to biofilm establishment, its synergistic cooperation was demonstrated when paired with *Shewanella japonica* and *Dokdonia donghaensis*, which resulted in a >167% increase in biomass over single species biofilms (Burmølle et al., 2006). Although *L. pneumophila* embedded in *Microbacterium* biofilms failed to grow, the concept of synergy may explain the tropism demonstrated by *L. pneumophila* for *Microbacterium* biofilms. Conversely, biofilms comprised of an unidentified *Pseudomonas* sp., *Corynebacterium glutamicum*, and *K. pneumoniae* were refractory to *Legionella* colonization, with the last species facilitating the detachment of *L. pneumophila* and impairing its ability to attach to potential biofilm surfaces (Mampel et al., 2006).

Mixed species interactions have proven to be of particular value in biological assays used for autoinducer detection. Studies of this kind provide support for the concept of heterologous, intercellular communication. For example, *Vibrio harveyi* BB170 is consistently cited for its utility in the discovery of AI-2 in new species, among them *E. coli* 0157:H7, *S. enterica* serovar Typhimurium, *C. perfringens*, *H. pylori*, and *Actinobacillus actinomycetemcomitans* (Ohtani et al., 2002; Joyce et al., 2000; Fong et al., 2001; Surrett et al., 1999). This concept has also been applied to AHSL detection, where biosensor detection encompasses multiple isoforms of the metabolite.

In accordance with our hypothesis for the ability of *L. pneumophila*'s to respond to heterologous autoinducers, intercellular signals are capable of gene regulation in recipient species that are incapable of the signals synthesis. A definitive example is the affect of AI-2 on the expression of virulence by *P. aeruginosa*, which does not possess a

luxS homologue (Duan et al., 2003). Several observations have illustrated the impact of AI-2 mediated interspecies communication the pathogenesis of *P. aeruginosa* including evidence that: i) virulence, in terms of lung pathology, is enhanced when the pathogen is co-inoculated with a *Streptococcus* sp. isolated from a cystic fibrosis (CF) patient, ii) as many as 214 promotors were upregulated when co-cultured with *Staphylococcus* or *Streptococcus* CF isolates, and iii) the expression of six virulence genes was upregulated, including those required for rhamnolipid, elastase, exotoxin, and phenazine synthesis, by exogenous AI-2 or a *Streptococcus* CF isolate (Duan et al., 2003). The fact that AI-2 can enhance virulence gene expression in *P. aeruginosa* without its concomitant production suggests a long-standing and complex relationship among prokaryotes. Duan et al (2003) suggested that an evolutionary advantage of microbial interaction and the subsequent perception of heterologous signals is the maintenance of virulence genes in the absence of a host.

AI-2 facilitated interspecies communication has also been cited as a contributing factor in biofilm genesis. *luxS* mutations sustained by multiple oral bacteria, i.e. *S. gordonii* and *P. gingivalis,* are associated with an inability to form mixed species biofilms or more specifically dental plaque; however, complementation of a single species (*S. gordonii*) with *luxS* is sufficient to facilitate biofilm formation with other *luxS* mutants (*P. gingivalis*) (McNab et al., 2003).

In a manner similar to that of *P. aerugionosa, E. coli* and *S. enterica* serovar *typhimurium* respond to, but cannot synthesize, AHSL. Along with *K. pneumoniae* and *Shigella flexneri*, these organisms possess a *luxR* homologue called *sdiA* (suppressor of cell division inhibition) (Ahmer et al., 1998, Michael et al., 2001, Yao et al., 2006). The

presence of *sdiA* is noteworthy given the fact that SdiA is a quorum sensing protein without a cognate, autoinducing metabolite. Thus, the absence of a *luxI* homologue in the genomes of these *Enterobacteriaciae* suggests that the SdiA transcriptional regulator is responsive to AHSL compounds synthesized strictly by other microorganisms. When grown in close proximity to AHSL synthesizing microorganisms, i.e. *Yersenia enterocolitica*, *Hafina alvei*, *Chromobacterium violaceum* and *Vibrio fischeri*, *sdiA* reporter strains increased. These findings support a role for gene regulation by the natural susceptibility of *sdiA* to heterologous autoinducers produced by unrelated microorganisms (Michael et al., 2001; Smith and Ahmer, 2003).

AHSL mediated interspecies communication has been implicated between *P. aeruginosa* and *B. cepacia* due to the ability of metabolites produced by the former to upregulate metabolite synthesis in the latter. Both of these pathogens are frequently isolated concomitantly from the lung tissue of individuals with cystic fibrosis, and the natural circumstance of their association suggests the plausibility of intercellular communication or cooperation via AHSL. Despite *B. cepacia*'s synthesis of N-octanoyl homoserine lactone (C_8-HSL) and to a lesser extent N-hexanoyl homoserine lactone (C_6-HSL), the production of its virulence factors, i.e. siderohore, lipase, and protease is significantly enhanced by *P. aeruginosa'*s exoproducts, presumably PAI1 and PAI2, rather than its own, native autoinducers.

Protein expression (proteomics) has been used as an approach to establishing the effects of quorum-sensing on gene regulation. This method has proven to be an asset in defining the quorum sensing regulon; however, this technology not been employed in the discovery intercellular signaling in novel microorganisms. This is also true for its use as

a means of determining the capacity for heterologous signal interception by neighboring prokaryotes. The objectives of the research that follows are to determine the response of *L. pneumophila* to various communication signals secreted by microorganisms known to participate in quorum sensing. The response was evaluated by focusing on the induction of protein expression in response to the supernatants of unrelated prokaryotes.

2. Materials and Methods

2.1 *Bacterial strains.*

L. pneumophila RI243 was evaluated for its ability to respond to heterologous intercellular signals. BCYE supplemented with L-cysteine (0.4g/L) and ferric pyrophosphate (0.25 g/L) and yeast extract broth (YEB) was used for cultivation *in vitro*. Other species used in this study included wild type strains of microorganisms known to produce intercellular signals. These included *Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia, Streptococcus pyogenes, Bacillus subtilis, Bacillus cereus,* and *Escherichia coli* O157:H7. *Escherichia coli* DH5α was also used in this study. The signaling capacity, or the lack thereof, was confirmed in *P. aeruginosa, B. cepacia, S. pyogenes, E. coli* O157:H7, and *E. coli* DH5a. All species, except *S. aureus* and *B. cepacia*, were obtained from the Department of Biological Sciences, Auburn University. A clinical isolate of *S. aureus* was provided by East Alabama Medical Center (EAMC; Opelika, AL) and *B. cepacia* was purchased from Presque Isle Cultures (Presque Isle, ME). All strains, except *S. pyogenes*, were cultured on TSA and 0.5X TSB. *S. pyogenes* was maintained on 5% sheep's blood agar, brain heart infusion (BHI) agar, and 0.5X BHI broth. Additional media used for *S. aureus* included mannitol salt agar (MSA); *E. coli*

O157:H7 and *E. coli* DH5α, were alternately cultured on SMAC and MAC, respectively. *L. pneumophila* RI243 and *S. pyogenes* were cultivated at 37 ºC in 3-5% CO_2. All other strains were cultured at 37 ºC in ambient air. All microorganisms were prepared for long term storage by adjusting overnight broth cultures to a final concentration of 15% glycerol and freezing at -70 ºC.

2.2 *Assays for L. pnemophila response to heterospecific, intercellular signals.*

For response assays, autoinducer containing supernatants were required. On the day prior to response assays, *L. pneumophila* and signal producing microorganisms were cultured simultaneously in 10 ml volumes. Signal secreting microorganisms were grown in 0.5X broth (TSB or BHI) for 18-24 hours, after which the cultures were pelleted (5 min; 14,000 x g) and the supernatant was passaged through a 0.45 μm filter. Supernatants were used within 24 hours of preparation and refrigerated until required for use. Following overnight growth of *Legionella*, the cultures were streaked on BCYE, BCYE with 100 mM NaCl, and TSA. This was incorporated for quality control, i.e legionellae cannot grow on TSA, and to ensure that the organisms had reached stationary phase, as organisms in stationary phase are sensitive to 100 mM NaCl (Byrne and Swanson, 1998). *L. pneumophila* was prepared for experimentation by washing the cells 3 times with 10 ml 0.5X PBS. Following the resuspension of washed *L. pneumophila* in the test medium (5ml 2X YEB and 5 ml conditioned supernatant), the culture was incubated for 3 hours at 37 ºC with aeration, after which it was streaked a second time on TSA, BCYE, and BCYE with 100mM NaCl. Each experiment was repeated at least twice. When required, *L. pneumophila* was also tested for its response to undiluted supernatants.

2.3 *Protein isolation and quantification.*

Following exposure of *L. pneumophila* to autoinducer containing supernatant, the cells were prepared for extraction of intracellular proteins. The cells were pelleted via centriguation at 14,000 x g for 5 minutes, after which the pellet was washed 3 times with 40 mM Tris-HCL pH 7.2 and resuspended in 100 µl of lysis solution composed of 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-HSL, and 0.2% Bio-Lyte 3/10 [Bio-Rad]). To minimize proteolysis, a protease inhibitor mix (Amersham Biosciences) was added at a final concentration of 10 µl/ml (v/v). After incubation on ice with agitation for 1 hour, the suspensions were transferred to a 1.5 ml microcentrifuge tube for centrifugation for 30 minutes at 8 °C, 18,000 x g. The supernatant, containing soluble protein, was decanted and stored at -20 °C (Park et al., 2003). All protein samples were quantified against a BSA standard by the method of Bradford (1976).

2.4 *Protein separation by 2-dimensional electrophoresis*.

Intracellular *L. pneumophila* proteins were resolved via 2-dimensional electrophoresis (2-DE). Proteins were separated in the first dimension by isoelectric focusing. This was accomplished by combining 300 µg of protein with rehydration buffer (8M urea, 2% CHAPS, 50mM DTT [dithiothreitol], and 0.2% 3-10 Bio-Lyte ampholytes, BioRad) to a final volume of 300 µl. To reduce streaking between protein spots as the result of the oxidation of disulfide groups, DeStreak Reagent (Amersham Biosciences) was added to the final solution in accordance with manufacturer recommendations. The solution was used to rehydrate an 18 cm, pH 4-7 IPG

(immobilized pH gradient; BioRad) strip for 16-24 hours. Rehydrated strips were focused in accordance with the manufacturer's recommendations using a Protean IEF Cell (BioRad). The following parameters were used for isoelectric focusing, culminating in a total of 50,000 volt-hours (V-hr): temperature of 20 ºC for electrophoresis, 30 minutes at 250V, followed by 2.5 hours at 10,000V, and 40,000 Volt-Hours at 10,000 volts (BioRad ReadyPrep 2-D Starter Kit Instruction Manual). Following IEF, IPG strips were equilibrated for 15 minutes each in equilibration buffer 1 (6M urea, 0.375M Tris pH 8.8, 2% SDS, 20% glycerol, 2% DTT) and equilibration buffer 2 (6M urea, 0.375M Tris pH 8.8, 2% SDS, 20% glycerol, 2.5% [w/v] iodoacetamide) with agitation, briefly rinsed in 1X Tris-glycine-SDS buffer, and applied to a 12% total monomer SDS-PAGE 20X20 cm gel (BioRad; Hercules, CA) for resolution in the second dimension by molecular weight. Proteins were resolved using a current of 20 mV for ~18 hours. Following electrophoresis, the proteins were fixed in the gel by submerging them in a solution of 10% methanol and 7% acetic acid (10/7) for 1 hour, washed with distilled, deionized water, and stained with SYPRO Ruby (BioRad; Hercules, CA) overnight. Prior to imaging using the Typhoon 9410 Scanner (Amersham Biosciences; Westborough, MA), the gels were destained using 10/7 methanol-acetic acid for 10 minutes.

2.5 *Proteomic analysis*

Each assay in which *L. pneumophila* RI243 was exposed to heterologous supernatants was completed twice, with a minimum of one gel produced per assay. This resulted in a minimum of 2 gels per experiment. 2-DE gels were analyzed using ImageMaster 2D Platinum (Amersham Biosciences; Westborough, MA). This program was used in the initial comparison of gels for replicate assays and between assays and the

proteome of the experimental control. Manual comparisons were conducted between the control and experimental gels using a UV box. Using this technique, spot matches in terms of differentially expressed proteins between the gels (proteomes) were established. Spots of interest, namely those that appeared to be induced following exposure to autoinducing supernatants, were selected for further analysis.

2.6 *Preparation of proteins for identification*

Spots of interest were excised from 2-DE gels, transferred to a 1.5 ml microcentrifuge tube, and cut into small cubes using a small scalpel. Gel pieces were washed 3 times with 150 µL of a solution composed of 10 mM NH_4HCO_3 (ammonium bicarbonate) and 50% acetonitrile (ACN) for 15-20 minutes. After washing, the gel slices were dried for 30 minutes in a Speed Vac with no heat. To initiate proteolytic digestion of the protein samples, the gel slices were rehydrated with 5 µL of a solution composed of a 5:1 mixture of 10 mM NH_4HCO_3 and proteomics grade trypsin (Sigma Aldrich; St. Louis, MO), 20 µg/mL. Gel slices were reconstituted to their original volume by adding 15-20 µl (or more) of 10mM NH_4HCO_3 to cover them, after which incubation was allowed to proceed for 1 hour at 37 ˚C. Following incubation, the gel slices were covered with an additional 15-20 µl of buffer (10mM NH_4HCO_3) and then incubated overnight. Following incubation, the supernatant was removed from the gel slices and saved. Peptides were extracted from the gel cubes by 3 iterations of incubation in ~100 µL of a buffer composed of 10 mM NH_4HCO_3/60% ACN for 20-30 minutes at RT. Following each incubation, supernatants were removed and combined with the supernatants retained from previous elutions. A final attempt at peptide extraction was completed by dehydrating the gel cubes completely with a small amount (100-150 µl) of isopropanol.

Following a brief incubation (~5 minutes), the supernatant was removed and pooled with the other supernatants. Combined supernatants were condensed in a Speed Vac (without heat) to a small volume (~10 μL) and resuspended in 30 μL of 0.1% sequencing grade trifluoroacetic acid (TFA). Samples were further acidified by the addition of 1 μL 10% TFA and desalted with the aid of a C_{18} ZipTip (Millipore) in accordance with the manufacturer's protocol. Peptide samples were euluted from the ZipTip by pipetting 2 μL of matrix/ACN (1.5 μl of matrix stock solution and 0.5 μl of ACN) into the ZipTip. Peptides were eluted directly onto a MSP 96 target polished steel microScout Target plate (Bruker Daltonics; Billerica, MA) and allowed to completely air dry before conducting peptide analysis. The MALDI matrix used for peptide deposition is α-cyano-4-hydroxy cinnamic acid (CHCA; Bruker Daltonics; Billerica, MA), and is prepared at a concentration of 10mg/ml in a solution composed of equal volumes of Solution A (30% ACN, 25% methanol, 2% acetic acid) and Solution B (100% ACN) (CDC protocol).

2.7 *Microflex matrix-assisted laser desorption-ionization time of flight [MALDI-TOF]) and Data Analysis.*

MALDI-TOF analysis was completed with the Microflex Mass Spectrometer (Bruker Daltonics; Billerica, MA). Peptides were analyzed in accordance with the manufacturer's recommendations using the positive reflectron mode (RP) for mass spectrometry. Following the characterization of peptide peaks, the consolidated spectra were formatted to remove background and baseline peaks, and subject to smoothing prior to entering the MASCOT search engine (www.matrixscience.com) for protein identification through its peptide mass spectrum database. With regard to the proteins in

the database, a value of 73 or greater was considered a significant match for protein identification.

To determine the confidence value of a given peptide score, MASCOT uses a database scoring algorithm called Mowse (www.matrixscience.com). MASCOT matches the spectrum derived from MALDI-TOF analysis to multiple sequence databases after which a score is produced. This score reflects the probability that the peptide-spectrum match observed between the unknown peptide and the protein identification in the database is a random event (Kall et al., 2008)

3. Results and Discussion

Forty proteins were selected for identification following the analysis of *L. pneumophila's* response to 5 heterospecific (non-*Legionella*) supernatants. Out of the 40 spots that were selected for MALDI analysis, 36 were differentially expressed. The BLAST search of the peptide spectra failed to *conclusively* identify (i.e. a MASCOT score of $\geq$73) 25 of the proteins. A total of 10 proteins did, however, yield significant values, 5 of which were differentially expressed. These proteins (annotated in Figures 4.1 through 4.9), identified as hypothetical proteins lpp1207, lpl0966, and *atpH*; global stress protein GspA; and a 24kDa macrophage induced protein. Additional proteins, all with peptide scores between 45-67 were also noted for differential expression, with the closest identifications being transcription factor GreA, hypothetical proteins lpl0966 and lpl1478 (identified twice), Hsp10 (a heat shock protein), and malate dehydrogenase, elongation factor EF-Ts, and 7 unidentified proteins. All proteins and MASCOT scores are outlined in Tables 4.1 through 4.4.

By virtue of enhanced protein expression, the data presented demonstrates the potential for *L. pneumophila* to detect the presence of unrelated microorganisms, either through heterospecific intercellular signals or another metabolic product. AHSL and AI-2 producing microorganisms were selected for the evaluation of the pathogen's response. In that AI-2 has been detected in numerous prokaryotes (both gram-positive and gram-negative), and is commonly referred to as a universal communication signal among microorganisms, the evaluation of these supernatants was the greater focus of analysis. While the absence of AI-2 in *Legionella*'s supernatant has been noted, the presence of this compound within habitats known to harbor legionellae suggests the potential for AI-2 signal interception whether they produce this compound or not. This is also substantiated by induction of gene activity (as inferred by the presence of multiple spots in the proteome) upon exposure to AI-2 supernatants, which strongly infers participation in cross-species communication.

Despite the fact that the majority of the induced spots could not be positively identified or attributed to any known biological function, those that were identified collectively represented a stress response mounted by the bacterium. This conclusion contradicts the original hypothesis where AHSL and AI-2 were thought to the enhance viability of the microorganism. However, this response, that includes a macrophage induced protein, heat shock protein, universal stress protein, and cold shock proteins constitutes a set of adverse reactions or stresses rather than the benefit of upregulation of survival or virulence gene expression that is commonly seen in other bacteria. The expression of these genes with regard to heterospecific signals suggests alternative roles

for these proteins in a stress response induced by the presence of competitors rather than environmental or host conditions.

Consistent with these findings is the expression of global stress protein GspA and Hsp10. GspA is induced in *L. pneumophila* following its exposure to *in vitro* stress stimuli, i.e. osmotic stress, heat shock, acid shock, and oxidative stress. The 19 kDa GspA protein is also expressed during intracellular growth in macrophages. The importance of the protein was demonstrated by its absence in *gspA* mutants, which are more susceptible to stress stimuli *in vitro* than the wild type (Kwaik et al., 1997). Likewise, heat shock proteins (Hsp) have been characterized in *Legionella poneumohila*. Likely similar to Hsp10, Hsp60 is expressed from the htpAB operon and its abundant expression has been noted during growth in eukaryotic host cells. The protein is also expressed and released from *L. pneumophila* following heat shock and has been noted to mediate adherence to HeLa cells (Garduno et al., 1998; Hoffman et al., 1990).

In that supernatants were used rather than purified compounds, the response cannot be solely limited to AHSL or AI-2 and could represent a reaction by *L. pneumophila* to another intercellular signal, a metabolic compound or waste product in the supernatant. Given the fact that the organisms used in this study are known to produce additional signals, these compounds *may* have also contributed to *L. pneumophila's* response. For example, *S. aureus* synthesizes a peptide signal AgrD, which controls the production of multiple virulence factors including beta-hemolysin, coagulase, and protein A (Ji et al., 1995, reviewed by Dunny and Leonard 1997). Thus, does the detection of AI-2 or AHSL (or other signaling compounds) present a significant advantage to *L. pneumophila* in the environment and/or during the genesis of

Legionnaire's disease? The answers to these questions may be partially resolved by comparing the responses of *L. pneumophila* to the wild type and a mutant that is incapable of AI-2 or AHSL production. Still a better approach to this analysis would be the use of purified AHSL or AI-2. In this manner, the response seen by *L. pneumophila* could have been solely relegated to the presence of the specified autoinducer. While this approach would provide indisputable evidence for the reaction of *L. pneumophila* to AHSL or AI-2, the data would not represent the pathogens response under natural conditions nor would it take into account of other, potentially intervening metabolites that may (or may not) affect the organisms final response. Additonal answers may also be provided by evaluating the organism's ability to populate biofilms, to persist, and to infect protozoa after disabling the genes responsible for the induced proteins.

A recent publication underscores the stress response demonstrated by *L. pneumophila* in the presence of autoinducing supertatants. The presence of the *P. aeruginosa* autoinducer 3-oxo-C_{12}-HSL is capable of suppressing the growth of *L. pneumophila* in a dose-dependent manner. C_{12}-HSL was not refractory to the growth of *Serratia marcescens*, *Proteus mirabilis*, or *E.coli*; however, a concentration of 50 μM inhibited the growth of *L. pneumophila* and suppressed continued biofilm formation when added to experimental biofilm cultures at days 5 and 7 after its initiation. 50 μM C_{10}-HSL also demonstrated inhibitory effects on *L. pneumophila*. Other AHSL molecules, namely C_4, had no inhibitory affect on the pathogen (Kimura et al., 2009). While this data partially supports conclusion of a *L. pneumophila* stress response to the presence of heterologous prokaryotes, the autoinducer concentrations used in this study are not physiologically relevant to those actually detected in biofilms, which have been

calculated at 14 nM (Charlton *et al*., 2000). Still, Kimura et al.'s (2009) findings support the application long chain AHSLs as a viable alternative to biocides in the control of legionellae in domestic water supplies.

Comparative proteomic analysis has been used to characterize quorum sensing regulated proteins in a modest number of microorganisms including *P. aeruginosa, B. cepacia, Pseudomonas putida* IsoF, *Sinorhizobium meliloti*, and *Neisseria meningitides* (Nouwens et al., 2003, Arevalo-Ferro et al., 2003 and 2005, Riedel et al., 2003, Chen et al., 2003, Schauder et al., 2005). An assessment of the *P. aeruginosa* PAO1 wild type and isogenic *lasRI, rhRl,* or *lasRI-rhRIl* mutant proteomes identified several novel proteins among quorum sensing regulated polypeptides, among them an aminopeptidase, an alklaline metalloproteinase (ArpA), lipase (lipA), a flagellar hook-associated protein (FlgK), the flagellin FliC, and multiple hypothetical proteins (Nouwens et al., 2003). Arevalo-Ferro et al (2003) identified 723 AHSL responsive proteins in the proteome of *P. aerugionosa* using a similar approach, identifying the reduced expression of multiple proteins in the *lasI-rhlI* double mutant background including several involved in the post-transcriptional regulation and modification of other proteins prior to maturation.

Regarding this form of regulation, quorum sensing contributes indirectly to the post-translational processing of an extracellular heme-binding protein, HasAp, by regulating the transcription of the extracellular proteases required for its modification. Other proteins, i.e. elastase (LasB), phenazine, and a chitin-binding protein (CbpD), were also noted for their diminished expression or complete absence in the mutant background, substantiating previous reports regarding their subjugation to AHSL regulation (Arevalo-Ferro et al., 2003). Beyond proteomic data, the information derived from DNA

microarray and transcriptome analysis regarding quorum sensing mediated gene and mRNA expression provide conclusive support for the application of intercellular communication to *P. aeruginosa*'s fitness (Wagner et al., 2003 and 2004).

Analysis of the *B. cepacia* quorum-sensing regulon revealed 55 quorum related proteins following comparative proteomics between the parent strain and an autoinducer deficient *cepI* mutant (Riedel et al., 2003). Chen *et al* demonstrated that the exposure of low density *S. meliloti* cultures to stationary phase concentrations of purified, native C_{14}-HSL and/or 3-oxo-C_{16}-HSL resulted in the upregulation of over 100 proteins, among them a NADP-dependent malic enzyme, an ABC transporter periplasmic binding protein, a flagellar hook protein, and putative heat shock protein GroEL (Chen et al., 2003). Periplasmic proteins were also identified among the 53 AHSL regulated proteins synthesized by sessile, biofilm dwelling forms of *P. putida* IsoF following a comparison of its proteomic signature to that of its planktonic counterpart. Other potential members of the *P. putida* quorum sensing regulon include outer membrane proteins OmpH and H1, a thioredoxin, ribosomal protein L24, and multiple hypothetical proteins (Arevalo-Ferro et al., 2005).

While the data support a general response by *L. pneumophila* to heterologous signals or metabolic products rather than a specific response to AHSL or AI-2, additional corroboration of the results would strengthen this argument. For example, replicate 2-DE gels of each experimental assay are typically conducted when performing proteome analysis. This is done to account for any anomalies in protein precipitation, polyacrylamide gel preparation, electrophoresis conditions, etc will affect the final outcome of the proteome. To reduce this variability, multiple gels are typically run from

the same sample, after which they are averaged together to ensure that a sufficient representation is produced (Hanes and Yates, 2000). Thus, the production of replicate gel serve to ensure that the data presented is the results of a response by *L. pneumophila* to heterologous microorganisms rather than the result of variations resulting from laboratory techniques. Furthermore, additional work using transcriptome or DNA microarray analysis to analyze the expression of a large number of *L. pneumophila* coding transcripts and genes responsive to the presence of heterologous supernatants would aid in substantiating the data presented here.

The identification of species-specific communication among legionellae remains elusive. While LAI-1 has been recently identified, its role in intercellular signaling has not yet been established. Furthermore, the gene responsible for LAI-1 (*lqsA*) was not detected via PCR in *L. bozemanii*, *L. rubrilucens*, or *L. taurinensis* indicating that this signal may not be prolific amongst legionellae (Spirig et al., 2008). Thus, much work remains where the identification of potential signals, cognate receptors, and the genes comprising a proposed regulon of genes responsive to autoinducing compounds. If such machinery exists, then to what extent can this information be used to control the presence of legionellae in the environment? Thus, in summary, although species-specific intercellular communication was not conclusively demonstrated as a capability of *L. pneumophila*, the data supports the likelihood that *Legionella* can sense AHSL, AI-2, and/or other intercellular communication signals metabolites.

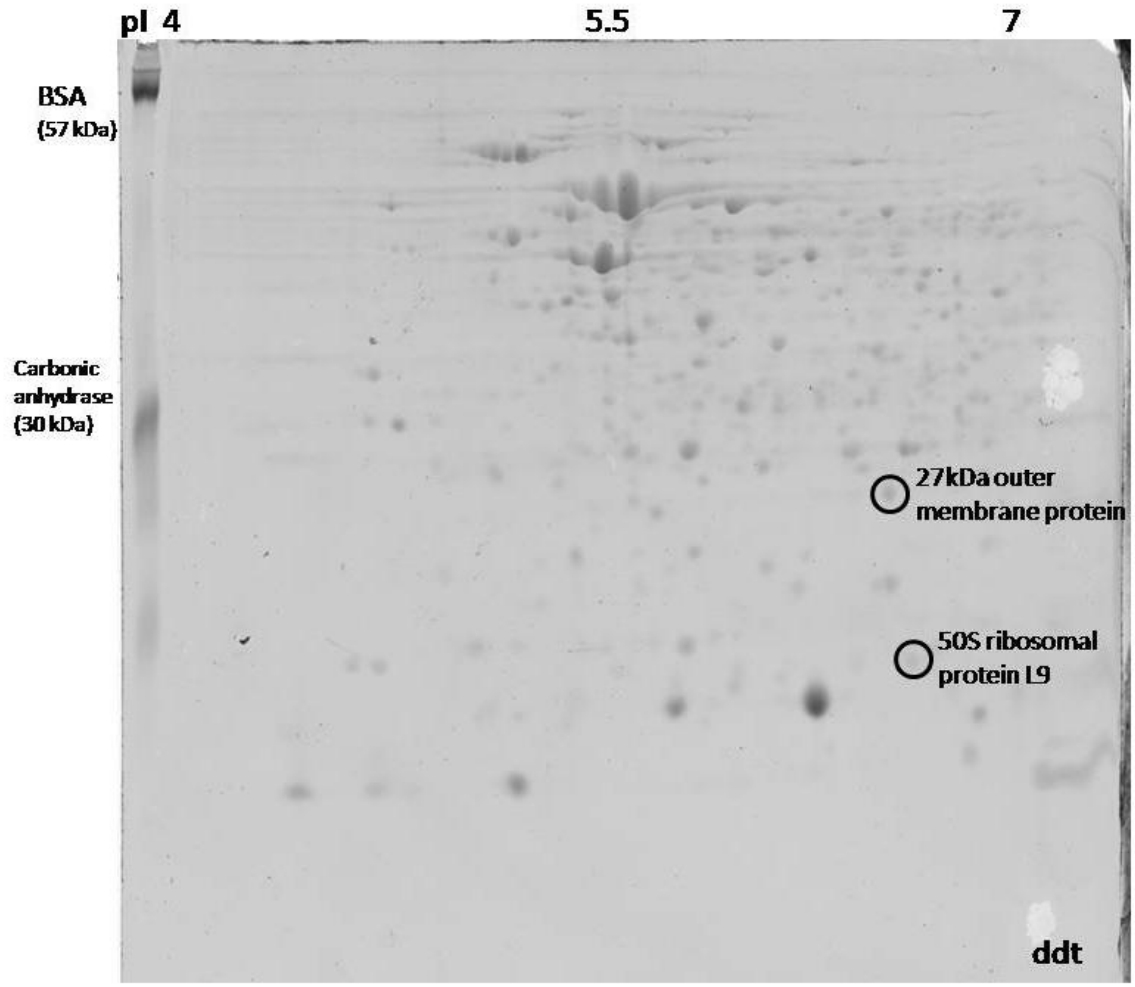

Figure 4.1. Control proteome of *L. pneumophila* RI243 following ~20 hours of growth in YEB. This gel served as the control for proteomes prepared using a pH 4-7 IPG strip. The proteins that are identified on this gel, i.e. 27 kDa outer membrane protein and 50S ribosomal protein L9, were used as markers during the analytical process of gel matching and protein spot selection.

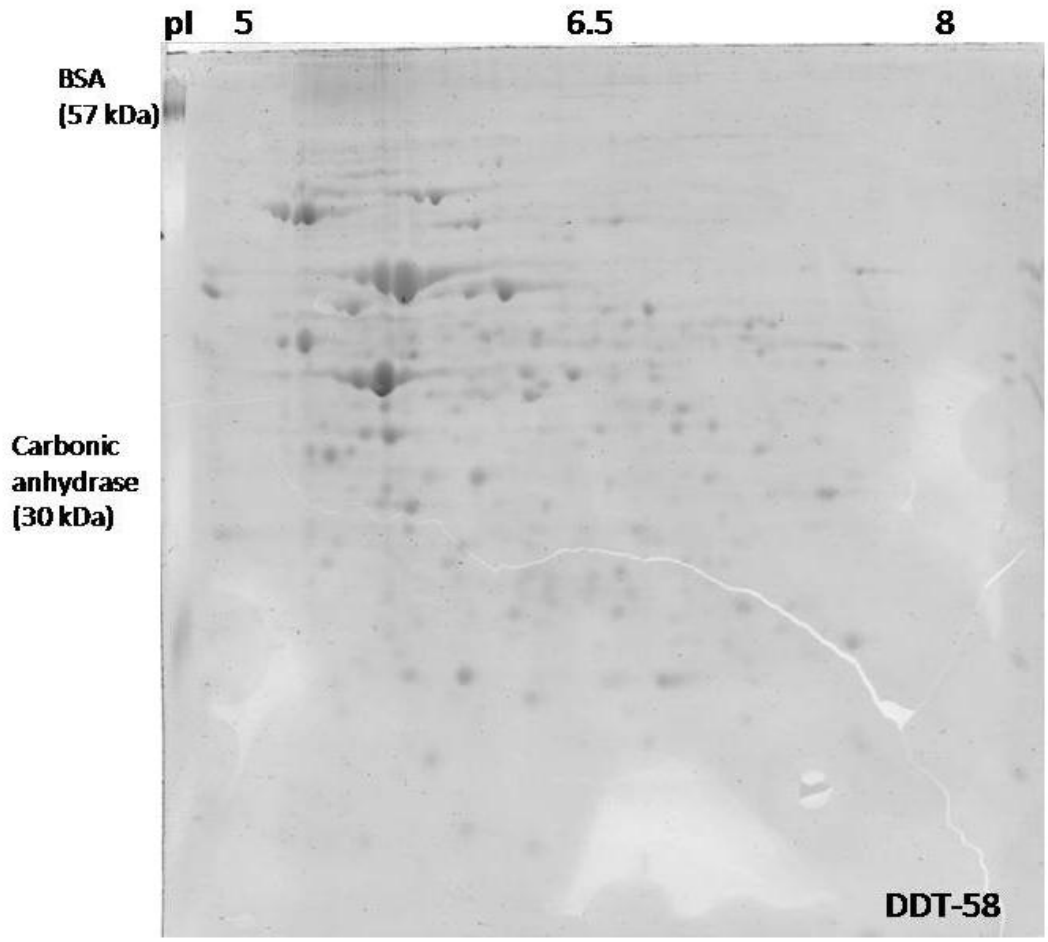

Figure 4.2. 2nd control proteome of *L. pneumophila* RI243 following ~20 hours of growth in YEB. This gel served as the control for proteomes prepared with a pH 5-8 IPG strip. Markers were not identified.

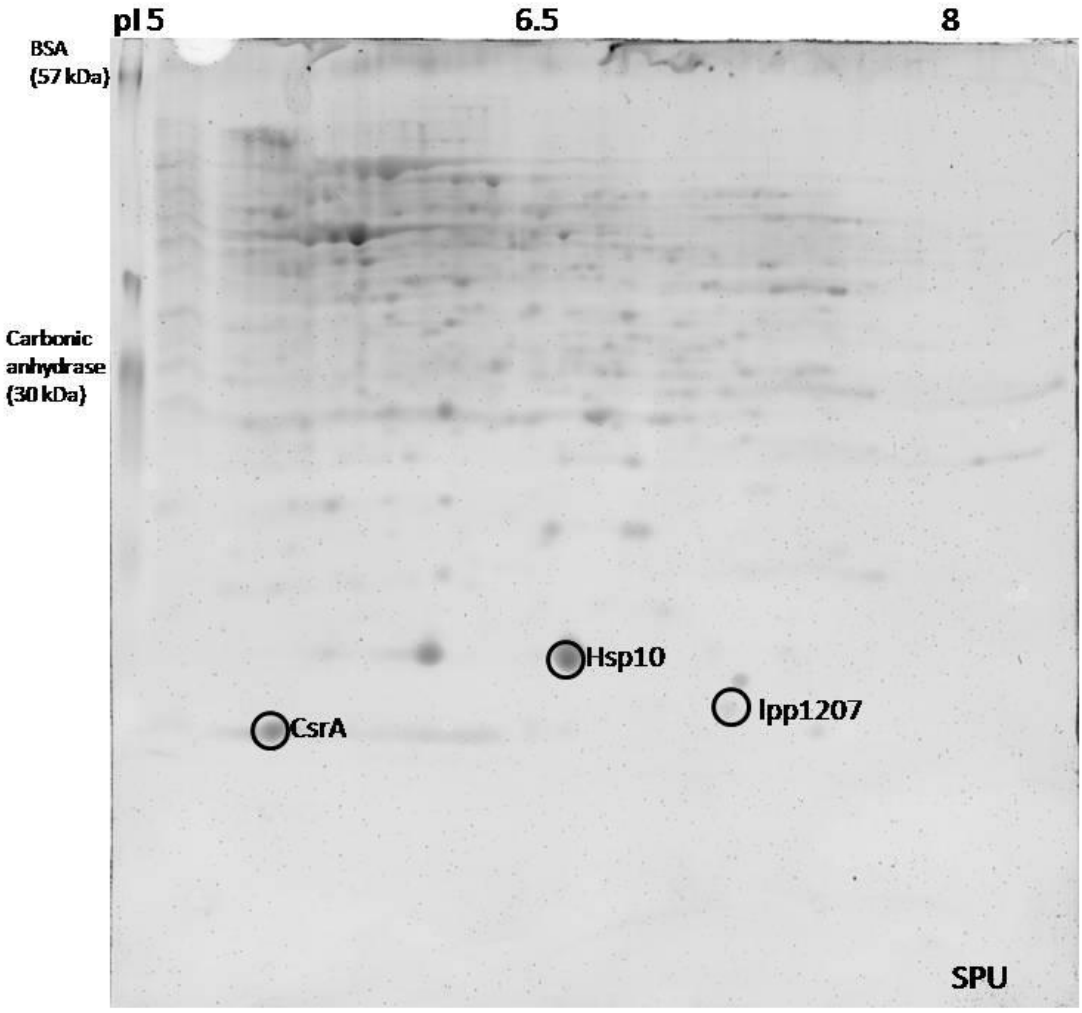

Figure 4.3. Proteome of *L. pneumophila* RI243 culture exposed to 24 hour cell-free supernatants prepared from *S. pyogenes*. Lpp1207 was identified for its absence from the control proteome (see Figure 1). The other proteins identified, i.e. CsrA and Hsp10, were used as markers to aid in spot matching during the analytical process.

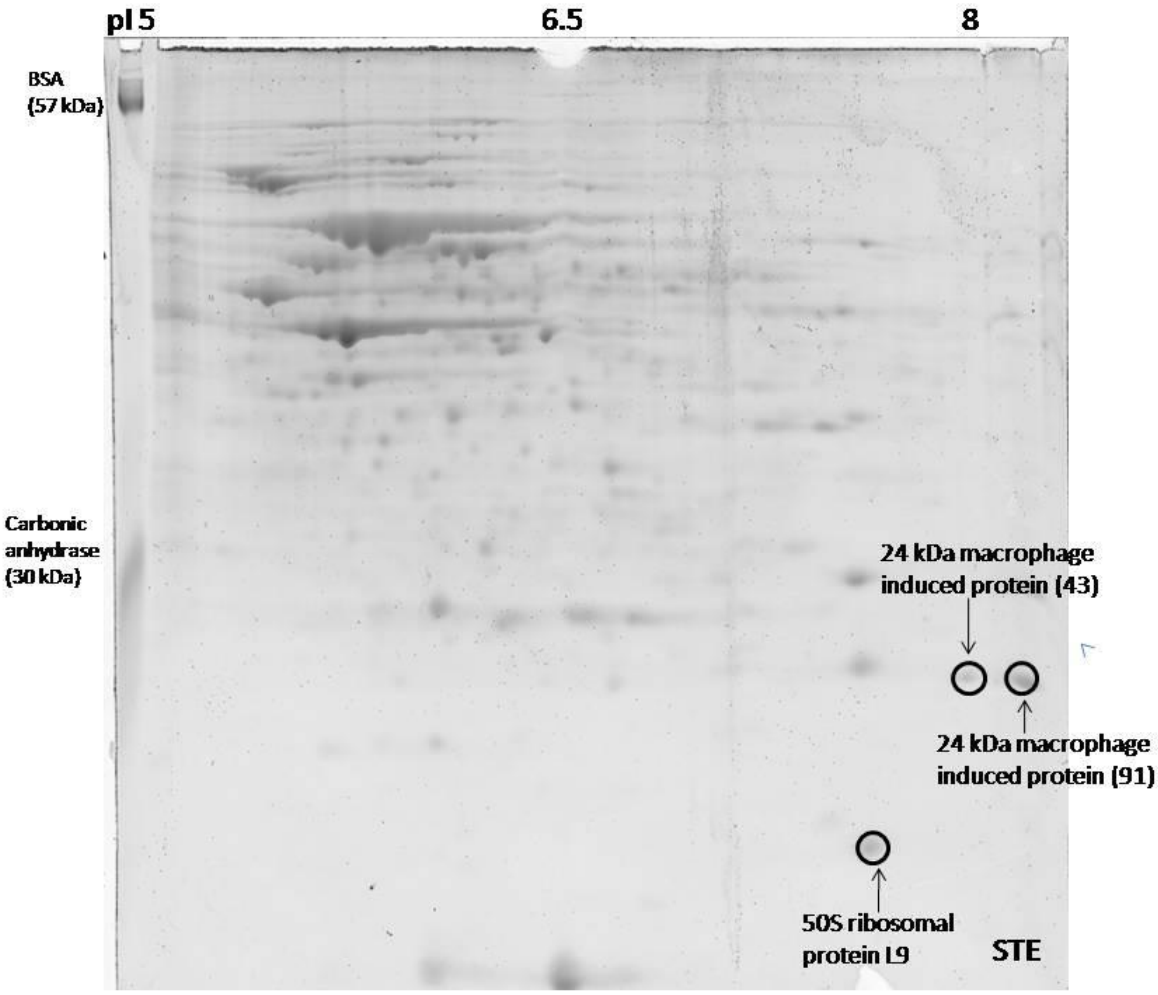

Figure 4.4. Proteome of *L. pneumophila* RI243 culture following exposure to 24 hour cell free supernatants prepared from *S. enterica* serovar Typhimurium. Both of the proteins listed as 24 kDa macrophage induced protein are annotated by their MASCOT scores in parenthesis. 50S ribosomal protein was used as a marker to aid in spot matching during the analytical process.

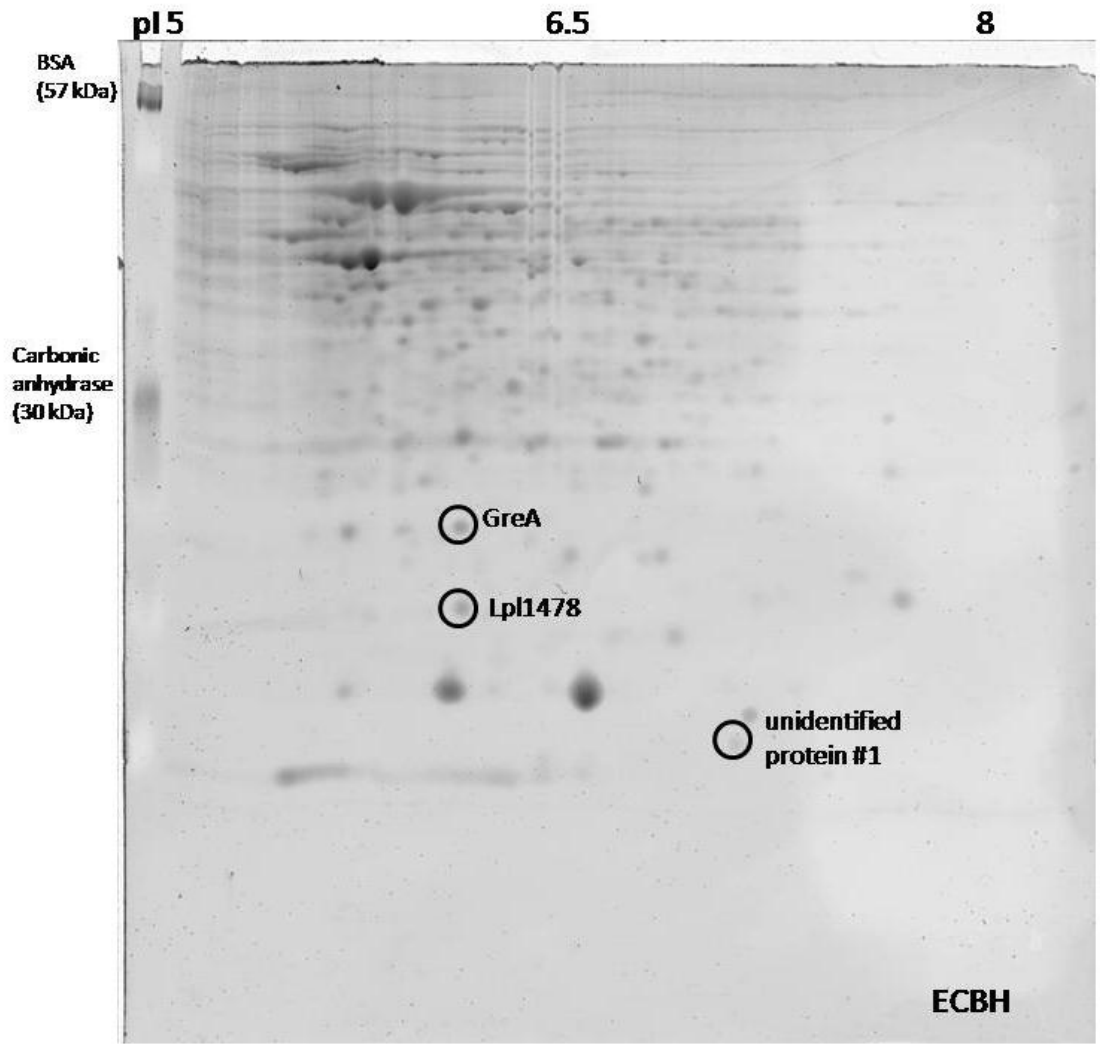

Figure 4.5. Proteome of *L. pneumophila* RI243 culture following exposure to 24 hour cell free supernatant prepared from *E. coli* O157:H7. 2 of the differentially expressed proteins were presumptively identified as GreA and Lpl1478. The third, unidentified spot (unidentified protein #1) is shown as one that was differentially expressed but could not be identified during MASCOT analysis.

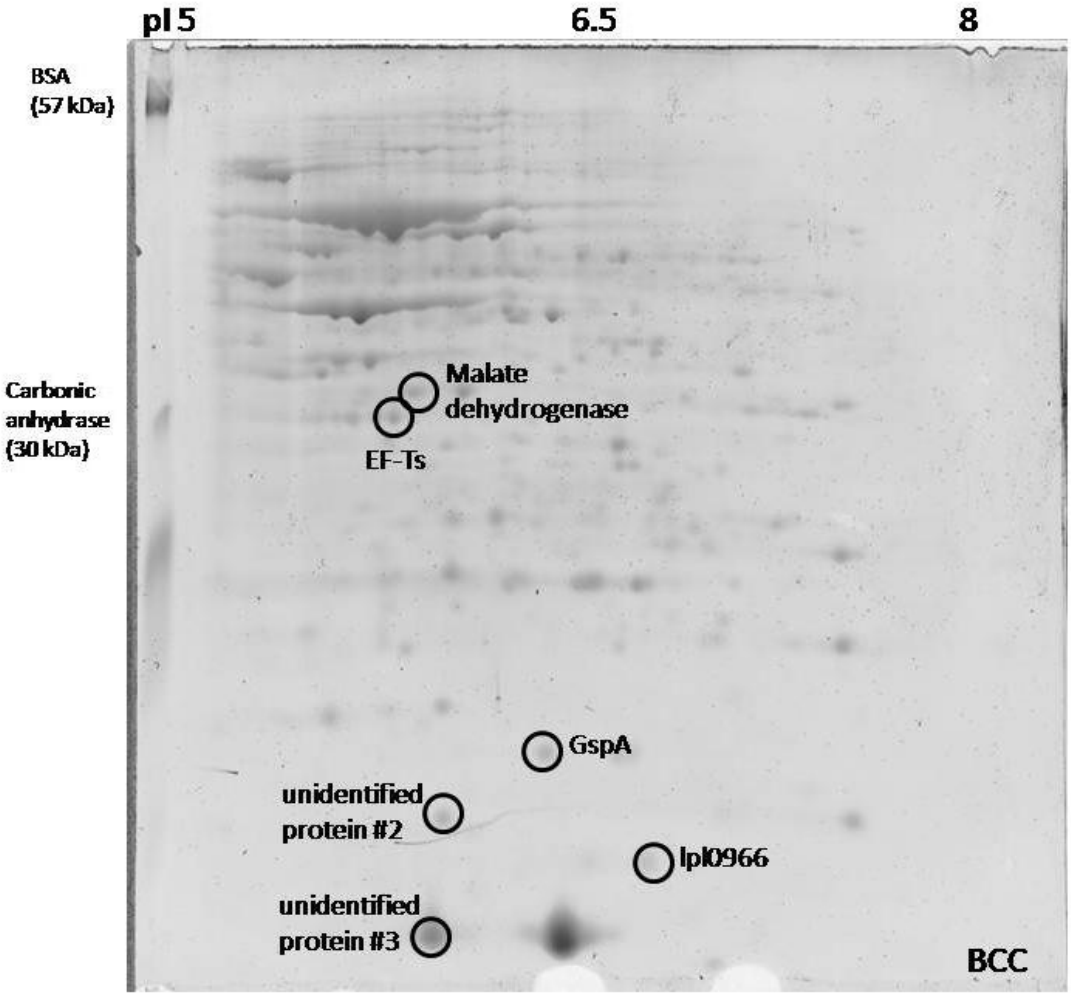

Figure 4.6. Proteome of *L. pneumophila* RI243 culture following exposure to 24 hour cell free supernatants prepared from *B. cereus*. The proteins, which were presumptively identified as malate dehydrogenase, EF-Ts, GspA, and hypothetical protein lpl0966 were differentially expressed. Unidentified protein #2 was also differentially expressed but could not be identified by MASCOT. The remaining protein, unidentified protein #3, was used as a marker to aid in gel matching during the spot selection process.

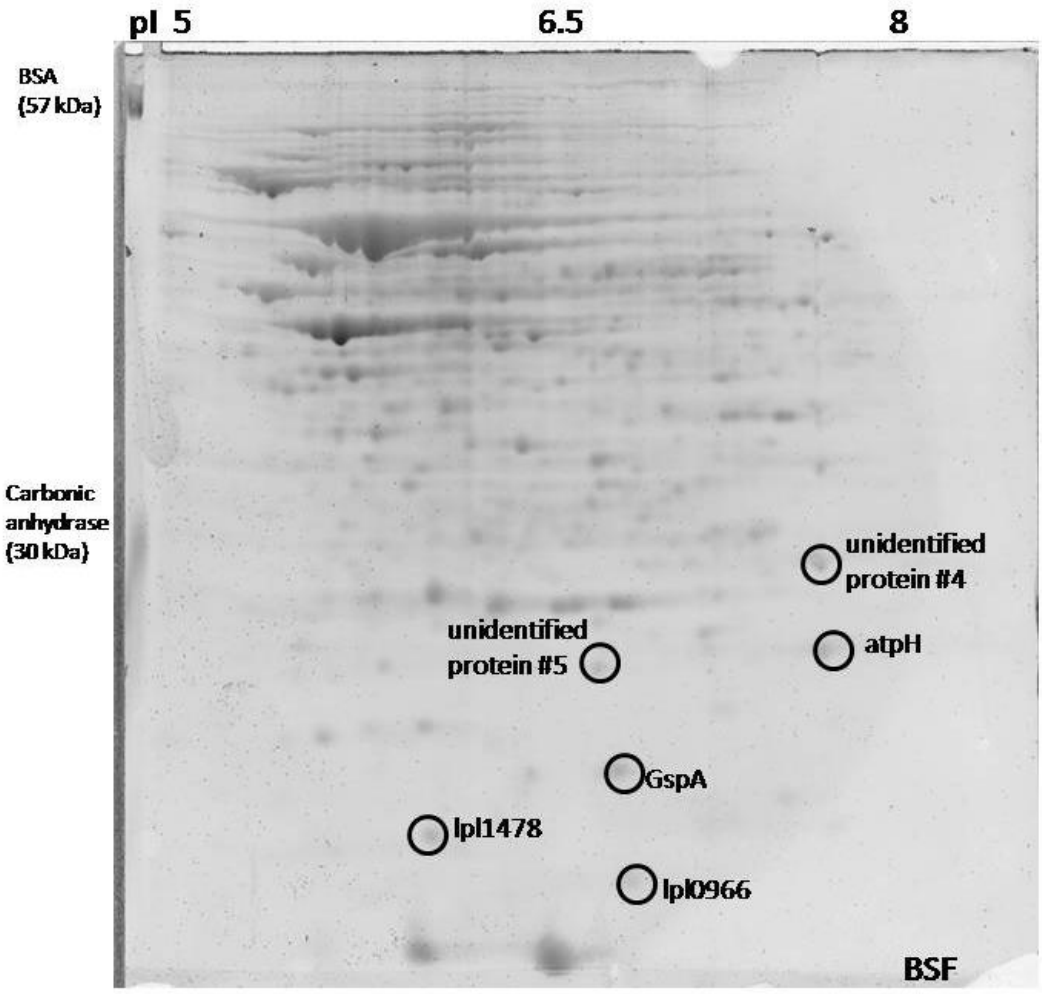

Figure 4.7. Proteome of *L. pneumophila* RI243 culture following exposure to 24 hour cell free supernatants prepared from *B. subtilis*. Hypothetical proteins lpl1478, lpl0966, and atpH were all selected and identified based on differential expression when compared to the control proteome. GspA was also differentially expressed. Proteins labeled #1 and #2 were also differentially expressed but could not be identified via MASCOT.

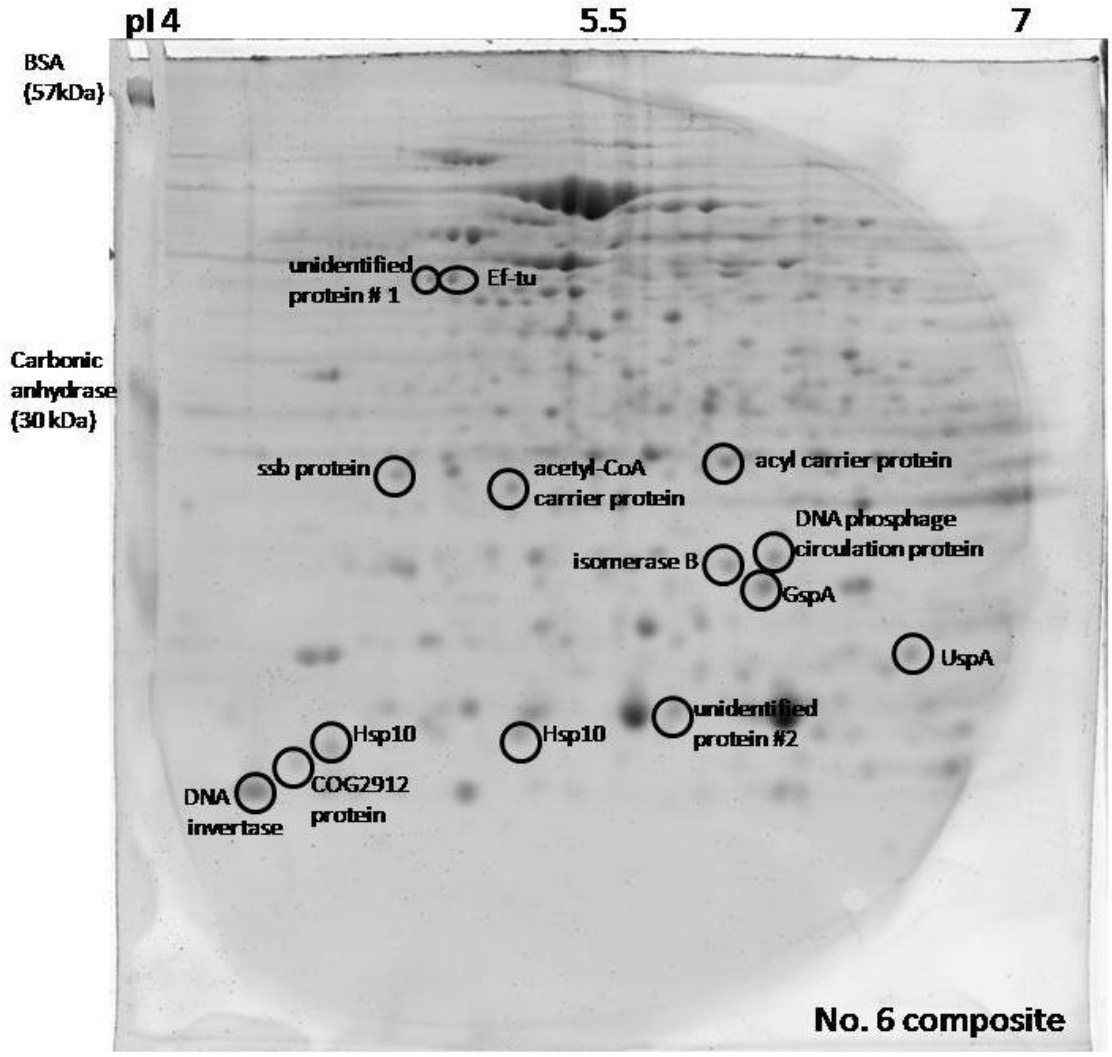

Figure 4.8. Composite proteome of *L. pneumophila* RI243 culture. This protein composite was prepared by combining 75µg of *L. pneumophila* proteins from each of 4 separate supernatant trials: *S. aureus* supernatant, autoclaved *S. aureus* supernatant, *E. coli* DH5α supernatant, and *E. coli* O157:H7 supernatant. 12 of the 14 proteins shown were presumptively identified. MASCOT scores are annotated in Table 4.2

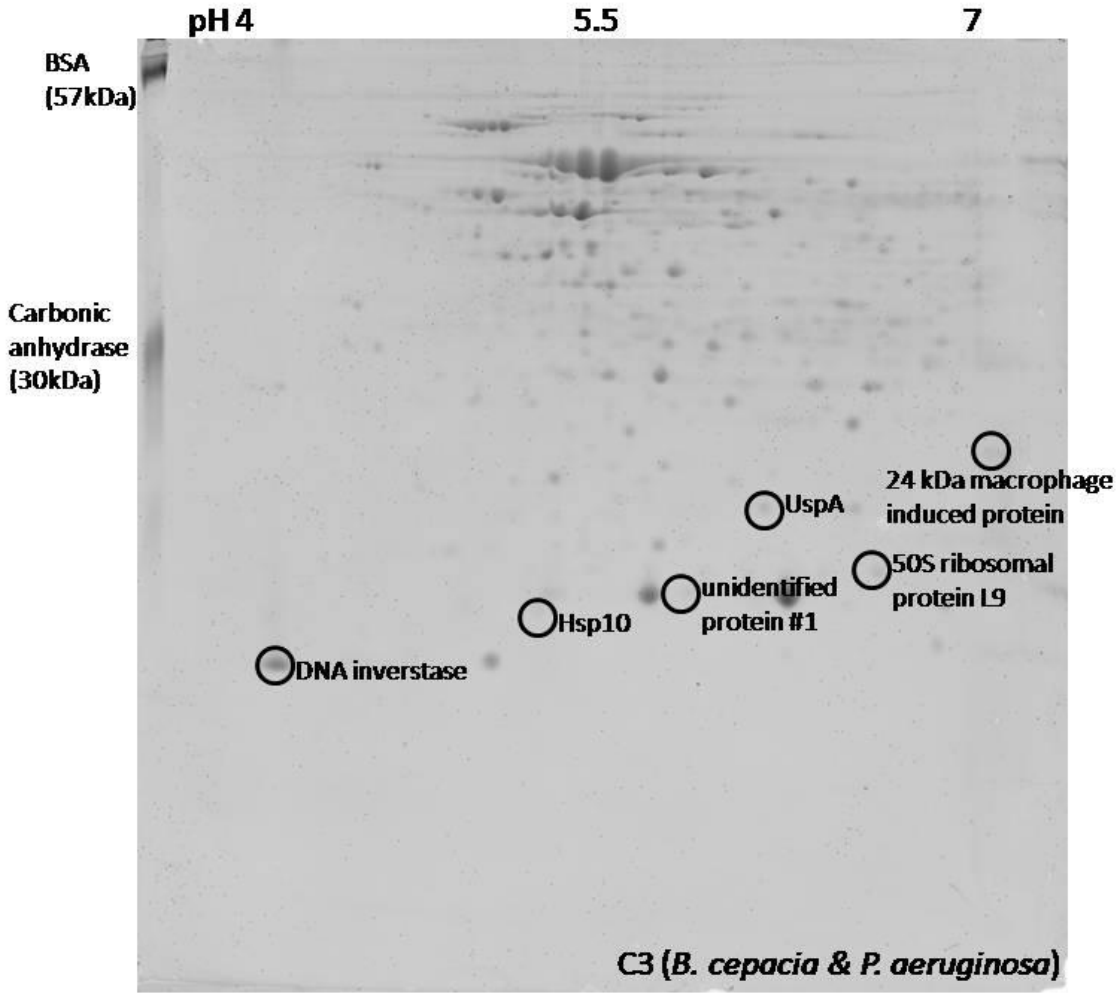

Figure 4.9. Composite proteome of *L. pneumophila* RI243 proteins. This composite was prepared by combining 150 µg of *L. pneumophila* protein from each of 2 separate trials in which the pathogen was exposed to *P. aeruginosa* and *B. cepacia* supernatants. 5 of the 6 proteins shown were presumptively identified. 50S ribosomal protein was identified as a marker and was not differentially expressed. MASCOT scores are annotated in Table 4.3.

Gel	Spot ID	MASCOT Number	Differential Expression
Control	27kDa outer membrane protein (*L. pneumophila* subsp. *pneumophila* subsp. Philadelphia)	88	No
	50S ribosomal protein L9 (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	60	No
S. pyogenes	Global regulator CsrA (*L. pneumophila* str. Paris)	90	No
	Hsp10, 10 kDa chaperonin GroES (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	78	No
	Hypothetical protein Lpp1207 (*L. pneumophila* str. Paris); cold shock domain family protein CspA (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	108; 91	Yes
S. enterica	50S ribosomal protein L9 (*L. pneumophila* str. Paris)	56	No
	24 kDa macrophage induced protein (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	91	Yes
	24 kDa macrophage induced protein (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	43	Yes

Table 4.1. Consolidated list of proteins identified from the *L. pneumophila* RI243 control gel and of *L. pneumophila* following exposure to the supernatants of signal producing microorganisms (Figures 4.1 through 4.6). A MASCOT score of $\geq$73 is considered a significant match.

Gel	Spot ID	MASCOT Number	Differential Expression
***E. coli* O157:H7**	Transcription elongation factor GreA (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	69	Yes
	Hypothetical protein lpl1478 (*L. pneumophila* str. Lens)	63	Yes
	Unidentified protein #1	NA	Yes
B. cereus	Malate dehydrogenase (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	42	Yes
	Elongation factor Ts (EF-Ts) (*L. pneumophila* str. Paris)	63	Yes
	GspA (global stress protein) (*L. pneumophila* str. Paris)	76	Yes
	Hypothetical protein lpl0966 *L. pneumophila* str. Lens)	35	Yes
	Unidentified #2	NA	Yes
	Unidentified #3	NA	No
B. subtilis	Hypothetical protein lpl1478 (*L. pneumophila* str. Lens)	56	Yes
	Global stress protein GspA *(L. pneumophila* str. Lens)	81	Yes
	Hypothetical protein Lpl0966 (*L. pneumophila* str. Lens) and universal stress protein A (UspA) (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	84 (both)	Yes
	Hypothetical protein atpH (*L. pneumophila* str. Lens)	73	Yes
	Unidentified #4	NA	Yes
	Unidentified #5	NA	Yes

Table 4.2. Continued list of proteins identified from the *L. pneumophila* RI243 control gel and of *L. pneumophila* following exposure to the supernatants of signal producing microorganisms (Figures 4.1 through 4.6). A MASCOT score of $\geq$73 is considered a significant match.

Gel	Spot ID	MASCOT Number	Differential Expression
No. 6 Composite	Putative DNA invertase (*P. aeruginosa*)	45	Yes
	COG2912 uncharacterized conserved protein (*Magnetospirillum megnetotacticum*)	47	Yes
	Hsp10, 10kDa chaperonin GroES (*L. pneumophila* subsp *pneumophila* strain Philadelphia)	56	Yes
	Hsp10, 10kDa chaperonin GroES (*L. pneumophila* subsp *pneumophila* strain Philadelphia)	61	Yes
	Universal stress protein (UspA) (*L. pneumophila* subsp. *pneumophila*)	63	Yes
	Global stress protein GspA (*L. pneumophila* str. Paris)	55	Yes
	Mu-like prophage DNA circulation protein *Vibrio cholera* 0395)	41	No
	Peptidyl-prolyl cis-trans isomerase B (cyclophillin-type PPIase family) (*L. pneumophila* str. Paris)	67	No
	3-oxoacyl-(acyl carrier protein) reductase (*Dienococcus geothermalis* DSM 11300)	54	No
	Acetyl-CoA carboxylase biotin carboxyl carrier protein (*L. pneumophila* str. Paris)	55	No
	Single strand binding protein (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	56	Yes
	Unidentified #1	N/A	Yes
	Translation elongation factor Tu (EF-Tu) (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	88	No
	Unidentified #2	N/A	Yes

Table 4.3. Consolidated list of proteins identified from the exposure of *L. pneumophila* to *S. aureus* and *E. coli* supernatants (Figure 4.7). A MASCOT score of $\geq$73 is considered a significant match. 12 of the 14 proteins listed here were presumptively identified. 9 of the proteins were induced in the presence of heterolgous supernatants.

Gel	Spot ID	MASCOT Number	Differential Expression
Gel C3	24 kDa macrophage induced protein (*L. pneumophila* str. Lens)	55	Yes
	50S ribosomal protein L9 (24 kDa macrophage induced protein (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	60	No
	Universal stress protein (UspA) (*L. pneumophila* subsp. *pneumophila*)	56	Yes
	Hsp10, 10 kDa chaperonin GroES	78	Yes
	Putative DNA invertase (*P. aeruginosa*)	45	Yes
	Unidentified protein #1	NA	Yes

Table 4.4. Consolidated list of proteins identified from the exposure of *L. pneumophila* to *P. aeruginosa* and *B. cepacia* supernatants (Figure 4.8). A MASCOT score of $\geq$73 is considered a significant match. Five out of the 6 proteins listed were presumptively identified. Five of the proteins were induced in the presence of heterologous supernatants. 50S ribosomal protein L9 was identified as a marker during the analysis process and for informational purposes.

V. EVALUATION OF INTERCELLULAR SIGNALING BETWEEN *LEGIONELL A PNEUMOPHILA* AND MULTIPLE PROTOZOAN HOSTS

Abstract

Legionella pneumophila interacts with with protozoa in its natural habitat. Protozoa support the growth requirements for this organism, which has not been conclusively demonstrated to grow in the absence of a suitable host in an environmental setting. In an effort to further define the pathogen's capacity for intercellular signaling and/or its ability to respond to host cell cues, assays were performed in which *L. pneumophila* was exposed to supernatants of protozoa that are known to support the intracellular growth of the pathogen, i.e. *Hartmannella vermiformi*s, two *Acanthamoeba* species, and two *Tetrahymena* species. Following a brief culture period, extracted proteins were resolved via 2-dimensional electrophoresis. A comparison of proteomes of control cultures to those exposed to host supernatants revealed the induction of multiple proteins in the latter. Thirty-six of these proteins were analyzed via MALDI-TOF. The peptide spectra were matched through BLAST searches at www.MASCOT.com, revealing identities that include multiple hypothetical proteins, a *Legionella* long chain fatty acid transporter, cold shock domain protein CspA, a potential sigma 54 modulation protein, and a potential malate dehydrogenase. The results support the hypothesis that *L. pneumophila* mounts a coordinated response to its host prior to establishing contact and that this pathogen can sense intercellular host signals or a secreted metabolic product.

1. Introduction

Legionella species are frequently isolated from microbial biofilms. Within this matrix, legionellae are closely associated with multiple species of prokaryotes and protozoa. Thus, legionellae, i.e. *L. pneumophila* strains, are potentially exposed to intercellular signals produced by neighboring microorganisms, and furthermore is subject to grazing by bacterivorous protozoa (Murga et al., 2001). Various species of protozoa support the intracellular growth of legionellae, including the members of the genera *Hartmannella, Acanthamoeba, Balamuthia, Dictyostellium, Echinamoeba,* and *Tetrahymena, Naegleria,* and *Vahlkampfia* (Holden et al., 1984; Fields et al., 1990; Tyndall and Domingue, 1982; Newsome et al., 1985; Shadrach, 2005; Fields et al., 1989; Rowbotham, 1986; Solomon et al., 2000). Infection appears to be mediated by both the pathogen and its host, where *Legionella* has been noted for its active pursuit of host cells and conversely, potential hosts have been observed feeding on legionellae. In the case of the former, Rowbotham (1983) observed the attraction of legionellae to extended *A. polyphaga* trophozoites prior to their phagocytosis. With regard to the latter, protozoa are known to be predators of bacteria, where grazing has been noted in soil and aquatic environments that include both biofilms and planktonic communities (Eisenmann et al., 1998; Matz and Jurgens, 2003; Parry, 2004; Weekers et al., 1993). The review that follows demonstrates that under many circumstances, the interactions between bacteria and protozoa are governed by chemical compounds that solicit a response by the recipient, and therefore may be more specifically classified as intercellular communication signals.

Bacteria susceptible to protozoan grazing are known to recognize the presence of these predators, and subsequently respond with “pre-ingestional” defense mechanisms

(Hahn et al., 1999; Jurgens et al., 1999; Matz et al., 2004; Matz and Jurgens, 2005). These include the formation of large, inedible microcolonies; the induction of larger, grazing resistant morphotypes; enhanced motility; and the production of anti-predatorial toxins. The intercellular signals involved in quorum sensing mediate some of these responses. Cytotoxic pigments produced by *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, and *Serratia marcescens* are both regulated by autoinducers and toxic to grazing protozoa (Castro, 1967; Matz et al., 2004; Matz et al., 2004a). Specific examples of prokaryotic perception of protozoa include microcolony formation by *P. aeruginosa* PAOI in response to the presence of *Tetrayhymena* species and the flagellate *Rhynchomonas nasuta* (Hahn et al., 2000; Matz et al., 2004). In the absence of grazing protozoa, newly formed *P. aeruginosa* biofilms formed have been characterized as flat, thin, and uniform with no microcolony formation. Conversely, those biofilms assembled in the presence of *R. nasuta* yielded large, inedible microcolonies that were an average of 27 μm in size (Matz et al., 2004).

Conversely, examples of signal perception by protozoa include the chemo-attraction of the ciliate *Pseudocohnilembus marinus* to cell free extracts from *Vibrio natriegens* and *Vibrio neries* (Snyder, 1991). The expression of a "CD59-like" protein by *Naegleria fowleri* in response to the presence of *Escherichia coli* and *P. aeruginosa* also supports this concept. CD59 protein functions by protecting mammalian cells from complement mediated lysis. Following coculture of *N. fowleri* with the aforementioned prokaryotes, the amoeba was noted for the upregulation of "CD59-like" protein expression. The fact that the organisms remained intact despite the fact that bacteria were observed multiplying within vacuoles within its cytoplasm suggests a protective

role for this protein. When *Acanthamoeba* species were observed under the same conditions, these organisms failed to express "CD59-like" protein and further, succumbed to intercellular infection by *P. aeruginosa*. These data, taken with the fact that "CD59-like" protein was also expressed by *N. fowleri* in response to the cell-free, conditioned supernatants of *P. aeruginosa*, suggests that extracellular factors do facilitate the interactions of bacteria and protozoa and that these interactions may qualify as a form of intercellular communication (Fritzinger and Marciano-Cebral, 2004).

Among ciliates, extracellular communication mediates both predator-prey interactions and sexual propagation. Prey organisms, such as the ciliate *Euplotes octocarinatus* exhibit morphological changes following the perception of "kairomones" or predator induced chemical signals. These alterations, which include spines, keels, and ridges, inhibit engulfment by potential predators. While the production of such signals seem contrary to the feeding requirements of predatory ciliates such as *Amoeba proteus*, *Lembadion bullinum*, and *Stenostomum sphagnetorum*, kairomones also prevent cannibalism amongst the species that secrete them. For example, the "A-factor" kairomone synthesized by *Lembadion* species inhibits the ingestion of asexually produced clonemates (Kusch, 1999; Gortz et al., 1999). Another signal, called a "gamone", facilitates preconjugant interactions among certain species of protozoa. As mating pheromones, gamones are excreted by various ciliates, including *Blepharisma*, *Euplotes*, and *Dileptus*. The mating interactions of *Blepharisma japonicum* have been elucidated with detail, revealing that the ciliate produces 2 types of gamones, with each variety tailored to the attraction of a specific class of mating type (reviewed by Gortz et al., 1999).

Given the evidence that protozoa are capable of producing and responding to both homologous and heterologous intercellular signals, and that *Legionella* species require these organisms as hosts, the existence of an intercellular communication between these entities is highly probable. In addition to the observations made by Rowbotham (1983), the hypothesis that *L. pneumophila* can sense the presence of potential hosts is supported by evidence from biofilm experiments conducted by Murga et al (2001). The data showed that *L. pneumophila* displayed a diminished capacity for substratum colonization in the absence of the amoeba *H. vermiformis*. Conversely, *H. vermiformis* has also been shown to be responsive to *L. pneumophila* upon contact. An evaluation of its proteome revealed that 33 proteins were induced (12 of which could not be detected in resting amoebae) and 11 proteins were repressed. These results could not be replicated using heat-killed legoinellae, suggesting the presence of a secreted, extracellular factor. When *H. vermiformis* was exposed to cell-free *L. pneumophila* supernatants, two proteins of the previously detected proteins were expressed (Abu Kwaik et al., 1994).

Significant data in support of the hypothesis that legionellae can respond to secreted protozoan factors was produced using *in vitro* culture models of *L. pneumophila*, human monocytic leukemia cells (Mono Mac 6 or MM6) cells, and *A. castellanii*. Using a Transwell insert to separate infected MM6 host cells from *A. castellanii*, Neumeister et al (2000) demonstrated that significant increase in the intracellular growth of five *Legionella* species. *L. micdadei*, *L. longbeachae*, *L. gormanii*, *L. steigerwaltii*, and *L. dumoffii* demonstrated enhanced growth in MM6 in the presence of *A. castellani*. In the case of *L. dumofii*, MM6 cells are permissive to its intracellular growth only in the presence of *A. castellanii*. With the exception of *L. steigerwaltii*, enhanced growth was

also observed for all legionellae-MM6 cultures in the presence of *A. castellanii* supernatants as well as those prepared from previous MM6-legionellae cocultures. A "secreted amoebal substance" was suggested for the growth stimulating factor in the amoeba supernatants (Neumeister et al., 2000). This effect was not observed with *L. pneumophila*, although a similar phenomenon with regard to the enhancement of growth and virulence has been reported in the presence of *H. vermiformis* when inoculated into mice (Brieland et al., 1996)

The eukaryotic host cell response to *L. pneumophila* has been well characterized. Once *L. pneumophila* has invaded its host, the pathogen initiates the expression of multiple virulence factors and alterations in its phenotype. While mechanisms that govern the intracellular replication of *L. pneumophila* have been the subject of numerous investigations, there is little documentation regarding *L. pneumophila*'s perception of its host prior to attachment and furthermore, if intercellular signals facilitate this interaction. Unanswered questions include: (i) can *L. pneumophila* perceive the presence of potential hosts, (ii) if host recognition does take place, is communication mediated through intracellular signals, and (iii) does this pathogen actively pursue a host niche or is the encounter merely one of chance?

Given the propensity of prokaryotes and protozoa to perceive one another, the objective of the following research was to determine the ability of *L. pneumophila* to respond to protozoa by evaluating its protein expression following exposure to cell-free protozoa supernatants.

2. Materials and Methods

2.1 Cultivation of bacteria

L. pneumophila RI243 was used to determine its response to protozoa. BCYE supplemented with L-cysteine (0.4g/L) and ferric pyrophosphate (0.25 g/L) and yeast extract broth (YEB) was used for cultivation *in vitro*. *L. pneumophila* was grown at 37℃ in a 3-5% CO_2 atmosphere for 3 days.

2.2 *Cultivation of protozoa*

Five species of protozoa were selected for response assays, *H. vermiformis*, *T. pyriformis* ATCC 30005, *T. thermophila* SB mutant 255, *A. castellanii*, and *A. polyphaga*. These species were selected for their ability to support the intracellular growth of *L. pneumophila*. *T. thermophila* SB 255 mutant does not have the ability to exocytose mucocysts and some variants possess two mouths (personal communication, C. Sundermann). *H. vermiformis* was supplied by Dr. Barry Fields (CDC, Atlanta, GA). Both species of *Tetrahymena* were provided by Dr. Christine Sundermann (Auburn University, Auburn, AL) and the *Acanthamoeba* species were supplied by Dr. Sang-Jin Suh (Auburn University, Auburn, AL). All protozoa cultures were maintained axenically.

H. vermiformis was cultured in axenic growth medium (ATCC #1034 PYNFH medium). The medium is composed of three components. The based medium was prepared by combining 10.0 g peptone, 10.0 g yeast extract, 1.0 g yeast nucleic acid, 15.0 mg folic acid, and 1.0 mg hemin in 880 mL of distilled, deionized water (ddH_2O). A buffer solution was prepared by combining 18.1 g KH_2PO_4 and Na_2HPO_4 in 1L of

ddH_2O. These components were sterilized separately by autoclaving (standard conditions). After adding 20 mL of the buffer solution to the base medium, the pH was adjusted to 6.5 and filter sterilized. The medium was adjusted to a final concentration of 10% (v/v) fetal bovine serum before use. PYNFH medium was stored under refrigeration and warmed before use. *H. vermiformis* was cultured in flat bottomed, T-75 tissue culture flasks in 40mL volumes until a dense monolayer could be observed on the flasks surface. This was typically observed following 5-7 days of growth at 35 ºC. When required, protozoa were dislodged from the flasks surface by tapping the flask sharply against a hard surface. New cultures were initiated by transferring 100 µL of the protozoan suspension to fresh growth medium. *H. vermiformis* was prepared for long-term storage by harvesting trophozoites in fresh PYNFH medium and replacing it with a freezing medium composed of PYNFH adjusted to 20% dimethylsulfoxide (DMSO). Trophozoites were frozen at -70 ºC (CDC protocol).

Tetrahymena species were grown in 1% (w/v) proteose peptone at room temperature. Protozoa were maintained in sterile, 50 mL conical tubes and were established by transferring 100 µL of the stock culture to fresh media. For assays, protozoa were grown in ~40 mL volumes until dense growth was observed, typically 5-7 days. Stock cultures were maintained in 1% proteose peptone and passaged to fresh media every 2-3 weeks.

Acanthamoeba cultures were grown in proteose-peptone-glucose medium (PPG). PPG is composed of 15.0 g proteose peptone and 18.0g D-glucose per liter of Page's Amoeba Saline (PAS). PAS is composed of 0.12 g NaCl, 0.004 g $MgSO_4 \cdot 2H_2O$, 0.004 g $CaCl_2 \cdot 2H_20$, 0.14 g Na_2HPO_4 monobasic, and 0.135 g KH_2PO_4 monobasic per liter of

ddH_2O. For growth of *A. castellanii*, the solution was filter sterilized, while the medium was autoclaved and then filtered for *A. polyphaga* growth. Both amoebae were cultured in flat bottomed, T-75 tissue culture flasks with *A. castellanii* being cultivated at 37 ºC and *A. polyphaga* at 30 ºC. Amoebae were cultured in 25-40 mL volumes of PPG for 5-7 days, which is the point at which a confluent monolayer was observed. For maintenance of stock cultures, amoebae were passaged every 2-3 weeks in PPG.

For the preparation of supernatants for assays, cultures suspensions were pelleted by centrifugation at 12,000 rpm, and the supernatants were rendered cell-free by passage through a 0.45 μm filter. Supernatants were stored under refrigeration until required for use, typically within 24 hours of preparation.

2.3 *Assays for L. pnemophila response to protozoan supernatants*

For response assays, protozoan supernatants were required. On the day prior to response assays, *L. pneumophila* was cultured in 10 ml of YEB. Following overnight growth of *Legionella*, the cultures were streaked on BCYE, BCYE with 100 mM NaCl, and TSA. This was incorporated for quality control, i.e legionellae cannot grow on TSA, and to ensure that the organisms had reached stationary phase, as organisms in stationary phase are sensitive to 100 mM NaCl (Byrne and Swanson, 1998). *L. pneumophila* was prepared for experimentation by washing the cells 3 times with 10 ml 0.5X PBS. Following the resuspension of washed *L. pneumophila* in the test medium (5ml 2X YEB and 5 ml conditioned protozoan supernatant), the culture was incubated for 3 hours at 37 ºC with aeration, after which it was streaked a second time on TSA, BCYE, and BCYE with 100 mM NaCl. Each experiment was repeated at least twice. When required, *L. pneumophila* was also tested for its response to undiluted supernatants.

2.4 *Protein isolation and quantification*.

Following exposure of *L. pneumophila* to protozoan supernatant, the cells were prepared for extraction of intracellular proteins. The cells were pelleted via centriguation at 14,000 x g for 5 minutes, after which the pellet was washed 3 times with 40 mM Tris-HCL pH 7.2 and resuspended in 100 µl of lysis solution composed of 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-HSL, and 0.2% Bio-Lyte 3/10 [Bio-Rad]). To minimize proteolysis, a protease inhibitor mix (Amersham Biosciences) was added at a final concentration of 10 µl/ml (v/v). After incubation on ice with agitation for 1 hour, the suspensions were transferred to a 1.5 ml microcentrifuge tube for centrifugation for 30 minutes at 8 °C, 18,000 x g. The supernatant, containing soluble protein, was decanted and stored at -20 °C (Park et al., 2003). All protein samples were quantified against a BSA standard by the method of Bradford (1976).

2.5 *Protein Separation by 2-Dimensional Electrophoresis.*

Intracellular *L. pneumophila* proteins were resolved via 2-dimensional electrophoresis (2-DE). Proteins were separated in the first dimension by isoelectric focusing. This was accomplished by combining 300 µg of protein with rehydration buffer (8M urea, 2% CHAPS, 50mM DTT [dithiothreitol], and 0.2% 3-10 Bio-Lyte ampholytes, BioRad; Hercules, CA) to a final volume of 300 µl. To reduce streaking between protein spots as the result of the oxidation of disulfide groups, DeStreak Reagent (Amersham Biosciences; Westborough, MA) was added to the final solution in accordance with manufacturer recommendations. The solution was used to rehydrate an

18 cm, pH 4-7 IPG (immobilized pH gradient; BioRad; Hercules, CA) strip for 16-24 hours. Rehydrated strips were focused in accordance with the manufacturer's recommendations using a Protean IEF Cell (BioRad; Hercules, CA). The following parameters were used for isoelectric focusing, culminating in a total of 50,000 volt-hours (V-hr): temperature of 20 ºC for electrophoresis, 30 minutes at 250V, followed by 2.5 hours at 10,000V, and 40,000 Volt-Hours at 10,000 volts (BioRad ReadyPrep 2-D Starter Kit Instruction Manual). Following IEF, IPG strips were equilibrated for 15 minutes each in equilibration buffer 1 (6M urea, 0.375M Tris pH 8.8, 2% SDS, 20% glycerol, 2% DTT) and equilibration buffer 2 (6M urea, 0.375M Tris pH 8.8, 2% SDS, 20% glycerol, 2.5% [w/v] iodoacetamide) with agitation, briefly rinsed in 1X Tris-glycine-SDS buffer, and applied to a 12% SDS-PAGE 20X20 cm gel (BioRad; Hercules, CA) for resolution in the second dimension by molecular weight. Proteins were resolved using a current of 20 mV for ~18 hours. Following electrophoresis, the proteins were fixed in the gel by submerging them in a solution of 10% methanol and 7% acetic acid (10/7) for 1 hour, washed with distilled, deionized water, and stained with SYPRO Ruby (BioRad; Hercules, CA) overnight. Prior to imaging using the Typhoon 9410 Scanner (Amersham Biosciences; Westborough, MA), the gels were destained using 10/7 methanol-acetic acid for 10 minutes.

2.6 Proteomic analysis

Each assay in which *L. pneumophila* RI243 was exposed to protozoan supernatants was completed twice, with a minimum of one gel produced per assay. This resulted in a minimum of 2 gels per experiment. 2-DE gels were analyzed using ImageMaster 2D Platinum (Amersham Biosciences; Westborough, MA). This program

was used in the initial comparison of gels for replicate assays and between assays and the proteome of the experimental control. Manual comparisons were conducted between the control and experimental gels using a UV box. Using this technique, spot matches in terms of differentially expressed proteins between the gels (proteomes) were established. Spots of interest, namely those that appeared to be induced following exposure to host cell supernatants, were selected for further analysis.

2.7 Preparation of proteins for identification

Proteins identification required that spots be digested, eluted from support matrices, and reapplied to a MALDI supportive matrix for identification by mass spectrometry. To accomplish this task, spots of interest were excised from 2-DE gels, transferred to a 1.5 ml microcentrifuge tube, and cut into small cubes using a small scalpel. Gel pieces were washed 3 times with 150 uL of a solution composed of 10 mM NH_4HCO_3 (ammonium bicarbonate) and 50% acetonitrile (ACN) for 15-20 minutes. After washing, the gel slices were dried for 30 minutes in a Speed Vac with no heat. To initiate proteolytic digestion of the protein samples, the gel slices were rehydrated with 5 μL of a solution composed of a 5:1 mixture of 10 mM NH_4HCO_3 and proteomics grade trypsin (Sigma Aldrich; St. Louis, MO), 20 μg/mL. Gel slices were reconstituted to their original volume by adding 15-20 μl (or more) of 10 mM NH_4HCO_3 to cover them, after which incubation was allowed to proceed for 1 hour at 37 ˚C. Following incubation, the gel slices were covered with an additional 15-20 μl of buffer (10 mM NH_4HCO_3) and then incubated overnight. Following incubation, the supernatant was removed from the gel slices and saved. Peptides were extracted from the gel cubes by 3 iterations of incubation in ~100 μL of a buffer composed of 10 mM NH_4HCO_3/60% ACN for 20-30

minutes at RT. Supernatants were removed after each incubation and combined with the supernatants retained from previous elutions. A final attempt at peptide extraction was completed by dehydrating the gel cubes completely with a small amount (100-150 µL) of isopropanol. Following a brief incubation (~5 minutes), the supernatant was removed and pooled with the other supernatants. Combined supernatants were condensed in a Speed Vac (without heat) to a small volume (~10 µL) and resuspended in 30 µL of 0.1% sequencing grade trifluoroacetic acid (TFA). Samples were further acidified by the addition of 1 µL 10% TFA and desalted with the aid of a C_{18} ZipTip (Millipore) in accordance with the manufacturer's protocol. Peptide samples were euluted from the ZipTip by pipetting 2 µL of matrix/ACN (1.5 µl of matrix stock solution and 0.5 µl of ACN) into the ZipTip. Peptides were eluted directly onto a MSP 96 target polished steel microScout Target plate (Bruker Daltonics; Billerica, MA) and allow completely air dry before conducting peptide analysis. The MALDI matrix used for peptide deposition is α-cyano-4-hydroxy cinnamic acid (CHCA; Bruker Daltonic; Billerica, MA), and was prepared at a concentration of 10mg/ml in a solution composed of equal volumes of Solution A (30% ACN, 25% methanol, 2% acetic acid) and Solution B (100% ACN) (CDC protocol).

2.8 *Microflex (matrix assisted laser desorption-ionization time of flight [MALDI-TOF]) and Data Analysis.*

MALDI-TOF analysis was completed with the Microflex Mass Spectrometer (Bruker Daltonics; Billerica, MA). Peptides were analyzed in accordance with the manufacturer's recommendations using the positive reflectron mode for mass spectrometry. Following the characterization of peptide peaks, the consolidated spectra

were formatted to remove background and baseline peaks, and subject to smoothing prior to entering the MASCOT search engine (http:www.matrixscience.com) for protein identification through the peptide mass spectrum. With regard to the proteins in the database, a value of 73 or greater was considered a significant match for protein identification.

To determine the confidence value of a given peptide score, MASCOT uses a database scoring algorithm called Mowse (www.matrixscience.com). MASCOT matches the spectrum derived from MALDI-TOF analysis to multiple sequence databases after which a score is produced. This score reflects the probability that the peptide-spectrum match observed between the unknown peptide and the protein identification in the database is a random event (Kall et al., 2008)

3. Results and Discussion

Thirty-six proteins were selected for analysis and identification, all of which were differentially expressed (Figures 5.1 through 5.6; Tables 5.1 through 5.4). Two of the proteins were identified with a MASCOT score of 73 or greater and were associated with *H. vermiformis* (Table 5.1). These were a long chain fatty acid transporter and cold shock domain family protein CspA, both of which are *L. pneumophila* proteins. One protein was presumptively identified with a MASCOT score of 71, i.e. a transcriptional regulator associated with *Mesorhizobium loti* (Table 5.2). All but 3 of the remaining proteins were presumptively identified with MASCOT scores between 38 and 69 (scores below 35 were not selected as presumptive identifications and were labeled as unidentified). Three of the proteins were labeled as unidentified (Table 5.1).

Although many of the factors that govern the relationship between legionellae (or other intracellular pathogens) and protozoa remain to be elucidated, a substantial amount of experimental data has been successfully applied to defining their coexistence. These include their natural association and the ability of legionellae to parasitize protozoan hosts and implicate protozoa in the furtherance of these microorganisms' pathogenicity. The latter concept has been has been eloquently elucidated using murine models and monocytic cell lines. The outcomes of *Legionella* infection in both is remarkably more effective either in the presence of certain protozoa or directly following egress from a protozoan host (Neumeister et al., 2000; Steinet et al., 1997; Cirilo et al., 1994). The data presented here complements the work done to date in that it helps to define this pathogens' behavior in the presence of protozoan hosts. If the mechanisms that favor *L.pneumophila*'s interaction with and subsequent infection of natural hosts can be elucidated, these facts can be exploited for the purposes of controlling and/or eradicating legionellae populations.

The present work was conducted to define the response by *L. pneumophila* in the phase prior to intracellular infection of its host. By working on the hypothesis that *L. pneumophila* is able to "sense" the presence of potential hosts either by intercellular signals or other chemical cues, a proteomics approach was selected to evaluate this concept. Given that the response to intercellular signals is identified by a particular phenotype which in turn is the product of gene expression, the bacterium's proteome was evaluated for changes that would be used as an indication of its ability to "respond" to intercellular cues from potential hosts. Thirty-six proteins were presumptively identified

as *a part* of the collective response by *L. pneumophila* to three protozoan genera, i.e. *Hartmannella*, *Tetrahymena*, and *Acanthamoeba*.

Only two of the proteins, i.e. a long chain fatty acid transporter and cold shock domain family protein CspA, were identified by MASCOT as a significant match (see Table 5.1). A third protein appeared to be closely identified with a transcriptional regulator of *Mesorhizobium loti* with a score of 71. The remaining proteins, including several *L. pneumophila* hypothetical proteins, a probable N-acetylgalactosaminyltransferase related to *Rhodopirellula baltica* SH 1, and three unidentified proteins were associated MASCOT scores between 38 and 69. Of note, the protein presumptively identified as COG1638 periplasmic component protein of *Magnetospirillum magnetotacticum* was identified in another analytical mode (termed "RP mode") as the autoinducer synthesis protein of *Rhodopseudomonas palustris* BisA531 (Table 4.2). While a MASCOT score of only 45 was assessed to this identification, the fact that the protein was detected as a response to *T. pyriformis* supernatants, and that it possesses a degree of similarity to autoinducing protein, suggests that this protein is a good candidate for further study.

While one of the more robust responses was noted with *H. vermiformis* in terms of *Legionella* proteins, the lack of true identifications suggests that the proteins that define the proteomic response of *L. pneumophila* to protozoan hosts is largely unchartered, and the proteins that constitute this response remain to be accurately identified. However, those proteins that were identified with some degree of confidence, i.e. CspA and CsrA, indicate the onset of a stress response. CsrA is a global repressor of multiple virulence traits, including motility, cytotoxicity, and heat and osmotic resistance.

Its expression during *L. pneumophila*'s replication period is hypothesized to promote growth and prevent the expression of transmission traits (i.e. motility, cytotoxicity, etc.) that would facilitate the infection of a new host (Molofsky and Swanson, 2003). Thus, the expression of this protein in response to *H. vermiformis* may be an indication that the pathogen recognized the presence of a suitable host, and in doing so, expressed the genes required to ensure a successful infection.

While the expression of cold shock protein CspA is consistent with a stress response, the appearance of this protein in the absence of cold temperatures, but in the presence of host supernatant suggests a function that is beneficial to the cell under both circumstances. Generally, the family of cold shock proteins facilitates measures that protect the microorganism from the effects of rapid temperature downshifts. These include decreasing membrane fluidity and destabilizing secondary RNA structures. While the function of CspA is believed to be that of an RNA chaperone capable of facilitating transcription and translation following a cold shock, CspA homolgues have been identified in a variety of host responses that include osmotic stress, the inhibition of replication, and resistance to antimicrobial peptides. Furthmore, two CspA homologues have also been identified in the hyperthermophilic bacterium *Thermotoga maritime*, expanding the function of CspA beyond its original application to the cold-shock response (reviewed by Phadtare, 2004). The diversity of CspA applications to the physiological state of microorganisms supports the plausibility that this protein can contribute to *L. pneumophila*'s "pre-ingestion" fitness with regard to host perception, which may result in its preparation for phagocytosis.

The perception of protozoan host signals or intercellular cues would confer multiple ecological advantages to *Legionella* sp., the most significant of which is the ability of legionellae to position themselves where they are most likely to be ingested. This concept was demonstrated by Murga *et al* (2001) in laboratory biofilm experiments. During this study, *L. pneumophila* was noted for its predilection for biofilms previously colonized with *H. vermiformis*. Although the bacterium was able to colonize biofilms devoid of protozoa, the organisms survived but failed to proliferate. The Neumeister (2001) study also supports this concept, particularly because it demonstrates that the mere presence of a permissive host, i.e. *A. castellanii*, is enough to stimulate the intracellular growth of legionellae in hosts previously unable to support the bacterium's reproduction.

While the data support a general response by *L. pneumophila* to protozoan signals or metabolic products, additional data would strengthen this argument. The use of replicate 2-DE gels of each experimental assay are used to verify both the consistency of the gels proteomic representation and reproducibility, which is highly subject to variability. Variables include anomalies in protein precipitation, polyacrylamide gel preparation, electrophoresis conditions, etc that will affect the final outcome of the proteome. To reduce this variability, multiple gels are typically run from the same sample, after which the gels are averaged together to ensure that a sufficient representation is produced (Hanes and Yates, 2000). Thus, the production of replicate gels serve to ensure that the data presented is the results of a response by *L. pneumophila* to eukaryotic hosts rather than the result of variations resulting from laboratory inconsistencies. Additional assays conducted using such techniques as transcriptome or DNA microarray analysis to analyze the expression of *L. pneumophila* coding transcripts

and genes responsive to the presence of heterologous supernatants would aid in substantiating the response of *L. pneumophila* to protozoan supernatants.

The data presented here represents a fraction of *L. pneumophila*'s response to the presence of protozoan hosts. Given that the emphasis of this research was both to establish the proteomics approach as a method by which the response potential intercellular signals may be measured and that a select component of the induced proteins was evaluated, this research represents only a partial response of *L. pneumophila* to the 5 protozoan hosts evaluated. The large number of permissive intracellular hosts, both of protozoan and mononuclear cell line in origin, underscores the fact that much of the pathogens "response phenotype" remains to be elucidated. That fact that most of the proteins could not be identified beyond "presumptive" suggests that the organism's behavior in the presence of its host remains unchartered. An understanding of its behavior in the phases prior to ingestion, however, will likely expose processes that can be exploited and thus, aid in novel approaches at controlling or eradicating *L. pneumophila.*

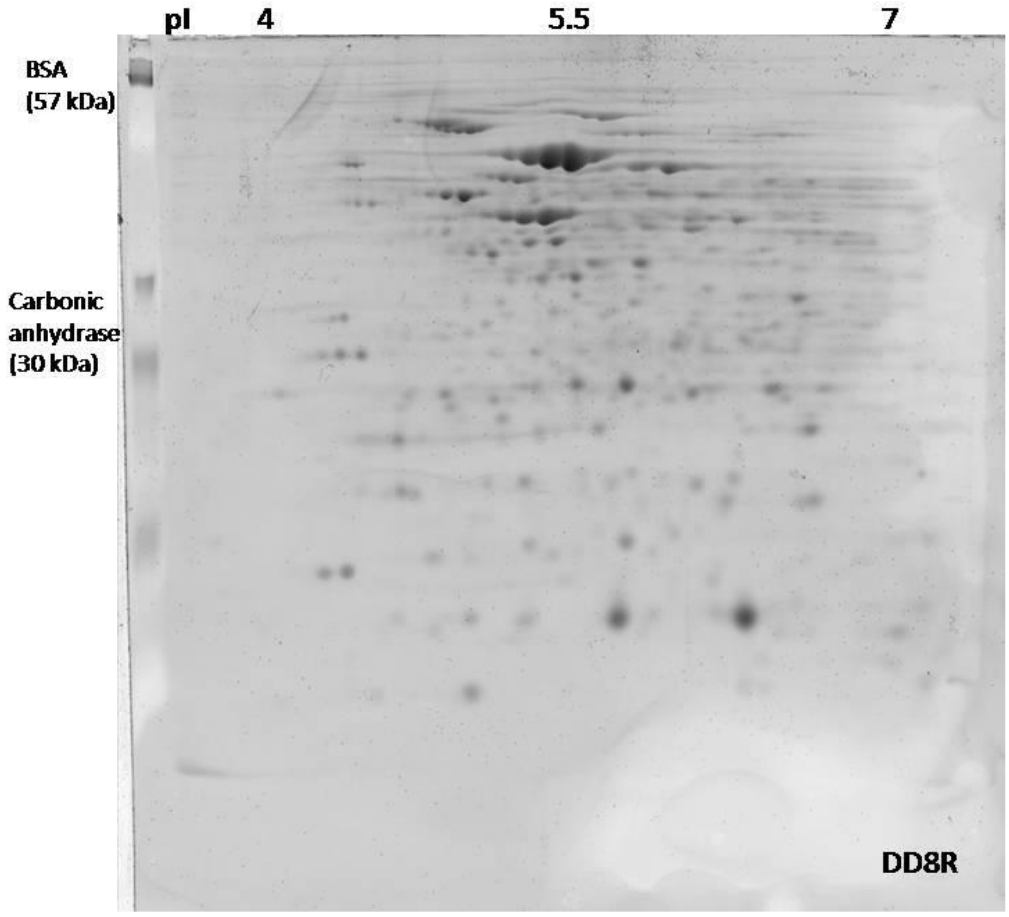

Figure 5.1. Proteome of *L. pneumophila* RI243 culture. (control). This proteome was used as the standard for comparison in the assessment of proteins that were induced following exposure to supernatants prepared from *H. vermiformis*, *T. pyriformis*, *T. thermophila*, *A. castellanii*, or *A. polyphaga*.

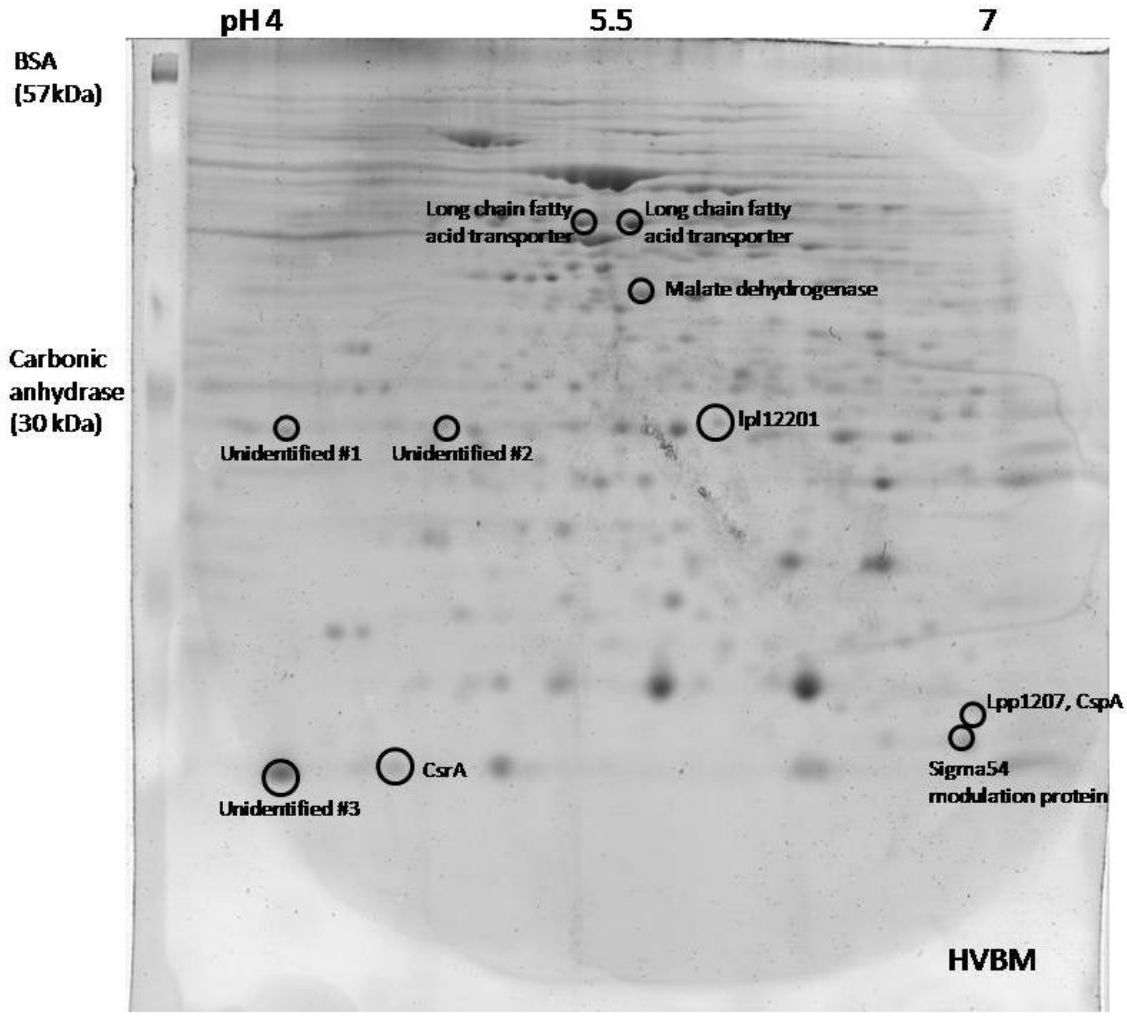

Figure 5.2. Proteome of *L. pneumophila* RI243 culture exposed to 5-7 day old supernatants prepared from *H. vermiformis*. A total of 10 proteins were selected for identification. With the exception of the long chain fatty acid transporter and CspA, the other protein identifications are presumptive. MASCOT scores are identified in Table 5.1.

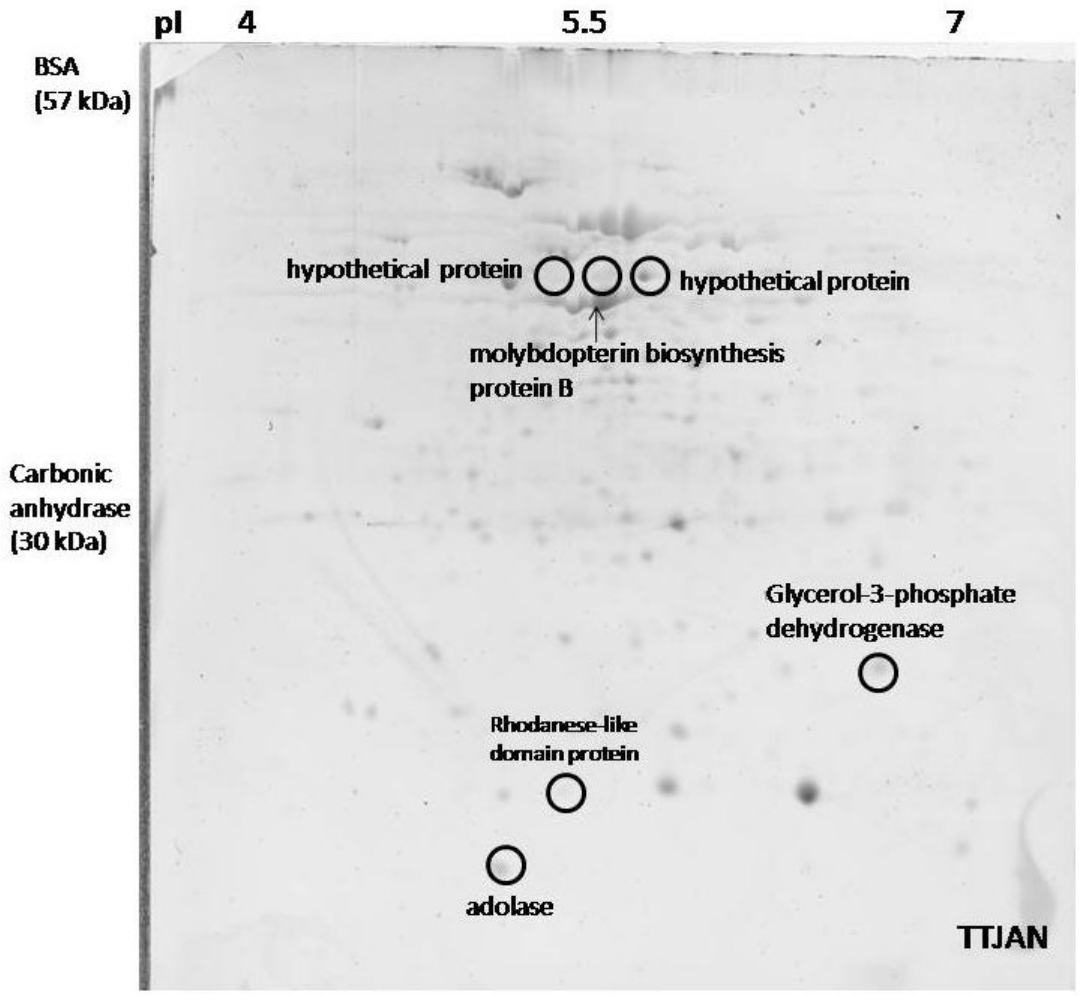

Figure 5.3. Proteome of *L. pneumophila* RI243 culture exposed to 5-7 day old supernatants prepared form *T. thermophila*. Six proteins were presumptively identified. MASCOT scores are identified in Table 5.3.

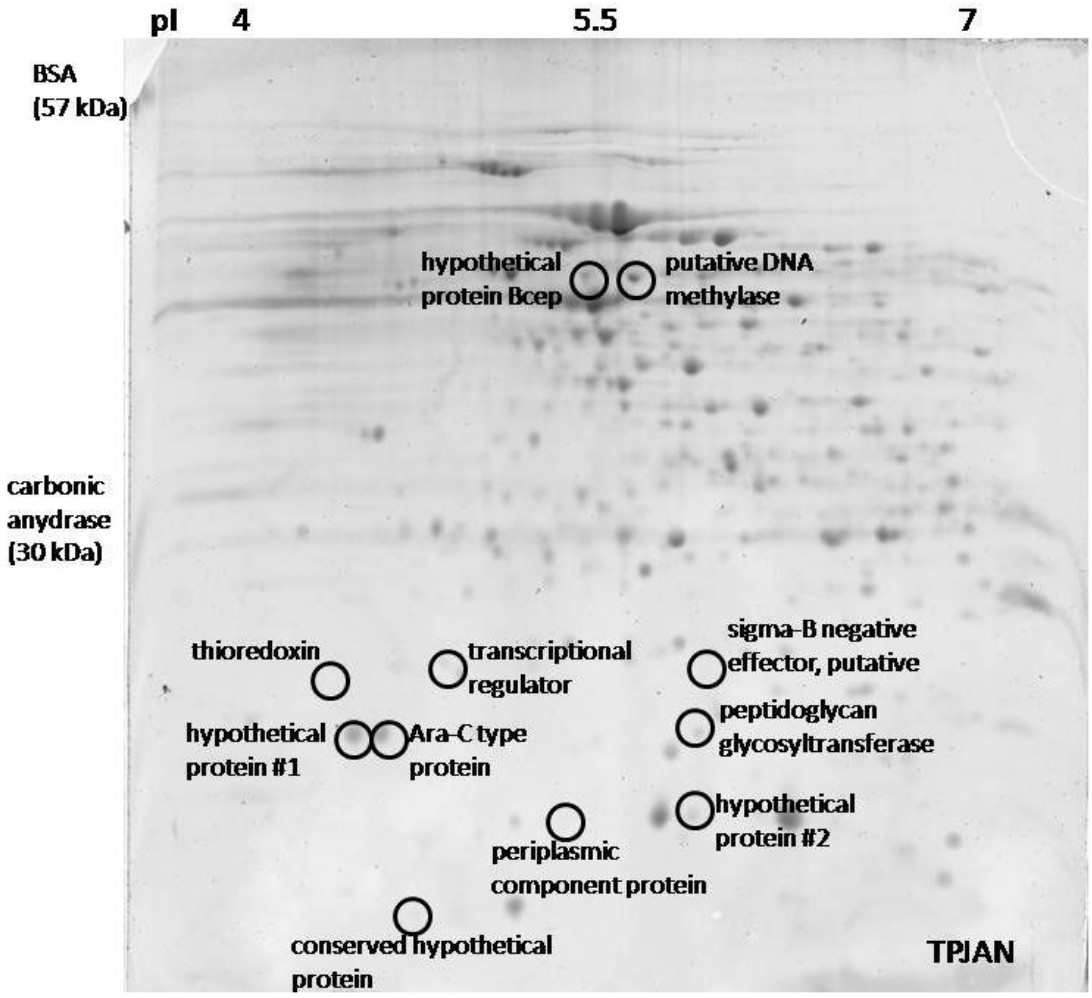

Figure 5.4. Proteome of *L. pneumophila* RI243 culture exposed to 5-7 day old supernatants prepared from *T. pyriformis*. Eleven proteins were presumptively identified. MASCOT scores are identified in Table 5.2.

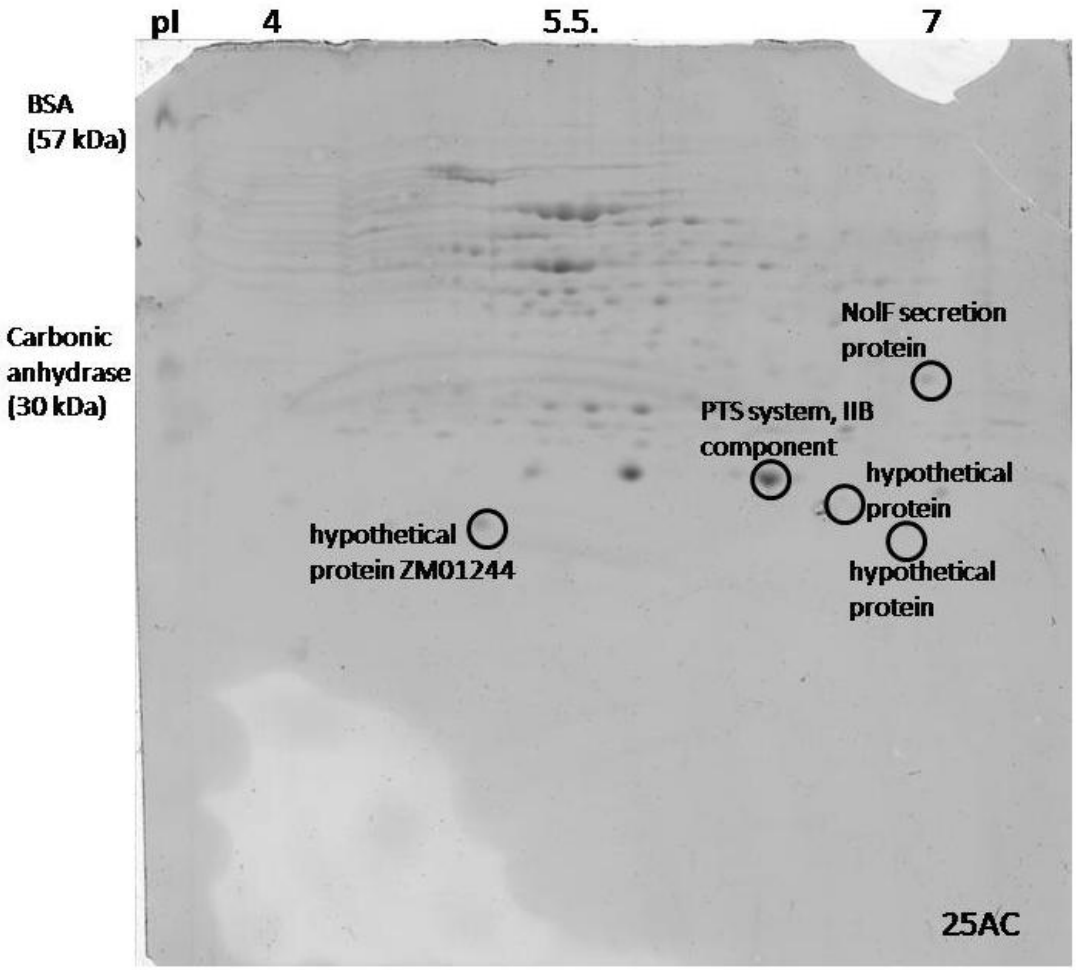

Figure 5.5. Proteome of *L. pneumophila* RI243 culture exposed to 5-7 day old supernatants prepared from *A. castellanii*. Five proteins were presumptively identified. MASCOT scores are identified in Table 5.4.

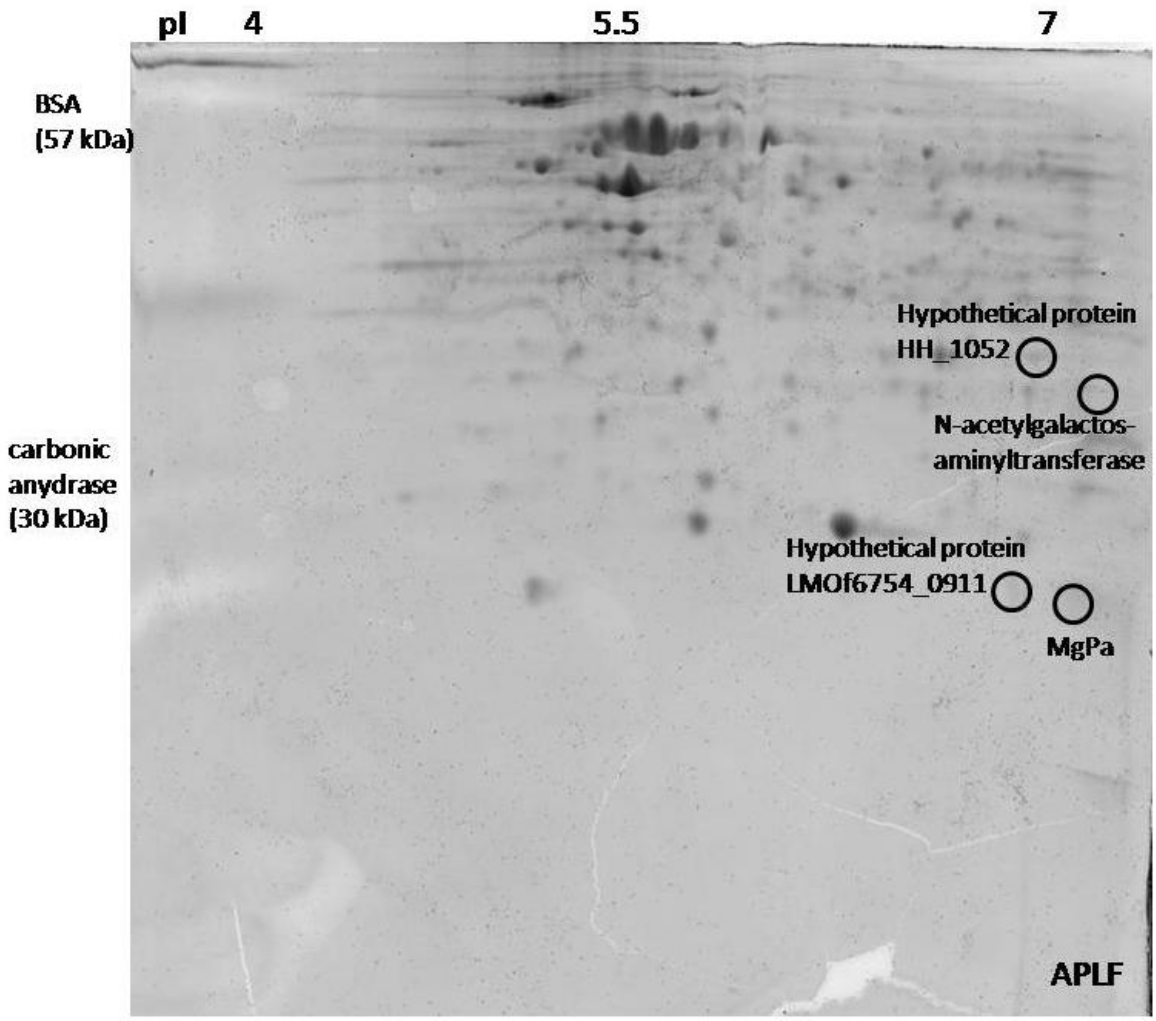

Figure 5.6. Proteome of *L. pneumophila* RI243 culture exposed to 5-7 day old supernatants prepared from *A. polyphaga*. Four proteins were presumptively identified. MASCOT scores are identified in Table 5.4.

Gel	Spot ID	MASCOT Number	Differential Expression
H. vermiformis (HVBM)	Unidentified protein #3	N/A	yes
	Global regulator CsrA (*L. pneumophila* str. Paris)	69	yes
	Unidentified protein #1	N/A	yes
	Unidentified protein #2	N/A	yes
	Long chain fatty acid transporter ((*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	66	yes
	Long chain fatty acid transporter ((*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	84	yes
	Malate deyhdrogenase (*L. pneumophila* subsp. *pneumophila* str. Philadelphia	55	yes
	Hypothetical protein lpg2275 (*L. pneumophila* subsp. *pneumophila* str. Philadelphia	59	yes
	Sigma-54 modulation protein (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	67	yes
	Hypothetical protein lpp1207 *(L. pneumophila* str. Paris); cold shock domain family protein CspA (*L. pneumophila* subsp. *pneumophila* str. Philadelphia)	110; 92	yes

Table 5.1. List of proteins identified from the *L. pneumophila* RI243 following exposure to *H. vermiformis* supernatants. A MASCOT score of $\geq$73 is considered a significant match.

Gel	Spot ID	MASCOT Number	Differential Expression
T. pyriformis (TPJAN)	Hypothetical protein #2 (*L. pneumophila* str. Paris)	62	yes
	COG1638: TRAP-type C4-dicarboxylate transport system, periplasmic component (*Magnetospirillum magnetotacticum* MS-1)	54	yes
	Conserved hypothetical protein (*Marinobacter aquaeolei* VT8)	54	yes
	Peptidoglycan glycosyltransferase (*Synechococcus* sp. WH 5701)	53	yes
	Sigma-B negative effector, putative (*Listeria monocytogenes* str. 1/2a F6854)	53	yes
	COG2207: AraC-type DNA-binding domain-containing proteins (*P. aerugionosa* C3719)	55	yes
	Hypothetical protein #1 (*L. pneumophila* str. Paris)	49	yes
	Thioredoxin (*Hahella chejuensis* KCTC 2396)	54	yes
	Putative DNA restriction-modification system , DNA methylase (*Corynebacterium jeikeim* K411)	59	yes
	Hypothetical protein Bcep1808DRAFT_6072 (*B. vietnamiensis* G4)	57	yes
	Transcriptional regulator (*Mesorhizobium loti* MAFF303099)	71	yes

Table 5.2. List of proteins identified from the *L. pneumophila* RI243 following exposure to *T. pyriformis* supernatants. A MASCOT score of $\geq$73 is considered a significant match.

Gel	Spot ID	MASCOT Number	Differential Expression
T. thermophila (TTJAN)	Hypothetical protein (*L. pneumophila* str. Paris)	42	yes
	Molybdopterin biosynthesis protein B (*Bradyrhizobium japonicum* USDA 110)	56	yes
	Conserved hypothetical protein (*Thermoanaerobacter tengcongensis* MB4)	49	yes
	2-dehydro-3-deoxyphosphooctonate aldolase (*Haemophilus influenza* 86-028NP)	59	yes
	Rhondanese-like domain protein (*Pelobacter carbinolicus* DSM 2380)	60	yes
	Glycerol-3-phosphate dehydrogenase (*Bacillus clausii* KSM-K16)	66	yes

Table 5.3. List of proteins identified from the *L. pneumophila* RI243 following exposure to *T. thermophila* supernatants. A MASCOT score of $\geq$73 is considered a significant match.

Gel	Spot ID	MASCOT Number	Differential Expression
A. castellanii (25AC)	PTS system, IIB component (*S. pyogenes* MGAS6180)	59	yes
	No1F secretion protein (*Sinorhizobium meliloti* 1021)	54	yes
	Hypothetical protein ZMO1244 (*Zymomonas mobilis* subsp. *mobilis* ZM4)	52	yes
	Conserved hypothetical protein (*Dechloromonas aromatic* RCB)	60	yes
	Truncated conserved hypothetical protein (*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305)	52	yes
A. polyphaga (APLF)	Probable N-acetylgalactosaminyltransferase (*Rhodopirellula baltica* SH 1)	62	yes
	Hypothetical protein LMOf6854_0911 (*L. monocytogenes* str. 1/2a F6854)	55	yes
	MgPa (*Mycoplasma genitalium*)	47	yes
	Hypothetical protein HH_1052 (*Helicobacter hepaticus* ATCC 51449)	38	yes

Table 5.4. List of 9 proteins presumptively identified from the *L. pneumophila* RI243 proteome following exposures to *A. castellanii* or *A. polyphaga* supernatants. A MASCOT score of $\geq$73 is considered a significant match.

VI. SUMMARY AND CONCLUSIONS

Summary

Many pathogens are capable of intercellular communications and appear to exploit this system for their own advantage. *Legionella* species were determined not produce AHSL, AI-2, or PQS; however, the data presented here demonstrate that *L. pneumophila* is capable of detecting the presence of both protozoan hosts and heterologous prokaryotes. Although AHSL, AI-2 and PQS autoinducers were not detected in *Legionella* species, the absence of these systems does not preclude legionellae from information exchange of this type. There is much diversity in prokaryotic communication systems, and it is likely that this phenomenon has yet to be discovered among legionellae. Although the capability of *Legionella* autoinducer 1 (LAI-1) as an intercellular signal has yet to be established, its presence does support this position. The fact that protein expression was induced in *L. pneumophila* in the presence of other organisms, which is consistent with the general concept of intercellular signaling, suggests the influence of unknown mechanisms on the organism's ability to persist between hosts, and when one is available, associate with that host and establish a successful intracellular infection.

Given the long-standing relationship that exists between legionellae, heterologous microorganisms, and protozoa, a natural consequence of their association would likely be the ability to exchange chemical cues. Contrary to the original hypothesis presented in this work, when taken together, the presumptive identification of differentially expressed

proteins collectively suggests that a stress response is mounted by *L. pneumophila* in the presence of other prokaryotes. For example, the appearance of a 24kDa macrophage induced protein, the global stress protein GspA, and the cold shock domain protein CspA might suggest that the association of legionellae with other bacteria may, in some cases, be less than beneficial and antagonistic. Many bacteria are known to produce bacteriocins or bacteriocin-like substances (BLS) that injur or kill prokaryotic competitors. A recent survey of BLS producing microorganisms demonstrated that several species, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Burkholderia cepacia* not only impede the growth of *L. pneumophila* but also interfere with its ability to persist in biofilms (Gurrieri et al., 2008). While the concept of bacteriocins or other antagonistic compounds may explain part the response observed by *L. pneumophila* to *S. enteric* serovar Typhimurium, *S. pyogenes*, *B. subtilis*, and *B. cereus* supernatants, the production of a bacteriocin would constitue only a fraction of the supernatant to which the organism responded.

The organisms that were used in this research were incorporated because of their ability to produce intercellular signals; however, a more realistic view of legionellae potential for synergistic interactions and/or intercellular communication may have been provided in its response to prokaryotes that are a component of the mixed species biofilms from which the organisms is typically isolated. Mampel et al (2006) demonstrated that *L. pneumophila* preferentially colonized *Microbacterium* sp. biofilms. The mechanism by which *Microbacterium* attracts the pathogen may be elucidated by evaluating *L. pneumophila's* proteome in the same manner as conducted in this research. Equally valuable information may be produced by proteomically characterizing

legionellae interactions with organisms that have the potential to repel the pathogen from biofilms, i.e., some *Pseudomonas* species, *Corynebacterium glutamicum*, and *Klebsiella pneumonia* (Mampel *et al* 2006). Exploiting the factors that both attract and repel legionellae from the resbite of biofilms may aid in identifying methods to prevent its colonization in both consumable and potable water supplies.

Conclusions

Given that pathogenic legionellae have not been implicated in person-to-person transmission, and that aqueous sources are implicated in outbreaks of legionellosis, the elimination of these organisms from human water sources is a necessary countermeasure in controlling the disease. Among the widely used approaches to controlling legionellae are chemical sanitization methods such as hyperchlorination and exposure to high temperatures (>65 ºC). While effective in the short term, these efforts fail to eradicate both legionellae and the protozoa that harbor them from water systems, suggesting that alternative biological control measures may provide a more efficient, long term solution. Given that AHSL is necessary to the development of robust biofilms, an indirect control measure for legionellae may be the destabilization of biofilms through the use of AHSL analogs or enzymes capable of AHSL hydrolysis.

With regard to the use of analogs in the disruption of quorum sensing related activities, an abundance of experimental observations supports the utility of this approach in the both the environment and clinical setting. Morohoshi *et al* (2007) evaluated the capacity of N-nonanoyl-cyclopentylamide (C_9-CPA) to inhibit AHSL mediated functions in *Serratia marcescens* AS-1 and found that effectively inhibited swarming motility and biofilm formation by this organisms. A similar compound, N-decanoyl cyclopentylamide

(C_{10}-CPA), is able to inhibit *las* and *rhl* activity in *P. aerugionosa*, and in turn prevents the organism from synthesizing multiple virulence factors and forming biofilms (Ishida *et al* 2007). Many other structural analogs have demonstrated effectiveness as inhibitors, including halogenated furanones, cinnamaldehyde and its derivatives, and N-phenylacetanoyl-L-homoserine lactone (Givskov *et al* 1996, Geske *et al* 2007, Brackman *et al* 2008).

Like biological mimics of AHSL and AI-2, enzymtatic hydrolysis of the quorum sensing signals has proven to be an effective method in disabling intercellular communication and the functions related to this activity. *Bacillus* species *B. cereus*, *B. mycoides*, *B. thuringiensis*, and *Bacillus* 240B1 produce an AHSL lactonase that inactivates AHSL by hydrolyzing the lactone bond (Dong *et al* 2002, Liu *et al* 2008). Quorum quenching activity has also been identified in *P. aeruginosa* and in the sera of mammals, where the activity in the former thought to be correlated to the modulation of its pathogenic behavior (Sio *et al* 2006, Yang *et al* 2005).

The examples provided here demonstrate only a few of the solutions that have the potential to interfere with the proliferation of pathogens like *L. pneumophila* where they are most likely to interface with human hosts. Given that AHSL has been detected in clinical samples, signal interference may one day revolutionize clinical applications in the treatment of microbial infections. Thus, the continued assessment of *L. pneumophila*'s (and other legionellae) response to heterologous microorganisms, host cells (protozoa and macrophages), and other environmental stresses will likely reveal a function that can be abrogated, and fully exploited in the eradication of legionellae from water sources or to a successful approach in the treatment of Legionnaire's disease.

Future Work

The prevalence of prokaryotic communication systems suggests that the interruption of this activity represents an effective strategy for controlling the presence of unwanted populations. To this end, the observations and conclusions outlined in this research yield significant research problems that will require additional analysis. These include further examination of the antagonism demonstrated by legionellae on the pigmentation of *Chromobacterium violaceum* (see Chapter 3). As some signaling compounds are known to antagonize others, the identification of antagonists produced by legionellae may reveal the presence of a novel signal. Other approaches include the proteome analysis of legionellae following exposure to prokaryotes that are known to both stimulate and repel biofilm attachment. In the same manner, evaluate the *Legionella* response in the presence of both permissive and non-permissive eukaryotic hosts (protozoa and cell lines). The differences in the response profiles as determined by DNA microarray, transcriptome, proteome analysis etc. for protagonistic and antagonistic organisms may yield pathways for the biological control of legionellae in biofilms. These data may also lead to alternatives to the treatment of legionellosis.

Additional work includes the response of unrelated prokaryotes (namely biofilm dwelling species) to legionellae and the evaluation of secreted factors that influence both the attachment and detachment of legionellae to biofilms; and the identification of *Legionella* signal receptors for LAI-1 and the elucidation of its role, if any, in biofilm development. The applications of this research to legionellae responses and requirements have a promising potential toward the discovery of antagonists which will in turn aid in

the reduction of legionellae in domestic water reservoirs and potential alternatives to antibiotic therapy.

BIBLIOGRAPHY

Abu Kwaik, Y., B.S. Fields, and N.C. Engleberg. 1994. Protein expression by the protozoan *Hartmannella vermiformis* upon contact with its bacterial parasite *Legionella pneumophila*. Infection and Immunity 62(5): 1860-1866.

Abu Kwaik, Y., L. Gao, O.S. Harb, and B.J. Stone. 1997. Transcriptional regulation of the macrophage-induced gene (*gspA*) of *Legionella pneumophila* and phenotypic characterization of a null mutant. Molecular Microbiology 24(3): 629-642.

Abu Kwaik, Y., L. Gao, B.A. Stone, C. Venkataraman, and O.S. Harb. 1998. Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. Applied and Environmental Microbiology 64(9): 3127-3133.

Ahmer, B.M.M., J. Van Reeuwijk, C.D. Timmers, P.J. Valentine, and F. Heffron. 1998. *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. Journal of Bacteriology 180(5): 1185-1193.

Allison, D.G., B.R. Ruiz, C. SanJose, A. Jaspe, and P. Gilbert. 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Microbiology Letters 167: 179-184.

Arevalo-Ferro, C., G. Reil, A. Gorg, L. Eberl, and K. Riedel. 2005. Biofilm formation of *Pseudomonas putida* IsoF: the role of quorum sensing as assessed by proteomics. Systematic and Applied Microbiology 28: 87-114.

Arevalo-Ferro, C., M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel, and L. Eberl. 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. Environmental Microbiology 5(12): 1350-1369.

Atkinson, S., J.P. Throup, G.S. Stewart,and P. Williams. 1999. A hierarchial quorum-sensing system in *Yersenia pseudotuberculosis* is involved in the regulation of motility and clumping. Molecular Microbiology 33: 1267-1277.

Atlas, R.M. 1999. *Legionella*: from environmental habitats to disease, pathology, detection and control. Environmental Microbiology 4: 283-293.

Bachman, M.A. and M.S. Swanson. 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. Molecular Microbiology 40(5): 1201-1214.

Barbaree, J.M., B.S. Fields, J.C. Feeley, G.W. Gorman, and W.T. Martin. 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. Applied and Environmental Microbiology 51(2): 422-424.

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. Molecular Microbiology 13(2): 273-286.

Bassler, B.L., E.P. Greenberg, and A.M. Stevens. 1997. Cross-species induction of luminescence in the quorum sensing bacterium *Vibrio harveyi*. Journal of Bacteriology 179(12): 4043-4045.

Bassler, B.L., M. Wright, R.E. Showalter, and M.R. Silverman. 1993. Intercellular signaling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Molecular Microbiology 9(4): 773-786.

Beeston, A.L. and M.G. Surette. 2002. *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar typhimurium. Journal of Bacteriology 184: 3450-3456.

Berendt, R.F. 1981. Influence of blue-green algae (cyanobacteria) on survival of *Legionella pneumophila* in aerosols. Infection and Immunity 32(2): 690-692.

Best, M.G., J.E. Stout, V.L. Yu, and R.R. Muder. 1985. *Tatlockia micdadei* (Pittsburgh pneumonia agent) growth kinetics may explain its infrequent isolation from water and the low prevalence of Pittsburgh pneumonia. Applied and Environmental Microbiology 49(6):1521-1522.

Bianchi, M. 1989. Unusual bloom of a star-like prosthecae bacteria and filaments as a consequence of grazing pressure. Microbial Ecology 17: 137-142.

Bloomfield, G. and C. Pears. 2003. Superoxide signaling required for multicellular development of *Dictyostelium*. Journal of Cell Science 116: 3387-3397.

Boettcher, K.J. and E.G. Ruby. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. Journal of Bacteriology 177: 1053-1058.

Boettcher, K.J., and E.G. Ruby. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. Journal of Bacteriology 177(4): 1053-1058.

Boles, B.R., M. Thoendel, and P.K. Singh. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Molecular Microbiology 57(5): 1210-1223.

Bosshardt, S.C., Benson, R.F., and B.S. Fields. 1997. Flagella are a positive predictor for virulence in *Legionella*. Microbial Pathogenesis 23: 107-112.

Brackman, G., T. Defoirdt, C. Miyamoto, P. Bossier, S. Van Calenbergh, H. Nelis, and T. Coenye. 2008. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. BMC Microbiology 8: 149-162

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Brieland, J., J. McClain, L. Heath, C. Chrisp, G. Huffnagle, M. LeGendre, M. Hurley, J. Fantone, and C. Engleberg. 1996. Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. Infection and Immunity 64(7): 2449-2456.

Burmølle, M., J.S. Webb, D. Rao, L.H. Hansen, S.J. Sørensen, and S. Kjelleberg. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and Environmental Microbiology 72(6): 3916-3923.

Byrne, B. and M.S. Swanson. 1998. Expression of *Legionella pneumophila* virlence traits in response to growth conditions. Infection and Immunity 66(7): 3029-3034.

Camilli, A. and B.L. Bassler. 2006. Bacterial small-molecule signaling pathways. Science 311: 1113-1116.

Castro, A.J. 1967. Antimalarial activity of prodigiosin. Nature 213(5079): 903-904.

CDC Protocol. 2004. Cultivation of amoebae. Provided by Dr. Barry Fields, CDC, Atlanta, GA.

CDC Protocol. 2006. Preparation of protein spots for MALDI-TOF analysis. Provided by Ms. Army Martin, CDC, Atlanta, GA.

Cha, C., P. Gao, Y. Chen, P.D. Shaw, and S.K. Farrand. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Molecular Plant-Microbe Interactions 11(11): 1119-1129.

Charlton, T.S., R. de Nys, A. Netting, N. Kumar, M. Hentzer, M. Givskov, and S. Kjelleberg. 2000. A novel and sensitive method for the quantification of N-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. 2(5): 30-541.

Chen, H., M. Teplitski, J.B. Robinson, B.G. Rolfe, and W.D. Bauer. 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to N-acylhomoserine lactone quorum-sensing signals and the transition to stationary phase. Journal of Bacteriology 185(17): 5029-36.

Chernin, L.S., Winson, M. K., Thompson, J.M., Haran, S., Bycroft, B.W., Chet, I., Williams, P., and G. S. A. B. Stewart. 1998. Chitinolytic activity in *Chromobacterium*

violaceum: substrate analysis and regulation by quorum sensing. Journal of Bacteriology 180(17): 4435-4441.

Chhabra, S.R., P. Stead, N.J. Bainton, G.P.C. Salmond, G.S.A.B. Stewart, and P. Williams. 1993. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of N-(3-oxohexanoyl)-L-homoserine lactone. The Journal of Antibiotics 46(3): 441-454.

Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., and E.W. Nester. 1974. *Agrobacterium tumifaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proceedings of the National Academy of Science USA 71: 3672-3676.

Chung, W.O., Y. Park, R.J. Lamont, R. McNab, B. Barbieri, and D.R. Demuth. 2001. Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. Journal of Bacteriology 183(13): 3903-3909.

Cirillo, D.M., E.J. Heffernan, L. Wu, J. Harwood, J. Fierer, and D.J. Guiney. 1996. Identification of a domain in Rck, a product of the *Salmonella typhimurium* virulence plasmid required for both serum resistance and cell invasion. Infection and Immunity 64(6): 2019-2023.

Cirillo, J.D., S. Falkow, and L.S. Tomkins. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infection and Immunity 62(8): 3254-3261.

Cloak, O.M., B.T. Solow, C.E. Briggs, C.Y. Chen, and P.M. Frantamico. 2002. Quorum sensing and production of autoinducer-2 in *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella enterica* serovar typhimurium in foods. Applied and Environmental Microbiology 68(9): 4666-4671.

Collier, D.N., L. Anderson, S.L. McKnight, T.L. Noah, M. Knowles, R. Boucher, U. Schwab, P. Gilligan, and E.C. Pesci. 2002. A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiology Letters 215: 41-46.

Conway, B.A., V. Venu, and D.P. Speert. 2002. Biofilm formation and acyl homoserine lactone production in *Burkholderia cepacia* complex. Journal of Bacteriology 184(20): 5678-5685.

Davies, D.G., M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, and E.P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280(5361): 295-298.

De Buck, E., L. Maes, E. Meyen, L. Van Mellaert, N. Geukens, J. Anne, and E. Lammertyn. 2005. *Legionella pneumophila* Philadelphia-1 tatB and tatC affect intracellular replication and biofilm formation. 331(4): 1413-1420.

De Kievit, T.R., Gillis, R., Marx, S., Brown, C., and B.H. Iglewski. 2001. Quorum sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Applied and Environmental Microbiology 67(4): 1865-1873.

Declerck, P., J. Behets, Y. Delaedt, A. Margineanu, E. Lammertyn, and F. Ollevier. 2005. Impact of non-*Legionella b*acteria on the uptake and intracellular replication of *Legionella pneumophila* in *Acanthamoeba castellanii* and *Naegleria lovaniensis*. Microbial Ecology 0: 1-14

DeKievit, T.R., R. Gillis, S. Marx, C. Brown, and B.H. Iglewski. 2001. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Applied and Environmental Microbiology 67(4): 1865-1873.

Diggle, S.P., K. Winzer, S.R. Chhabra, K.E. Worrall, M. Camara, and P. Williams. 2003. The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Molecular Microbiology 50(1): 29-43.

Dong, Y., A.R. Gusti, Q. Zhang, J. Xu, and L. Zhang. 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Applied and Environmental Microbiology 68(4): 1754-1759.

Duan, K., C. Dammel, J. Stein, H. Rabin, and M.G. Surette. 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Molecular Microbiology 50(5): 1477-1491.

Dunny, G.M. and B.A.B. Leonard. 1997. Cell-cell communication in gram-positive bacteria. Annual Reviews in Microbiology 51: 527-564.

Dunphy, G., C. Miyamoto, and E. Meighen. 1997. A homoserine lactone autoinducer regulates virulence of an insect-pathogenic bacterium, *Xenorhabdus nematophilus* (*Enterobacteriaciae*). Journal of Bacteriology 179(17): 5288-5291.

Eberhard, A., A.L. Burlingame, C. Eberhard, G.L. Kenyon, and K.H. Nealson. 1981. Structural identification of autoinducer of *Photobacterium fischeri*. Biochemistry 20:244-249.

Eberl, L. M.K. Winson, C. Sternberg, G.S.A.B. Stewart, G. Christiansen, S.R. Chhabra, B.W. Bycroft, P. Williams, S. Molin, and M., Givskov. 1996. Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behavior of *Serratia liquifaciens*. Molecular Microbiology 20:127-136

Edelstein, P.H. 1983. Culture diagnosis of *Legionella* infections. Zbl Bakt Hyg I Abt Orig A 255: 96-101

Eisenmann, H., H. Harms, R. Meckenstock, E. I. Meyers, and A.J.B. Zehnder. 1998. Grazing of *Tetrahymena* sp. on adhered bacteria in percolated columns monitored by *in situ* hybridization with fluorescent oligonucleotide probes. Applied and Environmental Microbiology 64(4): 1264-1269.

Engebrecht, J., and M. Silverman. 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proceedings of the National Academy of Sciences USA 81: 4154-4158.

Engebrecht, J., K. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell 32: 773-781

Erickson, D.L., R. Endersby, A. Kirkham, K. Stuber, D.D. Vollman, H.R. Rabin, I. Mitchell, and D.G. Storey. 2002. *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infection and Immunity 70(4): 1783-1790.

Fields, B.S., E.B. Shotts, J.C. Feeley, G.W. Gorman, and W.T. Martin. 1984. Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. Applied and Environmental Microbiology 47(3): 467-471.

Fields, B.S., G.N. Sanden, J.M. Barbaree, W.E. Morrill, R.M. Wadowsky, E.H. White, and J.C. Feeley. 1989. Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. Current Microbiology 18: 131-137.

Fields, B.S., Nerad, T.A., Sawyer, T.K., King, H., Barbaree, J.M., Martin, W.T., Morrill, W.E., and G.N. Sanden. 1990. Characterization of an axenic strain of *Hartmannella vermiformis* obtained from an investigation of nosocomial legionellosis. Journal of Protozoology 37(6): 581-583.

Fields, B.S., R.F. Benson, and R.E. Besser. 2002. *Legionella* and Legionnaires' Disease: 25 years of investigation. Clinical Microbiology Reviews 15(3): 506-526.

Fields, B.S., T.A. Nerad, T.K. Sawyer, C.H. King, J.M. Barbaree, T. Martin, W.E. Morrill, and G.N. Sanden. 1990. Characterization of an axenic strain of *Hartmannella vermiformis* obtained from an investigation of nosocomial legionellosis. Journal of Protozoology 37(6): 581-583.

Fliermans, C.B., W.B. Cherry, L.H. Orrison, S.J. Smith, D.L. Tison, and D.H. Pope. 1981. Ecological distribution of *Legionella pneumophila*. Applied and Environmental Microbiology 41(1): 9-16.

Fong, K.P., W.O. Chung, R.J. Lamont, and D.R. Demuth. 2001. Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. Infection and Immunity 69(12): 7625-7634.

Fraser, D.W., T.R. Tsai, W. Orenstein, W.E. Parkin, H.J. Beecham, R.G. Sharrar, J. Harris, G.F. Mallison, S.M. Martin, J.E. McDade, C.C. Shepard, and P.S. Brachman. 1977. Legionnaires' disease: description of an epidemic of pneumonia. New England Journal of Medicine 297: 1189-1197.

Freeman, J.A. and B.L. Bassler. 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Molecular Microbiology 31(2): 665-677.

Freeman, J.A., and B.L. Bassler. 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. Molecular Microbiology 35(1): 139-149.

Fritzinger, A.E., and F. Marciano-Cebral. 2004. Modulation of a "CD59-like" protein in *Naegleria fowleri* amebae by bacteria. Journal of Eukaryotic Microbiology 51(5): 522-528.

Fry, N. K., and T.G. Harrison. 1998. Diagnosis and epidemiology of infections caused by *Legionella* spp. Methods in Molecular Medicine, vol. 15: Molecular Bacteriology: Protocols and Clinical Applications. pp. 213-242.

Gallagher, L.A., S.L. McKnight, M.S. Kuznetsova, E.V. Pesci, and C. Manoil. 2002. Functions required for extracellular quinolone signaling by *Pseudomonas aerugionsa*. Journal of Bacteriology 184(3): 6472-6480.

Gao, L.Y. and Y. Abu Kwaik. 1999. Apoptosis in macrophages and alveolar epithelial cells during early stages of infection by *Legionella pneumophila* and its role in cytopathogenicity. Infection and Immunity 67: 862-870.

Garduno, R.A., G. Faulkner, M.A. Trevors, N. Vats, and P.S. Hoffman. 1998. Immunolocalization of Hsp60 in *Legionella pneumophila* cells. Journal of Bacteriology 180(3): 505-513.

Garduno, R.A., E. Garduno, M. Hiltz, and P.S. Hoffman. 2002. Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. Infection and Immunity 70(11): 6273-6283.C.

Geske, G.D., J.C. O'Neill, and H.E. Blackwell. 2007. N-Phenylacetanoyl-L-homoserine lactones can strongly antagonize or superagonize quorum sensing in *Vibrio fischeri*. ACS Chemical Biology 2(5): 315-319.

Givskov, M., R. De Nys, M. Manefield, L. Gram, R. Maximelien, L. Eberl, S. Molin, P.D. Steinberg, and S. Kjelleberg. 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. Journal of Bacteriology 178(22): 6618-6622.

Gonzalez Barrios, A.F., R. Zuo, Y. Hashimoto, L. Yang, W.E. Bentley, and T.K. Wood. 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulatory (MsqR, B3022). Journal of Bacteriology 188(1): 305-316.

Gortz, H.D., H.W. Kuhlmann, M. Mollenbeck, A. Tiedtke, J. Kusch, H.J. Schmidt, and A. Miyake. 1999. Intra- and intercellular communication systems in ciliates. Naturwissenschaften 86: 422-434.

Guerrieri, G., M. Bondi, C. Sabia, S. de Niederhausern, P. Borella, and P. Messi. 2008. Effect of bacterial interference on biofilm development by *Legionella pneumphila*. Current Microbiology 57: 532-536.

Guina, T., S.O. Purvine, E.C. Yi, J. Eng, D.R. Goodlett, R. Aebersold, and S.I. Miller. 2003. Quantitative proteomic analysis indicates increased synthesis of a quinolone by *Pseudomonas aeruginosa* isolates from cystic fibrosis airways. Proceedings of the National Academy of Sciences USA 100(5): 2771-2776.

Hagele, S., J. Hacker, and B.C. Brand. 1998. *Legionella pneumophila* kills human phagocytes but not protozoan host cells by inducing apoptotic cell death. FEMS Microbiology Letters 169: 51-58.

Hahn, M.W. and M.G. Höfle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiology Ecology 35:113-121.

Hahn, M.W., Moore, E.R.B., and M.G. Hofle. 1999. Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Applied and Environmental Microbiology 65(1): 25-35.

Hahn, M.W., Moore, E.R.B., and M.G. Höfle. 2000. Role of microcolony formation in the protistan grazing defense of the aquatic bacterium *Pseudomonas* sp. MWH1. Microbial Ecology 39: 175-185.

Hammer, B.K., Tateda, E.S., and M.S. Swanson. 2002. A two component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila.*. Molecular Microbiology 44(1):107-118.

Haynes, P.A. and J.R. Yates III. 2000. Proteome profiling-pitfalls and progress. Yeast 17: 81-87.

Herzberg, M., I.K. Kaye, W. Peti, and T.K. Wood. 2006. YdgG (TsqA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. Journal of Bacteriology 188(2): 587-598.

Hoffman, P.S., L. Houston, and C. A. Butler. 1990. *Legionella pneumohila htpAB* heat shock operon: nucleotide sequence and expression of the 60-kilodalton antigen in *L. pneumophila*-infected Hela cells. Infection and Immunity 58(10): 3380-3387.

Holden, E.P., H.H. Winkler, D.O. Wood, and E.D. Leinbach. 1984. Intracellular growth of *Legionella pneumohpila* within *Acanthamoeba castellanii* Neff. Infection and Immunity 45(1): 18-24.

Holden, M.T.G., S.R. Chhabra, R. de Nys, P. Stead, N.J. Bainton, P.J. Hill, M. Manefield, N. Kuman, M. Labatte, D. England, S. Rice, M. Givskov, G.P.C. Salmond, G.S.A.B. Stewart, B.W. Bycroft, S. Kjelleberg, and P. Williams. 1999. Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. Molecular Microbiology 33(6): 1254-1266.

Horowitz, M.A. 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell 36: 27-33.

Houdt, R.V., A. Aertsen, P. Moons, K. Vanoirbeek, and C.W. Michaels. 2006. N-acyl-L-homoserine lactone signal interception by *Escherichia coli*. FEMS Microbiology Letters 256: 83-89.

Hu, J.Y., Y. Fan, Y. Lin, H. Zhang, S.L. Ong, N. Dong, J. Xu, W.J. Ng, and L. Zhang. 2003. Microbial diversity and prevalence of virulent pathogens in biofilms developed in a water reclamation system. Research in Microbiology 154: 623-629.

Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl. 2001. The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 147: 2517-2528.

Huisman, G.W. and R. Kolter. 1994. Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. Science 265(5171): 537-539.

Huws, S.A., McBain, A.J., and P. Gilbert. 2005. Protozoan grazing and its impact upon population dynamics in biofilm communities. Journal of Applied Microbiology 98: 238-244.

Hwang, I., D.M. Cook, and S.K. Ferrand. 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. Journal of Bacteriology 177(2): 449-458.

Ishida, T., T. Ikeda, N. Takiguchi, A. Kuroda, H. Ohtake, and J. Kato. 2007. Inhibition of quorum sensing in *Pseudomonas aeruginosa* N-acyl cyclopentylamides. Applied and Environmental Microbiology 73(10): 3183-3188.

James, B.W., W.S. Mauchline, P.J. Dennis, C.W. Kevil, and R. Wait. 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Applied and Environmental Microbiology 65(2): 822-827.

Ji, G.Y., R.C. Beavis, and R.P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proceedings of the National Academy of Sciences USA 92: 12055-12059

Jones, S., B. Yu, N.J. Bainton, M. Birdsall, B.W. Bycroft, S.R. Chhabra, A.J.R. Cox, P. Glby, P.J. Reeves, S. Stephens, and M.K. Winson. 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudmonas aeruginosa*. EMBO Journal 12: 2477-2482.

Joyce, E.A.; B.L. Bassler, and A. Wright. 2000. Evidence for a signaling system in *Helicobacter pylori*: detection of *luxS*-encoded autoinducer. Journal of Bacteriology 182(13): 3638-3643.

Jurgens, K., J. Pernthaler, S. Schalla, and R. Amann. 1999. Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. Applied and Environmental Microbiology 65(3): 1241-1250.

Kall, L., J.D. Storey, M.J. MacCoss, and W.S. Noble. 2008. Assigning significance to peptides identified by tandem mass spectrometry using decoy databases. Journal of Proteome Research 7: 29-34.

K.P. Fong, W.O. Chung, R.J. Lamont, and D.R. Demuth. 2001. Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. Infection and Immunity 69(12): 7625-7634.

Kanamaru, K., K. Kanamaru, I. Tatsuno, T. Tobe, and C. Sasakawa. 2000. SdiA, an *Escherichia coli* homologue of quorum regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. Molecular Microbiology 38(4): 805-816.

Kaplan, H.B. and E.P. Greenberg. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. Journal of Bacteriology 163(3): 1210-1214.

Kikuhara, H., M. Ogawa, H. Miyamoto, Y. Nikaido, and S. Yoshida. 1994. Intracellular multiplication of *Legionella pneumophila* in *Tetrahymena thermophila*. Journal of the University of Occupational and Environmental Health 16(4): 263-275.

Kim, S.Y., S.E. Lee, Y.R. Kim, C.M. Kim, P.Y. Ryu, H.E. Choy, S.S. Chung, and J.H. Rhee. 2003. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Molecular Microbiology 48(6): 1647-1664

Kirwan, J.P. T.A. Gould, H.P. Schweizer, S.W. Bearden, R.C. Murphy, and M.E. Churchill. 2006. Quorum-sensing signal synthesis by the *Yersinia pestis* acyl-homoserine lactone synthase YspI. Journal of Bacteriology 188(2): 784-788.

Kuchta, J.M., J.S. Navratil, M.E. Shepherd, R.M. Wadowsky, J.N. Dowling, S.J. States, and R.B. Yee. 1993. Impact of chlorine and heat on the survival of *Hartmannella vermiformis* and subsequent growth of *Legionella pneumophila*. Applied and Environmental Microbiology 59(12): 4096-4100.

Kush, J. 1999. Self recognition as the original function of an amoeban defense-inducing kairomone. Ecology 80: 715-720.

Kwaik, Y.A., B.I. Eisenstein, and N.C. Engleberg. 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infection and Immunity 61(4): 1320-1329.

Lebeau, I., E. Lammertyn, E. DeBuck, L. Maes, N. Guekens, L. Van Mallaert, L. Arckens, J. Anne, and S. Clerens. 2005. First proteomic analysis of *Legionella pneumophila* based on its developing genome sequence. Research in Microbiology 156: 119-129.

Lewenza, S., B. Conway, E.P. Greenberg, and P.A. Sokol. 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. Journal of Bacteriology 181(3): 748-756.

Liu, D., J. Momb, P.W. Thomas, A. Moulin, G.A. Petsko, W. Fast, and D. Ringe. 2008. Mechanism of the quorum-quenching lactonase (AiiA) from *Bacillus thuringiensis*.1. product bound structures. Biochemistry 47: 7706-7714.

Llamas, I., Keshavan, N., and J.E. Gonzalez. 2004. Use of *Sinorhizobium meliloti* as an Indicator for Specific Detection of Long-Chain N-Acyl Homoserine Lactone. Applied and Environmental Microbiology 70(6): 3715-3723.

Lukomski, S., C.A. Montgomery, J. Rurangirwa, R.S. Geske, J.P. Barrish, G.J. Adama, and J.M. Musser. 1999. Extracellular cysteine protease produced by *Streptococcus pyogenes* participates in the pathogenesis of invasive skin infection and dissemination in mice. Infection and Immunity 67: 1779-1788.

Lynch, M.J., S. Swift, D.F. Kirke, C.W. Keevil, C.E.R. Dodd, and P. Williams. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environmental Microbiology 4(1): 18-28.

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*. Molecular Microbiology 42(1): 145-157.

Mampel, J., T. Spirig, S.S. Weber, A.J. Haagensen, S. Molin, and H. Hilbi. 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. Applied and Environmental Microbiology 72(4): 2885-2895.

Marciano-Cebral, F. 1988. Biology of *Naegleria* spp. Microbiological Reviews 52:114-133.

Marketon, M.M., M.R. Gronquist, A. Eberhard, and J.E. Gonzalez. 2002. Characterization of the *Sinorhizobium meliloti sinR*/*sinI* locus and the production of novel N-acyl homoserine lactones. Journal of Bacteriology 184(20): 5686-5695.

Marre, R., A.A. Medeiros, and A.W. Pasculle. 1982. Characterization of the beta-lactamases of six species of *Legionella*. Journal of Bacteriology 151(1): 216-221.

Marston, B.J., J.F. Plouffe, T.M. File, B.A. Hackman, S.J. Salstrom, H.B. Lipman, M.S. Kolczak, and R.F. Breiman. 1997. Incidence of community-acquired pneumonia requiring hospitalization-results of a population-based active surveillance study in Ohio. Archives of Internal Medicine 157: 1709-1718.

Matz, C. and K. Jürgens. 2003. Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. Microbial Ecology 45: 384-398.

Matz, C., and S. Kjelleberg. 2005. Off the hook-how bacteria survive protozoan grazing. Trends in Microbiology 13(7): 302-307.

Matz, C., Bergfeld, T., Rice, S.A., and S. Kjellegerg. 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environmental Microbiology 6(3): 218-226.

Matz, C., P. Deines, J. Boenigk, H. Arndt, L. Eberl, S. Kjelleberg, and K. Jurgens. 2004a. Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. Applied and Environmental Microbiology 70(3): 1593-1599.

Matz,C. and K. Jurgens. 2003. Interactions of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. Microbial Ecology 45: 384-398.

Matz,C. and K. Jurgens. 2005. High motility reduces grazing mortality of planktonic bacteria. Applied and Environmental Microbiology 71(2): 921-929.

McClean, K.H., M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S.A.B. Stewart, and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology 143:3703-3711.

McClean, K.H., Winson, J.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S, Bycroft, B.W., Stewart, G. S. A. B., and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143: 3703-3711.

McClean, R.J., M. Whitely, D.J. Stickler, and W.C. Fuqua. 1997a. Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiology Letters 154: 259-263.

McGrath, S., D.S. Wade, and E.C. Pesci. 2004. Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). FEMS Microbiology Letters 230: 27-34.

McKenney, D., K.E. Brown, and D.G. Allison. 1995. Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. Journal of Bacteriology 177(3): 6989-6992.

McKnight, S., B.H. Iglewski, and E.C. Pesci. 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. Journal of Bacteriology 182(10): 2702-2708.

McNab, R., S.K. Ford, 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.* Journal of Bacteriology 185(1): 275-284.

McNab, R.; S.K. Ford, 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*. Journal of Bacteriology 185: 274-284.

Merritt, J., F. Qi, S.D. Goodman, M.H. Anderson, and W. Shi. 2003. Mutation in *luxS* affects biofilm formation in *Streptococcus mutans*. Infection and Immunity 71(4): 1972-1979.

Meyer, R.D and S.M. Finegold. 1980. Legionnaires' disease. Annual Review in Medicine 31: 219-232.

Michael, B., J.N. Smith, S. Swift, F. Heffron, and B.M.M. Ahmer. 2001. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. Journal of Bacteriology 183(19): 5733-5742.

Miller, J.A. 1972. Experiment 48: Assay of β-Galactosidase. Experiments in molecular genetics (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 352-355.

Milton, D.L., A. Hardman, M. Camara, S.R. Chhabra, B.W. Bycroft, G.S.A.B. Stewart, and P. Williams. 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI*/*vanR* locus and identification of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone. Journal of Bacteriology 179: 3004-3012.

Mok, K.C., N.S. Wingreen, and B.L. Bassler. 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducer controls gene expression. EMBO Journal 22(4): 870-881.

Molmeret, M., D.M. Bitar, L. Han and Y.Abu Kwaik. 2004. Cell biology of the intracellular infection by *Legionella pneumophila*. Microbes and Infection 6: 129-139.

Molofsky, A.B. and M.S. Swanson. 2003. *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. Molecular Microbiology 50(2): 445-461.

Molofsky, A.B., and M.S. Swanson. 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Molecular Microbiology 53(1): 29-40.

Morohoshi, T., T. Shiono, K. Takidouchi, M. Kato, N. Kato, J. Kato, and T. Ikeda. 2007. Inhibition of quorum sensing in *Serratia marcescens* AS-1 by synthetic analogs of N-acylhomoserine lactone. Applied and Environmental Microbiology 73(20): 6339-6344.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S., and R.M. Dolan. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147: 3121-26.

Negron-Alvira A., I. Perez-Suarez, and T.C. Hazen. 1988. *Legionella* spp. in Puerto Rico cooling towers. Applied and Environmental Microbiology 54(10): 2331-2334.

Nelson, K.H., T. Platt, and J.W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. Journal of Bacteriology 104:313-322.

Neumeister, B., Reiff, G., Faigle, M., Dietz, K., Northoff, H., and F. Lang. 2000. Influence of *Acanthamoeba castellanii* on intracellular growth of different *Legionella* species in human monocytes. Applied and Environmental Microbiology 66(3): 914-919.

Newsome, A.L., R.L. Baker, R.D. Miller, and R.R. Arnold. 1985. Interactions between *Naegleria fowleri* and *Legionella pneumophila*. Infection and Immunity 50(2): 449-452.

Nguyen, T.M., D. Ilef, S. Jarraud, L. Rouil, C. Campese, D. Che, S. Haeghebaert, F. Ganiayre, F. Marcel, J. Etienne, and J.C. Desenclos. 2006. A community-wide outbreak of Legionnaire's disease linked to industrial cooling towers-how far can contaminated aerosols spread? Journal of Infectious Disease 193(1): 102-11.

Nouwens, A.S., S.A. Beatson, C.B. Whitchurch, B.J. Walsh, H.P. Schweizer, J.S. Mattick, J.S., and S. J. Cordwell. 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudmonas aeruginosa*. Microbiology 149: 1311-1322.

Ohtani, K., H. Hayashi, and T. Shimizu. 2002. The *luxS* gene is involved in cell-cell signaling for toxin production in *Clostridium perfringens*. Molecular Microbiology 44(1): 171-179.

Oldham, L.J. and F.G. Rodgers. 1985. Adhesion, penetration and intracellular replication in *Legionella pneumophila*: an *in vitro* model of pathogenesis. Journal of General Microbiology 131: 697-706.

Page, F.C. 1967. Taxonomic criteria for limax amoebae, with description of 3 new species of *Hartmannella* and 3 of *Vahlkamfia*. Journal of Protozoology 14: 499-521.

Park, M., E. Lee, Y. Kim, T. Jung, Y. Shin, G. Shin, H. Cha, and G. Kim. 2003. Reference map of soluble proteins from *Salmonella* enterica serovar enteritidis by two-dimensional electrophoresis. Journal of Veterinary Science 4(2): 143-149.

Parry, J.D. 2004. Protozoan grazing of freshwater biofilms. Advances in Applied Microbiology 54: 167-196.

Paszko-Kolva, C., M. Shahamat, and R.R. Colwell. 1992. Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiology Ecology 102:45-55

Pearson, J.P., L. Passador, B.H. Iglewski, and E.P. Greenberg. 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences USA 92:1490-1494.

Pearson, J.P., M. Feldman, B.H. Iglewski, and A. Prince. 2000. *Pseudomonas aerugionsa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infection and Immunity 68(7): 4331-4334.

Pearson, J.P., M.G. Kendall, L. Passador, K.D. Tucker, A. Eberhard, B.H. Iglewski, and E.P. Greenberg. 1994. Structure of the autoinducer required for expression of

Pseudmonas aeruginosa virulence genes. Proceedings of the National Academy of Sciences USA 91: 197-201.

Pearson, J.P.; E.C. Pesci, and B.H. Iglewski. 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum sensing systems in control of elastase and rhamnolipid biosynthesis genes. Journal of Bacteriology 179(18): 5756-5767.

Pernthaler, J., T. Posch, K. Simek, J. Vrba, R. Amann, and R. Psenner. 1997. Contrasting Bacterial Strategies to Coexist with a Flagellate Predator in an Experimental Microbial Assemblage. Applied and Environmental Microbiology 63(2): 596-601.

Pesci, E.C., J.B. Milbank, J.P. Pearson, S. McKnight, A.S. Kende, E.P. Greenberg, and B.H. Iglweski. 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Science USA 96: 11229-11234.

Phadtare, S. 2004. Recent developments in bacterial cold shock response. Current Issues in Molecular Biology 6: 125-136.

Pierson, E.A., W.D. Wood, J.A. Cannon, F.M. Blachere, and L.S. Pierson. 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Molecular Plant-Microbe Interactions 11: 1078-1084.

Qureshi, M.N, A.A. Perez, R.M. Madayag, and E.J. Bottone. 1993. Inhibition of *Acanthamoeba* species by *Pseudomonas aeruginosa*: rationale for their selective exclusion in corneal ulcers and contact lens care systems. Journal of Clinical Microbiology 31:1908-1910.

Reading, N.C. and V. Sperandio. 2006. Quorum sensing: the many languages of bacteria. FEMS Microbiology Letters 254:1-11

Reasoner, D.J., and E.E. Geldreich. 1985. R2A medium. Applied and Environmental Microbiology 49(1): 1-7.

Reinders, J., U. Lewandrowski, J. Moebius, Y. Wagner, and A. Sickmann. 2004. Challenges in mass spectrometry-based proteomics. Proteomics 4: 3686-3703.

Riedel, K., C. Arevalo-Ferro, G. Reil, A. Gorg, F. Lottspeich, and L. Eberl. 2003. Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. Electrophoresis 24(4): 740-750.

Rogers, J., A.B. Dowsett, P.J. Dennis, J.V. Lee, and C.W. Keevil. 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. Applied and Environmental Microbiology 60(5): 1585-1592.

Rogers, J. and C.W. Keevil. 1992. Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Applied and Environmental Microbiology 58(7): 2326-2330.

Rowbotham, T.J. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. Journal of Clinical Pathology 33: 1179-1183.

Rowbotham, T.J. 1983. Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. Journal of Clinical Pathology 36: 978-986.

Rowbotham, T.J. 1986. Current views on the relationships between amoebae, legionellae, and man. Israel Journal of Medical Sciences. 22: 678-689

Rumbaugh, K.P., J.A. Griswold, B. H. Iglewski, and A.N. Hamood. 1999. Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infection and Immunity 67(11): 5854-5862.

Sabria, M., J. Alvarez, A. Dominguez, A. Pedrol, F., G. Sauca, L. Salleras, A. Lopez, M.A. Garcia-Nunez, I. Parron, and M.P. Barrufet. 2006. A community outbreak of Legionnaires' disease: evidence of cooling towers as the source. Clinical Microbiology and Infection 12: 642-647

Sauer, K., A.K. Camper, G.D. Ehrlich, J.W. Costerton, and D.G. Davies. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology 184(4): 1140-1154.

Schaefer, A.L., D.L. Val, B.L. Hanzelka, J.E. Cronan, and E.P. Greenberg. 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proceedings of the National Academy of Sciences USA 93: 9505-9509.

Schaffer, A.L., B.L. Hanzelka, A. Eberhard, and E.P. Greenberg. 1996. Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. Journal of Bacteriology 178: 2897-2901.

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. Molecular Microbiology 41(2): 463-476.

Schauder, S., L. Penna, A. Ritton, C. Mannin, F. Parker, and G. Renauld-Mongenie. 2005. Proteomics analysis by two-dimensional differential gel electrophoresis reveals the lack of a broad response of *Neisseria meningitides* to *in vitro*-produced AI-2. Journal of Bacteriology 187(1): 392-395.

Shadrach, W.S., K. Rydzewski, U. Laube, M. Ozel, A.F. Kiderlen, and A. Flieger. 2005. *Balamuthis mandrillaris*, a free living ameba and opportunistic agent of encephalitis, is a potential host for *Legionella pneumophila* bacteria. Applied and Environmental Microbiology 71(5): 2244-2249.

Shaw, P.D., G. Ping, S.L. Daly, C. Cha, J.E. Cronan, K.L. Rinehart, and S.K. Farrand. 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-

layer chromatography. Proceedings of the National Academy of Science USA 94: 6036-6041.

Sheehan, K.B., J.M. Henson, and M.J. Ferris. 2005. *Legionella* species diversity in an acidic biofilm community in Yellowstone National Park. Applied and Environmental Microbiology 71(1): 507-511.

Singh, P.K., A.L. Schaefer, M.R. Parsek, T.O. Moninger, M.J. Welsh, and E.P. Greenburg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-764.

Sio, C.F., L.G. Otten, R. H. Cool, S. P. Diggle, P.G. Braun, P.G. Braun, R. Bos, M. Daykin, M. Camara, P. Williams, and W. J. Quax. 2006. Quorum quenching by an N-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. Infection and Immunity 74(3): 1673-1682.

Sircili, M.P., M. Walters, L. Trabulsi, and V. Sperandio. 2004. Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. Infection and Immunity 72(4): 2329-2337.

Sitnikov, D. M., J.B. Schineller, and T.O. Baldwin. 1996. Control of cell division in *Escherichia coli*: Regulation of transcription of *ftsQA* involves both rpoS and SdiA-mediated autoinduction. Proceedings of the National Academy of Sciences 93: 336-341.

Siuzdak, G. 1996. Mass Spectrometry for Biotechnology. Academic Press, Inc; San Diego, California.

Smith, J. N. and B.M.M. Ahmer. 2003. Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. Journal of Bacteriology 185(4): 1357-1366.

Smith-Somerville, H.E., V.B. Huryn, C. Walker, and A.L. Winters. 1991. Survival of *Legionella pneumophila* in the cold-water ciliate *Tetrahymena vorax*. Applied and Environmental Microbiology 57(9): 2742-2749.

Snyder, R.A. 1991. Chemoattraction of a bactivorous ciliate to bacteria surface compounds. Hydrobiologia 215: 205-213.

Solomon, J.M., A. Rupper, J.A. Cardelli, and R.R. Isberg. 2000. Intracellular growth by *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. Infection and Immunity 68(5): 2939-2947.

Sperandio, V. A.G. Torres, B. Jarvis, J.P. Nataro, and J.B. Kaper. 2003. Bacteria-host communication: the language of homromones. Proceedings of the National Academy of Sciences 100: 8951-8956.

Sperandio, V., A.G. Torres, J.A. Giron, and J.B. Kaper. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. Journal of Bacteriology 183(17): 5187-5197.

Spirig, T., A. Taiden, P. Kiefer, C. Buchrieser, J.A. Vorholt, and H. Hilibi. 2008. The *Legionella* autoinducer synthase LqsA produces an α-hydroxyketone signaling molecule. The Journal of Biological Chemistry 283(26): 18113-18123.

Steele, T.W. and A.M. McLennan. 1996. Infection of *Tetrahymena pyriformis* by *Legionella longbeachae* and other *Legionella* species found in potting mixes. Applied and Environmental Microbiology 62(3): 1081-1083.

Steele, T.W., C.V. Moore, and N. Sangster. 1990b. Distribution of *Legionella longbeachae* serogroup 1 and other legionellae in potting soils in Australia. Applied and Environmental Microbiology 56(10): 2984-2988.

Steele, T.W., J. Lanser, and N. Sangster. 1990a. Isolation of *Legionella longbeachae* serogroup 1 from potting mixes. Applied and Environmental Microbiology 56(1): 49-53.

Steinert, M., L. Emody, R. Amann, and J. Hacker. 1997. Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Applied and Environmental Microbiology 63(5): 2047-2053.

Stevens, A.M. and E.P. Greenberg. 1997. Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. Journal of Bacteriology 179(2): 557-562.

Stevens, A.M., K.M. Dolan, and E.P. Greenburg. 1994. Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain RNA polymerase to the *lux* promoter region. Proceedings of the National Academy of Sciences USA 91: 12619-12623.

Stevenson, B. and K. Babb. 2002. LuxS-mediated quorum sensing in *Borrelia burgdorferi*, the lyme disease spirochete. Infection and Immunity 70(8): 4099-4105.

Stone, B. and Y. Abu Kwaik. 1998. Expression of multiple pili by *Legionella pneumophila*: identification and characterization of type IV pilin gene and its role in adherence to mammalian and protozoan cells. Infection and Immunity 66:1768-1775.

Stoodley, P., K. Sauer, D.G. Davies, and J.W. Costerton. 2002. Biofilms as complex differentiated communities. Annual Review Microbiology 56:187-209.

Storey, D. G., E.E. Ujack, and H.R. Rabin. 1992. Population transcript accumulation of *Pseudomonas aeruginosa* exotoxin A and elastase in sputum from patients with cystic fibrosis. Infection and Immunity 60: 4687-4694.

Storey, M.V., J. Winiecka-Krusnell, N.J. Ashbolt, and T. Stenstrom. 2004. The efficacy of heat and chlorine treatment against thermotolerant acanthamoebae and legionellae. Scandinavian Journal of Infectious Diseases 36:656-662.

Stout, J.E., V.L. Yu, and M.G. Best. 1985. Ecology of *Legionella pneumophila* within water distribution Systems. Applied and Environmental Microbiology 49(1): 221-228.

Stroeher, U.H., A.W. Paton, A.D. Ogunniyi, and J.C. Paton. 2003. Mutation of LuxS of *Streptococcus pneumoniae* affects virulence in a mouse model. Infection and Immunity 71(6): 3206-3212.

Sun, J., R. Daniel, I. Wagner-Dobler, 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 Evolutionary Biology 4:36-46

Surette, M.G. and B.L. Bassler. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proceedings of the National Academy of Sciences 95: 7046-7050.

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 genes responsible for autoinducer production. Proceedings of the National Academy of Sciences 96: 1639-1644.

Surman, S.B., L.H.G. Morton, and C.W. Keevil. 1994. The dependence of *Legionella pneumophila* on other aquatic bacteria for survival on R2A Medium. International Biodeterioration & Biodegradation (1994) pp. 223-236.

Swartz, M. N. Clinical aspects of Legionnaire's disease. 1979. Annals of Internal Medicine 90: 492-495.

Swift, S., A.V. Karlyshev, E.L. Durant, M.K. Winson, S.R. Chhabra, P. Williams, S. Macintyre, and G.S.A.B. Stewart. 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologues AhyRI and AsaRI and their cognate signal molecules. Journal of Bacteriology 179: 5271-5281.

Swift, S., M.J. Lynch, L. Fish, D.F. Kirke, J.M. Tomas, G.S.A.B. Stewart, and P. Williams. 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infection and Immunity 67(10): 5192-5199.

Swift, S., M.K. Winson, P.F. Chan, N.J. Bainton, N.M. Birdsall, P.J. Reeves, C.E.D. Rees, S.R. Chhabra, P.J. Hill, J.P. Throup, B.W. Bycroft, G.P.C. Salmond, P. Williams, and G.S.A.B. Stewart. 1993. A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of the LuxR:LuxI superfamily in enteric bacteria. Molecular Microbiology 10: 511-520.

Taiden, A., T. Spirig, P. Carranza, H. Bruggemann, K. Riedel, L. Eberl, C. Buchrieser, and H. Hilbi. 2008. Synergistic contribution of the *Legionella pneumophila lqs* genes to pathogen-host interactions. Journal of Bacteriology 190(22): 7532-7547.

Taminiau, R., M. Daykin, S. Swift, M. Boschiroli, A. Tibor, P. Lestrate, X. De Bolle, D. O'Callaghan, P. Williams, and J.J. Letesson. 2002. Identification of quorum-sensing signal molecule in the facultative intracellular pathogen *Brucella melitensis*. Infection and Immunity 70(6): 3004-3011.

Thomas, V., T. Bouchez, V. Nicolas, S. Robert, J.F. Loret, and Y. Levi. 2004. Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. Journal of Applied Microbiology 97: 950-963

Thorpe, T.C. and R.D. Miller. 1981. Extracellular enzymes of *Legionella pneumophila*. Infection and Immunity 33(2): 632-635.

Throup, J.P., M. Camar, G.S. Briggs, M.K. Winson, S.R. Chhabra, B.W. Bycroft, P. Williams, and G.S.A.B. Stewart. 1995. Characterization of the *yenI*/*yenR* locus from *Yersenia enterocolitica* mediating the synthesis of two N-acylhomoserine lactone signal molecules. Molecular Microbiology 17(2): 345-356.

Tison, D.L. and R.J. Seidler. 1983. *Legionella* incidence and density in potable drinking water supplies. Applied and Environmental Microbiology 45(1): 337-339.

Tison, D.L., D.H. Pope, W.B. Cherry, and C.B. Fliermans. 1980. Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). Applied and Environmental Microbiology 39(2): 456-459.

Tomlin, K. L., R.J. Malott, G. Ramage, D.G. Storey, P.A. Sokol, and H. Ceri. 2005. Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms. Applied and Environmental Microbiology 71(9): 5208-5218.

Tyndal, R.L, and E.L. Domingue. 1982. Cocultivation of *Legionella pneumophila* and free-living amoebae. Applied and Environmental Microbiology 44(4): 954-959.

VanBogelen, R.A., E.E. Schiller, J.D. Thomas, and F.C. Neidhardt. 1999. Diagnosis of cellular states of microbial organisms using proteomics. Electrophoresis 20: 2149-2159.

Wadowsky, R.M., and R.B. Yee. 1985. Effect of non-legionellaceae bacteria on multiplication of *Legionella pneumophila* in potable water. Applied and Environmental Microbiology 49(5): 1206-1210.

Wagner, V.E., D. Bushnell, L. Passador, A.I. Brooks, and G.H. Iglewski. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. Journal of Bacteriology 185(7): 2080-2095.

Wagner, V.E., R.J. Gillis, and B.H. Iglewski. 2004. Transcriptome analysis of quorum-sensing regulation and virulence factor expression in *Pseudomonas aeruginosa*. Vaccine 22S: S15-S20.

Walker, J.T., A. Sonesson, C.W. Keevil, and D.C. White. 1993. Detection of *Legionella pneumophila* in biofilms containing a complex microbial consortium by gas chromatography-mass spectrometry analysis of genus-specific hydroxy fatty acids. FEMS Microbiology Letters 113(2): 139-144.

Waters, C.M. and B.L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annual Review of Cell Developmental Biology 21: 319-346.

Weekers, P.H.H., P.L.E. Bodelier, J.P.H. Wijen, G.D. Vogels. 1993. Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii, Acanthamoeba polyphaga,* and *Hartmannella vermiformis* on various bacteria. Applied and Environmental Microbiology 59(7): 2317-2319.

Weitere, M., Bergfeld, T., Rice, S.A., Matz, C., and S. Kjelleberg. 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology 7(10): 1593-1601.

Wen, A.T. and R.A. Burne. 2004. LuxS mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. Journal of Bacteriology 186(9): 2682-2691.

Westermeier, R. and T. Naven. 2002. Proteomics in Practice A Laboratory Manual. Wiley-VCH.

Whitehead, N.A., A.M.L. Barnard, H. Slater, N.J.L. Simpson, and G.P.C. Salmond. 2001. Quorum-sensing in gram-negative bacteria. FEMS Microbiology Reviews 25:365-404

Winfrey, M.R., Rott, M.A., and A.T. Wortman. 1997. Unraveling DNA molecular biology for the laboratory, p. 334. Prentice Hall, NJ.

Winzer, K., K.R. Hardie, N. Burgess, N. Doherty, D. Kirke, M.T.G. Holden, R. Linforth, K.A. Cornell, A.J. Taylor, P.J. Hill, and P. Williams. 2002. LuxS: its role in central metabolism and the *in vitro* synthesis of 4-hydroxy-5-methyl-3(*2H*)-furanone. Microbiology 148: 909-922.

Winzer, K., Y. Sun, A. Green, M. Delory, D. Blackley, K.R. Hardie, T.J. Baldwin, and C.M. Tang. 2002. Role of *Neisseria meningitides* luxS in cell-to-cell signaling and bacteremic infection. Infection and Immunity 70(4): 2245-2248.

Wood, D. and L.S. Pierson III. 1996. The phzI gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine production. Gene 268: 49-53.

Xavier, K.B., and B.L. Bassler. 2003. LuxS quorum sensing: more than just a numbers game. Current Opinion in Microbiology 6:191-197.

Yamamoto, H., Y. Hashimoto, and T. Ezaki. 1996. Study of nonculturable *Legionella pneumophila* cells during multiple-nutrient starvation. FEMS Microbiology Ecology 20: 149-154.

Yang, F., L. Wang, J. Wang, Y. Dong, J.Y. Hu, and L. Zhang. 2005. Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS Letters 579: 3713-3717.

Yao, Y., M.A. Martinez-Yamout, T.J. Dickerson, A.P. Brogan, P.E. Wright, and H. J. Dyson. 2006. Structure of the *Escherichia coli* quorum sensing protein SdiA: activation

of the folding switch by acyl homoserine lactones. Journal of Molecular Biology 355: 262-273.

Zhang, L.P., P.J. Murphy, A. Kerr, and M.E. Tate. 1993. *Agrobacterium* conjugation and gene regulation by N-acyl-L-homoserine lactones. Nature 362:446-448.

Zhu, H., R. Bandara, T.C.R. Conibear, S.J. Thuruthyil, S.A. Rice, S. Kjelleberg, M. Givskov, and M.D.P. Willcox. 2004. *Pseudomonas aeruginosa* with LasI quorum-sensing deficiency during corneal infection. Investigative Opthalmology & Visual Science 45(6): 1897-1903.

Zhu, J. and S.C. Winans. 1999. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. Proceedings of the National Academy of Science USA 96: 4832-4837.

Zhu, J. and S.C. Winans. 2001. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. Proceedings of the National Academy of Science USA 98(4): 1507-1512.