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EVIDENCE OF WATER TO HUMAN TRANSMISSION OF *Aeromonas hydrophila*: CRITICAL ROLE OF QUORUM SENSING IN BACTERIAL VIRULENCE & HOST RESPONSE

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EVIDENCE OF WATER TO HUMAN TRANSMISSION OF *Aeromonas hydrophila*: CRITICAL ROLE OF QUORUM SENSING IN BACTERIAL VIRULENCE & HOST RESPONSE

by

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Dissertation
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Dedicated to the memory of my father Late Saurendra M. Khajanchi and my eldest brother Late Subrata K. Khajanchi. To my brother Biraj K. Khajanchi for his continuous support and encouragement and my wife Taslima T. Lina for always inspiring me to reach this destination.

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EVIDENCE OF WATER TO HUMAN TRANSMISSION OF *Aeromonas hydrophila*: CRITICAL ROLE OF QUORUM SENSING IN BACTERIAL VIRULENCE & HOST RESPONSE

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Aeromonas hydrophila is a Gram-negative bacterium which produces a wide variety of virulence factors leading to both intestinal and extra-intestinal infections in humans. A large number of water and clinical *Aeromonas* isolates were molecularly finger-printed and our data provided the evidence of successful colonization and infection of humans with particular strains of certain *Aeromonas* species after consumption of water. Further, we delineated the role of N-*acyl* homoserine lactones (AHLs; autoinducer-1 [AI-1])-mediated quorum sensing (QS) in the virulence of a diarrheal isolate SSU of *A. hydrophila* by generating a Δ*ahyRI* mutant. Our data suggested that AI-1-mediated QS modulated the *in vitro* virulence of *A. hydrophila* by regulating type 6 secretion effectors, metallo-protease production, and biofilm formation. In a septicemic mouse model, the Δ*ahyRI* mutant was also attenuated as compared to the parental strain of *A. hydrophila*. In addition, we demonstrated that AHLs possess immunomodulatory and protective roles and AHL pretreatment modulates innate immune response in mice and enhances their survivability during *A. hydrophila* infection. Further, AHL treated animals exhibited a significantly reduced bacterial load in the blood and other mouse organs, as well as various levels of cytokines/chemokines as compared to control animals. Importantly, AHL pretreatment significantly elevated neutrophil numbers in the blood, suggesting that neutrophils quickly cleared bacteria either by phagocytosis or possibly other mechanism(s) during infection. These findings coincided with the fact that neutropenic animals were more susceptible to *A. hydrophila* infection than normal mice. We showed that animals challenged with *A. hydrophila* die because of kidney and liver damage, hypoglycemia, and thrombocytopenia, and, importantly, pretreatment of animals with AHLs prevented clinical sequelae, resulting in increased survivability of mice. Finally, we identified and characterized a new two-component based QseBC/AI-3 QS system in *A. hydrophila* SSU by generating a Δ*qseB* mutant. We noted that deletion of the *qseB* gene attenuated bacterial virulence in a septicemic mouse model of infection, had diminished swimming and swarming motility, and the mutant bacteria formed denser biofilms when compared to the parental strain of *A. hydrophila*. The decrease in the virulence of *A. hydrophila* Δ*qseB* mutant correlated with reduced production of protease and the cytotoxic enterotoxin, which has associated hemolytic activity.

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INTRODUCTION

Aeromonas species cause both intestinal and extraintestinal infections (68, 90, 238), the latter include septicemia, cellulitis, wound infections, urinary tract infections, hepatobiliary tract infections, soft tissue infections and, occasionally, meningitis and peritonitis (68, 90, 238). In immuno-compromised children, this pathogen can cause even more severe forms of infections such as hemolytic uremic syndrome (HUS) and necrotizing fasciitis (3, 62), although detailed studies are needed to establish such associations. Worldwide, the isolation rate of *Aeromonas* from diarrheic stools has been reported to be as high as 10.8%, compared to 2.1% from healthy controls (68, 100, 238). An increased isolation rate of *Aeromonas* species was reported in floodwater samples during Hurricane Katrina in New Orleans (170), and skin and soft tissue infections caused by *Aeromonas* species were among the most common in the survivors of the 2004 Tsunami in Southern Thailand (78). The ability of aeromonads among other bacteria to survive water chlorination when present in biofilm and their resistance to multiple antibiotics pose major public health concerns (129).

Further, *A. hydrophila* produces a wide battery of virulence factors, which function together to cause disease in the host (28, 124, 195). We characterized three different enterotoxins from a diarrheal isolate SSU of *A. hydrophila* (195). The most potent of these was the cytotoxic enterotoxin (Act) (61), which is secreted via the type 2 secretion system (T2SS) and functions as a hemolysin, a cytotoxin, or an enterotoxin, depending upon the target cells (28). Recently, we showed the contribution of two other secretion systems, namely T3- and T6-, in the virulence of *A. hydrophila* SSU (196, 197, 202, 203, 218-220), and reported the identification of a new T3SS effector, AexU, which led to the ADP-ribosylation of host cell proteins, resulting in their death *via* apoptosis

(203). Likewise, the virulence-associated genes *vasH* and *vasK* were essential components of the T6SS, and our laboratory provided evidence that isogenic mutants deleted for these genes were less virulent in a septicemic mouse model of *A. hydrophila* SSU infection (220). Recently, two T6SS-associated effectors, such as hemolysin co-regulated protein (Hcp) and the valine-glycine repeat G (VgrG) family of proteins, were characterized, and their roles in the virulence were established in *A. hydrophila* as well as several other pathogens (21, 150, 172, 173, 218, 219).

The production of such a wide array of virulence factors by *Aeromonas* species is indicative of its potential to cause severe diseases in humans. These virulence factor-encoding genes might be differentially expressed in *Aeromonas* species based on the prevailing environmental conditions, such as water versus the human host.

A cell-to-cell signaling system, known as quorum sensing (QS), might play an important role in sensing the physiological conditions and helping bacteria to express these virulence genes at an appropriate time and condition. Thus far in Gram-negative bacteria, at least three QS circuits have been identified and designated as LuxRI [autoinducer (AI)-1], LuxS/AI-2, and AI-3 epinephrine/norepinephrine. All these QS systems were detected in our clinical strain SSU of *A. hydrophila.*

In the first part of the study, 199 *Aeromonas* isolates, of which 146 were from water sources and 53 from human patients with diarrhea within the Unites States were identified and characterized in terms of their DNA finger prints and virulence signature. In addition, 28 reference and classical strains that were either obtained from various culture collections and/or isolated worldwide and from diverse sources, including water and clinical specimens, were also compared and characterized. All isolates were biochemically identified to the phenospecies Group level and examined for the presence of a set of 11 virulence factors by DNA colony blot hybridization, and fingerprinted by

pulsed-field gel electrophoresis (PFGE). Some of the selected virulence factors, including T6SS effectors, were also examined by functional assays. These data provided the first suggestive evidence of water-to-human transmission, i.e., of successful colonization of humans and infection with particular strains of certain *Aeromonas* species (107).

In my subsequent study, I characterized the role of AHL-mediated QS in modulating virulence of a clinical isolate SSU of *A. hydrophila*. AhyRI (LuxRI homolog) has been identified in clinical strain SSU as a result of our annotation of the genome sequence of an environmental isolate ATCC 7966 of *A. hydrophila* (193). We showed that disruption of the *ahyRI* genes in *A. hydrophila* SSU influenced metallo-protease production, secretion of the T6SS effectors (Hcp and VgrGs), biofilm formation, and mortality in a mouse model of infection, suggesting that AHL-mediated QS is a posivite regulator in controlling virulence of *A. hydrophila* (109).

Little is known as to how different AHLs modulate immune response during infection. Consequently, I demonastrated that AHLs such as butanoyl and hexanoyl homoserine lactones (C4- and C6-HSLs) as well as N-3-oxododecanoyl (3-oxo-C12)-HSL have immunomodulatory and protective roles during infection of mice challenged with *A. hydrophila* SSU (108). This study is the first to show that pretreatment of mice with AHLs prevents clinical sequelae to enhance survivability of mice after *A. hydrophila* infection, and that they trigger an innate immune response of mice to clear bacteria from the blood and different mouse tissues.

Finally, I identified and characterized the role of QseBC (AI-3) QS system in the regulation of virulence in *A. hydrophila* SSU. I demonstrated that QseBC system in *A. hydrophila* positively regulated both swimming and swarming motility, hemolytic activity of Act and protease production, while negatively modulating the biofilm formation. This study is the first to report a functional QseBC QS system in *A.*

hydrophila which may be networked to AI-1 and AI-2 QS systems in modulating bacterial virulence.

Overall, this study advanced our knowledge in better understanding the roles of AHLs and AI-3-mediated QS in regulation of bacterial virulence and also immunomodulatory roles of AHLs in modulating host response during infection with *A. hydrophila*.

CHAPTER 1

Review of the Literature

The Genus *Aeromonas*

In 1943, the taxonomy and classification of *Aeromonas* was defined and it was separated from other Gram-negative rods containing polar flagella (217). Subsequently, over the past several years, the genus *Aeromonas* has gone through a number of taxonomic and nomenclature revisions (1). Although originally it was placed in the family *Vibrionaceae* (236) together with the genera *Vibrio*, *Photobacteriam* and *Plesiomonas*, later phylogenetic studies suggested that the genus *Aeromonas* is not closely related to vibrios and hence it was proposed to have its own family *Aeromonadaceae* (38). Initially, there were only four *Aeromonas* species; however, through evolution into the molecular postgenomic era, there are currently 30 species of *Aeromonas* (http://www.baterio.cict.fr/), some of them are still under validation.

Based on the growth characteristics and biochemical features, historically, aeromonads have been divided in two distinctive groups: the mesophilic and the psychrophilic group (94). The mesophilic group is represented by the motile species that grow well at temperatures between 35 and 37°C, and include *A. hydrophila, A. caviae, A. sobria, A. veronii,* and *A. schubertii,* all associated with human infections. The psychrophilic group comprises non-motile strains, best represented by *A. salmonicida*, that grow at temperatures between 22 and 25°C and are generally associated with fish disease (94). Subsequent investigations redefined the mesophilic group by implementing DNA relatedness which revealed that multiple hybridization groups (HGs) existed within each of the mesophilic species (94). Each defined species has been assigned a specific number of HGs, such as *A. hydrophila* designated as HG1 (94).

A search in the PubMed using the word "*Aeromonas*" generated approximately 663 citations between 1980 and 1994 (94). However, to date, this number has increased to 5136, suggesting that over the years there is an enormous increase of interest in the scientific and medical community to study genus *Aeromonas*. Finally, the availability of the complete genome sequences of an environmental isolate *Aeromonas hydrophila* (ATCC7966^T) (193) and that of a fish isolate *A. slmonicida* A449 (180) represent key developments which would be very important tools in further investigating the taxonomy, pathogenecity, and the infection process associated with the genus *Aeromonas*.

Most Common Clinical Infections Associated with *Aeromonas*

Gastroentritis: The most common disease associated with aeromonads is gastroenteritis, which ranges from a mild self-limiting watery diarrhea (secretory) to a more severe, invasive *Shigella*-like dysenteric form. The watery diarrhea form is more common which accounts for 75% to 89% of all cases of *Aeromonas* gastroenteritis (94, 238). In contrast, the dysenteric form is less common with frequencies between 3% and 22% (238). *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria are diagnosed as most common species which account for more than 85% of *Aeromonas*-associated gastrointestinal infections (97). *Aeromonas* species are also one of the leading causes of traveler's diarrhea in adults, and an extensive study conducted in Japan revealed that *A. hydrophila* was isolated from 5.5% of traveler's returning from developing countries (257).

Another diarrhea outbreak study in Brazil revealed that among 582 stool samples examined, *Aeromonas* species were the most frequently isolated enteric pathogen (19.5%), with the dominant species isolates included *A. caviae*, *A. veronii* biovar sobria and *A. veronii* biovar veronii. Other pathogens were identified less frequently than *Aeromonas*, such as *V. cholerae* (3.1%), *Salmonella* species (1.4%), and *Shigella* species

(0.5%). These findings further indicate the predominant role of *Aeromonas* species in humans inflicted with diarrhea (80).

Although the subject of *Aeromonas*-related gastroenteritis remains somewhat controversial (63), the supporting evidence of association of *Aeromonas* in gastroenteritis stems from detailed case reports, recovery of *Aeromonas* as a sole pathogen from diarrheal patient, epidemiologic-controlled studies, and low colonization rates in asymptomatic human carrier (6, 78, 93, 97).

Wound infections: Wound infections are the second most common infection among the *Aeromonas*-associated clinical cases (163) and majority of the isolates (71%) from wound specimens were identified as *A. hydrophila* (105). Severity of wound infections caused by *Aeromonas* varies from mild, superficial infections (e.g., cellulitis and funculitis) to more serious deep-seated infections (e.g., ecthyma gangrenosum and myonecrosis) (71, 95, 105, 191, 241) and the susceptibility of would infections also vary from person to person, depending upon host immune status. During catastrophic conditions, such as 2004 Tsunami in Thailand, *Aeromonas*-associated wound infections were the most frequently encountered problem for the Tsunami survivors and majority of them became infected due to contamination with metal and wood debris (78). Wound infections caused by *Aeromonas* species involve several steps following introduction of bacteria through abrasions or penetrating injuries that occurs often during recreational activities in an aquatic environment or in soil, where this pathogen is present in high numbers (94).

Figure 1.1. Hypothetical model of *Aeromonas* wound infection

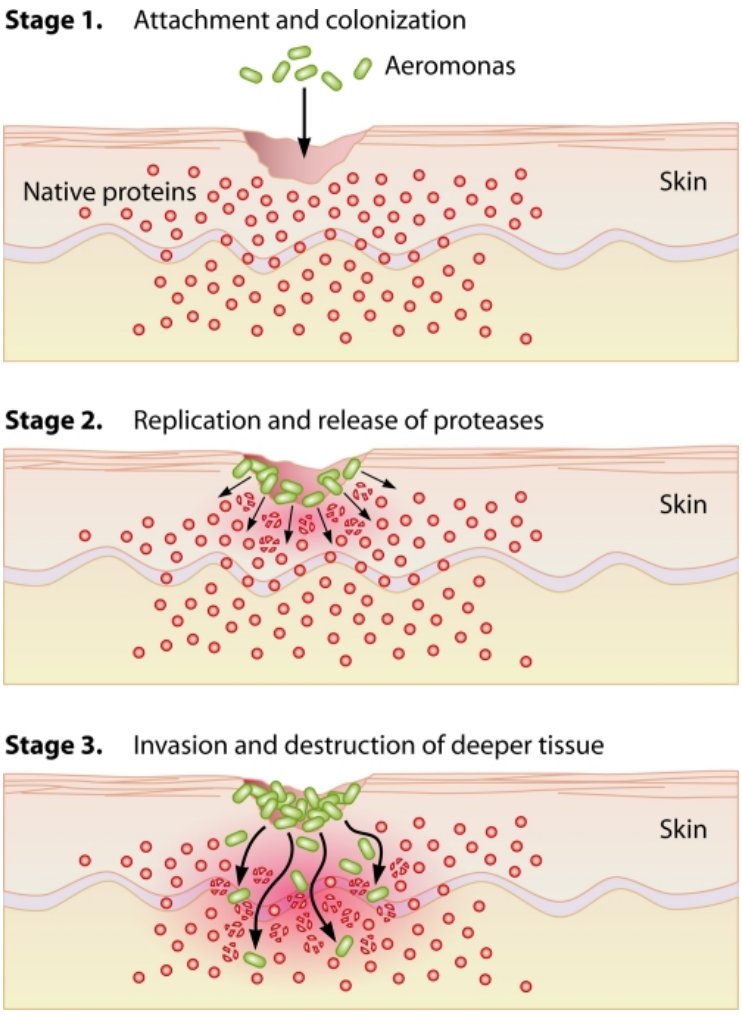

Fig. 1.1. Hypothetical model of *Aeromonas* wound infection. The process involves three major stages. (1) Attachment and initial colonization of wound site; (2) elaboration of proteases and degradation of proteinaceous material as an energy source, leading to multiplication of bacilli; and (3) migration of aeromonads into deeper tissues due to a gradient effect (higher concentration of proteins) via chemotactic motility. Janda *et. al.,* 2010, (94) © American Society for Microbiology, reproduced with permission.

Interestingly, most of the cases of water-borne *Aeromonas* wound infections occur in fresh water than the sea water (191). Figure 1.1 illustrates a hypothetical model for *Aeromonas* wound infections (94). In addition to adhesion or colonization, there are several important factors that play role in this process. Further, several proteases such as metallo proteases, serine proteases and aminopetidases that are produced by *Aeromonas* species help them to cause tissue injury and degradation of tissue and proteins provide energy source for subsequent multiplication of the bacteria. Other virulence factors, such as the type III and type VI secretion effectors and quorum sensing also might play roles in wound infection (94).

Septicemia: The majority of the *Aeromonas*-associated septicemia occurs in immunocompromised elderly individuals, having predisposing medical complications such as impaired hepatobiliary function and malignancy (23, 52, 143). It can also occur in other underlying disorders, such as trauma (9, 55, 110, 138, 161, 206), cardiac anomalies (155, 161), gastrointestinal disorders (24, 40), anemia (35, 227), and respiratory problems (55, 224). However, although it is rare, septicemia can occur in healthy adults usually from severe aeromonads wound infections (96, 183, 199). Four *Aeromonas* species such as *A. hydrophila*, *A. veronii*, *A caviae* and *A. jandei*, are more common cause of septicemia and 65% of those are caused by *A. hydrophila* as a mono-microbic infection (93). The mortality rate due to *Aeromonas*-associated septicemia ranges from 25% to 50% in infected adults with underlining medical conditions such as cancer and dibetes (52, 98). In case of septicemia that develops in patients from severe wound infections has a very high mortality rate of over 90% (96, 134).

Major Virulence Factors/Mechanisms of *Aeromonas* Species

The virulence of aeromonads is multifactorial as this pathogen produces a wide variety of virulence factors including those that code for aerolysin, hemolysin, enterotoxins (both cytotoxic and cytotonic), proteases, lipases, hemaggluttinins, T2SS, T3SSs and T6SSs, lipopolysaccharide (LPS), surface (S) layer, as well as flagella, type 4 pili, adhesins and biofilms (28, 30, 31, 144, 145, 197, 203, 220).

Cytotonic and Cytotoxic Enterotoxins: Thus far, three enterotoxins have been identified in *Aeromonas* species which included *Aeromonas* cytotoxic enterotoxin (Act), *Aeromonas* heat-labile (56ºC) cytotonic enterotoxin (Alt), and *Aeromonas* heat-stable cytotonic enterotoxin (Ast) (28). Alt consists of a single 44 kDa polypeptide chain and it is unrelated to cholera toxin (5). Alt from *A. hydrophila* SSU exhibited biologically activity in both *in vitro* and *in vivo* experiments (31). When Chinese Hamster Ovary (CHO) and intestinal epithelial cells were treated with Alt, it elevated cyclic AMP and prostaglandin levels (PGE_2). The toxin caused fluid accumulation in rat ligated ileal loops (31). Subsequent studies showed that PGE_2 levels in CHO cells treated with Alt were regulated by phospholipase A_2-activating protein (PLAA) (182). Additionally, it was noted that a significantly decreased fluid secretory response was observed in mice that were immunized with the purified recombinant Alt subsequently challenged with the WT *A. hydrophila* (31).

Ast, the second cytonic enterotoxin, is heat stable at 56ºC and was also identified in the SSU isolate of *A. hydrophila* (29). This toxin was found to be biologically active *in vivo* and it stimulated fluid in the rat ligated ileal loop assay (29). In addition, Ast induced elongation of CHO cells along with the elevation of cAMP *in vitro* assays (195).

A 52 kDa cytotoxic enterotoxin (Act) was found in a diarrheal isolate SSU of *A. hydrophila* that induced inflammation and intestinal damage both *in vitro* and *in vivo* models (61, 195, 256). Act is secreted by the T2SS and possesses several biological activities including hemolytic, cytotoxic and enterotoxic (28). Act is initially produced as pre-protoxin and subsequently the active and/or mature form of the toxin is generated by the removal of 23 amino acid residues at the NH_2-terminal end and the proteolytic cleavage of a 4-5 kDa peptide from the COOH-terminal end (30). Two active regions in the protein, amino acid residues 245-274 and 361-405 were found to be essential for the biological activity of Act. Additionally, it was shown that antibodies developed against these peptides significantly reduced the cytotoxic and hemolytic activity of the native Act toxin (61). Act is a pore forming toxin, which binds to cholesterol and induces pore formation (1.14-2.8 nm) in the host cell which allows the entry of water from the external milieu resulting in the lysis of the host cells (61).

In subsequent studies, the role of Act was investigated in terms of inhibition of phagocytosis, induction of proinflammatory cytokines, prostaglandin, and reactive oxygen species (ROS) production, and mortality in mice (28, 32, 33). Act induced the infiltration of macrophages and mononuclear cells at the site of infection, and it up-regulated the expression of genes encoding proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) (33, 182). It was shown that Act stimulated rapid mobilization of calcium from intracellular stores and caused influx of calcium that influenced the production of TNF-α and PGE_2 *via* activation of nuclear factor κB (NF-κB) and cyclic AMP-responsive element binding protein (CREB) transcription factors (33, 67, 182). Treatment of murine macrophages with Act also induced the expression of multiple pro-apoptotic encoding genes such as Bcl-10, BimEL, GADD45 and TDAG51

(69). In summary, these investigations revealed that Act plays a direct role in the induction of host inflammatory response.

Type III secretion system: The T3SS, which is present in many pathogenic Gram-negative bacteria, is a multi-protein, needle-like complex that allows injection of pathogenicity proteins (effectors) directly into the cytosol of eukaryotic cells. These effectors can exert multiple effects on the host cells, including alterations in the cytoskeleton architecture, programmed cell death, cell cycle progression, endocytic trafficking, and gene expression (197, 202, 203). The type III secretion apparatus consists of approximately 20 proteins, most of which are located in the inner membrane and many show homology to the flagellar system components (147). Several highly conserved inner membrane proteins including an ATPase and a series of integral membrane proteins are assembled together to form the T3SS in a highly regulated process (140). The T3SS effector proteins possess a secretion signal within the first 20-to-30 amino acids which is important for recognition of the effector that will be translocated via this secretion machinery (65).

The T3SS plays important roles in pathogenesis of many Gram-negative bacteria including *A. hydrophila*. Deletion of the *aopB* gene , which encoded an *Aeromonas* outer membrane protein was highly attenuated in its ability to induce cytotoxicity in cultured cells (i.e., RAW264.7 murine macrophages and HT-29 human colonic epithelial cells) and was avirulent in a murine model of infection (196). AopB is predicted to be crucial in the formation of the T3SS translocon in *A. hydrophila* (196). Further, an effector, designated as AexU, of the T3SS was recently characterized which caused ADP-ribosylation of host cell proteins, rounded phenotype in HeLa cells, inhibition of phagocytosis, induction of apoptosis, and mouse mortality (203). On the other hand, isogenic mutants of two potent virulence determinants of *A. hydrophila* (AexU and Act)

also showed high reduction in mortality in a mouse septicemic model as well as reduction in the lactate dehydrogenase (LDH) release *in vitro* models of *A. hydrophila* infection (196).

Type VI secretion system: The Type VI secretion system (T6SS) is a newly discovered bacterial protein delivery system which is similar to the phage-like machinery, and is present in at least one-fourth of the all sequenced Gram-negative bacteria (179). It was shown that proteins were secreted and/or translocated via the T6SS in a Sec- or Tat-independent manner as these proteins do not possess any signal peptide (51, 162). However, functions of majority of the proteins present in the T6SS cluster still are largely unknown (51). So far, two T6SS effector proteins, such as the hemolysin coregulated protein (Hcp) and members of the valine-glycine repeat protein family (VgrG) have been well characterized in many Gram-negative bacteria including *A. hydrophila* (21, 150, 172, 173, 218-220).

The T6SS-dependent secretion of Hcp was mostly studied in *V. cholerae* (21, 172, 173). It was shown that Hcp produced hexameric ring which polymerized into tubes forming a channel that could facilitate proteins to be secreted out through this channel (150, 173). The genome of *V. cholerae* contained three genes (*vgrG1*, *vgrG2* and *vgrG3*) that encoded VgrG proteins, and mutations in the *hcp* gene blocked secretion of all of these VgrG proteins (172). Likewise, inactivation of the *vgrG* genes blocked secretion of Hcp (172). This mutual dependence for secretion of Hcp and VgrGs was also reported in other pathogens, such as *Edwardsiella tarda* and enteroaggregative *Escherichia coli* (EAEC) (51, 171, 262). In contrast, some studies also showed that Hcp and VgrG were able to form channels independently (131, 150). In addition, these data also suggested that Hcp and VgrG proteins play dual role: i) to form the secretion apparatus and ii) functions as effectors. Hcp was detected in the clinical specimens of cystic fibrosis (CF)

patients and Hcp antibodies was also found in patient sera, indicating Hcp secretion is not only important *in vitro* but also *in vivo* (64).

The *vgrG* genes are present both in the T6SS gene cluster and outside the cluster and more often linked to the *hcp* gene (172). It was shown that VrgG protein shared common charecteristics with RhsG (Recombination hot spot) protein family (64, 246). It was also predicted that VgrG proteins share features with bacteriophage T4, particularly with the gp5 and gp27 proteins of the bacteriophage T4 tail spike (64, 171). Extensive analysis showed that VgrG proteins from different Gram-negative bacteria have common NH_2-terminal domains but they have distinct COOH-terminal domains (64, 171, 218). Some examples of COOH-terminal extensions include two VgrG proteins of *V. cholerae*. VgrG1 has a repeat in structural toxin A (RtxA) and VgrG3 contains a peptidoglycan binding domain (171). Similarly, VgrG from *P. aeruginosa* carried a zinc-metalloprotease domain, and in *Y. pestis*, VgrGs harbored tropomyosin-like, YadA-like, and pertactin-like domains (171). Recently a vegetative insecticidal protein-2 (VIP-2) domain was detected at the C-terminal of *A. hydrophila* VgrG1 (218). Both Hcp and VgrG proteins were extensively characterized in *A. hydrophila* SSU and our laboratory showed that these effector proteins translocate into the host cells and the T6SS mutants were less toxic to human and murine cell lines and more efficiently phagocytosed by macrophages. In addition, the T6SS mutant strains were less virulent in mouse model (220). Further, VgrG1 of *A. hydrophila* possesses actin ADP-ribosylating activity associated with its carboxy-terminal VIP-2 domain that induces cell rounding followed by host cell apoptosis (218). Hcp, on the other hand, modulates innate immunity by inhibiting phagocytosis of *A. hydrophila*, thus allowing bacterial multiplication and spread to different organs of mice resulting in their death (219).

Bacterial biofilm formation: In nature, bacteria live in a community and adhere to surface rather than as planktonic isolated cells (188). This complex microbial consortium is known as biofilm which is commonly associated with economic and health problems (188). Bacteria present in biofilms are more robust in nature in their ability to withstand chemical and physical stresses and are more resistant to host defenses than the free living or when in the planktonic state (42, 207). Biofilms are not only ubiquitous on the surface of water pipes but also important in medical field because more than 80% of bacterial chronic inflammatory diseases are associated with biofilms (251). Biofilm is generally held together by an extra-cellular polymeric substance (EPS) or exopolysaccharide. This matrix protects bacterial cells and facilitates their communication through chemical signaling. The formation of biofilm is a dynamic process and involves a coordinated series of molecular events, including adhesion, aggregation, and community expansion. Bacteria which are settled in biofilm community adopt different phenotypes as compared to the planktonic counterpart (188). Therefore, biofilm formation is considered as one of the most important virulence mechanisms that contributes to human disease transmission and pathogenesis (85, 160). Several studies also pointed out that QS plays a crucial role in controlling biofilm development and establishing efficient infection in both Gram-positive (160, 166) and Gram-negative bacteria (47, 265). It was shown that biofilm deficient mutant of *P. aeruginosa*, due to the lack of functional QS systems, were less virulent than the wild-type strains (46, 185). On the contrary, in a recent study on *Burkholderia cenocepacia,* it was observed that deletion of a sensor kinase regulator (*atsR*) resulted in a hyper-adherent phenotype (production of increased biofilm formation) both in abiotic surface and lung epithelial cells (8). Further, they demonstrated that the *atsR* mutant overexpressed and hypersecreted an Hcp-like protein (as compared to the WT *B. cenocepacia*) that is secreted by the T6SS and

required for the virulence of bacteria (8). Thus, measurement of *in vitro* and *in vivo* biofilm formation is one of the important factors in determining virulence of the bacterium.

Different Quorum Sensing Systems in Gram-negative Bacteria

QS, a bacterial communication system, involves the production, release, and subsequent sensing of chemical signaling molecules known as autoinducers (AI) that allow bacteria to regulate gene expression in response to changes in cell-population density (10, 177, 247). Bacteria constantly secrete low levels of autoinducers and sense them through their corresponding receptors. Once this critical threshold is reached, the population of bacteria respond by altering gene expression. These alterations can regulate physiological functions, such as sporulation, competency for transformation, turning on/off of various virulence factors, conjugation, antibiotic production, and biofilm formation (10, 177, 247).

Gram-negative bacteria form biofilms both in the environment and in the host gut for colonization, and this feature contributes greatly to the pathogenicity of the bacteria (136). In the intestine, colonization of pathogenic bacteria with a very low infectious dose could be autoinduced by autoinducers produced by nonpathogenic normal gut flora that aid them in causing disease (214). Since QS functions as a global regulator that controls number of virulence factors including biofilm formation in pathogenic organisms (214), QS systems represent an excellent target for therapeutic approach for many infectious diseases. Moreover, recent studies investigated QS as a novel target for infection therapy (99, 213). So far in Gram-negative bacteria, at least three QS circuits have been identified and are designated as LuxRI (AI-1), LuxS/AI-2, and AI-3 epinephrine/norepinephrine. Homoserine lactones (AHLs) function as autoinducers in the AI-1 system, and furanosyl

Figure 1.2. AHL inter-kingdom signaling

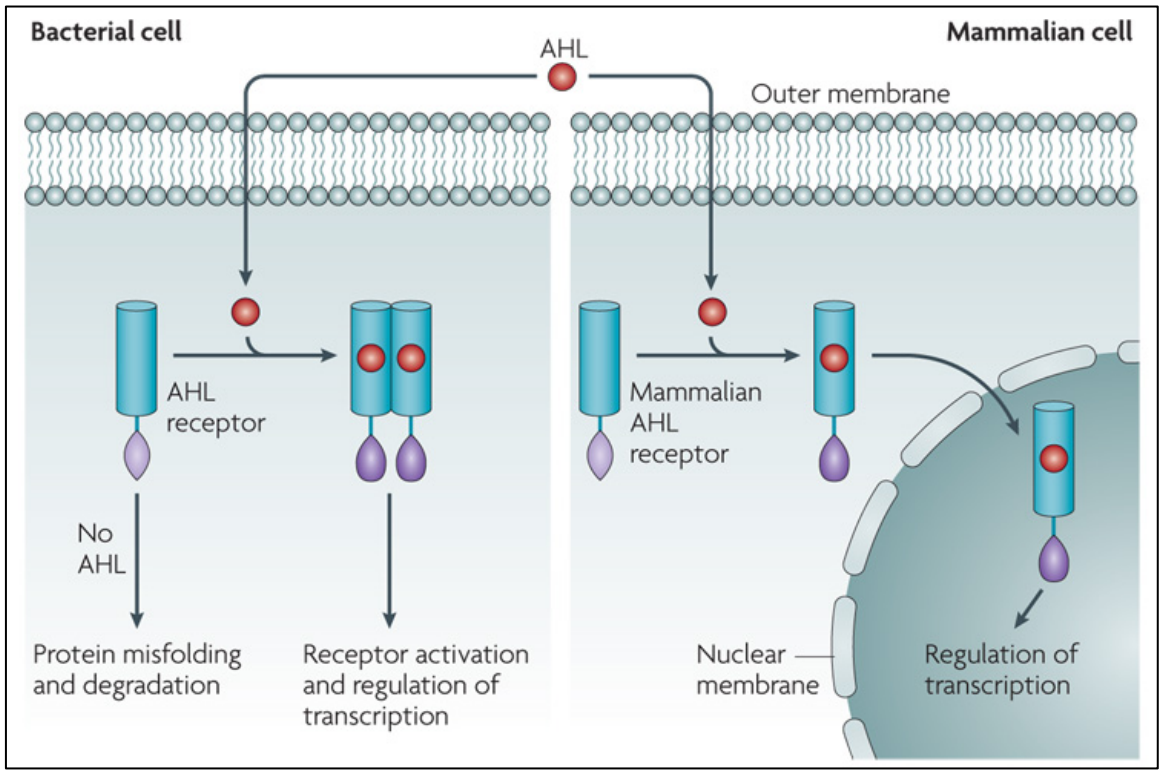

Fig. 1.2. AHL inter-kingdom signaling. In bacteria, AHLs cross the cell membrane and interact with cytoplasmic receptors of the LuxR family. Binding of AHL to the LuxR-type receptor allows proper folding of this protein, which allows the receptor to dimerize and bind to its target sequence on DNA to regulate gene expression. In the absence of signal, LuxR-type proteins misfold and are targeted for degradation. In mammalian cells, AHLs also gain access to the cytoplasm by crossing the plasma membrane. The identity of the mammalian receptor (or receptors) for AHLs in mammalian cells is unknown. However, if there are intracellular receptors, it is proposed that the interaction with the AHL ligand activates these receptors and thereby allows their transportation into the nucleus, where they could control gene expression. Huges *et al*., 2008 (84), © Nature Publishing Group, reproduced with permission.

borate diester and an aromatic compound (structure yet to determined) serve as autoinducers for AI-2 and AI-3 systems, respectively (106, 221).

LuxRI/A-I: The luxRI/AI-1 QS system is the best characterized QS system, and it was first described in the regulation of bioluminescence in *V. fischeri* (247). Later, a homolog of the LuxRI system was detected in many Gram-negative bacteria, including *A. hydrophila*, *Yersinia enterocolitica,* and *Pseudomonas* spp. (49, 136, 231). AHLs are synthesized by AHL synthases; the LuxI protein family, and, once produced, they diffuse in and out of the cell by passive, as well as active transport mechanisms (104, 165). AHLs are composed of a homoserine lactone ring (HSL) with an *acyl* side chain which varies in length from C4 to C18 (126, 139). In some AHL molecules, this *acyl* chain can be modified by a 3-oxo, a 3-hydroxy, or a terminal methyl branch with varying degrees of unsaturation (230). The *acyl* side-chain length and the substitutions on the side chain provide signal specificity (164). The concentrations of AHLs eventually reach a critical threshold, and then they are recognized by their cognate receptors, the LuxR protein family, which represent the second component of the system (34). Binding of AHLs to LuxR regulates expression of the *luxI* gene as well as that of genes that are involved in the virulence mechanisms of many pathogens, including *A. hydrophila,* which leads to various clinical manifestations in humans of all age groups (46, 77, 86, 109, 165). For example, mutation of the *yenI* gene in *Y. enterocolitica* influences motility by altering the expression of the *fleB* gene (7). Tateda *et al*. reported that AHLs in *P. aeruginosa* have a significant effect on their ability to cause disease as they influence bacterial colonization and dissemination in the lungs (228). Moreover, it was shown that a significantly lower numbers of bacteria were found in the lungs of mice infected with the QS mutant as compared to the WT bacterium (209).

Recent studies also unveiled the fact that QS signaling molecules, such as AHLs, are not only the regulators in bacteria, but also modulate different host cells (200, 208-210, 228, 249). However, the mechanisms of this modulation of host cells by AHLs are largely unknown. Although it was shown that peroxisome proliferator-activated receptors (PPARs) could be a mammalian receptor for AHL, the identity of the mammalian receptors for AHLs are largely unknown. Figure 1.2 shows interkingdom signaling of AHLs performing dual role to control bacterial virulence as well as modulate host cell functions.

An earlier study has shown that the *in vitro* effects of 3-oxo-C12-HSL on lymphocytes are immunosuppressive/anti-inflammatory at a low concentration (below 10 μM) and proinflammatory at a high concentration (20 μM and above) (39). Other investigators provided evidence that 3-oxo-C12-HSL promoted apoptosis in various cell types, including neutrophils, monocytes, fibroblasts, and breast carcinoma cells (133, 200, 228). Induction of apoptosis in various immune cells by AHLs might decrease inflammation by reducing the number of effective phagocytes and the mediators they produce (39). This apoptotic induction of host cells mediated by 3-oxo-C12-HSL occurred through a calcium-dependent signaling pathway, while the pro-inflammatory effect of 3-oxo-C12-HSL resulted through a calcium-independent and peroxisome proliferator-activated receptors (PPARs)-mediated pathway (89). The Toll-like receptor (TLR) pathways were not believed to be involved in AHL signaling (122).

LuxS/AI-2: Many bacteria use another autoinducer, AI-2, for interspecies cell-to-cell communication. In these bacteria, the *luxS* enzyme is responsible for the synthesis of AI-2, a furanosyl borate diester (187, 215, 250, 255). The LuxS enzyme is present in approximately one-half of all sequenced bacterial genomes, and this enzyme plays a role in the metabolic pathway of *S*-adenosyl-methionine into which LuxS converts *S*-ribosyl-

homocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable molecule that forms several different furanones after reacting with water (187, 215, 250, 255). In *luxS* mutant studies, it was observed that AI-2/LuxS system also regulates various genes and is responsible for phenotypic alteration of biofilm formation and motility in many bacteria (72, 174, 255). For instance, it was shown that the cell density dependent induction of the *invF* gene was abolished, and there was a decreased expression of the *Salmonella* pathogenecity island 1(SP-1) when the *luxS* gene was deleted (27). In addition, it has been demonstrated that the *luxS* QS system plays a critical role in global regulation of flagellar gene transcription in *Helicobacter pylori,* as altered flagellar morphology was observed in the LuxS mutant (174). In *Listeria monocytogenes,* it was shown that the *luxS*-deficient mutant produced a denser biofilm and attached to a glass surface19-fold better than the WT parental strain (190). However, some studies noted that the *luxS* QS system is more important for metabolic pathways rather than for the virulence of the pathogens (50, 181). Recently in our laboratory, a LuxS/AI-2 QS was identified as a negative regulator in controlling virulence of the clinical strain SSU of *A. hydrophila* (121).

AI-3/epinephrine/norepinephrine: In bacteria, two-component systems are widely used signal transduction mechanisms that facilitate in eliciting an adaptive response to various environmental stimuli, particularly through changes in the gene transcription (12, 79). The two component systems are typically composed of a membrane-associated sensor histidine kinase and a cytoplasmic transcriptional regulator (12, 79). In most cases, the stimuli sensed by these systems are transformed into a cellular signal via autophosphorylation of sensor kinase at the conserved histidine residue. The signal is then passed onto the response regulator, following phosphorylation at the aspartate residue, which results in its activation by undergoing conformational changes.

Figure 1.3. Schematic of the autophosphorylation of QseC in response to signals and phosphotransfer to QseB

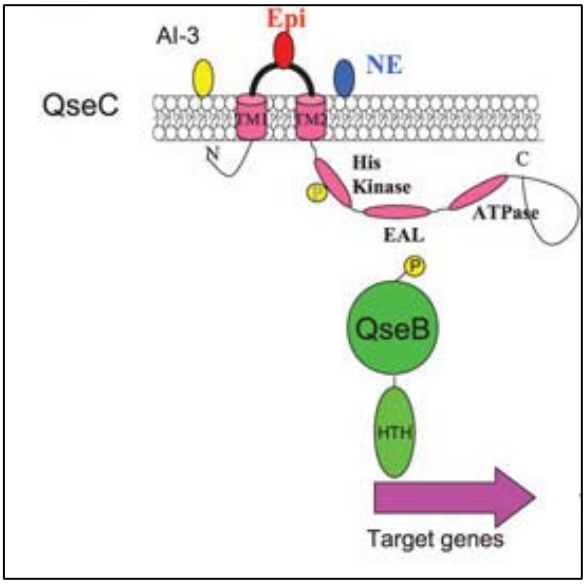

Fig. 1.3. Schematic of the autophosphorylation of QseC in response to signals and phosphotransfer to QseB. QseC is a sensor histidine kinase that responds to QS signal AI-3 and host hormone epinephrine (Epi) and Norepinephrine (NE). QseB is a response regulator, which is activated once phosphorylated and exerts its regulation on the transcription of various target genes. Rasko *et al*., 2008 (176) © American Association for the Advancement of Science, reproduced with permission.

The activated response regulator then exerts its regulation on the transcription of various target genes (**Fig.1.3**) (12, 79). One such two component system, QseBC that responds to QS signal AI-3 was first discovered in enterohemorrhagic *Escherichia coli* (EHEC) (216). In this two component system, QseC functions as a sensor kinase, and QseB acts as a response regulator (**Fig.1.3**). Later, this system was also found in other pathogens, such as *S. enterica* serovar Typhimurium (11), *E. tarda* (245), and uropathogenic *E. coli* (UPEC) (119). In addition to responding to AI-3, several studies have shown that QseC sensor kinase also responds to eukaryotic hormones typified by epinephrine and/or

norepinephrine (36, 243, 244). Further, by using adrenergic receptor antagonists, it is possible to block the effects of AI-3 and epinephrine/norepinephrine, suggesting a similar structure for these molecules and a similar signaling pathway (244). The role of QseBC system in the pathogenesis of EHEC, *Francisella tularensis*, and *S.* Typhimurium was recently reported (176), as deletion of the QseC histidine kinase-encoding gene attenuated virulence of the above-mentioned bacteria (176). Further, a synthetic compound (LED209) that interferes with the QseC signaling inhibited *in vitro* virulence of EHEC, *S.* Typhimurium and *F. tularensis* and also modulated *in vivo* virulence of the latter two pathogens (176). In UPEC, a recent study has shown that in addition to kinase activity, QseC has phosphatase activity that is critical in modulating regulatory activity of QseB (119). Recently, an association of biofilm formation and AI-3 mediated quorum sensing has also been reported (72). In *E. coli,* the T3SS gene transcription and protein secretion, encoded by the LEE pathogenecity Island, has been found to be regulated by the AI-3/epinephrine/norepinephrine QS system (214). This T3SS is regulated by another two component system, QseEF that controls the gene encoding QseA to regulate T3SS (178, 198).

CHAPTER 2

Distribution of Virulence Factors and Molecular Fingerprinting of *Aeromonas* Species Isolated from Water and Clinical Samples: Suggestive Evidence of Water-to-Human Transmission[1]

INTRODUCTION

Aeromonas-related gastroenteritis remains somewhat controversial (63, 93). The literature does contain a number of well-described cases and a few documented outbreaks, but it still is somewhat questionable as to whether all aeromonad fecal isolates from symptomatic persons are the actual cause of the diarrheal disease. One theory for this conundrum was posed in 2000 by two of the authors of this paper, which suggests that only specific subsets of *Aeromonas* strains within and between species are actually pathogenic for humans (101). This highlights the importance of the development of accurate biotyping, molecular fingerprinting, and virulence factor methods when striving to differentiate and compare environmental and clinical aeromonads from one another (101).

Of the 19 currently recognized *Aeromonas* species, *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria are the most common species known to cause the majority of human infections and account for more than 85% of all clinical isolates (91). The pathogenesis of *Aeromonas* infections is multifactorial, as this pathogen produces a wide variety of virulence factors, including hemolysins, cytotonic and cytotoxic enterotoxins,

[1] Khajanchi, B. K., A. A. Fadl, M. A. Borchardt, R. L. Berg, A. J. Horneman, M. E. Stemper, S. W. Joseph, N. P. Moyer, J. Sha, and A. K. Chopra. 2010. Distribution of virulence factors and molecular fingerprinting of *Aeromonas* species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. Appl Environ Microbiol **76:**2313-2325.

proteases, lipases, leucocidins, endotoxin, adhesions, and an S-layer, that act in concert to cause disease in the host (28, 30, 31, 144, 145). The production of such a wide array of virulence factors by *Aeromonas* species is indicative of its potential to cause severe diseases in humans. These virulence factor-encoding genes might be differentially expressed in *Aeromonas* species based on their environmental status such as the presence in water versus the human host.

The presence of any virulence gene in strains of *Aeromonas* isolated from water should be carefully scrutinized, as such genes could be better expressed in a human host and lead to devastating outcomes. In addition, it is possible that in the environment certain *Aeromonas* clones may predominate and cause human diseases more frequently than do other clones. Thus, it is important to determine the clonal variation of a range of *Aeromonas* species from various isolation sources and identify predominant clones by a polyphasic approach that includes biochemical phenotyping, virulence marker detection, and molecular fingerprinting techniques.

In the present study, we compared 199 *Aeromonas* isolates, of which 146 were from water sources and 53 from human patients with diarrhea within the Unites States. In addition, 28 reference and classical strains that were either obtained from various culture collections and/or isolated from worldwide and diverse geographical sources, including water and clinical specimens, were also characterized. All isolates were biochemically identified to the phenospecies Group level and examined for the presence of a set of 11 virulence factors by DNA colony blot hybridization and fingerprinted by pulsed-field gel electrophoresis (PFGE). Some of the selected virulence factors, including T6SS effectors, were also examined by functional assays. Our data provide the first suggestive evidence of water-to-human transmission, i.e., of successful colonization and infection with particular strains of certain *Aeromonas* species.

MATERIALS AND METHODS

Bacterial strains. The collection of strains examined in this study originated from multiple sources and collections. One set of 83 strains (originally 84 but NM 59 did not grow for biochemical studies) strains was selected from the larger Dr. Nelson Moyer (NM) collection of clinical and water isolates from his past epidemiological studies related to *Aeromonas* and diarrhea that were identified using conventional biochemical tests and ribotyped (151, 153, 154)**.** A second collection of 101 environmental strains were obtained from the USA Environmental Protection Agency (EPA), with their species Group identification "blinded" and these originated from a variety of water sources. A third smaller set of 17 clinical diarrheal stool isolates were received from Dr. Mark Borchardt (MB) and originated from patients at the Marshfield Clinic in Marshfield, Wisconsin. The last set of 28 reference and classical strains were from the collection of Dr. Amy Horneman**.** For the biochemical phenotypic tests, the strains were grown on SBA (Sheep Blood Agar, BDMS, Sparks, MD) plates and incubated at 37^{o}C. Stock cultures were maintained frozen at -80^{o}C in trypticase soy broth (TSB) with 30% (v/v) glycerol.

Genus identification of *Aeromonas* isolates. All strains were initially screened by using the following tests: Gram stain; oxidase activity (1% solution of N, N, N', N'-tetramethyl-ρ-aminodimethylaniline oxalate); acid production from glucose using a triple sugar iron (TSI) slant; motility using semi-solid agar; and resistance to O/129 Vibriostat (150 μg/ml, Oxoid; Ogensburg, NY). Only those strains that were motile, oxidase-positive, produced acid from gluocse, and were O/129-resistant and Gram-negative rods were considered to be members of the genus *Aeromonas*.

"Species-Group"identification. Each strain was then examined using a battery of biochemical tests from Aerokey II^{TM} (20), the 2003 Abbott and Janda Schema (1) and

selected supplementary biochemicals from *Bergey's Manual of Systematic Bacteriology*, including esculin hydrolysis, indole production (Kovac's method); methyl red, Voges-Proskauer; citrate utilization; the presence of lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase; acid and gas production from glucose; acid production from arabinose, cellobiose, lactose, mannitol, rhamnose, salicin, sorbitol, and sucrose; hydrolysis of urea; ONPG production; and the inability to grow in the presence of 6.5% NaCl.

Additionally, all isolates were tested for susceptibility to ampicillin (10 μg) and cephalothin (30 μg) by using the latest CLSI charts and guidelines. All of these tests were performed using conventional methods previously published (1, 20, 82). Using the test results for each isolate and the combined references, a "Species-Group" identification of either *A. hydrophila* Group, *A. caviae/media* Group, or *A. veroni/sobria* Group, was given to each isolate. Each "Species-Group" includes at least 3 different DNA hybridization groups and phenotypic species (1, 92). For example, the *A. hydrophila* Group includes *A. hydrophila, A. bestiarum* and *A. salmonicida* (both motile and non-motile species). Likewise, the *A. caviae* Group includes *A. caviae, A. media, and A. eucrenophila*, and the *A. veronii/sobria* Group includes *A. veronii* biovars sobria*, A. jandaei, A. schubertii*, and *A. trota.* The "Species Group" designation was used because of the preponderance of water isolates, where presumably all aeromonad species reside. However, there is not sufficient published data to support the actual species identification of such environmental isolates without additional uncommon biochemical tests. If an isolate could not be definitely placed into any one "Species Group", it was considered to be an "atypical" aeromonad.

Colony blot hybridization. *Aeromonas* cultures (clinical and water isolates) were inoculated atop nylon membrane filters in a grid pattern placed on Luria-Bertani

(LB) agar plates. The plates were incubated at 37°C for 4-8 h and then filters were removed and processed (5). After denaturation and neutralization, the filters were extensively washed with 1.5 M NaCl + 1 M Tris, pH 7.4 and chloroform under vacuum to remove cell debris. Then, the filters were dried, baked at 80°C for 2 h and prewashed with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, and 0.1% SDS at 42°C to remove additional cell debris. For hybridization, filters were first prehybridized in Quickhyb solution (Stratagene, La Jolla, CA) at 68°C for 2 h, and then hybridized by using Quickhyb and [α-^{32}P] dCTP-labeled gene probes under high-stringency conditions for 3 h at 68°C. The membranes were washed twice at 68°C in 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH-7.0) plus 0.1% SDS for 20 min, and then twice in 1x SSC plus 0.1% SDS for 20 min at 68°C. Subsequently, the filters were dried and the X-ray film was exposed to the filters (167).

The probes encompassed coding regions of the following genes: *act*, *alt, ast*, *ascV* (constitutes T3SS); *aexU* (an effector protein secreted *via* the T3SS); *gidA*; enolase; *dam*; *tagA*; *hlyA* (hemolysin); and *ahyRI* (autoinducer synthase/transcription regulator of AHL). The probes were prepared by polymerase chain reaction (PCR) using specific primers for each of the genes and the appropriate plasmid DNA as the template**.** Conditions for PCR included an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing of the primers at 55-68°C for 1 min, and extension at 72°C for 1 min. After final extension at 72°C for 10 min, the reaction mixtures were held at 4°C until the PCR products were analyzed by agarose gel electrophoresis. Probes were labeled with [α-^{32}P]dCTP (MP Biomedicals, Solon, OH) by using a random primer kit (Invitrogen, Carlsbad, CA) (57). For each colony blot DNA hybridization assay, *A. hydrophila* SSU and *E. coli* DH5α were used as a positive and a negative control, respectively.

Measurement of hemolytic activity. To examine the lysis of rabbit red blood cells (RBCs), the culture filtrates from various *Aeromonas* cultures (grown for 18 h in LB medium at 37°C with shaking [180 rpm]) were first treated with trypsin (final concentration, 0.05%) at 37°C for 1 h and then subjected to hemolytic assay (195). For the assay, 100 μl of 1X (Dulbeco's PBS) DPBS was added to each of the wells of a 96-well microtiter plate. The above-treated culture supernatants were added to the first well in each row of the microtiter plate followed by serial twofold dilution and the addition of 100 μl of 3% rabbit erythrocytes (Colorado Serum Co., Denver, CO). The negative control included 1X DPBS and trypsin alone. The plate was incubated at 37°C for 1 h and observed for hemolytic activity associated with Act and HlyA. We defined hemolytic titer as the reciprocal of the highest dilution of the culture filtrates that caused 50% lysis of the red blood cells. These hemolytic activity titers were divided by the optical density of the culture to obtain hemolytic activity/unit of growth. The results were presented per ml of the supernatant. The treatment of culture filtrates with trypsin was required to activate Act and HlyA for measuring hemolytic activity, as RBCs do not produce any of their own proteases (58, 59).

The limitation of the hemolytic activity assay used was that various hemolysins produced by *Aeromonas* species might have different affinities for rabbit erythrocytes. However, because of the number *Aeromonas* isolates that were examined in this study, it was not possible for us to employ RBCs from different animal species. Since most *Aeromonas* hemolysins can be detected by using rabbit erythrocytes, we opted to use only these RBCs. We successfully detected hemolytic activity of Act and HlyA (in the *act* minus strain) of *A. hyrophila* SSU after trypsin treatment using rabbit erythrocytes (59). However, an earlier study demonstrated that trypsin treatment might inactivate the biological activity of some hemolysins produced by *Aeromonas* species (157). In our

analyses of the *Aeromonas* isolates, we used culture filtrates both before and after trypsin treatment and detected only increased hemolytic activity after trypsin treatment associated with Act and HlyA. These data suggested that none of the *Aeromonas* isolates used in this study had trypsin-sensitive hemolysin(s).

Measurement of cytotoxic activity. The RAW 264.7 murine macrophage cell line (American Type Culture Collection, Manassas, VA) was used for the cytotoxicity assay (195). The culture filtrates from various *Aeromonas* cultures were serially diluted (2-fold dilution) and added to the macrophages. The cytotoxicity was measured by examining cells under the microscope after 18 h of incubation. The dead cells were rounded and detached from the monolayer. Alternatively, the adherent cells in the monolayer were stained with Giemsa and then examined under the microscope. We defined cytotoxicity titer as the reciprocal of the highest dilution of the culture filtrate that caused 50% destruction of RAW cells. These cytotoxic titer values were divided by the optical density of the bacterial culture to obtain cytotoxic activity/unit of growth. The results were calculated per ml of the supernatant.

Measurement of protease activity. An aliquot (200 µl) of overnight culture filtrates (in the presence of 0.2% arabinose) from various *Aeromonas* strains was added to disposable 6-ml, snap-cap tubes which contained 800 µl of the DPBS and 5 mg of Hide azure powder substrate (Calbiochem, La Jolla, CA). The tubes were incubated in a shaker incubator at 37°C for 1 to 3 h. As the proteinase in the culture filtrates catalyzed the substrate, blue color was released from the substrate and was quantified at $OD595_{nm}$. The proteinase activity was calculated per ml of culture filtrate and/or per 10^8 colony forming units (cfu). The hide azure powder substrate was used for measuring protease activity because of the sensitivity and rapidity of the assay. Further, this substrate could detect both metallo- and serine proteases, which are the two major classes of proteases

produced by *Aeromonas* species. The substrate incubated with DPBS alone served as a negative control.

N-*acyl* homoserine lactone (AHL) production. AHL production was detected by cross-streaking of various *Aeromonas* strains against the biosensor strain *Chromobacterium violaceum* CV026 on LB agar medium (141). The lactones produced by *Aeromonas* isolates diffused through the agar and induced the production of a pigment violacein in the biosensor strain (222), resulting in varying intensity of the purple color depending upon the amount of lactones produced by *Aeromonas* isolates after overnight incubation at 30^{o}C. The range of the scale for lactone production was designated as – (no lactone production), + (very weak), ++ (weak), +++ (moderate), ++++ (high), +++++ (very high level of lactone production) (**Fig. 2.1**). An isogenic *ahyRI* mutant of *A. hydrophila* SSU was used as a negative control, as this mutant was negative for lactone production (109). Although several reporter stains are available for measuring AHL production, *C. violaceum* CV026 is particularly sensitive for the detection of N-*acyl* chains varying in length from C4 to C8. Earlier studies have shown that *Aeromonas* species produce C4-C6 homoserine lactones (HSLs) as the major AI-1 autoinducer molecules (18, 222); consequently, in this study we used *C. violaceum* for measuring AHLs. However, some of the *Aeromonas* isolates might produce longer *acyl* chain HSLs and in that case *Agrobacterium tumefaciens* A136 (264) can be used for their detection.

PFGE for DNA fingerprinting of *Aeromonas* isolates. The PFGE procedure for *Aeromonas* was modified from methods previously described (225). Isolates were grown overnight in 5 ml of Brain Heart Infusion broth at 37^{o}C, harvested by centrifugation, and washed with 1 ml PIV buffer (10 mM Tris-HCl [pH 7.6], 1 M NaCl). Pelleted cells were adjusted to a concentration of 1 x 10^{9} cfu/ml in PIV buffer by using a Vitek colorimeter (Hach Co., Loveland, CO). The cells were mixed with an equal volume of 2% low

melting agarose (FMC Bioproducts, Rockland, ME), dispensed into plug molds (Bio-Rad Laboratories, Hercules, CA), and allowed to solidify for 10 min at room temperature. Plugs were incubated in 3 ml of the lysis buffer (6 mM Tris-HCl, 1.0 M NaCl, 0.1 M EDTA, 0.5% Brij 58, 0.5% sarkosyl, 0.2% deoxycholate, 1 mg/ml lysozyme) at 37°C for 4 h. Lysis buffer was replaced with proteinase K solution (0.5 M EDTA, 1% N-lauroyl sarcosine, 1 mg/ml proteinase K) followed by incubation at 55°C overnight. Plugs were washed 4 times in TE buffer (10 mM Tris-HCl, 0.1 M EDTA [pH 8.0]) and stored at 4°C.

The genomic DNA (gDNA) was digested with 30 U *Xba*I (Promega, Madison, WI) at 37°C overnight. Electrophoresis was performed in 1% Seakem Gold agarose (FMC Bioproducts) by using the CHEF-DRIII system (Bio-Rad) in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM and EDTA [pH 8.0]) at 14°C. The running parameters were 6 V/cm for 20 h with 0.5 - to 20-sec pulses. *Salmonella* serotype Braenderup strain H9812 digested with *Xba*I was run in multiple lanes of each gel as a DNA global reference for standardizing runs and to determine DNA band size. DNA banding patterns were visualized with 0.1% ethidium bromide and digitally photographed. BioNumerics software (version 4.01 Applied Maths, Austin, TX) was used to compare the genetic similarity among isolates and to construct a similarity dendrogram using the Dice coefficient and the UPGMA algorithm (unweighted pair-group method with arithmetic mean) with a position tolerance of 1.0% (16).

Westernblot analysis. Western blot analysis was performed according to established procedures. Overnight cultures of *Aeromonas* strains were diluted 1:20 in fresh LB medium and grown for 2 h (absorbance ~0.8 at OD_{600}) and 4 h (absorbance ~1.4 at OD_{600}) at 37°C with shaking at 180 rpm. To measure protein levels in the cells, 2 ml of culture was pelleted (10000 *g* for 10 min at 4°C) and dissolved in 200 µl of 2x sample buffer. To measure secretion of the proteins in the LB medium, 2 ml supernatants were

separated from the pellet and filtered through a 0.22-μm membrane filter. Proteins present in the supernatant fraction were precipitated with trichloroacetic acid (TCA) (10% final concentration) and pelleted by high- speed centrifugation at 14000 *g* for 15 min at 4ºC. The pellet was re-suspended in 50 μl of 2x sample buffer. Briefly, equal amounts of total protein were loaded and separated on SDS-10% PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and washed in 1X Tween (0.1%)-Tris-buffered saline (TTBS) three times for 5 min each. Primary antibodies diluted 1:1000 or 1:5000 in 5% milk or bovine serum albumin (BSA) (prepared in 1X TTBS) were allowed to incubate overnight at 4^0C. After washing, HRP-conjugated secondary antibody (Cell Signaling Technology, Denvers, MA) was diluted 1:20,000 in 5% milk and applied to membranes. Subsequently, membranes were washed and a chemiluminescence substrate (Pierce Biotechnology, Rockford IL) was applied and allowed to incubate at room temperature for 5 min, before exposing the membranes to X-ray films.

Statistical analysis. The prevalence of species groups among the isolates was summarized by isolate source (stool, water, and water source). Standard descriptive statistics were used to summarize factors related to virulence by species group and source. Species group prevalence and the presence/absence of virulence factors were compared by source using Fisher's exact test. Comparisons of activity levels for the virulence factors by species group were made with the Wilcoxon rank-sum test (analogous to the t-test or ANOVA with two groups). Results were deemed statistically significant at the 5% level ($p < 0.05$) with no correction for multiple comparisons. Adjustment for multiple comparisons is always potentially controversial, because adjustment to reduce false positives always increases the false negative rate, thus reducing the power.

Table 2.1. Identification of *Aeromonas* species from water and clinical samples

Species Identification	Water (%)[a]	Stool (%)[a]
A. hydrophila Group	87 (59.5)	7 (13.2)
A. veronii/*sobria* Group	23 (15.7)	3 (5.6)
A. caviae/*media* Group	21 (14.3)	38 (71.6)
Atypical aeromonads	15 (10.2)	5 (9.4)
Total	146	53

[a] Numbers in parentheses indicate percentages of isolates of a specific species group among the total number of isolates from a given source (e.g., water versus stool)

Table 2.2. Water source from which *Aeromonas* isolates were obtained

Species Identification	Source of water					
	SW	GW	DW	WW	WHHW	KTW
A. hydrophila Group	36	34	15	0	0	2
A. veronii/*sobria* Group	13	8	1	1	0	0
A. caviae/*media* Group	0	6	6	5	2	2
Atypical aeromonads	4	6	2	2	1	0

Abbreviation: SW, surface water; GW, ground water; DW, distribution water; WW, well water; WHHW, well head hydrant water; KTW, kitchen tap water. Indicated numbers denote number of *Aeromonas* species Group isolates obtained from the designated water source

RESULTS

Biochemical properties of *Aeromonas* isolates. All of the *Aeromonas* strains produced medium-sized tan to buff-colored colonies on Trypticase Soy Agar (TSA) with 5% Sheep Blood when incubated at 35^{o}C for 2-5 days. The overwhelming majority of the strains likewise displayed beta-hemolysis on the TSA blood agar plates, with this beta-hemolysis being significantly stronger (3-4+) with the *A. hydrophila* and *A. veronii/sobria* Group strains and somewhat "weaker" (1-2+) or alpha-hemolytic or gamma-hemolytic with the *A. caviae* Group strains.

The breakdown of "Species Group" numbers for each set of isolates was as follows: The NM collection (n=81, only constituting water isolates as there were 2 isolates obtained from food) included n=41 *A. caviae/media* Group strains, n=17 *A. hydrophila* Group, and n=12 *A. veronii/sobria* Group, and n=11 "atypical aeromonads". The MB collection (n=17) included n=12 *A. caviae/media* Group strains, n=3 *A. hydrophila* Group, n=2 atypical aeromonad strains and no *A. veronii/sobria* Group strains. The largest group, the EPA collection (n=101), included n=6 *A. caviae/media* Group strains, n=74 *A. hydrophila* Group, n=14 *A. veronii/sobria* Group strains and n=7 atypical aeromonad strains (data not shown).

Sources of species groups. A total of 227 *Aeromonas* isolates were included in this study, which encompassed 53 clinical stool isolates, 146 water isolates, and 28 reference and classical strains from environmental, clinical and veterinary sources. The majority of the water and clinical stool isolates were collected from different geographical locations within the United States, with the remainder originating from worldwide locations. Among water samples, the *A. hydrophila* Group was the most prevalent and accounted for 59.5% (n=87), followed by the *A. veronii*/*sobria* (15.7%;

n=23) and the *A. caviae*/*media* Group (14.3%; n=21). The least prevalent of all were the atypical aeromonads 10.2% (n=15) **(Table 2.1)**.

Water isolates were obtained from different sources, such as surface water (SW), ground water (GW), well water (WW), well head hydrant water (WHHW), kitchen tap water (KTW) and municipal drinking water distribution systems (DW). The *A. hydrophila* and *A. veronii/sobria* Groups were more commonly found in SW or DW and GW, whereas the *A. caviae/media* Group was commonly isolated from DW, GW and WW. Atypical aeromonads was more common in GW and SW **(Table 2.2)**.

In clinical samples, the *A. caviae*/*media* Group was the predominant group consisting of 71.6% (n=38) of the total isolates, followed by the *A. hydrophila* Group at 13.2% (n=7), the atypical aeromonads at 9.4% (n=5), and the *A. veronii*/*sobria* Group at 5.6% (n=3) **(Table 2.1)**.

Hemolytic activity. The hemolytic activity associated with *Aeromonas* isolates was measured by using rabbit RBCs. In water isolates, the highest percentage of the positive strains (97%) was found in the *A. hydrophila* Group, whereas the lowest percentage (10%) was in the *A. caviae/media* Group. Similarly for the clinical isolates, these percentages were 100% for the *A. hydrophila* Group and 3% for the *A. caviae/media* Group. Importantly, for the *A. veronii/sobria* Group, all of the stool isolates (100%) were hemolytic, while only 61% of the water isolates were positive for the hemolytic activity. For atypical aeromonads, 67% of the water isolates were positive for hemolytic activity, while 40% of the stool isolates had this activity (data not shown).

The level of hemolytic activity detected in the water *A. hydrophila* Group ranged from very low to very high (4 to 7,314 units). This activity was in a low and narrow range (19-33 units) in the *A. caviae/media* Group isolated from the water samples. On the other hand, among the clinical (stool) strains, the *A. veronii/sobria* Group produced the highest

hemolytic activity (750-1,765 units), followed by the *A. hydrophila* Group (20-1,406 units), atypical aeromonads (320-457 units), and the *A. caviae/media* Group (50 units).

We performed statistical analysis on the prevalence of hemolysin(s) (based on functional biological assay) in water and stool *Aeromonas* isolates across all three groups (*A. hydrophila*, *A. caviae/media*, and *A. veronii/sobria*). The mean hemolytic activity of isolates from water samples was 336.5, with a standard deviation and median of 850.4 and 80, respectively. The corresponding numbers were 133.7, 364.5, and 0, respectively, for the stool isolates. The water isolates had a statistically significant higher hemolytic activity compared to that in the stool isolates ($p<0.001$).

However, the trend of data was reversed when we considered different groups of *Aeromonas* separately. For example, the mean hemolytic activity of *A. hydrophila* Group isolates from water samples was 376.1 units, which increased to 525.5 units for the clinical isolates of the *A. hydrophila* Group **(Table 2.3)**. Likewise, in the *A. veronii/sobria* Group, the mean hemolytic activity was 854.5 units for the clinical isolates and 501.0 units for the water isolates **(Table 2.3)**. Although these data were not statistically significant, an upward trend in the hemolytic activity was clearly noted for the clinical isolates. In the case of the *A. caviae/media* Group, however, the clinical and water isolates had mean hemolytic activities of 1.3 and 2.4 units, respectively. These numbers were very low for both the water and stool isolates, which suggested to us that the *A. caviae/media* Group is either a low or non-producer of hemolysins **(Table 2.3)**. The mean hemolytic activity of reference *Aeromonas* strains was 1160.8 for clinical samples and 279.5 for water samples.

Cytotoxic activity. The cytotoxic activity of various *Aeromonas* isolates was measured by using a macrophage RAW 264.7 cell line. Both in water and stool samples, 98-100% of the isolates belonging to the *A. hydrophila* Group produced cytotoxic

activity. A minimal number of *A. caviae/media* Group isolates (water or stool) caused cytotoxicity in RAW 264.7 macrophages, and nearly no cytotoxicity was detected in the stool isolates (3%) of the *A. caviae/media* Group. All of the *A. veronii/sobria* Group stool isolates, on the other hand, had associated toxicity, while only 61% of this group of isolates from water samples exhibited cytotoxic activity, while that associated with the percentage of atypical aeromonads of water and stool isolates was 73 and 40%, respectively.

Like hemolytic activity, the prevalence of cytotoxic activity in *Aeromonas* isolates when examined across three species groups (*A. hydrophila*, *A. caviae/media*, and *A. veronii/sobria*) was significantly greater in water isolates than in stool isolates. For example, the mean cytotoxic activity for the water isolates was 11,391 units (standard deviation of 27,379 and median of 1190.7) compared to 6,328 units (standard deviation of 17,071 and median of 0) for the stool isolates and was statistically significant ($p<0.001$). However, when each group of *Aeromonas* species was examined separately, an opposite scenario emerged. For instance, the mean cytotoxic activity of clinical isolates of the *A. hydrophila* Group was significantly higher (29,820 units, $p=0.021$) compared to that of the water sample isolates (13,716 units) **(Table 2.3)**. In the case of the *A. veronii/sobria* Group, the mean cytotoxic activity for clinical and water isolates was 19,516 and 12,944 units, respectively ($p = 0.065$) **(Table 2.3)**. Likewise, the mean

Table 2.3. Statistical analysis on hemolytic and cytotoxic activities produced by water and stool isolates of *Aeromonas* species

Species	Source (n)	Activities/O.D. unit/ml							
		Hemolytic				Cytotoxic			
		Mean	S.D.	Median	p-value	Mean	S.D.	Median	p-value
A. hydrophila Group	Water (87)	376.1	911.9	116.7	0.105	13716	29717	2382.7	0.021
	Stool (7)	525.5	487.3	405.0		29820	32572	15437	
A. veronii/*sobria* Group	Water (23)	501.0	945.7	123.1	0.269	12944	29046	1280.0	0.065
	Stool (3)	854.5	863.0	750.0		19516	11712	24904	
A. caviae/*media* Group	Water (21)	2.4	8.0	0.0	0.268	254.8	876.6	0.0	0.037
	Stool (38)	1.3	7.9	0.0		640.1	4047.7	0.0	

S.D.: Standard deviation

cytotoxic activity associated with the *A. caviae/media* Group water isolates was 254.8 units and 640.1 units for the stool isolates, indicating a significant increase in the activity for the latter isolates (p=0.037) **(Table 2.3)**. In reference strains, the mean cytotoxic activity of *Aeromonas* species for clinical and water samples isolates was 32,393 and 21,497, respectively.

Protease activity. The distribution of the strains that had protease activity was similar between the water and clinical strains. Like the cytotoxic activity data, in the *A. caviae/media* Group, the mean protease activity was greater in clinical strains (7.1 units) than it was in the water isolates (2.9 units), but the data did not reach statistical

significance (p = 0.079). In the *A. hydrophila* and *A. veronii*/*sobria* Groups, the mean protease activity was either similar or greater in the water sample isolates when these were compared to the clinical sample isolates but the data were not statistically significant. The mean protease activity for the reference strains was 4.7 for clinical isolates and 3.95 for water isolates (data not shown).

N-*acyl* homoserine lactone production. Comparing AHL production between water and stool isolates among the three *Aeromonas* species Groups using the Fisher's exact test, a greater percentage of stool isolates of *A. hydrophila* and *A. caviae/media* (p=0.019-0.028) produced more AHLs than water isolates, whereas the reverse was true for *A. veronii/sobria* Group; the percentage of water isolates producing AHLs was higher than for stool isolates **(Fig. 2.1)**.

Distribution of virulence factors. All *Aeromonas* isolates were examined for the presence of 11 virulence genes by using specific probes in colony blot DNA hybridization under high stringency conditions. In water samples, 79% or more of the *A. hydrophila* Group strains harbored six virulence factor genes such as *alt*, *dam*, *tagA*, *gidA*, *ahyRI*, and *enolase*, while among the clinical samples, all the *A. hydrophila* Group isolates contained these six virulence factor genes. Approximately, 45-57% of the *A. hydrophila* Group isolates obtained from both water and clinical samples were positive for *hlyA*, *ascV* and *aexU* genes (data not shown). However, the *act* gene was more prevalent in water isolates (83%) than in clinical isolates (57%) of the *A. hydrophila* Group. In the *A. caviae/media* Group, *alt*, *dam*, and *ahyRI* genes were more prevalent in clinical isolates than in water isolates. In addition to *alt* and *dam* genes in the *A. veronii/sobria* Group, *act*, *gidA* and *enolase* genes were more common in clinical than in water isolates. Further, T3SS genes (*ascV* and *aexU)* were found in both water and clinical isolates of *A. hydrophila* and *A. vernonii/sobria* Groups, while none of the

Figure 2.1. Hue diagram showing levels of N-*acyl* homoserine lactones (AHLs) produced by different species Groups of *Aeromonas*

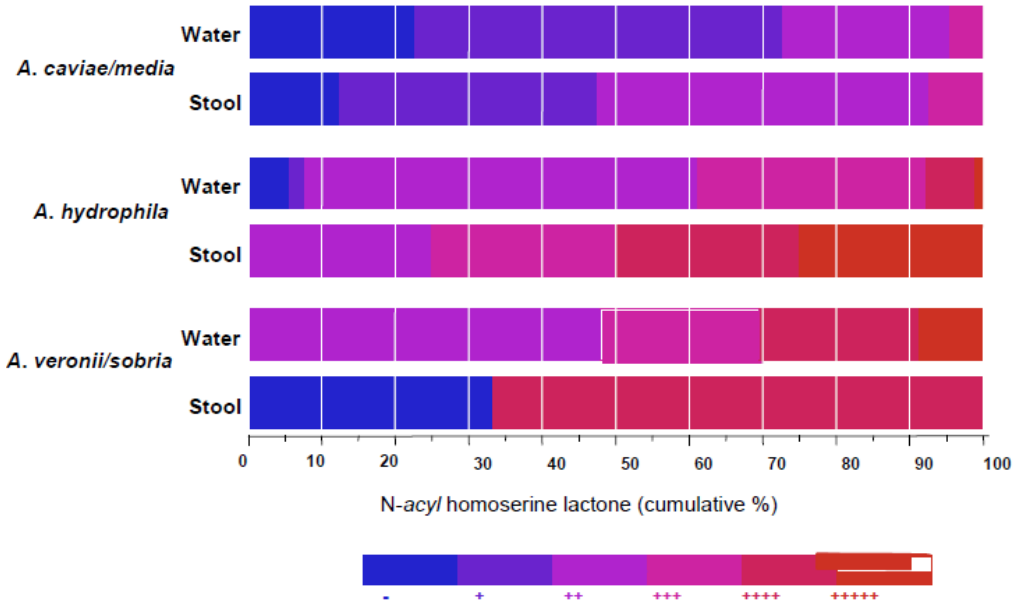

Fig. 2.1. AHL production was detected using *Chromobacterium violaceum* CV026 biosensor strain. Symbols + to +++++ represent different levels of AHLs produced and are described in materials and methods section.

water and stool isolates in the *A. caviae/media* Group harbored these T3SS genes. In reference strains, 61.5% of the clinical isolates and 16.7% of the water isolates harbored *act,* while 38.5% of the clinical isolates and 50% of water isolates had both *ascV* and *aexU* genes.

PFGE analysis. Of the 227 study isolates, 226 were typeable by PFGE and are reported as a similarity dendrogram. The one non-typeable isolate, EPA-3, was isolated from water and identified as *A. veronii/sobria* Group. The *Xba*I digestion resulted in 15-

24 well-resolved genomic DNA bands ranging in size from approximately 35-780 kb. Among the 226 isolates, there were 177 distinct pulsotypes exhibiting extensive genetic diversity (data not shown). Analyzing all pairwise comparisons, the median similarity among all isolates was 66.7% (range 37.8% to 100%, n = 25,425), and among the three species groups, the median similarities were 66.7% for *A. hydrophila* Group (range 43.9% to 100%, n = 5,460), 70.8% for *A. veronii/sobria* Group (range 55.8% to 100%, n = 406), and 66.7% for *A. caviae/media* Group (range 37.9% to 100%, n = 2,016).

To determine the clonal relationships between the water and clinical isolates, we compared virulence clusters with the cluster analysis of the PFGE data and found three sets of isolates from stool and water samples indistinguishable by PFGE and having the same or similar virulence signatures **(Fig. 2.2)**. This is not surprising since one of the authors (Nelson Moyer) had previously reported that the pair NM-14 and NM-35 had the same conventional biochemical identification of *A. caviae* and a matching ribotype pattern (152). Likewise, the pair of strains NM-22 and NM-33 had the same conventional biochemical identification of *A. caviae* and a different albeit matching ribotype pattern (152).

Epidemiological data. NM-22 was isolated in 1988 from the stool of a child in Iowa that was ill with gastroenteritis and had presumably been exposed to water from the family well. NM-33 was isolated from the suspected infection source, the private household well used by the child's family. This epidemiologic linkage, the indistinguishable pulsotypes **(Fig. 2.2)**, and the identical virulence patterns strongly suggested these isolates were truly identical and the child's *Aeromonas* infection

Figure 2.2. PFGE profile analysis of three sets of water and clinical *Aeromonas* isolates

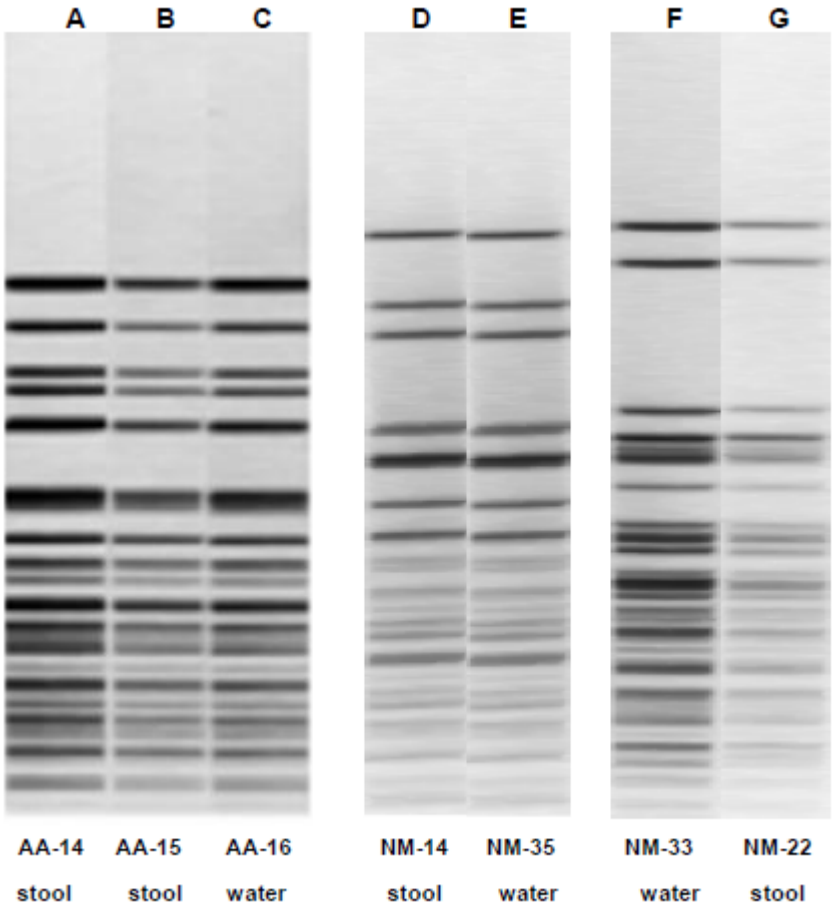

Fig. 2.2. PFGE profile analysis of three sets of water and clinical *Aeromonas* isolates that had indistinguishable PFGE patterns. Lane A (AA-14, *A. caviae/media* Group); Lane B (AA-15, *A. caviae/media* Group); Lane C (AA-16, *A. caviae/media* Group); Lane D (NM-14, *A. caviae/media* Group); Lane E (NM-35, *A. caviae/media* Group); Lane F (NM-33, *A. caviae/media* Group); Lane G (NM-22, *A. caviae/media* Group).

was the probable result of waterborne transmission **(Table 2.4)**. The second pulsotype and virulence group, NM-14 with NM-35 were not quite as tightly linked epidemiologically in that NM-14 was a stool sample from a child in Iowa with diarrhea

that was collected in early August of 1987 and NM-35 was isolated from a different private farm well in another geographic area of Iowa in 1986, a year prior to NM-14 isolation **(Table 2.4)**. Most interesting was the fact that a third strain of *A. caviae* (which was not available for this study) had the same ribotype as NM-14 and NM-35 and had been submitted from a local hospital laboratory to a reference bacteriology laboratory in 1988 (N. Moyer, personal communication). This suggested that genotypically identical strains, all belonging to the *A. caviae/media* Group, were present in the environment and causing disease in patients between 1986 and 1988 (well sample in 1986 and stool sample in 1987, and hospital laboratory referral in 1988).

The matching trio of strains, AA-14, AA-15, and AA-16 were collected during a "dysentery" outbreak in rural villages in the Sudan in January 1984 **(Table 2.4)**. AA-14 was isolated from a 1 year-old male with acute lymphocytic leukemia in the Kassala area (capital city of Sudan south of Port Sudan) with watery and bloody diarrheal stools (7 stools within the last 24 h) that were also positive for fecal leucocytes and negative for other common enteric pathogens, including parasites and viruses. AA-15 was isolated from a 27 year-old female in the Port Sudan area with fever and diarrhea (less than 3 stools in the last 24 h and without blood and white cells) and also negative for other common enteric pathogens, including parasites and viruses. AA-16 was a well-water isolate from the rural town of Sinkat, which is just outside of Kassala on the road from Port Sudan. Once again, although this trio of *A. caviae/media* Group strains was not strictly epidemiologically linked, their indistinguishable PFGE pulsotypes **(Fig. 2.2)**

Table 2.4. Virulence patterns of three sets of *Aeromonas* isolates that are indistinguishable by pulse-field gel electrophoresis (PFGE)

Isolate	Source	Species	HA	CA	PA	*ahyRI*	*alt*	*dam*	*gidA*	*enol*	T6SS effectors	
											Hcp	VgrG2/3
AA-14	Stool	*A. caviae/media* Group	0	0	3.4	+	+	+	+	+	+	+
AA-15	Stool	*A. caviae/media* Group	0	0	3.7	+	+	+	+	+	+	+
AA-16	Water	*A. caviae/media* Group	0	0	2.2	+	+	+	+	+	+	+
NM-14	Stool	*A. caviae/media* Group	0	0	1.2	+	+	+	+	+	+	+
NM-35	Water	*A. caviae/media* Group	0	0	0.3	+	+	+	+	+	+	+
NM-22	Stool	*A. caviae/media* Group	0	0	0.6	+	+	+	+	+	-	-
NM-33	Water	*A. caviae/media* Group	0	0	1.0	+	+	+	+	+	-	-

Abbreviation: HA, hemolytic activity; CA, cytotoxic activity; PA, proteolytic activity; enol; enolase; Hcp, hemolysin-coregulated protein; VgrG, valine-glycine repeat G protein

combined with their identical or nearly identical virulence patterns **(Table 2.4)** suggested that these isolates represent possible water-to-human transmission.

Detection of T6SS effectors. We then performed Western blot analysis on those 7 isolates of *A. caviae/media* Group that exhibited similar DNA fingerprinting and the virulence signature for the detection of T6SS effectors (Hcp and VgrG2/3) in the culture supernatant. As noted from **Table 2.4**, 5 out of these 7 isolates (AA-14, AA-15, and AA-16 as well as NM 14 and NM 35) which represented water and stool pairs synthesized

and secreted Hcp and VgrG2/3 effectors of T6SS in the culture supernatant**.** We did not detect T6SS effectors in the NM 22 and NM 33 paired water and stool strains.

DISCUSSION

Aeromonas species inhabit various aquatic environments, and this pathogen can infect humans in several ways, including via recreational or occupational activities in water, e.g., fishing, swimming and consumption of contaminated food or water. Previous studies have shown that *Aeromonas* species isolated from water possess various virulence factors and that these isolates can cause human diseases (22, 111, 252). However, the mode of transmission of this pathogen from water to human and the set of bacterial virulence factors versus host responses that eventually lead to *Aeromonas*-associated diseases are still not clearly understood. Many investigators, including us, have been continuously seeking new virulence factors in *Aeromonas* species to better establish a relationship between the presence of virulence factors and disease state in the host. Thus, it is important to examine the distribution of these newly identified virulence factors in water and clinical *Aeromonas* isolates and to characterize their role in the virulence of *Aeromonas* species. In this study, we examined the presence of 11 virulence genes and clonal relatedness among a large number of water and clinical strains of different *Aeromonas* species isolated from various regions within the United States and around the world. Further, our aim was to determine a subset of *Aeromonas* species prevalent in water and capable of causing human disease.

Two of the earliest studies presenting somewhat compelling epidemiological data to link aeromonads to gastrointestinal disease were the work of Echeverria *et al*., who studied the causes of Travelers' Diarrhea in Peace Corp Volunteers in Thailand in 1981 (53), followed by a study in the United States by Holmberg *et al*., in 1984 (81). This latter study by investigators at CDC in Atlanta, Georgia, evaluated 34 cases of

gastroenteritis where *Aeromonas* species had been isolated from fecal specimens. Their conclusions were that some strains of *Aeromonas* could have caused diarrhea in previously normal hosts and that the organisms were most likely acquired by the drinking of untreated water. This was followed by a study in 1987 by one of the authors of our paper (Nelson Moyer) on the clinical significance of *Aeromonas* species isolated from patients with diarrhea. This study examined 248 strains of *Aeromonas* isolated from 3,334 human fecal specimens submitted to a State Public Health Laboratory over a 20-year period for culturing of enteric pathogens. His conclusions were that some *A. hydrophila, A. sobria (*now considered to be *A. veronii* biovar sobria) and *A. caviae* strains were "capable of causing diarrhea and that antibiotic therapy and the drinking of untreated water were significant risk factors for susceptible hosts" (151). At the same time, researchers at the University of Maryland, College Park and NMRI (Naval Medical Research Institute) in Bethesda, Maryland, were revisiting and re-identifying previous published aeromonad strain collections from both clinical and environmental sources to promote and prove the value of accurate differential biotyping of aeromonads and plesiomonads in the environment (102).

In a somewhat later study in 1997, it was noted that only a small subset of *Aeromonas* strains belonging to specific hybridization groups (HG) can cause gastroenteritis in humans (125). Further, both Kirov *et al.* (115) and Hänninen (75) indicated that HG1 was associated with clinical specimens, while HG3, and, to a lesser extent, HG2, predominated in water and environmental samples. In 1997-1998, we (in collaboration with the International Centre for Diarrhoeal Disease Research, Bangladesh [ICDDR,B]) investigated the distribution of enterotoxin genes (*alt, ast, act*) in aeromonads isolated from children with diarrhea (n=115), healthy matched controls (n=27) and different water sources (n=120) in Bangladesh in order to establish a link

between virulence properties of aeromonads and cause of gastroenteritis in human (5). In this study, 125 children were positive for aeromonads and other enteric pathogens (7.2%) out of 1,735 diarrheal children and 28 (22%) of these children only had *Aeromonas* species in their stool. Only 27 (3.3%) control children out of 830 enrolled in this study were positive for aeromonds. Among 2,120 environmental samples tested, 600 (28.3%) were positive for aeromonads and every fifth sample was subsequently examined for the distribution of enterotoxin genes. Our findings revealed that a number of isolates positive for both the *alt* and *ast* genes were significantly higher in diarrheal children than from control children and the water sources. Further, patients who had isolates positive for both the *alt* and *ast* genes had watery diarrhea while those isolates positive for only the *alt* gene had loose stools (5). In the United States, another study reported that 43, 70, and 30% of the *Aeromonas* isolates obtained by the US EPA (a total of 205 examined) from drinking water from 16 utilities in 4 states harbored *alt, act*, and *ast* genes, respectively (192). In addition, a recent study from Bangladesh reported that certain *Aeromonas* clones persisted in different sewage water treatment plants and the same clones were found in fish and also in children suffering from diarrhea (175). They concluded that certain genotypically and phenotypically stable clonal lineages of *Aeromonas* might spread from hospitalized children suffering from diarrhea to fish produced for human consumption through the sewage water treatment system (175).

Despite the association of virulence factors with aeromonads isolated from drinking water, there is increasing evidence that strains isolated from the environment generally belong to different hybridization groups than do clinically associated strains. For example, Havelaar *et al*. (76) examined 187 *Aeromonas* strains from human diarrheal stools and 263 strains from drinking water in the Netherlands and observed little similarity between the two. Likewise, Borchardt *et al*. (16) noted that stool and water

isolates were genetically unrelated. Therefore, it is still unclear whether the same strains that are most prevalent and virulent in the environment are responsible for causing human diseases. Consequently, the current study was very crucial in resolving this contradiction by investigating the virulence potential and clonal relationship between water and clinical isolates of *Aeromonas* species isolated from different locations within the United States and around the world.

In addition to *alt, act*, and *ast* genes, we examined eight additional virulence factor genes, including some of the newly identified ones, in *Aeromonas* isolates by colony blot DNA hybridization. Since we used gene probes developed based on the sequences from one strain of *A. hydrophila* SSU, variable intensity signals with other strains and species of *Aeromonas* due to possible divergence in the sequences of these genes are expected. However, we obtained very clean and strong signals in our blots by using a technique that we modified and standardized in our laboratory. Although, there are some arguments against the reproducibility of PCR-based typing methods (2, 233, 234), many investigators used such techniques for the detection of different virulence factor-encoding genes (192, 204). However, optimization of primers and PCR conditions are very crucial to obtain best results (204), which is only feasible when the number of strains to be tested are minimal. Since in our study, we examined more than 200 isolates, colony blot DNA hybridization was possibly the best option to screen various virulence factor genes although this approach has some limitations.

In our first step during statistical analysis of the data, we compared the prevalence of discriminating variables (i.e., the genotypic and phenotypic traits) one at a time between isolates collected from the two sources of interest, water and stool. We reasoned that the three species groups, namely, *A. caviae/media*, *A. hydrophila*, and *A. veronii/sobria,* could be grouped for the analysis because these species are commonly

found in water, and together they comprise 85% of clinical infections (91). Initially, we assumed that grouping across species would be the best method for statistical analysis as because it would increase the sample size and improve statistical power. However, we later found different outcomes when we analyzed subsets by species. In elaboration, when data were grouped across species, the stool and water isolates were statistically different in the prevalence of 11 genetic traits. For 10 of these genes, the prevalence was always much greater in the isolates collected from water. For only the *dam* gene, the prevalence was higher in stool isolates. Likewise, three phenotypic traits, e.g., protease, hemolytic and cytotoxic activity measurements were much higher in the isolates from water, and hemolytic and cytotoxicity measurements were highly significantly different from those of the stool isolates.

However, when we analyzed the subsets of the species, a surprising result emerged. Many of the genetic virulence traits in these three groups of *Aeromonas* isolates were more prevalent in clinical isolates than in water isolates. Moreover, phenotypic traits, e.g., cytotoxic activity, were significantly higher for the stool isolates of the *A. caviae* and *A. hydrophila* Groups and were also higher, albeit of border line significance, for stool isolates from the *A. veronii* Group **(Table 2.3)**. Other investigators also reported that clinical isolates of *A. hydrophila* harbored a higher proportion of hemolysin genes (22) and produced greater cytotoxicity in CHO-K_1 cells (124).

The fact that the pattern was identical for the three species when they were analyzed separately, but then the pattern reversed when the species were combined into one group is a little known paradox called the reversal of inequalities or the Simpson's paradox (232). The paradox arises when groups of unequal size are combined and an unknown variable called a lurking variable differs between the groups. The lurking variable weighs the results in the direction opposite from the groups considered

separately. In the present study, this was best illustrated by the cytotoxic data. Examining the three species separately, in each case, the stool isolates produced substantially more cytotoxic activity than the water isolates. Even though this pattern was consistent for all three species, when the species were combined, the water isolates appear to produce more cytotoxic activity than the stool isolates. Examining the cytotoxin levels by species instead of source, we noticed that *A. caviae/media* Group produced less cytotoxin by two orders of magnitude compared to the other two species Groups, and moreover, the majority of isolates from stool, 72%, were *A. caviae/media* Group. Most of the *A. hydrophila* Group and *A. veroni/mediai* Group isolates were from water. It is this association between species and source of isolates that is the lurking variable, and in the combined analysis, the large number of low cytotoxin producing *A. caviae/media* Group in stool weighs the average downward to where it appears the water isolates produce more cytotoxin than the stool isolates.

In fact, the three species were significantly different in nearly all the genotypic and phenotypic traits and it was these differences and the association between species and source that could have led to the erroneous conclusion that virulence traits were more prevalent in water isolates than in stool isolates. The lesson is that it is important to correctly identify the isolates to the proper *Aeromonas* "species Group" before applying any predictive model for discriminating stool and water isolates.

The majority of the water (>78%) and stool (>90%) isolates from the *Aeromonas* species Groups (*A. hydrophila*, *A. caviae/media, A. vernonii/sobria*) possessed three virulence factor-encoding genes, i.e., *dam*, *gidA*, and *enolase.* From these data, we could hypothesize that these three genes were essential for the *Aeromonas* species to survive in the environment, as well as to cause human disease. Further, these three genes could be used as molecular identification markers of *Aeromomas* species in the event of

difficulties in serological and biochemical identification. *A. caviae/media* Group strains were found to contain the smallest number of virulence genes in comparison to those of other *Aeromonas* species Groups, and these results agreed with the data presented in a previous study (252). The *alt* and *dam* genes were more common in clinical strains than in water isolates in all three groups. Further, in the clinical strain groups, the *alt* gene was found in 92% of *A. caviae/media* Group strains, 100% of *A. hydrophila* Group and 67% of *A. veronii/sobria* Group strains, while these percentages were 76, 97 and 57 for water isolates, respectively. This is in accordance with our previous study (5) implicating the *alt* gene encoding heat-labile cytotonic enterotoxin as one of the major virulence factors of *Aeromonas*-associated diarrheal disease.

We and others (44, 196, 203, 260) have investigated the contribution of the T3SS towards the virulence of the *Aeromonas* species. However, Silver *et al.,* recently demonstrated that the functional T3SS of *A. veronii* was crucial for both beneficial and pathogenic colonization of the host, for example, in the leech versus the mouse model, respectively (205). Other investigators also reported that the T3SS was required for intercellular beneficial associations, such as in the insect endosymbiont *Sodalis glossinidius* (45) and *Rhizobium*-leguminous plant (240). Therefore, further studies are warranted to obtain a clear understanding on the role of bacterial T3SS in pathogenic and beneficial associations in different host environments.

In our study, irrespective of the *Aeromonas* species, we found that the T3SS genes were more prevalent in water isolates (for *aexU*, 68 were positive out of 199 isolates and for *ascV,* 60 were positive out of 199) than in the clinical *Aeromonas* isolates (for *aexU,* 5 were positive out of 53 isolates and for *ascV,* 5 were positive out of 53), a finding in agreement with another recent study (4). However, these genes were more prevalent in clinical isolates than in water isolates specifically in the *A. hydrophila* Group. Based on

these results, it is plausible that the T3SS might play a role in the ability of *A. hydrophila* Group isolates to cause human diarrhea. However, we should cautiously interpret these data, as the number of clinical isolates in the study was limited. In a study conducted in Thailand, 11% and 30% of the clinical isolates of *A. caviae* complex harbored *ascV* and *aexT* (*aexU*-like) genes, respectively (252). However, in our study, none of the clinical and water isolates of *A. caviae/media* Group contained these genes. These differing results can be explained by the fact that the pathogenic mechanisms of disease caused by *Aeromonas* species may differ by geographic region. Nevertheless, in future work, a comparative analysis of the isolates from these different geographical locations would be important in understanding pathogenesis of *Aeromonas*-associated infections.

AHL is an autoinducer molecule for QS regulation and it has been reported that AHL mediated QS is a global regulator and controller of a number of virulence factors in many pathogens (13, 86, 211). As found with cytotoxic activity, the highest AHL levels were detected from stool isolates compared to water isolates, in both the *A. hydrophila* and *A. caviae* Groups **(Fig.2.1)**. Our recently published data (109) indicated that deletion of the *ahyRI* genes from *A. hydrophila* SSU decreased the biofilm formation and affected production of metalloprotease and secretion of T6SS effectors, Hcp and VgrG2/3. Further, the *ahyRI* mutant was less virulent in a mouse septicemic model. Therefore, increased production of AHLs by stool isolates in *A. caviae/media* and *A. hydrophila* Groups could directly or indirectly modulate bacterial virulence. In addition, the *A. caviae/media* Group paired isolates from water and stool (e.g., NM-14 and NM-35) synthesized and secreted Hcp and VgrG2/3 effectors of T6SS **(Table 2.4)** and also synthesized AHLs, indicating that these lactones, in part, might be involved in regulating T6SS effectors in these strains.

The PFGE analysis and colony blot DNA hybridization data revealed that three sets of isolates from stool and water samples had indistinguishable PFGE patterns and harbored the same/similar virulence signatures **(Table 2.4)**. Based on our biochemical identifications, all seven strains were identified as belonging to the *A. caviae/media* Group. Further, these strains in the *A. caviae/media* Group harbored only four virulence/regulatory genes (*alt*, *dam*, *gidA* and *enolase*) among the 11 genes tested. These strains were also unable to produce cytotoxic and hemolytic activities, as they did not have the *act* gene **(Table 2.4)**. Nevertheless, 5 out of the 7 paired water and stool strains possessed the functional T6SS **(Table 2.4)**, which is one of the major virulence mechanisms of *A. hydrophila* SSU characterized recently in our laboratory (218, 220). Therefore, in future studies, it would be interesting to examine in details the virulence mechanism(s) of these selected *A. caviae/media* Group isolates.

Finally, in the present study, we observed that *A. hydrophila* and *A. veronii*/*sobria* Groups possessed a greater number of virulence genes and had more cytotoxic and hemolytic activities, when compared with these characteristics of the *A. caviae/media* Group. Thus, it seems possible *A. hydrophila* and *A. veronii/sobria* Groups have a much greater potential to cause human diseases than does *A. caviae/media* Group. One limitation of the present study was that there were fewer clinical isolates, particularly in the *A. hydrophila* and *A. veronii/sobria* Groups. Therefore, further investigation with a large number of clinical and water isolates of the *Aeromonas* species is crucial to better understand the pathogenic mechanism of the *Aeromonas* species. In conclusion, our data suggest that there is water-to-human transmission, at least in the *A. caviae/media* Group, and that such exposure leads to gastroenteritis in humans.

CHAPTER 3

N-*acyl* homoserine lactones involved in quorum sensing control type VI secretion system, biofilm formation, protease production, and *in vivo* virulence from a clinical strain of *Aeromonas hydrophila*[2]

INTRODUCTION

The N-*acyl* homoserine lactone (AHL) based QS system has been identified in both human (118, 209, 222) and plant pathogens (86, 135). An earlier study indicated that *A. hydrophila* produced two types of AHLs, namely N-3-butanoyl-DL-homoserine lactone (C4-HSL) and N-3-hexanoyl-DL-homoserine lactone (C6-HSL), of which C4-HSL was the predominant type (222). Importantly, AhyR functions as both a negative and a positive regulator of the *ahyI* gene in *A. hydrophila* (113).

Microarray analyses revealed the differential transcription of 26% of the genome in *Pectobacterium atrosepticum* (a plant pathogen) and 6% of the genome in *Pseudomonas aeruginosa* in EspI and LasI-RhlI mutants, respectively, compared to their corresponding parental strains. These data suggested that AHL-mediated QS was a master regulator for many genes in these pathogens (135, 189). In addition, the role of an AHL-mediated QS control on the T3- and T6- secretion systems of *P. aeruginosa* and other pathogens was investigated and reported (15, 70, 77, 135, 189). Although in microarray analysis, it was shown that the expression of the *hcp* gene was reduced in the *lasR-rhlR* mutant, when compared to that in the WT *P. aeruginosa*, it was not known at that time that Hcp was an effector of the T6SS (189).

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[2] Khajanchi, B. K., J. Sha, E. V. Kozlova, T. E. Erova, G. Suarez, J. C. Sierra, V. L. Popov, A. J. Horneman, and A. K. Chopra. 2009. N-acyl homoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and in vivo virulence in a clinical isolate of *Aeromonas hydrophila*. Microbiology 155:3518-3531.

Specifically, we showed that AHL production was significantly decreased when we deleted two major virulence factor-encoding genes, such as the *act* and an outer membrane protein (*aopB),* an important component of the T3SS in *A. hydrophila* SSU (196). We also observed that lactone production was modulated by regulatory genes such as *dam* (DNA adenine methyltransferase) and *gidA* (glucose-inhibited division A) in *A. hydrophila* SSU (57). These data prompted us to further investigate the regulatory role of AHL-mediated QS in virulence of *A. hydrophila* SSU.

Our studies were substantiated by previous reports showing that AHL-mediated QS regulated exoprotease production (223) and biofilm formation (136) in *A. hydrophila*. In addition, the *ahyR* mutant of *A. hydrophila* J-1 was significantly attenuated in a fish infection model (14). However, the role of AHL-mediated QS in clinical isolates of *A. hydrophila* has never been tested in a mouse model of infection, and the role of QS in modulating T3- and T6- secretion systems is largely unknown in this pathogen.

Consequently, in this study, we identified AhyRI (LuxRI homolog) in a clinical isolate SSU of *A. hydrophila* as a result of our annotation of the genome sequence of an environmental isolate ATCC 7966 of *A. hydrophila* (193). We showed that disruption of the *ahyRI* genes in *A. hydrophila* SSU influenced metallo-protease production, secretion of the T6SS effectors (Hcp and VgrGs), biofilm formation, and mortality in a mouse model of infection. Our study is the first to document that AHL mediated the QS-regulated secretion of the newly discovered T6SS effectors in *A. hydrophila* SSU and that the production of a metallo-protease was specifically reduced in the Δ*ahyRI* mutant based on zymography.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals. The bacterial strains and plasmids used in this study are listed in **Table 3.1**. Chemically synthesized C4-HSL and C6-HSL

were obtained from Fluka (St. Louis, MO). Stock solutions (50 mM) of AHLs were prepared by dissolving them in acetonitrile (far-UV grade), and stocks were stored at -20^{o}C.

Generation and characterization of the Δ*ahyRI* mutant of *A. hydrophila* SSU. Based on DNA sequences of the *ahyI*/*ahyR* genes (homologs of the *luxI*/*luxR* genes) in the *A. hydrophila* ATCC 7966 strain, two primers (*ahyR*, 5'-TTATTGCATCAGCTTGGGGAAG-3' and *ahyI*, 5'-TTATTCGGTGACCAGTTCGCG-3') were synthesized. By using these primers, a 1.5-kb DNA fragment was PCR amplified from the genome of *A. hydrophila* SSU, and subsequently cloned in the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). DNA sequence analysis revealed that this fragment was 1469 bp long and contained the entire *ahyRI* operon. By using restriction enzyme *Stu*I (generated blunt ends), we removed a 443-bp DNA fragment from within the *ahyRI* operon harbored in the TA cloning vector, and replaced it with the blunt-ended 2.0-kb Sm/Sp^{r} gene cassette from plasmid pHP45Ω (169), which was obtained by *Sma*I restriction enzyme digestion. This strategy resulted in a 185- and 196-bp deletion from the start codons of the *ahyI* and *ahyR* genes, respectively. The Sm/Sp^{r}-truncated *ahyRI* genes were removed from the pCR2.1 vector by *Kpn*I/*Xba*I digestion and ligated to the pDMS197 suicide vector at compatible restriction enzyme sites. The resulting plasmid (pDMS197*ahyRI*Sm/Sp) was transformed into *E. coli* SM10, which contained *λpir* (54). The recombinant *E. coli* (pDMS197ahyRISm/Sp) cells were then conjugated with WT *A. hydrophila* SSU-R (195). The transconjugants were selected based on resistance to appropriate antibiotics and sucrose and their genomic DNA (gDNA) subjected to Southern blot analysis for their correct identification.

Southern blot analysis. Chromosomal DNA from isogenic mutants, as well as from WT *A. hydrophila*, was isolated, and an aliquot (10 μg) was digested with

appropriate restriction enzymes and subjected to 0.8% agarose gel electrophoresis (195). Next, the digested DNA was transferred to a nylon membrane and baked at 80°C for 2 h. Three DNA probes were used for Southern blot analysis. These probes represented the coding region of the target gene, a 2.0-kb Sm/Spr gene cassette from plasmid pHP45Ω obtained by *BamH*I restriction enzyme digestion, and a suicide vector, pDMS197 (6.0 kb) (195). The prehybridization and hybridization conditions were the same as those mentioned earlier for the colony blot hybridization.

Complementation of the *A. hydrophila* SSU Δ*ahyRI* mutant. The *ahyRI* genes were PCR amplified by using gDNA of *A. hydrophila* as a template and two primers (*ahyRI*-N/*Sal*I: 5'-GGGGTCGACAGCAGCTTGTAATCCAACGC-3' and *ahyRI*-C/*Eco*RI: 5'-GGGGAATTCATGAACCGTCCAGCAGAGTGA-3'; indicated restriction endonuclease sites are underlined). We included 293 bp of the upstream and 210 bp of the downstream DNA-flanking sequences containing potential promoter regions of divergent *ahyRI* genes for complementation studies. This DNA fragment (1972 bp) was cloned in pBR322 vector (Tc^r Ap^r) at *Sal*I-*Eco*RI sites and transformed into the *E. coli* DH5α strain. The pBR322/*ahyRI* (Tc^s Ap^r) recombinant plasmid was isolated from the *E. coli* strain and electroporated into an *A. hydrophila* Δ*ahyRI* mutant.

Complementation of the *ahyR* gene in *A. hydrophila* SSU Δ*ahyRI* mutant. To complement the *ahyR* gene in the double mutant Δ*ahyRI* of *A. hydrophila,* the *ahyR* gene was amplified with the following primers: *ahyR*-N/*Sca*I: 5'-GGGAGTACTATGAAACAAGACCAACTGCTT-3' and *ahyR*-C/*Pst*I: 5'-GGGCTGCAGTTATTGCATCAGCTTGGGGA-3'. The DNA fragment (783 bp) was cloned in pBR322 vector (Tc^r Ap^r) at *Sca*I-*Pst*I sites and transformed into *E. coli* DH5α strain. The pBR322/*ahyR* (Tc^r Ap^s) recombinant plasmid was isolated from the *E. coli* strain and electroporated into *A. hydrophila* Δ*ahyRI* mutant.

Lactone production. AHL production was detected by cross-streaking of WT, Δ*ahyRI* mutant and the complemented strains of *A. hydrophila* SSU on LB agar medium against the biosensor strain *Chromobacterium violaceum* CV026 as described in Chapter 2.

Measurement of the protease activity. Protease activity was measured in culture filtrates of overnight-grown cultures of WT *A. hydrophila*, Δ*ahyRI* mutant and the complemented strains (Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$ and Δ*ahyRI*/*ahyR*$^+$) as described in Chapter 2. To determine the nature of proteases, we used metallo- and serine- protease inhibitors EDTA and phenylmethylsulphonyl fluoride (PMSF) at a final concentration of 100 and 10 mM, respectively. The proteases were inactivated with their corresponding inhibitors for 2 h at 37^{o}C before measuring the activity.

Zymography. To characterize the nature of protease(s) and to delineate which specific protease(s) was affected in the Δ*ahyRI* mutant, we performed casein zymography using established procedures (156, 223). Briefly, equal amounts of total protein were loaded and separated on SDS-12% PAGE gel and subsequently soaked in 2.5% Triton X-100 solution to remove the SDS. Then , the gel was placed in a 2% (w/v) casein solution dissolved in 0.05 mol/liter Tris-HCl buffer (PH 7.5) at 7-8^{0}C for 30 min to allow the casein to enter thorough the gel and the gel was then kept at room temperature for 90 min for enzyme activity. To visualize the protease bands, the gel was stained with Coomassie blue dissolved in a mixture of 50% (v/v) ethanol and 12% (w/v) TCA followed by 2 h of destaining in a solution containing 30% (v/v) ethanol, 7.5% (v/v) acetic acid and 5% (v/v) TCA.

Measurement of the hemolytic activity. The hemolytic activity associated with Act of WT *A. hydrophila* SSU and its Δ*ahyRI* mutant strain was measured accoding to procedure described in Chapter 2. For neutralization assay, culture filtrates of WT and

Δ*ahyRI* mutant strains were mixed with either pre-immune (control) or hyper-immune rabbit sera (laboratory stock, 1:10 dilution) containing antibodies to Act (59).

Measurement of lactate dehydrogenase (LDH) activity. RAW 264.7 murine macrophages were infected at a multiplicity of infection (MOI) of 5 with the WT *A. hydrophila* SSU and its Δ*ahyRI* mutant strain. During infection, cell morphology was monitored, and at various time points after infection, host cell cytotoxicity associated with Act or T3- and T6- secretion system effectors, was measured by the release of lactate dehydrogenase (LDH) enzyme using CytoTox 96® kit (Promega, Madison, WI) in the tissue culture supernatant (196).

Swimming and swarming motility assay. LB medium with 0.3% Difco Bacto-agar (Difco Laboratories, Detroit, MI) was used to characterize the swimming motility (121), while Difco nutrient broth with 0.5% Eiken agar (Eiken Chemical Co., Ltd., Tokyo, Japan) was employed for measuring swarming motility (117) of WT *A. hydrophila* SSU and its Δ*ahyRI* mutant strain. *A. hydrophila* ATCC 7966 strain (193), which does not possess genes encoding the lateral flagellum, was used as a negative control in the swarming motility assay. The overnight cultures grown in the LB medium were adjusted to the same optical density and equal numbers of cfu (10^8) of WT, mutant and complemented strains were stabbed into swimming and swarming plates. Swimming and swarming plates were incubated at 37°C and 30°C, respectively, for 18-24 h, and the motility was assayed by examining migration of bacteria through the agar from the center towards the periphery of the plate.

Table 3.1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristic (s)	Source or reference
A. hydrophila SSU		CDC, Atlanta, Ga
SSU-R	Rifampin resistance (Rif^r) strain of *A. hydrophila* SSU	Laboratory stock
Δ*ahyRI*	*ahyRI* gene deletion mutant of *A. hydrophila* SSU-R strain Rif^r Sm^r Sp^r	This study
Δ*ahyRI*/pBR322-*ahyRI*	*ahyRI* mutant complemented with *ahyRI* genes via pBR322 Rif^r Sm^r Sp^r Ap^r	This study
Δ*ahyRI*/pBR322-*ahyR*	*ahyRI* mutant complemented with *ahyR* gene via pBR322 Rif^r Sm^r Sp^r Tc^r	This study
E. coli		
DH5α	Production of recombinant plasmids. *recA, gyrA.*	Life Technologies
SM10	Km^r, *λpir*	(54)
Chromobacterium violaceum CV026	ATCC 31532 derivative, *cviI*::Tn5*xylE;* Km^r Sm^r	(141)
Plasmids		
pCR2.1	TA cloning vector Ap^r Km^r	Invitrogen
pCR2.1/*ahyRI*	TA cloning vector carrying *ahyRI* gene Ap^r Km^r	This study
pHP45Ω	pHP45 plasmid contains a 2-kb Sm/Sp^r gene cassette (Ω element)	(169)
pCR2.1/*ahyRI*- Sm/Sp	TA cloning vector harboring *ahyRI* gene disrupted by Ω element, Ap^r Sm^r Sp^r	This study
pDMS197	A suicide vector; R6K *ori, sacB,* Tc^r	(54)
pDMS197/ *ahyRI*-Sm/Sp	Suicide vector containing *ahyRI* gene with Ω element,$Tc^r$$Sm^r$ Sp^r	This study
pBR322	Ap^r Tc^r	Amersham
pBR322/*ahyRI*	Contains *ahyRI* gene, Ap^r	This study
pBR322/*ahyR*	Contains *ahyR* gene, Tc^r	This study

Crystal violet biofilm assay. As a modification of the biofilm ring assay (159), WT, Δ*ahyRI* mutant and the Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$ and Δ*ahyRI*/*ahyR*$^+$ complemented strains of *A*. *hydrophila* were grown directly from the -80^{o}C stock in 3 ml LB medium contained in polystyrene tubes at 37^{o}C for 24 h with shaking. The Δ*ahyRI*/*ahyR*$^+$ complemented strain was grown in the presence of 20 μM C4-HSL. Biofilm formation was quantified according to the procedure described earlier (149). Finally, the biofilm formation results were normalized to 1 x 10^9 cfu to account for any differences in the growth rates of various bacterial strains used. The experiment was repeated independently three times.

Scanning electron microscopy (SEM) biofilm experiments. SEM on biofilm formation was performed using 13-mm-diameter thermanox plastic cover slips. After 48 h of incubation, unattached cells were removed, the cover slips were fixed, stained with ruthenium red and samples were examined in a Hitachi S4700 field emission scanning electron microscope (Hitachi High Technologies America) according to the established procedure (121).

Western blot analysis. Western blot analysis was performed using polyclonal antisera against Hcp, and VgrG as described in Chapter 2.

Translocation of AexU. The eukaryotic HT-29 cells were seeded in 6-well tissue culture plates. Before infection, the cells were washed twice with Dulbecco's modified essential medium (DMEM) (Invitrogen) without serum, and 1 ml of fresh DMEM was added to each well. After 30 min incubation, the cells were infected with various *A. hydrophila* strains as prepared above at an MOI of 10. After 2 h of infection at 37°C with 5% CO_2, the tissue culture supernatants were collected and centrifuged to separate unbound bacteria and floating eukaryotic cells. The supernatants which might contain the secreted AexU toxin were filter-sterilized (0.22 μm) and precipitated with 10% TCA

(final concentration). The infected eukaryotic cells were washed twice with PBS, and 100 μl of water (containing mammalian cell protease [P-8430] and phosphatase [P-2850] inhibitor cocktail [Sigma]) or the lysis buffer (0.1% TX-100, 200 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 10 mM Tris-HCl, pH 7.0) was added to each well of the plate. The lysed cells were scraped off, and the suspensions were centrifuged to separate the soluble and insoluble fractions. The cell pellets, which were separated from the tissue culture supernatants, as described above, were combined with their corresponding insoluble fractions and washed twice with PBS. These combined cell pellets contained bound and unbound bacteria as well as the eukaryotic cell debris. The soluble fractions represented cytoplasmic fractions of the eukaryotic cells, as the water or 0.1% TX-100 only lysed eukaryotic cells, but not the bacteria. Western blot analysis was performed to detect the expression and production of AexU using specific antibodies.

Animal Experiments. Groups of 10 Swiss Webster mice (Taconic Farms, CA) were infected by the intraperitoneal route (i.p.) with 3 x 10^7 cfu (WT or its Δ*ahyRI* mutant) in accordance with the approved animal care protocol. One group of mice was inoculated with DPBS (n = 10) and served as a control. Deaths were recorded for 16 days post infection. This bacterial dose used represented approximately 2X LD_{50} of WT *A. hydrophila* (256).

Statistics. Wherever applicable, at least three independent experiments were performed, and the data analyzed by using the student's t test, with p values of ≤ 0.05 considered significant. The animal data were analyzed by using Fisher's exact test.

Nucleotide Sequence Accession Number. The sequences of the *A. hydrophila* SSU *ahyRI* genes were deposited in the GenBank database under accession number DQ398101.

RESULTS

Characterization of the AhyRI QS regulon in *A. hydrophila* SSU. In a previous study (222), a LuxRI homolog was identified in *A. hydrophila* A1 strain and designated as AhyRI (Accession no. X89469). By our sequence annotation of *A. hydrophila* ATCC 7966 (193), we identified *ahyR* and *ahyI* genes in a clinical isolate *A. hydrophila* SSU, which had a 97% and 94% sequence homology with the corresponding genes of the *A. hydrophila* A1 strain, respectively. The *ahyR* and *ahyI* genes in *A. hydrophila* SSU were oppositely oriented with a 59-bp intergenic region. Further, a Pfam protein sequence search (http://pfam.sanger.ac.uk/) revealed that AhyR had two domains; one for autoinducer binding and the other a regulatory domain (Lux family).

To characterize the role of AHL-mediated QS in the regulation of virulence in *A. hydrophila* SSU, we deleted both the *ahyR* and *ahyI* genes by double crossover homologous recombination and subsequently generated a complemented strain with both of the *ahyRI* genes (Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$). For further confirmation of interaction between AhyR and signaling molecules (AHLs) in modulating virulence factors, we also complemented the *ahyR* gene in the Δ*ahyRI* mutant strain (Δ*ahyRI*/*ahyR*$^+$) and examined the restoration of phenotypic changes by the exogenous addition of AHLs. As expected, the Δ*ahyRI* mutant was unable to produce AHLs, which was examined by using *C. violaceium* CV026 as a biosensor strain. Lactone production was restored in the Δ*ahyRI* complemented strain.

The Δ*ahyRI* mutant produced a reduced level of protease. Earlier studies indicated that the pathogenic and virulence characteristics of *A. hydrophila* were associated with the production of exoenzymes (e.g., proteases and lipases) (28, 93). Consequently, we measured protease production and the Δ*ahyRI* mutant strain produced a significantly reduced level of protease compared to that of the WT *A. hydrophila* strain

(Table 3.2). Further, the protease production was restored to the WT level in the Δ*ahyRI* complemented strain (Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$) **(Table 3.2)**. For further confirmation of complementation, protease production was also measured in the Δ*ahyRI*/*ahyR*$^{+}$ complemented strains when they were simultaneously supplied with two different exogenous AHLs (C4-HSL and C6-HSL). We observed that addition of both of the exogenous lactones restored protease production **(Table 3.2)**, which suggested to us that AHL molecules interacted with AhyR to control protease production in *A*. *hydrophila* SSU.

Based on casein zymography, we identified three protein bands (with sizes of 61, 52, and 19 kDa) in the cultures filtrates of WT *A. hydrophila* SSU and its Δ*ahyRI* complemented strain (Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$) with protease activity. Importantly, 61- and 52- kDa protease-associated protein bands were missing in the Δ*ahyRI* mutant **(Fig. 3.1)**. Further, we noted that the 61-kDa band represented a metallo-protease, as treatment of the culture supernatants from WT *A*. *hydrophila* and its Δ*ahyRI* complemented strain with EDTA resulted in complete disappearance of this band **(Fig. 3.1)**. The nature of proteases associated with 52- and 19- kDa bands is unknown. Based on our enzyme assay, EDTA resulted in 81% loss of the total protease activity, while PMSF had minimal effect on the protease activity.

Hemolytic and cytotoxic activities associated with Act were unaltered in the Δ*ahyRI* mutant. The level of hemoglobin release from rabbit erythrocytes was identical in both the WT and the Δ*ahyRI* mutant strain of *A*. *hydrophila* SSU. To demonstrate that the lack of detection in reduction of Act-associated hemolytic activity was not due to the up-regulation of other genes encoding hemolysin; we performed hemolytic activity assay after neutralization of Act in the culture supernatant using specific antibodies.

Table 3.2. Measurement of protease activity in culture supernatants and biofilm mass on polystyrene plastic of *A. hydrophila* SSU, Δ*ahyRI* mutant, and complemented strains

Strain	Protease activity (OD_{595nm}/ml/10^8 cfu) (Mean ± SD†)	Biofilm formation (OD_{570nm}) (Mean ± SD)
A. hydrophila SSU	1.20 ± 0.23	3.70 ± 0.28
Δ*ahyRI*	0.11 ± 0.04*[1]	0.55 ± 0.07*[1]
Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$	1.98 ± 0.40*[2]	2.50 ± 0.28*[2]
Δ*ahyRI*/*ahyR*$^+$+C4-HSL	1.41 ± 0.18*[3]	3.90 ± 0.14*[3]
Δ*ahyRI*/*ahyR*$^+$+C6-HSL	0.56 ± 0.03*[4]	ND‡

†SD; standard deviation

Protease activity:*[1]statistically significant differences between the Δ*ahyRI* mutant and the WT bacteria by student's *t* test (p=0.001); *[2]between the Δ*ahyRI* mutant and Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$-complemented strain (p=0.001); *[3,4]statistically significant differences between the Δ*ahyRI* mutant and Δ*ahyRI*/*ahyR*$^+$ complemented strains with exogenous supply of C4-HSL or C6-HSL (p<0.001).

Biofilm formation: *[1]statistically significant differences between the Δ*ahyRI* mutant and the WT bacteria (p=0.004); *[2]between the Δ*ahyRI* mutant and Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$-complemented strain (p=0.01); *[3]statistically significant differences between the Δ*ahyRI* mutant and Δ*ahyRI*/*ahyR*$^+$ complemented strain with exogenous supply of C4-HSL (p=0.001).

‡ND, not determined

Figure 3.1. Casein zymogram of supernatant proteins of WT *A. hydrophila* SSU, Δ*ahyRI* mutant, and its complemented strain

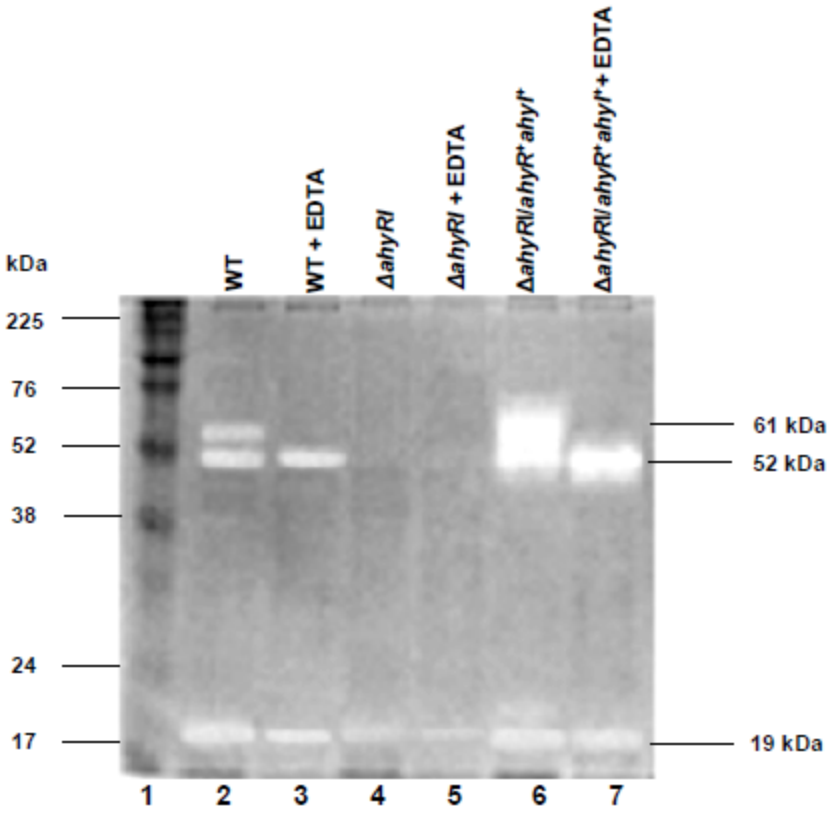

Fig. 3.1. Casein zymogram of supernatant proteins of WT *A. hydrophila* SSU, Δ*ahyRI* mutant, and its complemented strain. After incubation of the SDS-polyacrylamide gel (12%) in 2% casein solution, Coomassie blue staining showed activity associated with proteases, which appeared as clear bands due to hydrolysis of casein on a blue background. **Lane 1**, molecular weight standard; **lane 2**, supernatant from WT *A. hydrophila* SSU; **lane 3**, supernatant from WT *A. hydrophila* SSU treated with EDTA; **lane 4**, supernatant from the Δ*ahyRI* mutant; **lane 5,** supernatant from Δ*ahyRI* mutant treated with EDTA; **lane 6**, supernatant from complemented strain (Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$); **lane 7,** supernatant from complemented strain (Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$) treated with EDTA.

We noted that the level of residual hemolytic activity in the WT versus Δ*ahyRI* mutant strains remained unchanged, indicating that the expression of other hemolysin genes, in general, was not altered by deletion of the *ahyRI* genes. In addition, based on Western blot analysis, similar levels of Act were noted in the WT and the Δ*ahyRI* mutant strain of *A. hydrophila* SSU. Likewise, the Δ*ahyRI* mutant produced cytotoxic activity at a level similar to that of the WT bacteria (data not shown). These data suggested to us that the AhyRI QS regulon had no effect on Act-mediated biological activities in *A. hydrophila* SSU.

Swimming and swarming motility of the Δ*ahyRI* mutant was unaffected. *A. hydrophila* SSU WT strain had both swimming and swarming motility; however, the Δ*ahyRI* mutant migrated in a manner similar to that of the parental strain on the swimming and swarming agar plates (data not shown), which indicated that the swimming and swarming motility were not regulated by the AHL-mediated QS in *A. hydrophila* SSU.

Crystal violet (CV) staining biofilm assay demonstrated attachment deficiency in the Δ*ahyRI* mutant. To measure the solid surface-associated biofilm formation, we performed a CV staining assay after 24 h growth of WT, Δ*ahyRI* mutant, and the Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$ and Δ*ahyRI*/*ahyR*$^+$ (with exogenous AHLs) complemented strains in LB medium **(Table 3.2)**. The Δ*ahyRI* mutant formed a significantly decreased solid-surface-associated biofilm in polystyrene tubes **(Table 3.2),** with a 86% reduction in the CV staining when compared to that of the WT *A. hydrophila* SSU strain. The Δ*ahyRI*/*ahyR*$^+$*ahyI*$^+$-complemented strain adhered to the polystyrene tube in a manner similar to that of the WT bacteria. Addition of exogenous C4-HSL (20 μM) also restored biofilm formation in the Δ*ahyRI*/*ahyR*$^+$-complemented strain **(Table 3.2).** These data

suggested to us that AHL QS had a positive regulatory role on the biofilm formation in *A. hydrophila* SSU.

A distinct architecture of biofilm was observed in the Δ*ahyRI* mutant when examined by SEM. Exopolysaccharide (EPS), which consists of polysaccharides, DNA and proteins, plays an important role in determining biofilm's architecture (47). To investigate the surface architecture of bacterial cells aggregated in biofilm formed by WT and the Δ*ahyRI* mutant, we performed SEM. Ruthenium red staining is an excellent method for visualization of surface properties of bacteria (60), and, hence, we stained bacterial aggregates with ruthenium red. As expected, SEM results revealed that WT bacteria formed a structured biofilm in which cells were well connected by filaments **(Fig. 3.2 a, c and e)**. On the contrary, the Δ*ahyRI* mutant was less filamentous, and it was not properly aggregated as was the parental strain **(Fig. 3.2 b, d and f)**. In addition, EPS produced by WT bacteria **(indicated by arrow, Fig. 3. 2 e)** was thick and tightly attached to the surface of the bacteria. On the other hand, EPS produced **(indicated by arrow, in Fig.3.2 f)** by the Δ*ahyRI* mutant were loosely bound to bacterial cells. The Δ*ahyRI*-complemented strains produced biofilms similar to that of the WT bacteria (data not shown). Overall, SEM images indicated that the Δ*ahyRI* mutant was defective in the production of mature biofilm compared to that of the parental strain, which suggested to us that AHL-mediated QS has a regulatory role in the biofilm development of *A. hydrophila* SSU.

AHL mediated the QS-controlled secretion of Hcp, a T6SS effector. T6SS is a novel secretion mechanism, and very little is known as to how effector proteins are secreted and translocated to host cells through this system. We recently demonstrated that secreted Hcp could bind to the murine RAW 264.7 macrophages from outside and that it could also translocate into host cells, resulting in their apoptosis (220).

Figure 3.2. Scanning electron micrograph (SEM) images of biofilm formation by WT *A. hydrophila* SSU and its Δ*ahyRI* mutant

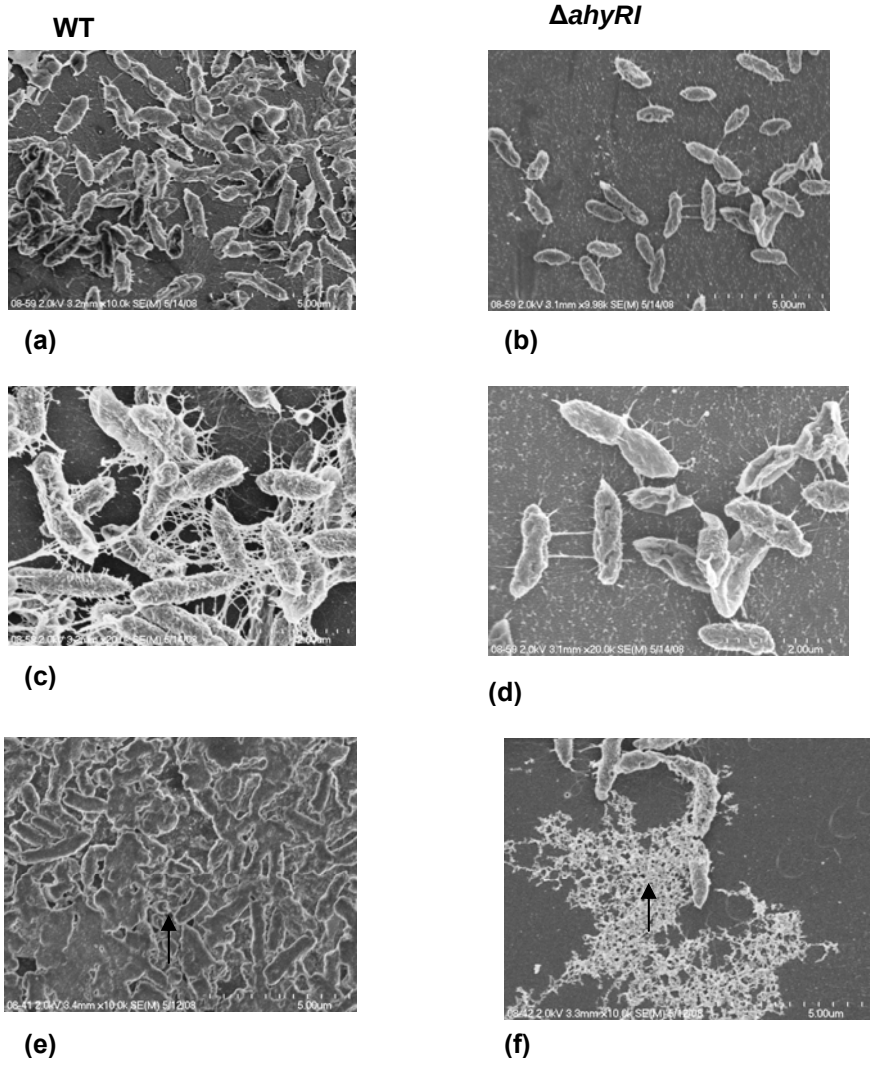

Fig. 3.2. SEM images of biofilm formation by WT *A. hydrophila* SSU and its Δ*ahyRI* mutant after 48 h of cultivation at 37^{o}C on thermonox cover slips stained with ruthenium red. **(a and c)** compact aggregation and cells were well connected with filaments in biofilm formed by the WT strain. **(b and d)** less aggregated cells in the biofilm, which were connected with fewer filaments compared to that of the biofilm formed by the WT bacteria in the Δ*ahyRI* mutant biofilm. **(e)** a thick exopolysaccharide (EPS) was produced by the WT strain that was tightly bound on the surface of the cells (indicated by an arrow). **(f)** the Δ*ahyRI* mutant produced a distinct type of EPS, compared to that of the WT strain, and it (EPS) was loosely bound to the surface of the cells (indicated by an arrow).

In this study, to examine the role of AHL-mediated QS in the regulation of the T6SS, we performed Western blot analysis to determine the production and secretion of Hcp at different time points (2 h and 4 h) in WT, Δ*ahyRI* mutant and the Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$ and Δ*ahyRI*/*ahyR*$^{+}$-complemented strains of *A*. *hydrophila* SSU. We only showed data on expression and secretion of Hcp in WT, mutant and the complemented strains at the 4-h time point.

We found that Hcp2 could be detected in bacterial cell pellets in both the WT and its Δ*ahyRI* mutant at 2 h (data not shown) and 4 h **(Fig. 3.3, lanes 2 and 4)**. However, in the WT bacteria, a significant amount of Hcp2 was secreted into the medium at both 2 h (data not shown) and 4 h **(Fig. 3.3, lane 1)**. On the contrary, the Δ*ahyRI* mutant was unable to secrete Hcp2 at 2 h (data not shown) and 4 h **(Fig. 3.3, lane 3)**. The Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$-complemented strain secreted Hcp2 to the WT level **(Fig. 3.3, lane 5)**. Also, addition of C4-HSL to the Δ*ahyRI*/*ahyR*$^{+}$-complemented stain restored Hcp2 secretion **(Fig. 3.3, lane 7),** which suggested to us that AHL mediated the QS-regulated secretion of Hcp in *A*. *hydrophila* SSU.

AHL-mediated QS also regulated secretion of another T6SS effector, VgrG. As in *V*. *cholerae* (172), there were three copies of the *vgrG* gene-encoded effector proteins, namely VgrG1, VgrG2 and VgrG3 in *A*. *hydrophila* SSU (220). Further, the role of VgrGs in cytotoxicity and virulence was recently reported in *V*. *cholerae* (172, 173). However, the regulation of these VgrG effector molecules is currently unknown. In the present study, we examined whether AHL-mediated QS regulated the production and secretion of these VgrGs in *A*. *hydrophila* SSU.

Figure 3.3. Western blot analysis showing production of Hemolysin coregulated protein 2 (Hcp2) in WT *A. hydrophila* SSU, Δ*ahyRI* mutant, and the complemented strains

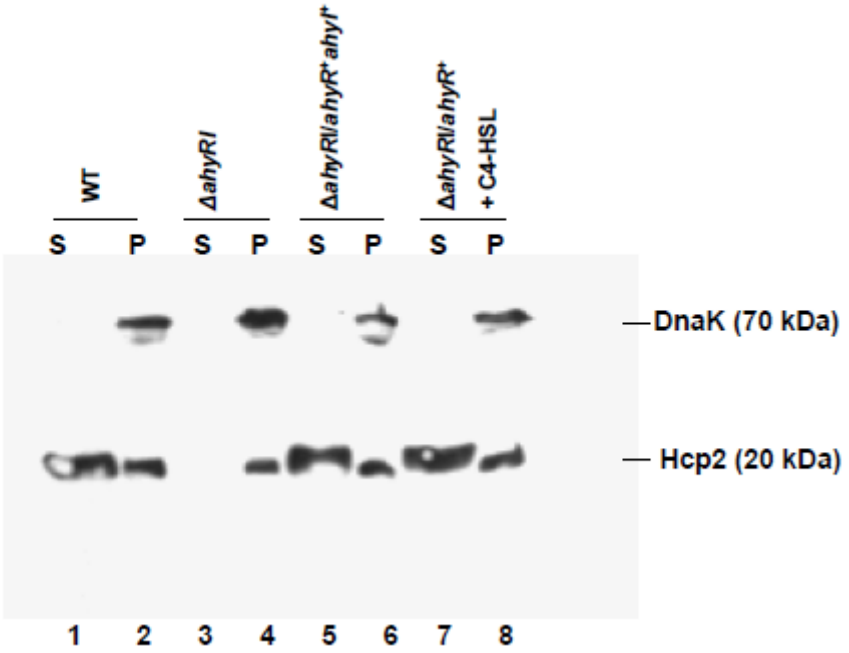

Fig. 3.3. Western blot analysis showing production of Hcp2 in the cell pellet (P) and secretion of Hcp2 in the culture supernatants (S) of WT *A. hydrophila* SSU, Δ*ahyRI* mutant, and the complemented strains. **Lane 1,** supernatant of WT strain; **Lane 2**, cell pellet of WT strain; **Lane 3,** supernatant of the Δ*ahyRI* mutant; **Lane 4,** cell pellet of the Δ*ahyRI* mutant; **Lane 5,** supernatant of the Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$-complemented strain; **Lane 6,** cell pellet of the Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$-complemented strain; **Lane 7,** supernatant of the Δ*ahyRI*/*ahyR*$^{+}$-complemented strain; **Lane 8,** cell pellet of the Δ*ahyRI*/*ahyR*$^{+}$-complemented strain. The Δ*ahyRI*/*ahyR*$^{+}$-complemented strain was grown in the LB medium supplemented with 20 µM C4-HSL. Polyclonal antibody against Hcp2 (at 1:1000 dilution) and secondary antibody (1:10000 dilution, goat anti-mouse IgG) conjugated with horseradish-peroxidase (HRP) were used. The blots were developed after reaction with super signal west pico chemiluminescence followed by X-ray film exposure. Anti-DnaK antibodies were used to measure the intactness of bacterial cells. Three independent experiments were performed.

Figure 3.4. Western blot analysis showing Valine Glycine repeat G family of proteins, VgrG1 and VgrG2 in WT *A. hydrophila* SSU, Δ*ahyRI* mutant and the complemented strains

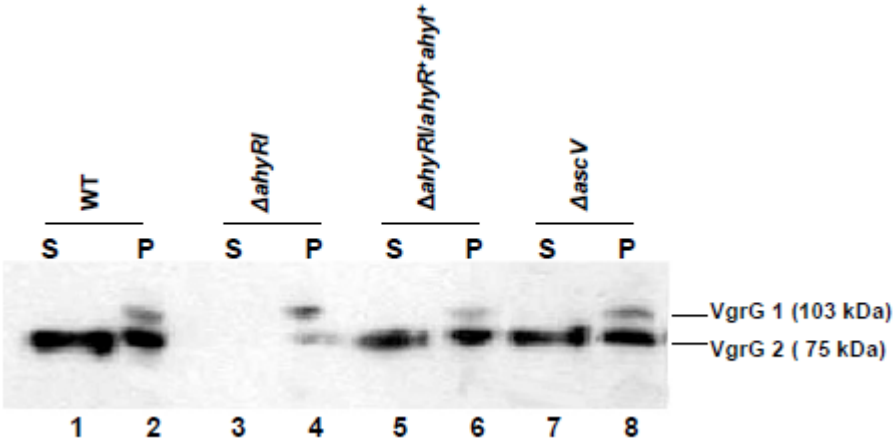

Fig. 3.4. Western blot analysis showing VgrG1 and VgrG2 production in the cell pellet (P) and secretion of VgrGs in the culture supernatants (S) of WT *A. hydrophila* SSU, Δ*ahyRI* mutant and the complemented strains. **Lane 1**, supernatant of the WT strain; **Lane 2**, cell pellet of the WT strain; **Lane 3**, supernatant of the Δ*ahyRI* mutant; **Lane 4**, cell pellet of the Δ*ahyRI* mutant; **Lane 5**, supernatant of the Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$- complemented strain; **Lane 6,** cell pellet of the Δ*ahyRI*/*ahyR*$^{+}$*ahyI*$^{+}$- complemented strain; **Lane 7**, cell pellet of the Δ*ascV* mutant; **Lane 8**, supernatant of the Δ*ascV* mutant. The *ascV* gene constitutes a component of the T3SS and was used as a control. Polyclonal antibody against VgrG2 (at 1:1000 dilution) and secondary antibody (1:10000 dilution, goat anti-mouse IgG) conjugated with HRP were used. The blots were developed after reaction with west femto chemiluminescence substrate followed by X-ray film exposure. Results were reproduced through three independent experiments.

In Western blot analysis, the production of VgrG2 effector was noted both in the WT and in the Δ*ahyRI* mutant of *A. hydrophila* SSU (**Fig.3.4, lanes 2 and 4, lower band)**. A significant level of VgrG2 effector protein was secreted in the supernatant collected from the WT *A. hydrophila* SSU strain **(Fig.3.4, lane 1);** however, the Δ*ahyRI* mutant was unable to secrete VgrG2 in the medium **(Fig.3.4, lane 3)**. Importantly,

secretion of VgrG2 effector protein was restored in the Δ*ahyRI*-complemented strain **(Fig.3.4, lane 5)**. Deletion of the *ascV* gene that codes for an inner membrane component of the T3SS channel from *A. hydrophila* SSU had no effect on the expression and secretion of these effector proteins **(Fig.3.4, lanes 7 and 8)**, which suggested that production and secretion of VgrGs were T3SS independent. Overall, these results suggested to us that AHL mediated the QS controlled secretion of VgrG2 effector of T6SS in *A. hydrophila* SSU.

Expression and translocation of AexU, a T3SS effector, was not affected in the Δ*ahyRI* mutant of *A. hydrophila* SSU. To demonstrate regulation of the AHL-mediated QS on T3SS effector translocation, we examined expression of the *aexU* gene in bacterial cell pellets and translocation of AexU in human colonic epithelial (HT-29) cells in WT and its Δ*ahyRI* mutant grown in Dulbecco's Modified Eagle Medium (DMEM). We found that the Δ*ahyRI* mutant had similar levels of production of AexU compared to that of WT bacteria. Similarly, no difference in the translocation of AexU in HT-29 cells was noted after infection of the latter with the Δ*ahyRI* mutant and the WT bacteria (data not shown). These data indicated that AHL-mediated QS had no effect on the expression and translocation of the T3SS effector, AexU.

The Δ*ahyRI* mutant showed decreased virulence in an animal model. By using *in vitro* experiments, we demonstrated that deletion of the *ahyRI* genes from *A. hydrophila* SSU resulted in the decreased production of protease, prevented secretion of T6SS effectors, such as Hcp and VgrGs, and, the mutant was unable to produce mature biofilms. To examine whether these changes in virulence factors regulated by AHL-mediated QS had any influence on *in vivo* virulence of *A. hydrophila* SSU, we injected mice intraperitoneally with the Δ*ahyRI* mutant and the WT strain of *A. hydrophila* at a lethal dose of 3×10^7 cfu **(Fig.3.5).** We noted that 100% of the animals infected with the

WT *A. hydrophila* died within 6 days. However, mice infected with the Δ*ahyRI* mutant strain exhibited significantly lower mortality (only 50%) over a tested period of 16 days, which suggested to us that bacterial attenuation occurred when we deleted the *ahyRI* genes from *A. hydrophila* SSU.

DISCUSSION

In the present study, we examined the regulation of AHL-mediated QS in modulating various virulence factors, including the T6SS and biofilm formation in a clinical isolate of *A. hydrophila* SSU. Interestingly, we showed that deletion of the *luxS* gene (AI-2 mediated QS) increased the overall virulence of *A. hydrophila* SSU (121). On the contrary, deletion of the *ahyRI* genes decreased the virulence of this pathogen. Therefore, in *A. hydrophila* SSU, QS systems have both a positive and negative regulation of its virulence. A role of protease in *Aeromonas*-associated tissue damage has been reported (186), and, in experimental animal models, the protease null mutants exhibited a decreased virulence both with *A. hydrophila* and *A. salmonicida* (132, 186) compared to that of the WT bacteria. Moreover, the early expression of exoprotease may stimulate host defense (223), and, therefore, regulation of protease production by QS could be an important step in modulating host defense as well as in establishing an infection.

We observed, in agreement with earlier studies (14, 223), that AHL mediated QS-regulated protease production in *A. hydrophila* SSU. Based on casein zymogram analysis, three protein bands of sizes 61, 52 and, 19 kDa were associated with protease activity of *A. hydrophila* SSU, and two of these higher molecular bands were missing from the Δ*ahyRI* mutant strain. We provided evidence that the 61 kDa metallo-protease contributed to the majority of the protease activity (81%) in *A. hydrophila* SSU. On the

Figure 3.5. AhyRI QS regulon of *A. hydrophila* SSU contributes to *in vivo* virulence

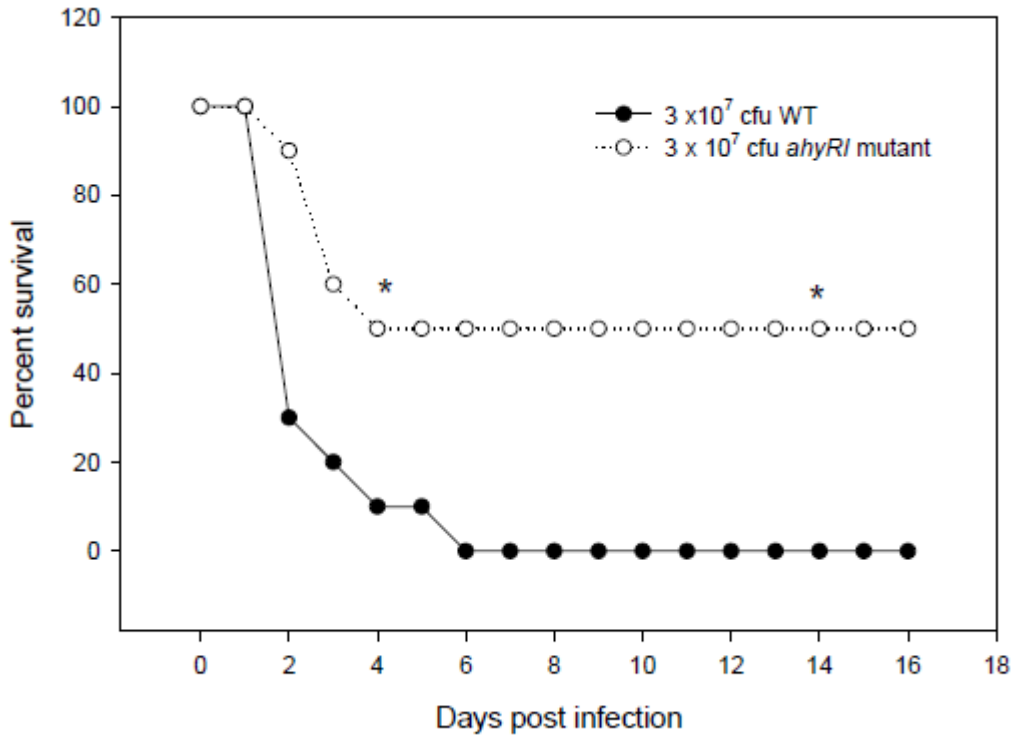

Fig. 3.5. AhyRI QS regulon of *A. hydrophila* SSU contributes to the virulence of the bacterium. Swiss Webster mice (n = 10 per group) were injected intraperitoneally with two 50% lethal doses of WT *A. hydrophila* SSU. The same dose was used to infect mice with the Δ*ahyRI* mutant, and both groups were monitored for death over a 16-day period. The data were statistically analyzed by using Fisher's exact test. Three independent experiments were performed, and data from a typical experiment are shown. * denotes statistically significant differences between the *ahyRI* mutant and WT bacteria ($P \leq 0.05$).

contrary, a previous study showed that serine protease contributed approximately 60% and metalloprotease accounted for approximately 30% of the total protease activity in *A. hydrophila* AH-1N strain (223). By zymogram analysis, they demonstrated that the Δ*ahyI* mutant did not produce the serine protease band (223). Interestingly, in our study, addition of serine protease inhibitor minimally affected the total protease activity of *A. hydrophila* SSU. The nature of other two proteases needs to be further elucidated.

We provided evidence that deletion of the *act* gene, which is secreted through the T2SS in *A. hydrophila* SSU, decreased the production of AHL molecules (196). Consequently, we tested whether AHL-mediated QS regulated the function of Act. However, our study did not reveal any role of this AI-1 QS system in controlling the hemolytic and cytotoxic activities of this toxin in *A. hydrophila* SSU. We demonstrated that Act, T3- and T6- secretion system-associated effectors contributed to cytotoxity in host cells in *A. hydrophila* SSU (196, 220). Although, AHL mediated the QS-regulated T6SS, we could not differentiate between the level of cytotoxicity in the Δ*ahyRI* mutant and its parental bacterium. These results indicated to us that cytotoxicity associated with Act and T3SS effectors could be masking T6SS-associated cytotoxic effects. In future studies, we will determine the cytotoxicity of the Δ*ahyRI* mutant in *act* and *ascV* mutant strains of *A. hydrophila* SSU to discern the effect of T6SS on host cell toxicity. It will also be important to evaluate how Act might be modulating AHL levels in *A. hydrophila* SSU.

Motility is an important virulence factor of Gram-negative bacterial pathogens, as it helps them to reach to the target host tissue to colonize and cause disease (68). We noted a significantly decreased swimming motility in the Δ*luxS* mutant of *A. hydrophila* SSU compared to that in the WT strain (121). However, deletion of the *ahyRI* QS regulon did not affect the swimming and swarming motility of *A. hydrophila* SSU, which was in

accordance with a previous study on *P. syringae* in which the AHL-mediated QS regulon did not affect motility in this plant pathogen (112). On the contrary, in other pathogens (e.g., *Erwinia chrysanthemi* and *Yersinia enterocolitica*), deletion of the *luxRI* homologs produced either enhanced motility (86) or decreased motility (7).

Biofilms are adherent aggregates of bacterial cells growing on biotic and abiotic surfaces. Biofilm-forming bacteria are less susceptible to host immune responses and various antimicrobial agents (42). Importantly, biofilms are often associated with chronic infection, such as cystic fibrosis, caused by *P. aeruginosa* and catheter-associated biofilms of *Staphylococcus epidermidis* (265). In biofilms, EPS is a key component that determines its physico-chemical and biological properties (128). Indeed, EPS was required for the initial attachment of *V. cholerae* (248) and *S. epidermidis* to the solid surface (142).

In the present study, a CV-binding assay showed that the Δ*ahyRI* mutant of *A. hydrophila* SSU was defective in the solid surface attachment, and SEM images further confirmed that the Δ*ahyRI* mutant strain produced a defective EPS on its surface which resulted in the formation of unstructured biofilms. Similar results were reported in *P. aeruginosa* (46) and other pathogens, e.g., *Serratia liquifaciens* (127), in which deletion of the homologous genes of the LuxRI QS system showed unstructured and frail biofilm formation.

More importantly, we noted that the Δ*ahyRI* mutant of *A. hydrophila* SSU was unable to secrete Hcp and that it was also defective in the biofilm formation, suggesting that the secretion of this protein might play an important role in the development of the biofilm. Indeed, a role of Hcp in biofilm development was noted in *P. aeruginosa* (212) and *V. parahaemolyticus* (56). When these studies were performed, it was not known that Hcp is a T6SS effector. However, the underlying mechanism(s) that modulates biofilm

formation through the T6SS effector Hcp is far from clear and needs further in-depth studies, not only in *A. hydrophila* SSU, but also in other pathogens.

Although the T6SS was recently identified in several Gram-negative bacterial pathogens (21, 150, 172, 173), the mechanism(s) of secretion and translocation of its effectors is still in its infancy. Thus far, only the Hcp and VgrG family of proteins were shown to be secreted and translocated into the eukaryotic cells by using this T6SS (137). Our recent study showed that *A. hydrophila* SSU possesses a functional T6SS, and that the effector protein Hcp is translocated into eukaryotic cells through this system and plays an important role in the virulence of this pathogen (220).

VgrG proteins have different COOH-terminal extensions, which contained domains with different activities. For example, VgrG1 and VgrG3 from *V. cholerae* carried a repeat in structural toxin A (RtxA) and peptidoglycan-binding domains, respectively, while VgrG from *P. aeruginosa* carried a zinc metallo-protease domain (171). Further, it was reported that VgrG1 of *V. cholerae* had actin cross-linking activities in eukaryotic cells which were associated with cell-rounding phenotypes (137, 171, 172). Since T6SS has complex regulatory machinery, an optimal timing of gene expression of this cluster is necessary for its optimal function. We believe that AHL-mediated QS plays a crucial role in controlling this complex secretion machinery in *A. hydrophila* SSU.

We noted that the secretion of Hcp2 and VgrG2 in the supernatant was impaired when we deleted the AhyRI QS regulon from *A. hydrophila* SSU, which suggested to us that AHL mediated the QS- regulated secretion of these effector proteins. However, these effectors could still be detected in the cell pellet of the Δ*ahyRI* mutant strain at a similar level to that of the WT *A. hydrophila* SSU. We speculated that the absence of intercellular accumulation of T6SS effectors in the Δ*ahyRI* mutant strain could be due

either to their rapid degradation and/or alternatively to the reduced expression of the corresponding genes, which need to be further investigated.

Further, there are two copies of the *hcp* gene (*hcp1* and *hcp2*) in the genome of *A. hydrophila* SSU, and because they are identical (98% homology) and similar in size, it is possible that we could also be detecting Hcp1 on the Western blots when using polyclonal antibodies against Hcp2. Likewise, VgrG2 has high homology with VgrG3, and they are similar in size; consequently, we might be detecting VgrG3 along with VgrG2 on the Western blots by using VgrG2 antibodies. However, it is not known whether genes encoding *hcp1* and *2* are expressed and regulated similarly, as the *hcp1* gene is not located within the T6SS gene cluster (220). However, both the *vgrG*2 and -3 genes are located within the T6SS gene cluster (220). In *A. hydrophila* ATCC 7966 strain, the AHA gene designations are as follow: *hcp1* (AHA_1118); *hcp2* (AHA_1826); *vgrG1* (AHA_1119); *vgrG2* (AHA_1827); and *vgrG3* (AHA_1848).

In a recent study on plant pathogen *P. atrosepticum*, it was shown for the first time that AHL mediated QS-regulated T6SS and its putative effectors Hcp and VgrGs (135). In addition, they showed by microarray analysis that 11 of the 18 genes of the T6SS cluster were expressed at significantly lower levels in the *expI* mutant than in the WT *P. atrosepticum* (135). This study is very provocative and provided the first clue that AhyRI regulon might affect bacterial virulence by modulating T6SS.

Since VgrGs have a high homology between them, we detected VgrG1 in the bacterial cell pellet by using antibodies against VgrG2 (**Fig.3.4, lanes 2 and 4, upper band)**. However, we could not detect VgrG1 in the culture supernatant of either the WT or its Δ*ahyRI* mutant strain (**Fig.3.4, lanes 1 and 3)**. Nonetheless, the production of VgrG1 was similar in the WT versus its Δ*ahyRI* mutant strain in bacterial pellets. It was recently shown that deletion of the *clpV* gene, an ATPase which provided energy for the

secretion of T6SS effectors, inhibited their secretion while expression and production of these effector proteins were unaltered (150). Likewise, we demonstrated in the *vasK* mutant that the secretion, but not the expression/production and translocation of Hcp, were affected, and that the *vasK* mutant was highly attenuated in a septicemic mouse model of *A. hydrophila* infection.

In addition, we showed that mice infected with WT *A. hydrophila* SSU had circulating antibodies to Hcp, and animals immunized with recombinant Hcp were protected from subsequent challenge with WT bacterium (220). These data clearly suggested to us that secreted Hcp played an important role in the virulence of this pathogen (220). Therefore, we speculate that AHL-mediated QS might regulate secretion of T6SS effectors by modulating other T6SS components, such as *vasK* and *clpV* genes. However, further detailed studies are needed to delineate the mechanistic basis of how AHL-mediated QS regulates T6SS in *A. hydrophila* SSU.

The T3SS was identified in several *Aeromonas* species, and its role in the establishment of infection in the host was determined (19, 196, 197, 239). Further, studies have shown that QS modulates T3SS either positively in enteropathogenic and enterohemorrhagic *E. coli* (214) or negatively in *P. aeruginosa*, *Y. pestis*, and *V. harveyi* and *V. parahemolyiticus* (15, 70, 77). However, in the present study, we noted that AHL-mediated QS system had no role in the regulation of the T3SS effector, AexU, in *A. hydrophila* SSU. Similarly, we showed that deletion of the *luxS* gene from *A. hydrophila* SSU had no effect on the expression and translocation of AexU (121). In enterohemorrhagic *E. coli,* the T3SS is regulated by the AI-3-mediated QS (214), and recently, we identified the AI-3 QS in *A. hydrophila* SSU (unpublished data). In the future, we will examine the role of AI-3-mediated QS in regulating T3SS genes in *A. hydrophila* SSU.

There is limited information on the role of an AHL-mediated QS *in vivo* model of infection. Our study on the septicemic model of mouse infection indicated that the Δ*ahyRI* mutant of *A. hydrophila* SSU was significantly less virulent than that of the WT bacterium, which suggested to us that this AHL-mediated QS regulon contributed to the virulence of *A. hydrophila* SSU. The *in vivo* role of AHL-mediated QS was well established in a report of an acute lung infection model of *P. aeruginosa* (209). Further, AHLs were detected in lung tissues of mice infected with *P. aeruginosa* (254), which was a further evidence that these signaling molecules may play a role in bacterial pathogenesis. In addition, deletion of LasRI and/or RhlRI QS systems from *P. aeruginosa* were found to result in its significant attenuation in terms of its ability to colonize hosts, induce inflammation, and cause mortality (165, 185, 209, 226, 253). In a future study, we will delineate the direct role of AHL molecules in the pathogenesis of *A. hydrophila* SSU in a mouse model of infection.

In conclusion, we demonstrated that AHL-mediated QS plays a crucial role in modulating the virulence of *A. hydrophila* SSU, and these findings validated the notion that interference by AHL-mediated QS would be a promising target for the development of new-generation antimicrobial therapeutics.

CHAPTER 4

Immunomodulatory and Protective Roles of Quorum Sensing Signaling Molecules N-*acyl* Homoserine Lactones during Infection of Mice with *Aeromonas hydrophila*[3]

INTRODUCTION

AHLs, such as the N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL), is not only important in the regulation of bacterial virulence genes, but also interacts with various eukaryotic cells to modulate immune responses (200, 208-210, 228, 249). An earlier study has shown that the *in vitro* effects of 3-oxo-C12-HSL on lymphocytes are immunosuppressive/anti-inflammatory at a low concentration (below 10 μM) and proinflammatory at a high concentration (20 μM and above) (39). Likewise, Telford *et al*., reported that 3-oxo-C12-HSL inhibited the lipopolysaccharide (LPS)-dependent activation of tumor necrosis factor (TNF)-α and interleukin (IL)-12 in murine peritoneal macrophages (229). Later studies further confirmed that 3-oxo-C12-HSL inhibited the proliferation and function (cytokine production) of both mitogen- and antigen-stimulated (26, 184) T lymphocytes. Contrary to these studies, Smith *et al*. reported that 3-oxo-C12-HSL stimulated the production of neutrophil-attracting chemokines, e.g., IL-8, cyclooxigenase (Cox)-2, and prostaglandin E_2 (PGE_2) synthase in human lung fibroblasts and epithelial cells (208). However, Kravchenko *et al*. did not observe such an induction of cytokine or chemokine production in primary respiratory epithelial cells (123).

[3] Khajanchi, B. K., M. L. Kirtley, S. M. Brackman, and A. K. Chopra. 2011. Immunomodulatory and protective roles of quorum sensing signaling molecules N-acyl homoserine lactones during infection of mice with *Aeromonas hydrophila*. Infect Immun 79:2646-2657.

A chemotactic role of 3-oxo-C12-HSL for neutrophils has been shown *in vitro* (266), and apart from IL-8 production and neutrophil attraction, other investigators provided evidence that 3-oxo-C12-HSL promoted apoptosis in various cell types, including neutrophils, monocytes, fibroblasts, and breast carcinoma cells (133, 200, 228). Induction of apoptosis in various immune cells by AHLs might decrease inflammation by reducing the number of effective phagocytes and the mediators they produce (39). This apoptotic induction of host cells mediated by 3-oxo-C12-HSL occurred through a calcium-dependent signaling pathway, while the pro-inflammatory effect of 3-oxo-C12-HSL resulted through a calcium-independent and peroxisome proliferator-activated receptors (PPARs)-mediated pathway (89). The Toll-like receptor (TLR) pathways were not believed to be involved in AHL signaling (123).

As mentioned earlier, several *in vitro* studies reported varied effects of 3-oxo-C12-HSL on different mammalian cells; however, there is limited information available on the effects of different types of AHLs *in vivo*. For example, Smith *et al*. showed that dermal injection of 3-oxo-C12-HSL in mice induced the production of inflammatory mediators, such as IL-1α, IL-6, macrophage inflammatory protein 2 (MIP-2), monocyte chemotatic protein 1 (MCP-1), MIP-1β, and inducible protein 10 (IP-10) (209). On the contrary, Kravchenko *et al*. reported that injection of 3-oxo-C12-HSL in the peritoneum of mice suppressed an LPS-induced immune response (122). Currently, it is not known how these immunomodulatory effects of different AHL signaling molecules influence host immune response during bacterial infection.

Consequently, we investigated the immunomodulatory role of three AHLs, namely C4-HSL, C6-HSL, and 3-oxo-C12-HSL, on the innate immune response in response to *A*. *hydrophila* infection in a septicemic mouse model. Our study is the first to show that pretreatment of mice with AHLs prevents clinical sequelae to enhance

survivability of mice after *A. hydrophila* infection, and that they trigger an innate immune response of mice to clear bacteria from the blood and different mouse tissues.

MATERIALS AND METHODS

Bacterial strain and chemicals. The clinical strain *A. hydrophila* SSU, isolated from a diarrheal patient, was used in this study. All of the N-*acyl* homoserine lactones (C4-, C6-, and 3-oxo-C12 HSLs) used in this study were purchased commercially (Sigma, St. Louis, MO) and were of the highest quality grade (98-100% pure) based on high-performance liquid chromatography (HPLC) analysis. Stock solutions (50 mM) of AHLs were prepared by dissolving them in acetonitrile (for C4-HSL and C6-HSL) and dimethyl sulfoxide (DMSO) (for 3-oxo-C12-HSL), and stocks were stored at -20^{o}C. Before injection in mice or treatment of RAW 264.7 murine macrophages, appropriate concentrations of AHLs were prepared by dilution in phosphate buffered saline (PBS) or DMSO. We used 200 μM and 250 μM of each AHL for *in vitro* and *in vivo* studies, respectively, unless otherwise stated. Cyclophosphamide was purchased from Sigma and was dissolved in water.

Mice survivability. Female 5- to 6- week-old Swiss-Webster mice (average weight, approximately 20 g) were purchased from Charles River Laboratories (Wilmington, MA). Mice were treated intraperitoneally (i.p.) with 250 μM per 100 μl dose of each C4-HSL, C6-HSL, 3-oxo-C12-HSL or a combination of C4-HSL and C6-HSL, and/or PBS (vehicle control) for 12 h and 6 h before *A. hydrophila* challenge (i.p.) with either 3 x 10^7 or 3.5 x 10^7 cfu. In some experiments, we also injected into mice lower amounts of C4- and C6- HSLs (25 and 100 μM per 100 μl dose of each) prior to *A. hydrophila* infection. Deaths were recorded for 16 days post infection (p.i.). All animal

experiments were performed by using protocols approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Blood chemistry. To determine changes in the blood chemistry, we pretreated mice with various AHLs or vehicle for 12 h and 6 h before challenge with *A. hydrophila* at a dose of 3 x 10^7 CFUs. A group of mice (n=5, each group) were only treated with either AHLs or vehicle without *A. hydrophila* infection. At 4 h (post infection or post treatment), animals were euthanized by using isoflurane, and blood was collected immediately *via* cardiac puncture in blood collection tubes with lithium heparin (Becton Dickinson, Cockeysville, MD). To evaluate changes in the blood chemistry at a later stage in *A. hydrophila* infection, blood was collected from different groups of mice (n=5, each time point) at 0, 12, and 24 h post infection with *A. hydrophila* at a dose of 3 x 10^7 CFUs. From each blood tube, 100 µl of heparinized whole blood was carefully dispensed to the Vetscan comprehensive diagnostic profile reagent rotor, and blood chemistry profiles were analyzed by using an automated Vetscan chemistry analyzer (Abaxis, Union City, CA). The VetScan rotor used in this study provided *in vitro* determination of alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), amylase (AMY), total calcium (CA), creatinine (CRE), globulin (GLOB), Glucose (GLU), phosphorus (PHOS), potassium (K), sodium (NA), total bilirubin (TBIL), total protein (TP), and urea nitrogen (BUN) in the blood. The data from all animals in each group were averaged, and the standard deviations were calculated.

Determination of bacterial burden. Mice were pretreated with various AHLs as described above prior to infection with *A. hydrophila* at a dose of 3.5 x 10^7 CFUs. To determine bacterial load in the blood, at 2 h and 4 h p. i., mice were euthanized by using isoflurane and the blood collected immediately *via* cardiac puncture in blood collection tubes containing potassium EDTA (Becton Dickinson). The animals were then humanely

sacrificed by CO_2 and cervical dislocation. To examine bacterial dissemination in different tissues, at 48 h p.i., the lungs, liver, and spleen were removed, weighed, and homogenized by using tissue grinders (Kendall, Mansfield, MA) in 1 ml of phosphate buffered-saline (PBS). The blood and tissue homogenates were serially diluted and cultured on Luria-Bertani (LB) agar plates supplemented with rifampin (200 μg/ml; we used a spontaneous mutant strain of SSU resistant to this antibiotic). After incubation of the plates at 37^0C for 12-18 h, bacterial colonies were counted, and the CFUs per gram of tissue or per ml of blood were calculated.

***In vivo* cytokine/chemokine analysis.** To determine cytokine/chemokine production in different tissues, mice were pretreated with various AHLs prior to *A. hydrophila* infection with 3.5 x 10^7 CFUs. As controls, mice were only treated with various AHLs without any bacterial challenge but given PBS. At 48 h post infection or post treatment with AHLs, the lungs, liver, and the spleen were removed, weighed, and homogenized in 1 ml of PBS. The tissue homogenates were centrifuged at 13,000 rpm for 10 min and the supernatants collected in 1.5-ml micro-centrifuge tubes. The levels of cytokines/chemokines were measured by using a multiplex assay (Bio-Rad, Hercules, CA) (194).

Blood cell population counts. Mice were pretreated with various AHLs and then challenged with *A. hydrophila* as described above. We determined different cell populations, including neutrophils and platelets, in the blood of the above-mentioned animals, as well as from those mice that were only treated with AHLs or vehicle control without bacterial infection to serve as a control. At 4 h post infection or post treatment with AHLs/vehicle, mice were euthanized and blood collected immediately in blood collection tubes containing lithium heparin (Becton Dickinson). The blood cell counts were analyzed immediately by using a Drew Scientific Hemavet 950 hematology system

(Drew Scientific, Inc., Dallas, TX). The number of neutrophils and platelets from all animals were averaged, and the data presented as means ± standard deviations.

***In vivo* neutrophil depletion.** To further characterize the role of neutrophils in *A. hydrophila* infection, mice were depleted of neutrophils before *A. hydrophila* challenge. Animals were rendered neutropenic by either i.p. injection of 250 mg of cyclophosphamide/kg body weight (two doses: one on day 0, 150 mg/kg body weight; and the second on day 4, 100 mg/kg weight) or by injection of 250 μg of purified anti-Gr-1 monoclonal antibody [No azide/low endotoxin (NA/LE); RB6-8C5, BD Pharmingen], 1 day prior to bacterial challenge. The control mice were given either PBS or an equivalent amount of purified rat immunoglobulin G (rIgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Both of these methods have been successfully used by others to generate neutropenic conditions in mice (43, 87, 130, 235).

To confirm neutrophil depletion, the blood was collected by retro-orbital bleeding after mice were anesthetized by using a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). In case of animals depleted for neutrophils by using a cyclophamide procedure, we challenged normal and neutropenic mice with three different doses of *A. hydrophila*: 1 x 10^6, 5 x 10^6 and 1.5 x 10^7 CFUs (n=5 each group). Since RB6-8C5 antibodies are expensive, we challenged normal and neutropenic mice with only 1.5 x 10^7 CFUs of *A. hydrophila* (n=5 each group). Mice were observed for survivability up to16 days post infection.

Bacterial phagocytosis by murine macrophages. RAW 264.7 murine macrophages were seeded in six-well plates and incubated until they reached 60 to 70% confluence (approximately 3 x 10^6 cells/well). Macrophages were treated with either 200 μM of C4-HSL, C6-HSL, C4-HSL and C6-HSL together, or vehicle for 1 h, and then the host cells were infected with *A. hydrophila* at a multiplicity of infection (MOI) of 10.

Phagocytosis assay was performed according to the established procedure (194). Briefly, after infection, the plates were centrifuged at 1,500 rpm for 10 min to facilitate binding of the bacteria to the host cells. After 30 min of incubation, extracellular bacteria were killed by gentamicin treatment (150 μg/ml) for 1 h, and then the macrophages were washed and lysed with 300 μl of 0.1% triton-X100 solution. Suspensions of lysed macrophages were serially diluted, and bacterial colonies were counted after incubation at 37^0C for 12-18 h.

Statistics. Wherever applicable, at least three independent experiments were performed, and the data analyzed by using the student's *t* test or one-way ANOVA, with *p* values of ≤ 0.05 considered statistically significant. The animal data were analyzed by using the Kaplan-Meier's survivability test with *p* values of ≤ 0.05 considered statistically significant.

RESULTS

AHL pretreatment provides protection to mice against infection. Animals that were pretreated with 250 μM of C4-HSL, C6-HSL, or 3-oxo-C12-HSL at 12 h and 6 h before *A. hydrophila* challenge with 3.5 x 10^7 CFUs were provided 40% (p=0.03), 50% (p=0.01) and 60% (p=0.004) protection, respectively, compared to control mice (100% death rate) that were treated with PBS and then infected with the same dose of bacteria **(Fig. 4.1)**. Importantly, 100% (p=0.004) of the mice pretreated with a combination of 250 μM each of C4-HSL and C6-HSL 12 h and 6 h before *A. hydrophila* challenge with 3 x 10^7 CFUs were protected, compared to the control animals that were pretreated with PBS. In the latter group, 60% of the animals died at this dose **(Fig. 4.2)**. Importantly, similar protection of mice was noted when they were pretreated with lower tested amounts of HSLs (C4-HSL + C6-HSL combined) (100 and 25 μM of each) (data not shown). For most of our subsequent studies, we opted to use higher amounts of HSLs,

i.e., 250 μM. In addition, to further dissect the significance of HSLs in *A. hydrophila* infection, we pretreated mice with C4- and C6- HSLs (250 μM each) in combination at 12 h and 6 h prior to challenge with the *ahyRI* double knockout QS mutant of *A. hydrophila* SSU that is unable to produce its HSLs and also does not respond to exogenous HSLs (109). Mice that were given PBS instead of HSLs but challenged with the mutant were used as a control. We found that HSL pretreatment (C4 + C6) also protected mice (75%) from subsequent infection with the QS *ahyRI* mutant (data not shown). These data suggested that immunomodulation by HSLs protected mice irrespective of whether or not *A. hydrophila* produced its own QS signaling molecules.

Further, we performed experiments in which HSLs were given to mice at the same time of bacterial challenge and no protection was observed, suggesting that HSLs acted in a prophylactic manner (data not shown). We injected 250 μM each of HSL or 500 μM in combination of C4- and C6- HSLs in control mice without infection, and there were no signs and symptoms of discomfort in animals during the duration of the experiment (16 days), and none of the mice died indicating that HSLs themselves were not toxic to the animals **(Fig. 4.1-4.2)**. Although 3-oxo-C12-HSL is mainly produced by *Pseudomans aeruginosa* and not *A. hydrophila,* we also examined the protective and immunomodulatory role of this lactone in our study to compare the efficiency and the mechanism of protection of animals by different AHL QS signaling molecules during *A. hydrophila* infection.

Changes in blood chemistry following *A. hydrophila* infection of mice with and without AHL pretreatment. To study systemic effects in mice at different time points following *A. hydrophila* infection and to evaluate the influence of AHL pretreatment on the blood chemistry**,** we analyzed blood samples at 0, 4, 12, and 24 h post infection **(Table 4.1)**.

Table 4.1. Alteration in blood chemistry during *Aeromonas hydrophila* infection

Blood component[e] (concentration)	Vehicle[a] ± SD[d]	AHLs[a] ± SD[d]	Vehicle + *A. hydrophila*[b] ± SD[d]	AHLs + *A. hydrophila*[c] ± SD[d]
ALB (g/dl)	3.82 ± 0.2	3.78 ± 0.2	3.46 ± 0.3	2.94 ± 0.6
ALP (U/l)	142.8 ± 36.8	136.4 ± 30.1	89.4 ± 13.9	77 ± 26.8
ALT (U/l)	51 ± 11.3	42 ± 7.3	76.6 ± 18.4	48.8 ± 12.8
AMY (U/l)	1106 ± 372.9	1149 ± 230.5	1543.6 ± 486.8	1092.6 ± 173.1
TBIL (mg/dl)	0.2 ± 0.0	0.22 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
BUN (mg/dl)	14.2 ± 1.8	17.4 ± 6.0	14.2 ± 3.6	10.6 ± 2.8
CA (mg/dl)	12.3 ± 0.7	12.88 ± 0.6	12.04 ± 0.1	11.36 ± 0.2
PHOS (mg/dl)	12.14 ± 0.6	12.56 ± 1.0	16.06 ± 1.6	13.84 ± 1.5
CRE (mg/dl)	0.26 ± 0.1	0.22 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
GLU (mg/dl)	390.2 ± 44.0	375.6 ± 102.6	226.4 ± 21.7	389.4 ± 122.9
NA+ (mg/dl)	154 ± 1.2	154.2 ± 3.0	155.2 ± 1.6	152.8 ± 4.1
K+ (mg/dl)	7.6 ± 1.0	7.68 ± 1.0	8.14 ± 0.4	7.6 ± 1.5
TP (g/dl)	5.2 ± 0.3	5.44 ± 0.3	4.78 ± 0.2	4.36 ± 0.8
GLOB (g/dl)	1.36 ± 0.2	1.7 ± 0.1	1.34 ± 0.1	1.4 ± 0.2

[a,] blood chemistry was analyzed from mice that were only treated with vehicle or AHLs at 12 h and 6 h without *A. hydrophila* infection.

[b,] blood chemistry was analyzed from mice that were treated with vehicle at 12 h and 6 h before challenge with *A. hydrophila* (3×10^7 CFUs). Blood was collected 4 h post infection.

[c,] blood chemistry was analyzed from mice that were treated with C4-HSL and C6-HSL in combination at 12 h and 6 h before challenge with *A. hydrophila* (3×10^7 CFUs). Blood was collected at 4 h post infection.

[d,] data are shown as an average mean value of five mice and standard deviations (SD) were determined.

[e,] abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; TBIL, total bilirubin; BUN, urea nitrogen; CA, total calcium; PHOS, phosphorus; CRE, creatinine; GLU, Glucose; NA^+, sodium; K^+, potassium; TP, total protein; and GLOB, globulin.

Figure 4.1. Survival of Swiss-Webster mice that were pre-treated with C4-HSL, C6-HSL and 3-oxo-C12-Homoserine Lactones

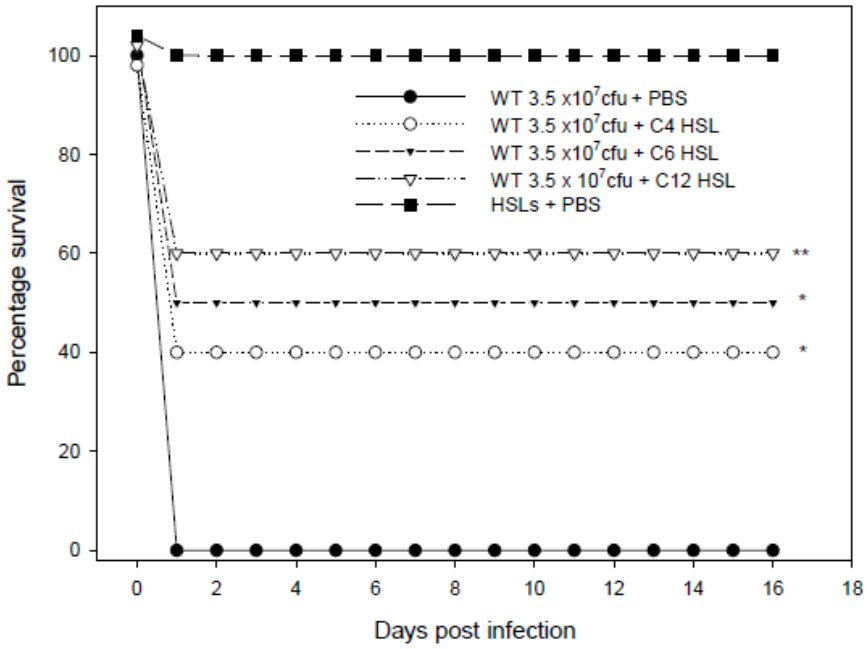

Fig. 4.1. Survival of Swiss-Webster mice that were pre-treated with AHLs by the i.p. route prior to *A. hydrophila* challenge. Percentages of surviving mice over time are shown, and the data were analyzed by using the Kaplan-Meier's survival estimates. Mice (n=10 each group) were treated with 250 µM per 100 µl dose each of C4-HSL, C6-HSL, 3-oxo-C12-HSL, or PBS (control) 12 h and 6 h before *A. hydrophila* challenge by the i.p. route with 3.5 x 10^7 CFUs. Mice (n=5 each group) were also treated with 250 µM each of C4-HSL, C6-HSL, and 3-oxo-C12-HSL without bacterial infection and served as controls for determining toxicity of HSL molecules. The survival percentages were statistically significant between control and C4-HSL (*) (p=0.03), C6-HSL (*) (p=0.01) and 3-oxo-C12-HSL (**) (p=0.004) treated mice after *A. hydrophila* infection.

Figure 4.2. Survival of Swiss-Webster mice that were pre-treated with combined doses of C4-HSL and C6-HSL

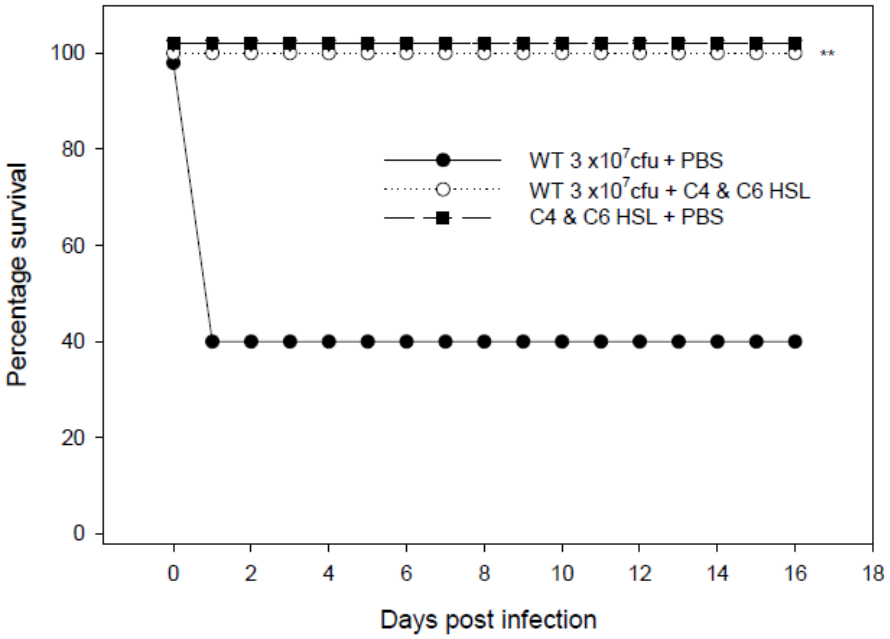

Fig. 4.2. Survival of Swiss-Webster mice that were pre-treated with AHLs by the i.p. route prior to *A. hydrophila* challenge. Percentages of surviving mice over time are shown, and the data were analyzed by using the Kaplan-Meier's survival estimates. Mice were treated with combined doses of C4-HSL and C6-HSL (250 µM each) or PBS (n=10 each group) 12 h and 6 h before *A. hydrophila* challenge with 3 x 10^7 CFUs. Mice were observed for deaths for 16 days post infection. Mice (n=5) were also treated with combined doses of C4-HSL and C6-HSL (250 µM each) without bacterial infection as a toxicity control. ** denotes statistically significant difference in the survival rate of mice between control and combined treatment of C4-HSL and C6-HSL (p=0.004) after bacterial infection.

As mentioned in the materials and methods section, animals were pretreated by the i.p. route with either C4-HSL and C6-HSL in combination or vehicle control 12 h and 6 h prior to challenge with 3 x 10^7 CFUs of *A. hydrophila*.

During *A. hydrophila* infection of mice without AHL pretreatment, the level of ALT was increased from 51 (U/L) at 0 h to 76.6 (U/L) at 4 h **(Table 4.1)**, 127.4 (U/L) at 12 h, and 159.25 (U/L) at 24 h post infection (data not shown). Likewise, the level of AMY was increased from 1106 (U/L) at 0 h to 1543.6 (U/L) at 4 h **(Table 4.1)** and reached to 1930.5 (U/L) at 24 h (data not shown). The phosphorus level increased from 12.14 (mg/dL) at 0 h to 16.06 (mg/dL) at 4 h and remained at a similar level or was slightly decreased at 12 h and 24 h post infection. On the other hand, the GLU level decreased over the course of *A. hydrophila* infection; its level was 226.4 (mg/dL) at 4 h post infection **(Table 4.1)**; this level dropped to 87.2 (mg/dL) at 12 h and dipped further to 70.5 (mg/dL) at 24 h post infection (data not shown). The levels of ALB and ALP also decreased over the time course of infection.

These changes in blood chemistry indicated kidney and liver damage, hypoglycemia, and a nutritional disorder in mice due to *A. hydrophila* infection, leading to their demise. Interestingly, blood chemistry results revealed that prior treatment of animals with combined C4-HSL and C6-HSL rescued them from these devastating systemic effects of *A. hydrophila* infection. For instance, the ALT level remained normal at 48.8 (U/L) at 4 h post infection when mice were pretreated with AHLs, while this value was higher (76.6 U/L) in mice infected with *A. hydrophila* without AHL pretreatment **(Table 4.1)**. In addition, AHL pretreatment helped mice to retain glucose, amylase, and phosphorus levels at normal levels after 4 h of *A. hydrophila* infection. These data indicated that AHL pretreatment was beneficial to mice when they were subsequently infected with *A. hydrophila* **(Table 4.1)**. Treatment of mice with only AHLs

at 12 h and 6 h without the *A. hydrophila* infection did not result in any alterations in blood chemistry **(Table 4.1)**.

AHL pretreatment rescues mice from thrombocytopenia evoked by *A. hydrophila*. The number of platelets significantly dropped when animals were challenged with *A. hydrophila* without AHL pretreatment as compared with that in vehicle control- and AHL- treated control mice without the infection, indicating that *A. hydrophila* induced thrombocytopenia in animals **(Fig. 4.3)**. Thrombocytopenia is a key feature of HUS (73, 201), which could be one of the major causes of animal death after *A. hydrophila* infection. Pretreatment of mice with C4-HSL, C-6 HSL, 3-oxo-C12-HSL, or a combination of C4- and C6-HSL, increased platelet counts of animals to a level within the normal range **(Fig. 4.3)** which further supported our notion that AHL pretreatment improved mice survivability during *A. hydrophila* infection.

AHL pretreatment enhances bacterial clearance from mice. To examine whether an enhanced survivability of AHL-pretreated animals following *A. hydrophila* infection was due to rapid clearance of bacteria from different tissues (lungs, liver, and spleen), we determined bacterial load in these organs. Briefly, we pretreated mice with 250 μM of C4-HSL, combination of C4-HSL and C6-HSL, and/or 3-oxo-C12-HSL 12 h and 6 h before *A. hydrophila* challenge with 3.5 x 10^7 CFUs. All of the mice that were only infected with bacteria without the HSL treatment developed acute infection. At 48 h post infection, a significantly reduced number of bacteria was found in the lungs **(Fig. 4.4A)**, liver **(Fig. 4.4B),** and spleen **(Fig. 4.4C)** of mice pretreated with C4-HSL, C4-HSL and C6-HSL in combination, or 3-oxo-C12-HSL, compared to those animals given PBS and then infected with the bacteria.

Figure 4.3. Prior AHL (N-*acyl* homoserine lactones) treatment rescued mice from thrombocytopenia induced by *A. hydrophila*

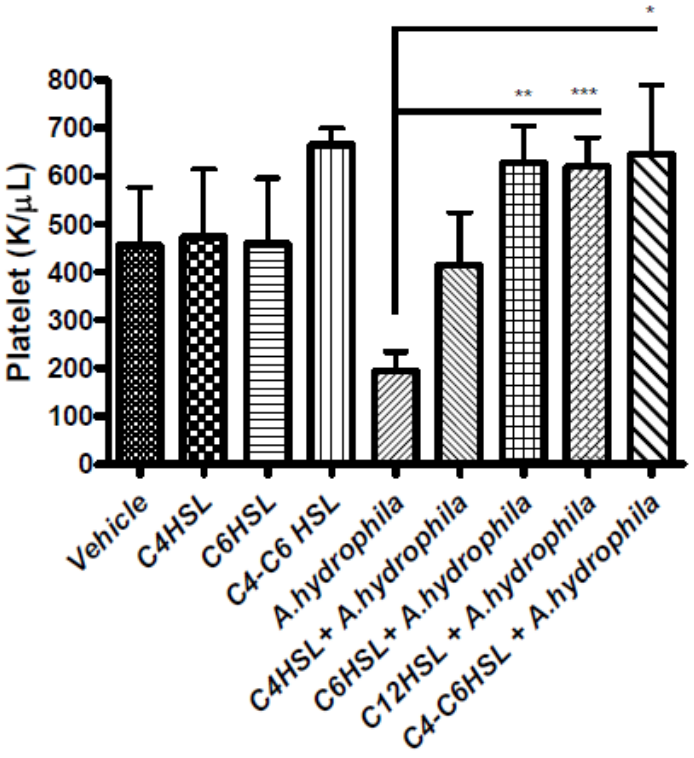

Fig. 4.3. Prior AHL treatment rescued mice from thrombocytopenia induced by *A. hydrophila*. Platelets were counted in the blood collected from mice (n=5, each treatment group) that were pretreated (i.p.) with C4-HSL, C-6 HSL, a combination of C4-HSL and C-6 HSL, or 3-oxo-C12-HSL and/or vehicle for 12 h and 6 h with and/or without *A. hydrophila* infection. AHL pretreatment without *A. hydrophila* infection did not influence platelet numbers. During infection without AHL treatment, the platelets were significantly decreased (thrombocytopenia) while AHL pretreatment before infection (C4-HSL [p=0.096], C6-HSL** [p=0.001], 3-oxo-C12-HSL*** [p=0.0004], C4- and C6-HSLs* [p=0.016]) normalized the platelet numbers to the normal range. Asterisks denote statistically significant platelets count differences between control (without the AHL treatment) and with AHL pretreatment followed by the infection.

The average number of bacterial counts in the tissues (lungs, liver and spleen) of control mice at 24 h post infection (n=4) was approximately 5 x 10^7 CFUs per gram of tissue, and this number was as high as 1 x 10^9 CFUs per gram of tissue (n=3) at 48 h of post infection **(Fig. 4.4 A-C)**. On the contrary, on average, there were approximately 1 x 10^4 CFUs in AHL-pretreated mice (n=3-4) at 48 h of post infection **(Fig. 4.4 A-C)**. Since *A. hydrophila* infection leads to bacteremia, we also investigated bacterial load in the blood at the early time points of 2 h and 4 h. For these experiments, mice were pretreated with 250 μM each of C4-HSL and C6-HSL in combination prior to *A. hydrophila* challenge. At 2 h and 4 h post infection, the bacterial load was determined in AHL- and PBS- treated mice. The bacterial counts in control mice were higher, i.e., 3.3-4.6 x 10^3 CFUs/ml of blood after 2-4 h of infection, as compared to those in the AHL-pretreated mice **(Fig. 4.4D)**. The average bacterial counts in the AHL-pretreated mice at 2 h were approximately 1.3 x 10^3 CFUs/ml, and this number was significantly reduced at 4 h (5 x 10^2 CFUs/ml) **(Fig. 4.4D)**.

AHL pretreatment of mice reduces cytokine/chemokine level in different tissues induced by bacterial infection. Increased levels of proinflammatory cytokines/chemokines may cause detrimental effects in the host, and, thus, we measured and compared their levels in different tissues between control and AHL- pretreated mice to determine the extent of inflammation by using a Bioplex assay. Mice were pretreated with C4-HSL, a combination of C4-HSL and C-6 HSL, and/or 3-oxo-C12-HSL for 12 h and 6 h before *A. hydrophila* challenge with 3.5 x 10^7 CFUs. The levels of 23 cytokines/chemokines were determined in the lungs, liver, and spleen of control and AHL-pretreated mice. Irrespective of the types of AHLs used for the pretreatment and tissues examined, in general, most of the cytokine/chemokine levels were reduced when

Figure 4.4. AHL pretreatment enhanced bacterial clearance from different mouse tissues and the blood

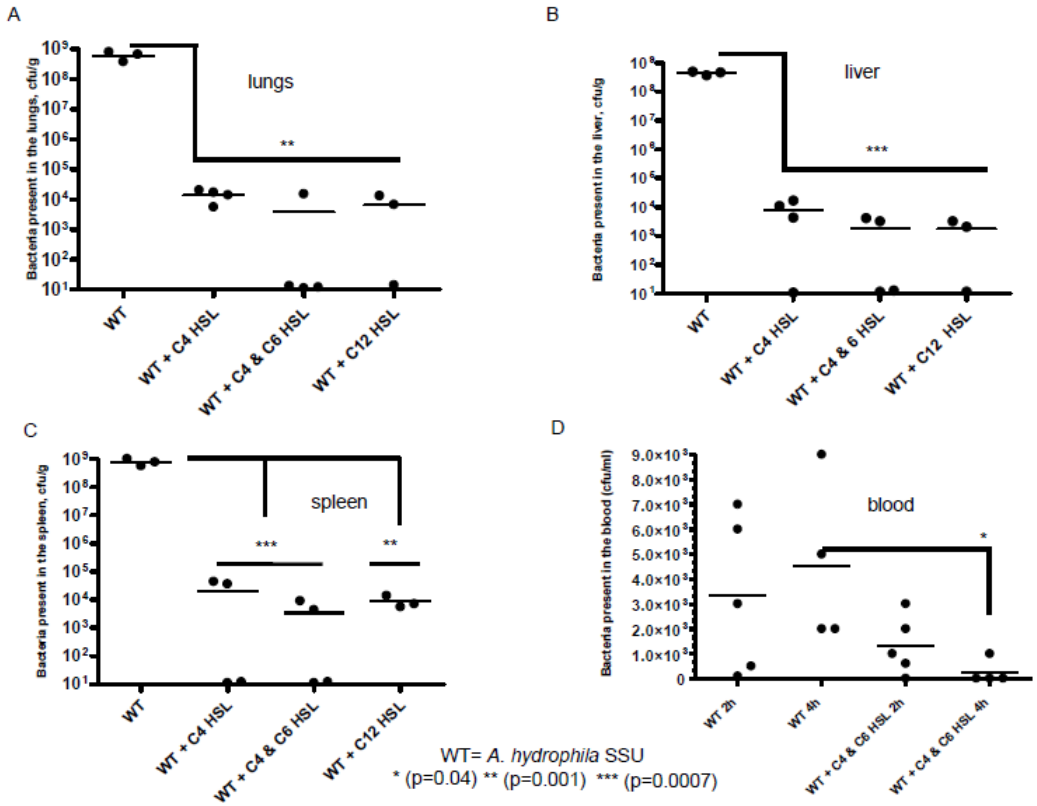

Fig. 4.4. AHL pretreatment enhanced bacterial clearance from different mouse tissues and the blood. To measure bacterial burden, we randomly obtained tissues from 7 out of 10 animals [with n=4 at 24 h p.i. (data not shown) and n=3 at 48 h p.i.] that were only infected with *A. hydrophila* without HSL pretreatment. We randomly obtained tissues from 3-4 animals at 48 h p. i., that responded to different HSL pretreatment. As evident from Figure 1, some mice did not respond to HSL pretreatment and died after *A. hydrophila* infection. These animals were excluded while plotting data. Mice were pretreated (i.p.) with C4-HSL, C4-HSL and C-6 HSL combined, 3-oxo-C12-HSL, or PBS 12 h and 6 h before *A. hydrophila* challenge (i.p.) with 3.5 x 10^7 CFUs. At 48 h p.i., bacterial loads were determined in the lungs (A), liver (B), and the spleen (C) after culturing on the LB agar plates. (D) mice were pretreated (i.p.) with the combined dose of C4-HSL and C-6 HSL or PBS (control) 12 h and 6 h before *A. hydrophila* challenge (i.p.) with 3.5 x 10^7 CFUs. At 2 h (n=5 each group) and 4 h p.i. (n=4 each group), the bacterial load was determined in the blood. Statistical analysis was performed between AHL- and PBS- treated infected animals using the student's t test and p values of ≤ 0.05 were considered significant.

mice were pretreated with AHLs compared to those in PBS-treated and infected control mice at 48 h post infection. Further, our data indicated that in the lungs, 3 cytokines/chemokines, such as granulocyte macrophage colony stimulating factor (GMCSF), MCSF, and interferon (IFN)-γ significantly reduced in mice when pretreated with C4-HSL, C4-HSL and C-6 HSL combined, and/or 3-oxo-C12-HSL as compared to findings in control animals at 48 post infection with *A. hydrophila* **(Fig. 4.5)**. Although IL-1α, IL-1β, and lipopolysaccharide-inducible CXC chemokine (LIX) were also reduced in the HSL-pretreated and infected mice compared to control animals, the results were not statistically significant **(Fig. 4.5)**. Likewise, in liver and spleen, while the levels of five cytokines/chemokines such as GMCSF, MSCF, IL-1α, IL-1β, and LIX, were significantly reduced in HSL pretreated mice as compared to that of control mice at 48 h post infection, the IFN-γ was also reduced in these tissues but the data were not statistically significant **(Fig. 4.6-4.7)**. In addition, the levels of cytokines and chemokines such as GMCSF, MCSF, and LIX in these tissues of control mice (infected but not AHL pretreated) were similar at 24 h and 48 h post infection; however the levels of IFN-γ, IL-1α, and IL-1β were 3-5 times lower at 24 h as compared to 48 h post infection (data not shown). Importantly, there were no significant changes in the cytokine/chemokine levels in these tissues from mice that were treated with AHLs alone compared to that of the PBS-treated control animals without the *A. hydrophila* challenge (data not shown).

AHL pretreatment induces neutrophil recruitment in the blood of infected mice. To determine the specific mechanism(s) as to how AHL-pretreated mice were clearing the bacteria, we evaluated various cell counts in blood collected from AHL-pretreated and control (vehicle-treated) mice with and without *A. hydrophila* infection by using an automated hematology system. After 4 h of bacterial challenge, the number of

Figure 4.5. AHL pretreatment reduced cytokine/chemokine levels in lungs

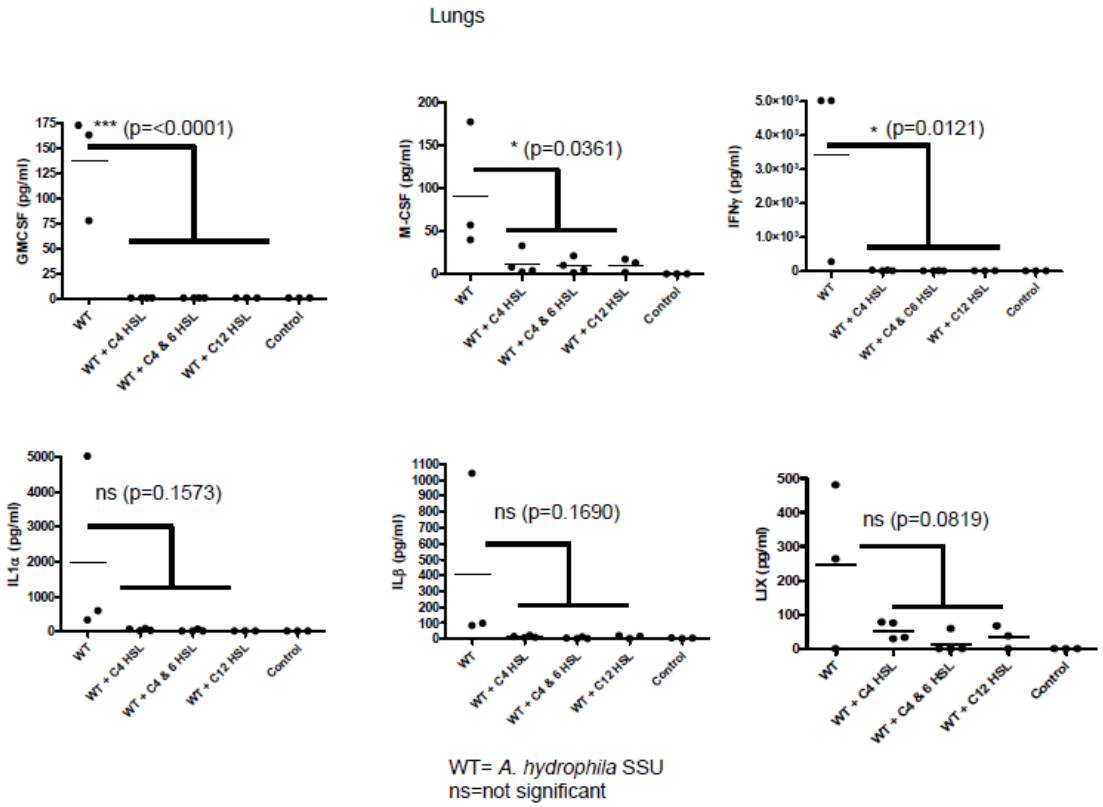

Fig.4.5. AHL pretreatment reduced cytokine/chemokine levels in lungs. To measure cytokines and chemokines levels, we randomly obtained tissues from 7 out of 10 animals [with n=4 at 24 h p.i. (data not shown) and n=3 at 48 h p.i.] that were only infected with *A. hydrophila* without HSL pretreatment. We randomly obtained tissues from 3-4 animals at 48 h p.i., that responded to different HSL pretreatment. As evident from Figure 1, some mice did not respond to HSL pretreatment and died after *A. hydrophila* infection. These animals were excluded while plotting data. Mice were pretreated (i.p.) with C4-HSL, C4-HSL and C-6 HSL combined, 3-oxo-C12-HSL, or PBS 12 h and 6 h before *A. hydrophila* challenge (i.p.) with 3.5 x 10^7 CFUs. Cytokines/chemokines were measured after 48 h p.i. by using the BioPlex assay. The data are shown for the level of six different cytokines/chemokines, such as GMCSF, MCSF, IFN-γ, IL1-α, IL1-β, and LIX of AHL-pretreated and PBS-treated infected mice. The basal level of cytokines/chemokines was also measured from non-infected mice that were only treated with PBS (control). Statistical analysis was performed between AHL- and PBS- treated infected animals by using one-way ANOVA and p values of ≤ 0.05 were considered significant.

Figure 4.6. AHL pretreatment reduced cytokine/chemokine levels in liver

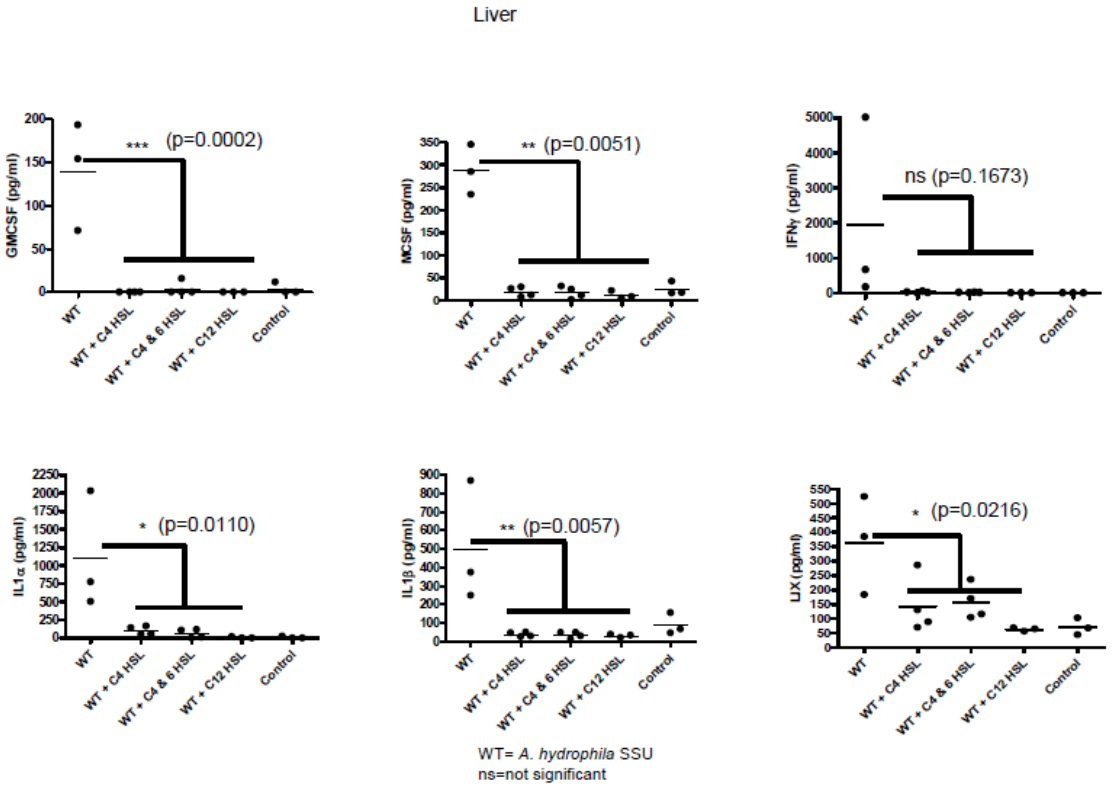

Fig.4.6. AHL pretreatment reduced cytokine/chemokine levels in liver. To measure cytokines and chemokines levels, we randomly obtained tissues from 7 out of 10 animals [with n=4 at 24 h p.i. (data not shown) and n=3 at 48 h p.i.] that were only infected with *A. hydrophila* without HSL pretreatment. We randomly obtained tissues from 3-4 animals at 48 h p.i., that responded to different HSL pretreatment. Mice were pretreated (i.p.) with C4-HSL, C4-HSL and C-6 HSL combined, 3-oxo-C12-HSL, or PBS 12 h and 6 h before *A. hydrophila* challenge (i.p.) with 3.5 x 10^7 CFUs. Cytokines/chemokines were measured after 48 h p.i. by using the BioPlex assay. The data are shown for the level of six different cytokines/chemokines, such as GMCSF, MCSF, IFN-γ, IL1-α, IL1-β, and LIX of AHL-pretreated and PBS-treated infected mice. The basal level of cytokines/chemokines was also measured from non-infected mice that were only treated with PBS (control). Statistical analysis was performed between AHL- and PBS- treated infected animals by using one-way ANOVA and p values of ≤ 0.05 were considered significant.

Figure 4.7. AHL pretreatment reduced cytokine/chemokine levels in spleen

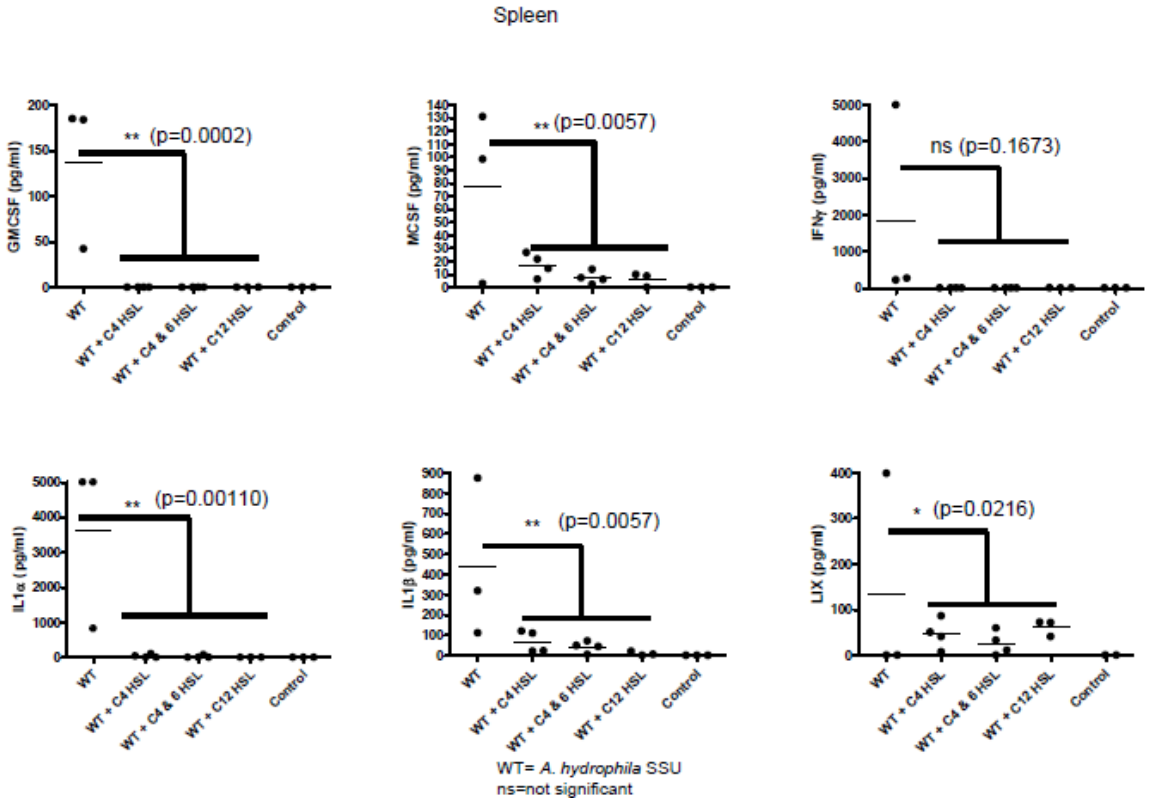

Fig.4.7. AHL pretreatment reduced cytokine/chemokine levels in spleen. To measure cytokines and chemokines levels, we randomly obtained tissues from 7 out of 10 animals [with n=4 at 24 h p.i. (data not shown) and n=3 at 48 h p.i.] that were only infected with *A*. *hydrophila* without HSL pretreatment. We randomly obtained tissues from 3-4 animals at 48 h p.i., that responded to different HSL pretreatment. Mice were pretreated (i.p.) with C4-HSL, C4-HSL and C-6 HSL combined, 3-oxo-C12-HSL, or PBS 12 h and 6 h before *A*. *hydrophila* challenge (i.p.) with 3.5 x 10^7 CFUs. Cytokines/chemokines were measured after 48 h p.i. by using the BioPlex assay. The data are shown for the level of six different cytokines/chemokines, such as GMCSF, MCSF, IFN-γ, IL1-α, IL1-β, and LIX of AHL-pretreated and PBS-treated infected mice. The basal level of cytokines/chemokines was also measured from non-infected mice that were only treated with PBS (control). Statistical analysis was performed between AHL- and PBS- treated infected animals by using one-way ANOVA and p values of ≤ 0.05 were considered significant.

neutrophils in the blood collected from C6-HSL-pretreated mice was significantly higher compared to that of the control animals that were given only vehicle **(Fig. 4.8)**. Pretreatment with C4-HSL slightly increased neutrophil counts compared to those in controls, but the data were not statistically significant, whereas 3-oxo-C12-HSL prior treatment did not induce neutrophil recruitment in the blood of mice when challenged with *A. hydrophila*. Without the bacterial challenge, the number of neutrophils in blood from AHL- and vehicle- treated mice was similar **(Fig. 4.8)**, which suggested to us that neutrophil recruitment in the blood required both the AHL treatment and exposure to different antigens of *A. hydrophila*. The other cell types, such as monocytes, eosinophils, basophils in the blood were not affected by the AHL pretreatment and infection (data not shown).

Neutropenic mice are more susceptible to *A. hydrophila* infection. To further investigate the role of neutrophils in *A. hydrophila* infection, we depleted this cell population from animals by either cyclophosphamide or anti-Gr-1 monoclonal antibody. Both of these treatments specifically depleted neutrophil population from mice **(Fig.4.9-4.10)**.

To determine susceptibility to *A. hydrophila* infection, normal and neutropenic mice (generated by cyclophosphamide treatment) were challenged with three different doses of bacteria: 1×10^6, 5×10^6 and 1.5×10^7 CFUs. Interestingly, 60% of the neutropenic mice died at 48 h post infection and the remaining 40% survived 16 days of the observation period when challenged with 5×10^6 CFUs of *A. hydrophila.* All of the normal mice were healthy and survived the duration of the experiment at this dose. This survival rate between normal and neutropenic mice was statistically significant ($p=0.05$) **(Fig. 4.9)**. At the dose of 1.5×10^7 CFUs, all of the neutropenic mice died at 48 h post infection, while 80% of the normal mice survived **(Fig. 4.9)**, and these data were

Figure 4.8. Prior C4-HSL and C6-HSL treatment enhanced neutrophil recruitment in the blood

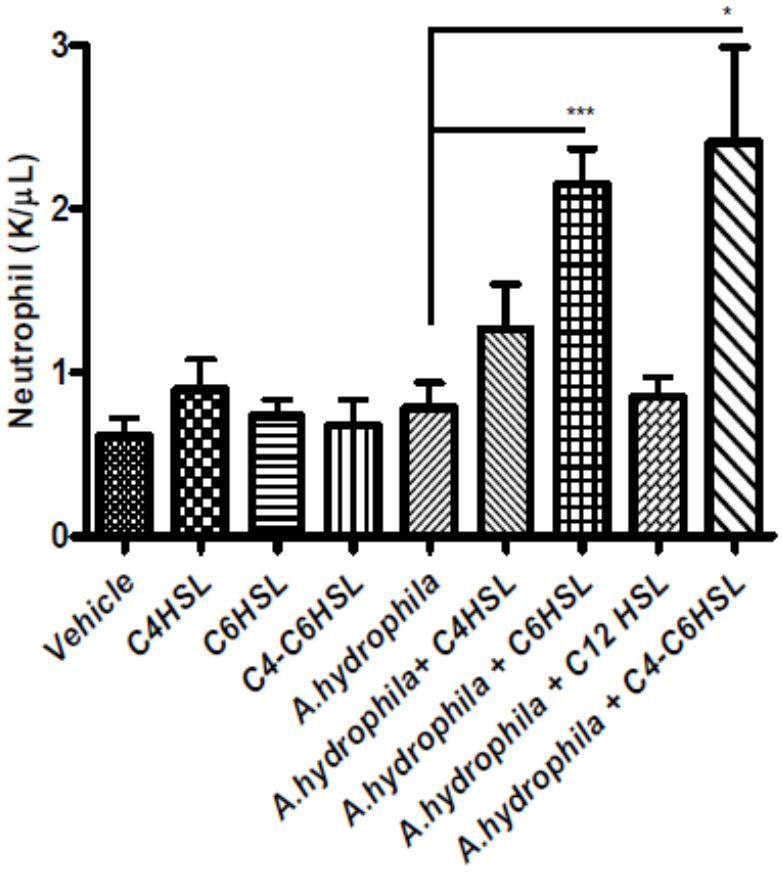

Fig.4.8. Prior C4-HSL and C6-HSL treatment enhanced neutrophil recruitment in the blood. Neutrophils were counted in the blood collected from mice (n=5, each treatment group) pretreated (i.p.) with C4-HSL, C-6 HSL, C4-HSL and C-6 HSL combined, or 3-oxo-C12-HSL and/or vehicle for 12 h and 6 h with and/or without *A. hydrophila* infection. AHL pretreatment without *A. hydrophila* infection did not increase neutrophils, while AHL pretreatment, specifically in C6-HSL-treated, and infected animals, significantly increased neutrophil numbers in the blood. Statistical analysis was performed between AHL- and vehicle- treated infected animals by using the student's *t* test and *p* values of ≤ 0.05 were considered significant. Increased neutrophil recruitment in the blood with C6-HSL*** (p=0.0008) and C4-HSL and C-6 HSL* in combination (p=0.0273) was statistically significant as compared to the vehicle treated and infected control mice.

statistically significant (p=0.014). However, none of the neutropenic and normal mice died when they were challenged with 1 x 10^6 CFUs of *A. hydrophila*. Since by using cyclophosphamide, we also reduced the number of other immune cells, such as monocytes (data not shown) in the blood, we specifically depleted the neutrophil population in the blood by using anti-Gr-1 monoclonal antibody RB6-8C5. When neutropenic mice generated by this antibody method were challenged with 1.5 x 10^7 CFUs of *A. hydrophila*, all of the neutropenic mice died within 24 h post infection, whereas all the normal mice survived (p=0.003) and remained healthy for the duration of the experiment at this dose **(Fig. 4.10)**.

AHL pretreatment induces bacterial phagocytosis by murine macrophages *in vitro*. Our data provided evidence that at earlier time points, AHL pretreatment stimulated neutrophil recruitment in the blood. Since bacterial dissemination to different tissues (lungs, liver, and spleen) also decreased in mice pretreated with AHLs, we hypothesized that at the later time course of *A. hydrophila* infection, AHL pretreatment might trigger other immune cells, such as monocytes/macrophages in different tissues to clear bacteria. To address this scenario, we performed an *in vitro* phagocytosis assay by using RAW 264.7 murine macrophages after treatment with C4-HSL, C6-HSL, C4-HSL and C6-HSL combined, or vehicle only for 1 h. Interestingly, we observed that treatment of either type of AHLs or in combination increased the phagocytosis of *A. hydrophila* by murine macrophages **(Fig. 4.11)**. For example, the number of phagocytosed bacteria was approximately double when treated with C4-HSL; this number was approximately 4 times higher in the case of treatment with C6-HSL; and the number was 6 times higher when macrophages were treated with C4-HSL and C6-HSL in combination, as compared with the findings in PBS- treated control macrophages **(Fig. 4.11)**.

Figure 4.9. Effect of inducing neutropenia by using cyclophosphamide on the survival rates of mice after challenge with *A. hydrophila*

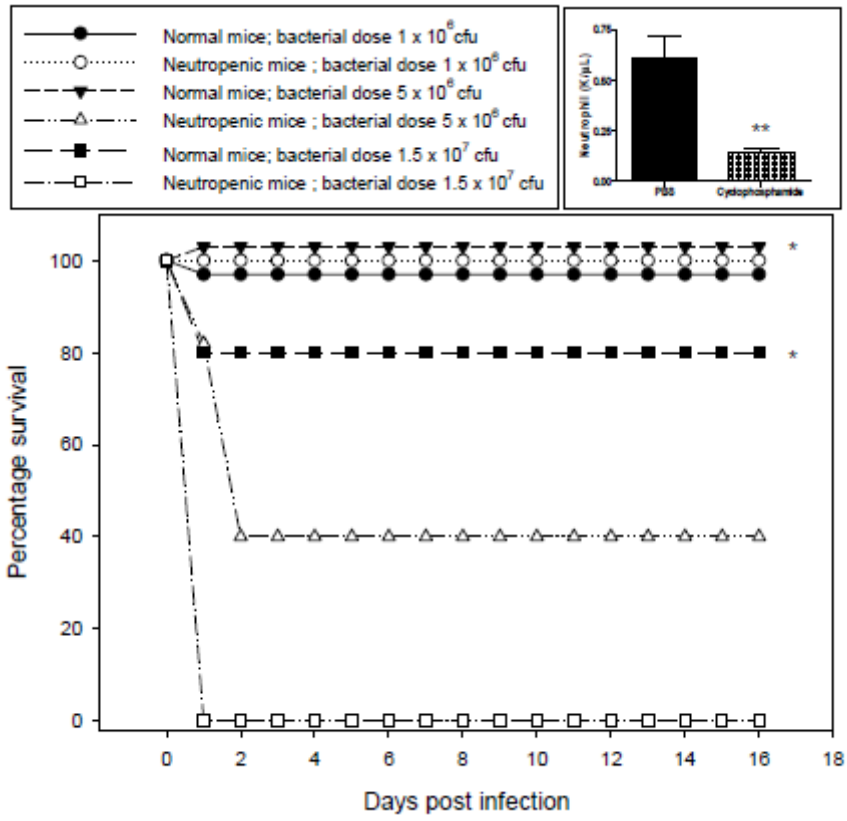

Fig.4.9. Neutrophils were depleted by the i.p. injection of 250 mg of cyclophosphamide/kg body weight and control mice were pretreated with PBS (n=5 each group). Neutrophil depletion was confirmed and was significant (**) by counting neutrophil numbers in the blood after treatment with cyclophosphamide. Normal and neutropenic mice were challenged with three different doses of *A. hydrophila*: 1 x 10^6, 5 x 10^6 and 1.5 x 10^7 CFUs (n=5 each group) and deaths were observed for 16 days post infection. Percentages of surviving mice over time are shown, and the data were analyzed by using the Kaplan-Meier's survival estimates. *denotes statistically significant differences in the percentage of neutropenic and normal mice surviving, following challenge with 5 x 10^6 (p=0.05) and 1.5 x 10^7(p=0.014) CFUs of *A. hydrophila.*

Figure 4.10. Effect of inducing neutropenia by using anti-Gr-1 monoclonal antibody (RB6-8C5) on the survival rates of mice after challenge with *A. hydrophila*

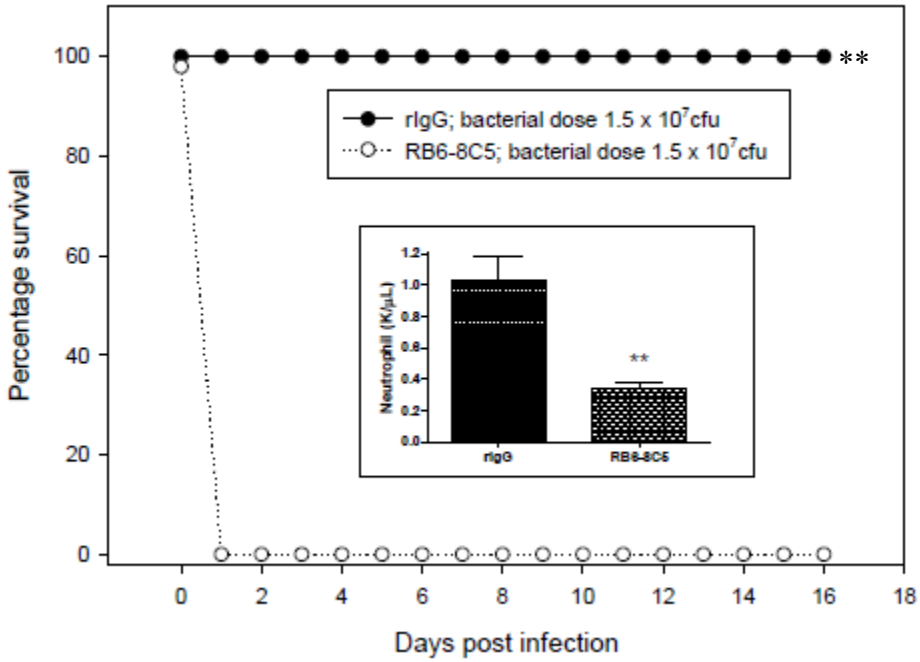

Fig. 4.10. Neutrophils were depleted by the i.p. injection of 250 µg of RB6-8C5, and control mice were given equivalent amounts of purified rat immunoglobulin G (rIgG) (n=5 each group). (Inset) Neutrophil depletion was confirmed and was significant (**) by counting neutrophil numbers in the blood obtained by retro-orbital bleeding of normal and neutropenic mice prior to challenge with *A. hydrophila* at a dose of 1.5 x 10^7 CFUs (n=5 each group) and deaths were observed for 16 days post infection. Percentages of surviving mice over time are shown, and the data were analyzed by using Kaplan-Meier's survival estimates. **denotes statistically significant difference in survival rate between neutropenic and normal mice (p=0.003).

Figure 4.11. AHL prior treatment of murine macrophages induced bacterial phagocytosis

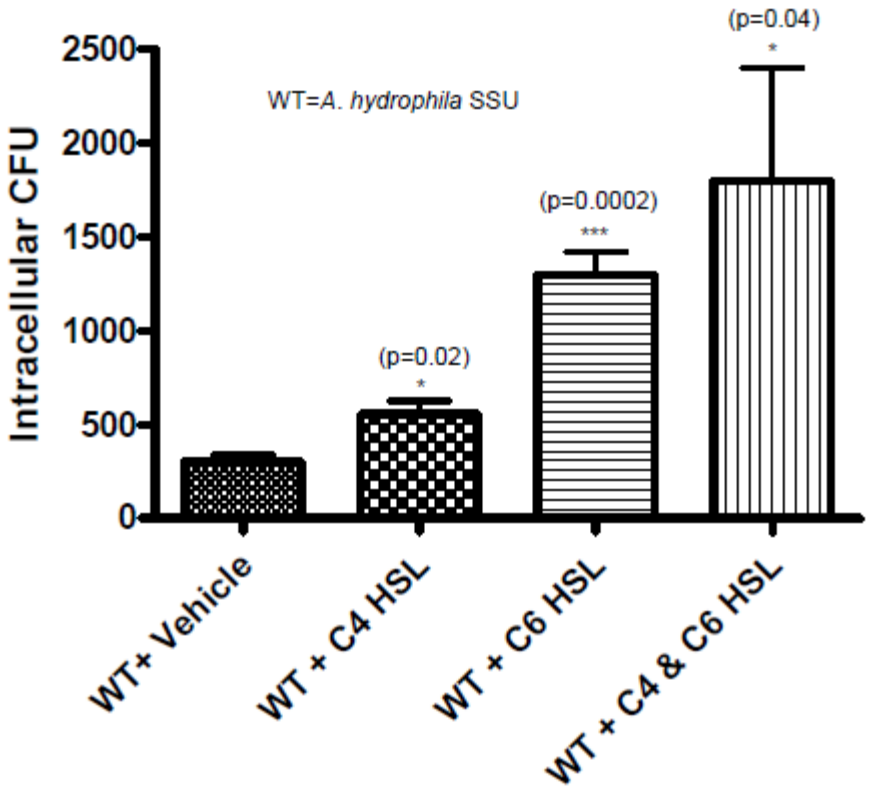

Fig. 4.11. RAW 264.7 murine macrophages were treated with 200 μM of C4-HSL, C6-HSL, and C4-HSL and C6-HSL together, or vehicle for 1 h. Macrophages were infected with *A. hydrophila* at a MOI of 10, and, after killing extracellular bacteria by the gentamicin treatment, host cells were lysed, and the intracellular bacteria counted on the LB agar plates. Data from at least three independent experiments (each treatment as triplicate) were averaged, and the standard deviations are shown. Asterisks indicate a statistically significant difference in phagocytosis between AHL-treated and vehicle-treated control murine macrophages.

DISCUSSION

Communication through the QS systems facilitates a bacterial population to coordinate activities in the environment as well as in the host. QS represents a global regulator in bacteria, because it controls many important biological activities, including motility, virulence factor production, and biofilm formation (46-48, 77, 86, 127). Recent studies also unveiled the fact that QS signaling molecules, such as AHLs, are not only the regulators in bacteria, but also modulate different host cells (200, 208, 209). However, the mechanisms of this modulation of host cells by AHLs are largely unknown.

It was recently shown that immunization of mice with 3-oxo-C12-HSL-bovine serum albumin conjugate induced antibody responses which protected animals from lethal *P. aeruginosa* lung infection (146). However, currently, it is not known whether AHL QS signaling molecules induce protective innate immune responses against bacterial infection in an animal model. Consequently, the present study examined the immunomodulatory role of different types of AHLs in a septicemic mouse model of *A. hydrophila* infection. The novel findings of our study are that pretreatment of mice with AHLs (C4-, C6- or 3-oxo-C12-HSL) prevents clinical sequelae during *A. hydrophila* infection, and, thus, providing protection to mice against mortality and that AHLs modulate innate immune response of animals to clear bacterial infection in a prophylactic manner.

Commercially available HSLs, such as C4-HSL and C6-HSL were purified from *Chromobacterium violacium,* and 3-oxo-C12 HSL was purified from *P. aeruginosa*. These HSL molecules and their receptor protein LuxR are well conserved in different Gram-negative bacteria. In our recent study, we showed that exogenous addition of both of the C4- and C6- HSLs to an autoinducer synthase (*ahyI*) mutant (*ahyI* is a homolog of *luxI*) of *A. hydrophila* SSU restored phenotypic properties (such as the biofilm formation,

metallo-protease production, and secretion of the type 6 secretion effectors) that are controlled by the AhyRI QS system, to the level of wild-type bacteria (109). These data clearly suggested that C4- and C6- HSLs produced by *A. hydrophila* SSU are similar functionally to those produced by other bacteria.

We used 250 μM amounts of HSLs in our animal studies as *P. aeruginosa* can produce as high as 600 μM of 3-oxo-C12 HSLs in biofilms (25). Although *in vivo* concentration of HSLs are noted to be in the nanomolar to low micromolar range, it has been reported that physiological pH and temperature conditions can reduce detectable levels of HSLs, and, thus, the actual concentration of HSLs in the tissues appears much lower (258). Importantly, however, we also demonstrated that lower amounts of HSLs (25 μM each of C4- and C6- in combination) provided a similar level of protection in mice as the 250 μM dose.

In our study, treatment of RAW cells with as high as 400 μM of C4-HSL and C6HSL in combination for 2-4 hrs did not induce any cell cytotoxicity. Therefore, the use of HSLs at a concentration of 250 μM *in vivo* and 200 μM *in vitro* in the present study was relevant when used as an option for treatment. Further, these doses were comparable to other earlier reports in which similar concentrations of HSLs were employed (89, 200).

A. hydrophila induced thrombocytopenia in mice (Fig. 4.3), which is a major feature of HUS caused by *E. coli*; however, the mechanism by which the platelets are depleted by this pathogen is not known. A recent study showed that the down regulation of platelet surface CD47 expression was associated with shiga toxin-producing *E. coli*, which could possibly represent the mechanism of HUS associated with such *E. coli* strains (73). Strikingly, our data showed that AHL pretreatment of mice normalized systemic effects, including platelet numbers, and improved animal survival rates following *A. hydrophila* infection.

A. hydrophila produces an array of virulence factors, and the ability of this pathogen to disseminate to any organ *via* blood contributes to its pathogenesis (33). Therefore, induction of an innate immune response and the clearance of bacteria from the blood and other organs would be crucial as the first line of host defense against *Aeromonas* infection. Our data provided evidence that the AHL pretreatment of mice increased bacterial clearance from the blood, as well as other tissues (Fig. 4.4), which correlated with enhanced mouse survivability (Fig. 4.1 and 4.2).

In general, the level of various cytokines/chemokines was very high in the lungs, liver, and spleen when mice were challenged with *A. hydrophila* without AHL pretreatment, suggesting that animals would die because of host tissue damage as a result of a proinflammatory cytokine storm (Fig. 4.5-4.7). Interestingly, the level of these cytokines/chemokines was decreased in tissues of mice pretreated with AHL, resulting in reduced inflammatory response and improved survivability of mice. Previous studies also reported that AHLs, specifically 3-oxo-C12-HSL treatment, reduced proinflammatory cytokine production in various host cells *in vitro* (229), as well as inhibited LPS-induced proinflammatory cytokine production in a mouse model by disrupting NF-κB signaling (122).

On the contrary, Smith *et al.,* showed that dermal injection of 3-oxo-C12-HSL in mice induced the production of various proinflammatory cytokines (209). These discrepancies in results could be attributed to differences in concentrations of AHLs that were used, differences in host cell types and mouse strains employed, and/or different routes by which AHL treatment was administered to animals. We noted that only AHL treatment in mice without *A. hydrophila* infection did not influence cytokine production, suggesting that other bacterial components might be necessary in modulating this inhibitory effect of AHLs. Earlier studies reported that small *acyl* chains, such as C4-

HSL, did not show any immunomodulatory activity *in vitro* (228, 266); however, in contrast, our *in vivo* data indicated that all three AHLs, namely C4-HSL, C6-HSL, and 3-oxo-C12-HSL, had immunodulatory activity (Figs.4.1-4.7).

Since the intensity of an inflammatory response can be directly related to the number of organisms present in various tissues of an infected animal, it is possible that the reduction in cytokine/chemokine levels we noted in different organs of infected mice after AHL pretreatment could be due to the reduction of bacterial load. However the direct role of these HSLs on cytokine/chemokine production in infected animals cannot be ruled out.

Neutrophils are the first line of host defense cells which are recruited from the blood to infection sites (17, 74). Several studies pointed out the crucial role that neutrophils play in the innate immune response in clearing invading pathogens (130, 235); however, the role of neutrophils in *Aeromonas* infection has not been studied. Intrigued by our findings of rapid clearance of *A. hydrophila* from the blood and other mouse tissues, we were prompted to examine the recruitment of various host defense cells in the blood after intraperitoneal treatment of mice with different types of AHLs. Importantly, C6-HSL pretreatment significantly increased neutrophil numbers at 4 h post infection with *A. hydrophila,* while C4-HSL pretreatment marginally increased neutrophil numbers in the blood (Fig. 4.8). However, 3-oxo-C12-HSL pretreatment did not induce neutrophil recruitment in the blood at all. These data indicate that different AHLs might operate *via* different mechanisms.

These results are in contrast to studies in which 3-oxo-C12-HSL was shown to induce directed migration (chemotaxis) of human polymorphonuclear neutrophils (PMNs) *in vitro* (266), and it also upregulated the expression of receptors known to be involved in host defense, such as adhesion proteins CD11b/CD18 and the

immunoglobulin receptors CD16 and CD64 (242). These data further reflect that immunomodulation by different AHLs may vary depending on *in vitro* and *in vivo* conditions. In addition to neutrophil recruitment in the blood, we speculate that AHL treatment might also stimulate various antimicrobial functions of neutrophils to clear bacteria from animals.

In our future studies, we plan to investigate the influence of AHL treatment on bacterial killing mechanisms of neutrophils, such as production of antimicrobial enzymes (e.g., myeloperoxidase and elastase), antimicrobial peptides (e.g., defensins and cathelicidins), as well as neutrophil extracellular trap (NET) formation. In addition, it can be speculated from our data that different AHLs might activate different immune cells to clear bacteria from the host. Therefore, it would be interesting to investigate in the future whether C4 HSL, C6 HSL, or 3-oxo-C12-HSL also activate other immune cells, such as natural killer (NK) cells and/or dendritic cells (DCs) *in vitro* as well as *in vivo*.

The *acyl* side-chain length as well as the substitutions on the side chain of HSLs provide signal specificity and several studies showed that immunomodulation by different HSLs varies depending on the length of the side chain (228, 266). Likewise, in our study, we observed that the degree of neutrophil recruitment in the blood varied when treated with different lactones (Fig. 4.8) and the survivability of mice also varied with different HSL pretreatment (Fig. 4.1). Therefore, it is plausible that different lactones are recognized by different pattern recognition of the host cells.

Our results showed that the number of neutrophils was increased in the blood of infected mice that were treated with C4- and C6- HSLs but not with 3-oxo-C12 HSL, although a reduction in the number of bacteria was seen with all of the AHLs (Fig. 4.4). We believe that the protective effect of HSLs seen in mice is a result of combination of several factors that culminated in reduction of the number of bacteria and cytokine and

chemokines levels in different tissues (Figs. 4.5-4.7). Since by using the neutropenic mouse model, we observed that neutrophils played an important role in the host defense against *A. hydrophila* infection, recruitment of these cells to the blood by C4 and C6-HSLs could be one of the several mechanisms as to how mice were clearing bacteria from the blood at the earlier course of infection.

In the phagocytosis assay (Fig. 4.11), the uptake rate of bacteria by macrophages was 2 times higher in the case of C6-HSL pretreatment compared to that of C4-HSL, which matched with our *in vivo* neutrophil data whereby C6-HSL pretreatment recruited more neutrophils in the blood than did C4-HSL treatment (Fig. 4.8). Interestingly, we also observed that C6-HSL pretreatment provided better protection than C4-HSL in terms of mice survivability (Fig. 4.1). From these data, we concluded that C-6 HSL was a more effective immunomodulator than C4-HSL in terms of inducing a protective immune response against *A. hydrophila* infection. We were not able to perform a phagocytosis assay after treatment of macrophages with 3-oxo-C12-HSL, as it induced cell lysis, which was in accordance with findings from a previous study by Tateda *et al*. (228) in which they showed that 3-oxo-C12-HSL treatment accelerated apoptosis of macrophages and neutrophils isolated from mice.

On the contrary, other investigators reported that 3-oxo-C12-HSL treatment stimulated the phagocytic activity of human monocyte-derived macrophages (237) and human PMNs (242). Our *in vitro* phagocytosis results by using murine macrophages warrant further investigation through the evaluation of phagocytosis of bacteria by monocytes/macrophages in mice during infection (168) following pretreatment of animals with AHLs.

In conclusion, our study reveals that QS signaling molecules, such as AHLs, can stimulate protective innate immune responses in mice infected with *A. hydrophila*, which

underscores the necessity of examining the immunotherapeutic potency of AHL molecules against other bacterial pathogens.

From an evolutionary standpoint, it is intriguing why bacteria would produce molecules that can be used in QS but also result in their own killing? When bacteria infect host, microbes only express their virulence machineries when they sense favorable conditions in terms of bacterial population governed by a threshold level of lactones. By doing so, pathogens overcome host defenses to cause disease. On the other hand, pretreatment of host (in our case mice) with different HSLs helps them to prepare against bacterial infections by activating different host defense/immune cells (e.g., neutrophils, macrophages) to efficiently clear bacteria from different tissues. This dual role of HSLs is not surprising as many antibiotics produced by microbes under natural environment function as cell signaling molecules, including QS (259), in addition to their therapeutic use.

CHAPTER 5

Two-Component QseBC System Involved in AI-3 Quorum-Sensing Regulates *In Vitro* and *In Vivo* Virulence of *Aeromonas hydrophila*[4]

INTRODUCTION

In Gram-negative bacteria, three QS circuits have been identified, of which AI-3/QseBC is least studied. Recently, we characterized the role of N-*acyl*-homoserine lactone-mediated (AI-1) and LuxS-based (AI-2) QS systems in the regulation of virulence factors in *A. hydrophila* SSU (109, 121). However, the role of a newly identified two-component based QseBC/AI-3 QS system in the regulation of bacterial virulence is not well understood. Consequently, only a limited number of studies have shown that this QS system also contributes to bacterial pathogenesis.

In this study, we identified and characterized the role of QseBC (AI-3) QS system in the regulation of virulence in *A. hydrophila* SSU by generating a Δ*qseB* mutant. We demonstrated that QseBC system in *A. hydrophila* positively regulated both swimming and swarming motility, hemolytic activity of Act, and protease production, while negatively modulating the biofilm formation. Since QseBC functions as both positive and negative regulator in controlling virulence of *A. hydrophila*, we indeed observed a marginal attenuation of virulence in an *in vivo* septicemic mice model when *qseB* gene was deleted. Our study is the first to report a functional QseBC QS system in *A. hydrophila* which may be networked to AI-1 and AI-2 QS systems in modulating bacterial virulence. This study is important in better understanding of the functional role

[4] Khajanchi B. K., E. V. Kozlova, J. Sha, V. L. Popov and A. K. Chopra. Two-component QseBC system involved in AI-3 quorum-sensing regulates *in vitro* and *in vivo* virulence of *Aeromonas hydrophila*. Microbiology, June 2011 (submitted).

of QseBC QS system in the regulation of bacterial virulence in general and of *A. hydrophila* in particular.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals. The bacterial strains and plasmids used in this study are listed in **Table 5.1**. The LB medium was supplemented with L-arabinose (0.2%) when the *ggdef* gene was expressed from the pBAD/Myc-HisB::*ggdef* plasmid **(Table 5.1)** under the control of an arabinose-inducible pBAD promoter (120).

Generation of an isogenic *qseB* mutant of *A. hydrophila* SSU. To generate the *qseB* knockout mutant of *A. hydrophila*, this gene (690 bp) was amplified by polymerase chain reaction (PCR) employing genomic DNA (gDNA) of *A. hydrophila* SSU-R **(Table 5.1)** as the template and a pair of primers (qseB-N: 5'-ATGCGGATCCTGTTGGTGGAAGA-3' and qseB-C: 5'CATGCCCGGTGGTCCCGGCGCTG-3'). The PCR product was then cloned in the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Subsequently, the pCR 2.1-*qseB* recombinant plasmid (r-plasmid) was transformed into *E. coli* DH5α **(Table 5.1)**. Within the *qseB* coding region, a unique *blp*I restriction site exists; the pCR 2.1-*qseB* r-plasmid was thus linearized by *blp*I digestion. A kanamycin (Km^r) gene cassette flanked by the *blp*I restriction site from the plasmid pUC4K (GE Healthcare, Uppsala, Sweden) was inserted at the *blp*I site of pCR 2.1-*qseB* r-plasmid to generate a pCR 2.1-*qseB*-Km plasmid **(Table 5.1)**. After digestion with the *Kpn*I/*Xba*I restriction enzymes, the DNA fragment from the pCR 2.1-*qseB*-Km r-plasmid was ligated to a pDMS197 suicide vector at the *Kpn*I/*Xba*I sites, and the resulting plasmid (pDMS197-*qseB*-Km) was transformed into an *E. coli* SM10 λ*pir* strain (54) **(Table 5.1)**. The recombinant *E. coli* [pDMS197-*qseB*-Km] was then conjugated with the WT *A. hydrophila* SSU-R. The transconjugants

Table 5.1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristic (s)	Source or reference
A. hydrophila SSU		CDC[a]
ATCC 7966	Environmental isolated strain	ATCC[b]
SSU-R	Rif^r strain of *A. hydrophila* SSU	Laboratory stock
Δ*qseB*	*qseB* gene deletion mutant of *A. hydrophila* SSU-R strain Rif^r Km^r	This study
Δ*ahyRI*	*ahyRI* gene deletion mutant of *A. hydrophila* SSU-R strain Rif^r Sm^r Sp^r	(109)
Δ*luxS*	*luxS* mutant of *A. hydrophila* SSU-R strain Rif^r Km^r	(121)
Δ*qseB/* pBR322 -*qseBC*	*qseB* mutant complemented with *qseBC* genes (ATCC) via pBR322 Rif^r Km^r Ap^r	This study
E. coli		
DH5α	Production of recombinant plasmids. *recA, gyrA*	Life Technologies
SM10	Km^r, *λpir*	(54)
Plasmids		
pCR2.1	TA cloning vector Ap^r Km^r	Invitrogen
pCR2.1/*qseB*	TA cloning vector carrying *qseB* gene Ap^r Km^r	This study
pUC-4K	Contains a 1.2-kb kanamycin^r gene cassette	Amersham
pCR2.1/*qseB*-Km	TA cloning vector harboring *qseB* gene disrupted by Km cassette- Ap^r Km^r	This study
pDMS197	A suicide vector; R6K *ori, sacB,* Tc^r	(54)
pDMS197/*qseB*-Km	Suicide vector containing *qseB* gene with Km cassette, Tc^r Km^r	This study
pBR322	Ap^r Tc^r	Amersham
pBR322-*qseBC*	Contains *qseBC* genes (ATCC); Ap^r	This study
pBAD/*Myc*-HisB	vector, *ara* BAD promoter Ap^r	Invitrogen
pBAD:: *ggdef*	GGDEF domain encoded gene of *A. hydrophila* cloned into pBAD/*Myc*-HisB *ara* Km^r Ap^r	Laboratory stock

Abbreviations: Rif, rifampin; Km, kanamycin, Sm, streptomycin, Sp, spectinomycin, Ap, ampicillin, Tc, tetracycline

[a] Center for Disease Control and Prevention

[b] American Type Culture Collection

were selected based on resistance to appropriate antibiotics and sucrose and subjected to further analysis (195). The identity of the *qseB* mutant was confirmed by Southern blot analysis as described in the previous chapter. PCR products of *qseB* gene, Km^r gene cassette, and the digested pDMS197 vector using the *Xba*I-*Kpn*I enzymes were used as probes.

Complementation of the *A. hydrophila* SSU Δ*qseB* mutant. The *qseBC* genes were PCR amplified by using gDNA of *A. hydrophila* ATCC 7966 as the template and two primers (QseBCN-*Hin*dIII: 5'-GGGAAGCTTGCATCGACCCCAACTTCTTCT-3'

and QseBCC-*Nhe*I:5'-GGGGCTAGCTGGAGCACATGGTGACGGT-3', the indicated restriction endonuclease sites are underlined). Since *qseBC* genes are highly homologous between *A. hydrophila* SSU and ATCC strains, we used *qseBC* from ATCC strain for complementation as we annotated the genome of this strain and the upstream sequence containing the promoter regions for these genes were available (193). We included 249 bp flanking upstream DNA sequence containing the potential promoter regions of the *qseBC* genes for complementation studies. This DNA fragment (2648 bp) was cloned in pBR322 vector (Tc^r Ap^r) at the *Hin*dIII-*Nhe*I sites and transformed into the *E. coli* DH5α strain **(Table 5.1)**. The pBR322/*qseBC* (Tc^s Ap^r) recombinant plasmid was isolated from the *E. coli* strain and electroporated into an *A. hydrophila* SSU Δ*qseB* mutant (195) **(Table 5.1)**.

Motility, hemolytic activity, protease production, biofilm formation, and Western blot analysis: Motility, hemolytic activity, protease production, biofilm formation and Western blot analysis which were performed to characterize WT and its *qseB* mutant of *A. hydrophila* SSU have been described in previous chapters.

Animal Experiments. Groups of 10 Swiss Webster mice (Taconic Farms, CA) were infected by the intraperitoneal route (i.p.) with 5 x 10^7 cfu (WT or its Δ*qseB* mutant)

in accordance with the approved animal care protocol. One group of mice was inoculated with DPBS (*n*=10) and served as a control. Deaths were recorded for 16 days post infection. The animal experiments were repeated three times.

Statistical analysis. All of the experiments were performed in triplicate, and wherever appropriate, the data were analyzed by using the Student's t test, with a p value of ≤ 0.05 considered significant. The data were presented as an arithmetic mean ± standard deviation. The animal data were analyzed by using the Kaplan-Meier's survival estimates.

Nucleotide Sequence Accession Number. The sequences of the *A. hydrophila* SSU *qseBC* genes were deposited in the GenBank database under accession number JF922013.

RESULTS

Characterization of the two-component QseBC QS system from *A. hydrophila* SSU. By our sequence annotation of *A. hydrophila* ATCC 7966 strain (193), we identified *qseB* and *qseC* genes in a clinical isolate SSU of *A. hydrophila*, which exhibited 99% and 96% homology at the amino acid level with the corresponding genes of *A. hydrophila* ATCC 7966 strain. A 4-bp overlap with the ATGA motif was found in the open reading frames (ORFs) for *qseB* and q*seC* genes in which translation stop codon of QseB overlapped with the translation start codon for QseC. A similar genomic organization for *qseBC* genes was identified in EHEC (37), however, QseB exhibited 51 % and QseC showed 31 % identity with the corresponding proteins of *E. coli*. Further, by analyzing the protein sequences of QseB and QseC at the NCBI conserved domains database, a Pfam protein sequence search (http://pfam.sanger.ac.uk/) and SMART analysis (http://smart.embl-heidelberg.de/), it was revealed that QseB possesses two domains: a receiver domain (REC) and a transcriptional regulatory protein/C-terminal

helix-turn-helix domain (HTH). QseC, on the other hand, harbors three domains: a HAMP /transmembrane domain, a His-kinase (HisKA), and a ATPase domain.

To characterize the role of QseBC system in the regulation of virulence of *A. hydrophila* SSU, we deleted the *qseB* gene by double crossover homologous recombination and subsequently generated a complemented strain with both of the *qseBC* genes that were supplied *in trans*.

Deletion of the *qseB* gene from *A. hydrophila* SSU diminishes both swimming and swarming motility. Motility is considered to be an important virulence factor in the pathogenesis of *Aeromonas*- associated infections as it facilitates pathogen to adhere and invade the host cells (114, 116, 117). *Aeromonas* species possess polar flagella for swimming motility and lateral flagella for swarming motility (114, 116, 117). To cause infection by avoiding the host defense, the motility of the pathogens needs to be tightly regulated. In this study, we examined whether two component QseBC system involved in QS regulates motility in *A. hydrophila*. Motility assay revealed that the Δ*qseB* mutant had significantly reduced migration both in the swimming and the swarming agar plates as compared to that of the WT *A. hydrophila* SSU **(Fig. 5.1 & 5.2)**, which indicated that both the swimming and swarming motility in *A. hydrophila* SSU were regulated by the QseBC QS system. In addition, swimming motility was restored in the *qseBC* complemented strain **(Fig.5.1)**. Similar to QS, cyclic diguanosine monophosphate (c-di-GMP), the bacterial intracellular second messenger, palys crucial role to control bacterial virulence. In addition, GGDEF domain proteins synthesize c-di-GMP while EAL domain proteins degrade c-di-GMP. Consequently, we examined the influence of c-di-GMP on the functions of QseBC QS system in controlling virulence mechanisms of *A. hydrophila*. We noted that the Δ*qseB* mutant with overproduced GGDEF protein (AHA0701h) (120) resulted in loss of the swimming motility **(Fig.5.3)**.

Figure 5.1. Swimming motility of WT *A. hydrophila* SSU, Δ*qseB* mutant, and the complemented strain

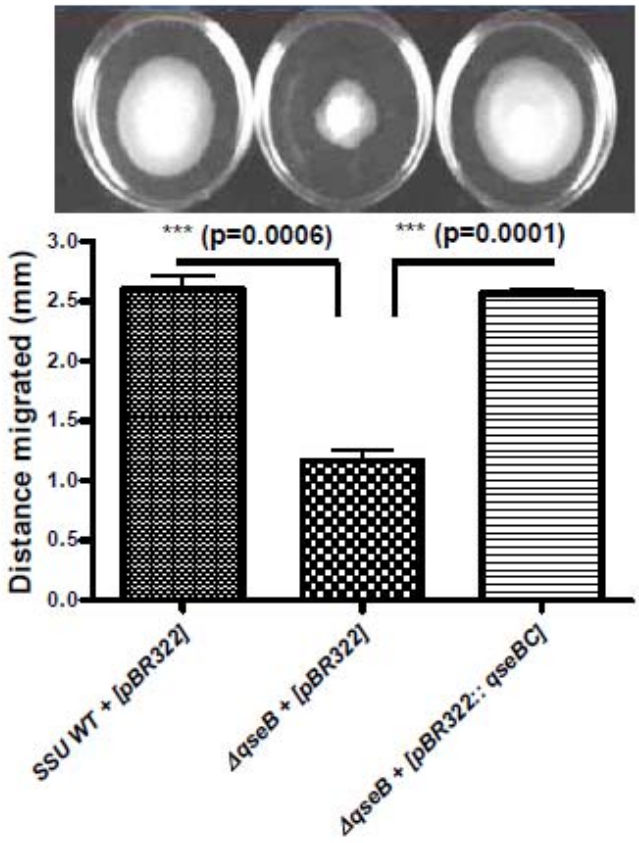

Fig. 5.1. Swimming motility of WT *A. hydrophila* SSU, Δ*qseB* mutant and the complemented strain. LB medium with 0.3% Difco Bacto-agar [supplemented with ampicillin (450 µg/ml) for complemented strain] was used to characterize the swimming motility. The Δ*qseB* mutant showed significantly decreased migration as compared to the WT of *A. hydrophila* SSU (p=0.0006). The *qseBC* complemented strain migrated similar to the WT strain. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted.

Figure 5.2. Swarming motility of WT *A. hydrophila* SSU and its Δ*qseB* mutant

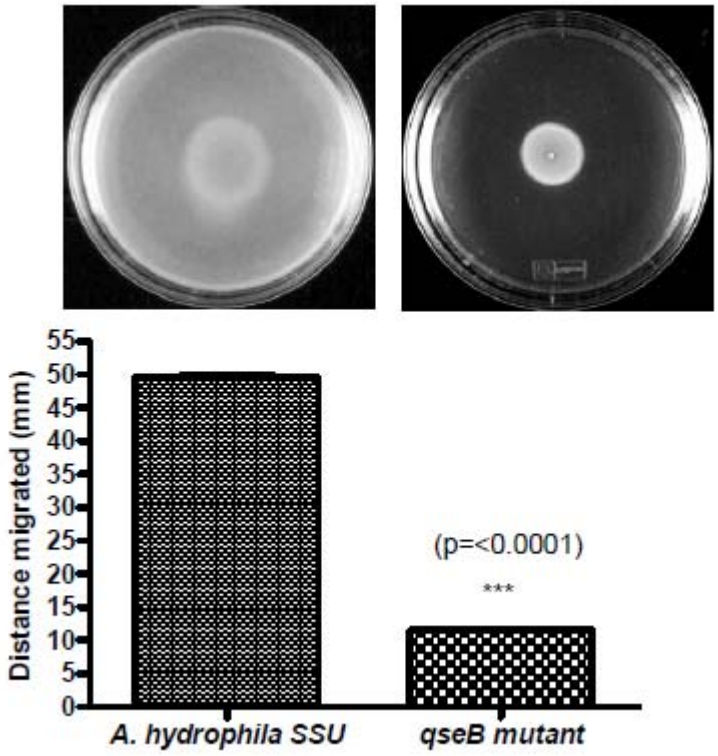

Fig. 5.2. Swarmming motility of WT *A. hydrophila* SSU and its Δ*qseB* mutant. Difco nutrient broth with 0.5% Eiken agar was used to determine swarming motility. Strong swarming response was observed for *A. hydrophila* SSU WT strain while reduced swarming motility was noted for the Δ*qseB* mutant. Asterisks (***, p<0.0001) indicate significant difference of migration between *A. hydrophila* SSU WT and its Δ*qseB* mutant. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted.

Figure 5.3. Influence of *ggdef* overexpression in the Δ*qseB* mutant on swimming motility

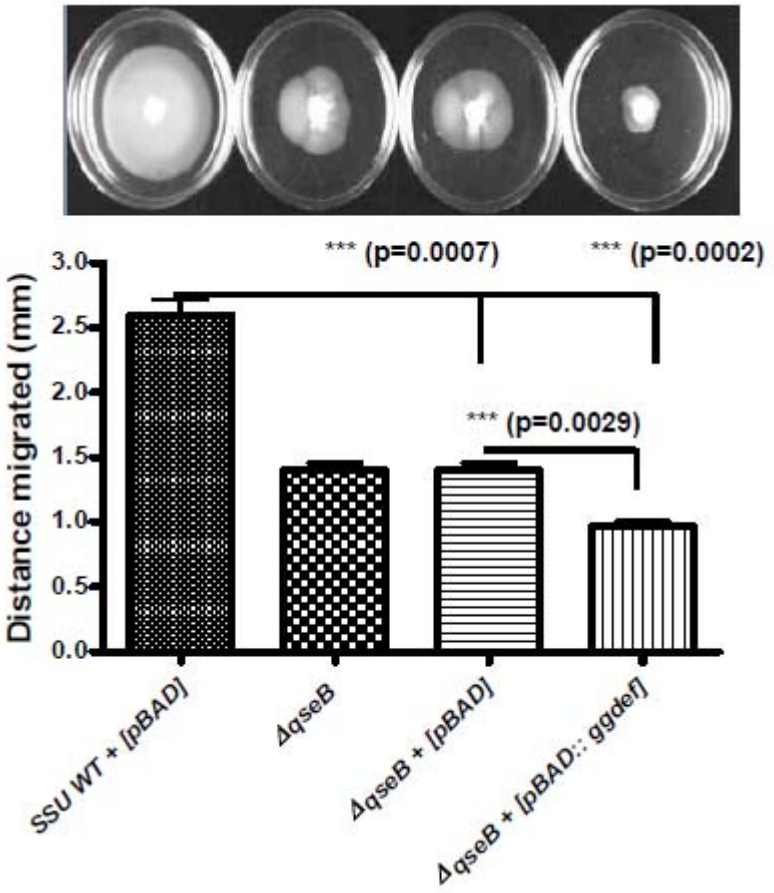

Fig. 5.3. Swimming motility of WT *A. hydrophila* SSU, Δ*qseB* mutant and the Δ*qseB* mutant overexpressing *ggdef*. The Δ*qseB* mutant with *ggdef*-overexpression did not show any migration but grew at the inoculation site. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. Asterisks (***) indicate significant statistical differences between WT and *qseB* mutant and *qseB* mutant and *qseB* mutant with overexpressed *ggdef* gene.

Hemolytic activity of T2SS-associated Act is significantly reduced in the Δ*qseB* mutant. We earlier showed that Act is one of the most potent virulence factors

that contributed to the pathogenesis of *A. hydrophila* SSU (33, 66, 195). To examine whether QseBC-system regulates Act production, we measured hemolytic activity associated with Act in the culture supernatant of WT and the Δ*qseB* mutant strain of *A. hydrophila* SSU. Interestingly, we found that the hemoglobin release from rabbit RBCs was significantly reduced when culture filtrate from the Δ*qseB* mutant was used compared to culture filtrate from the WT strain **(Fig. 5.4)**. To demonstrate that majority of this hemolytic activity was associated with Act, we neutralized the toxin by using specific antibodies. Indeed we noted much reduced and similar residual hemolytic activity in both the culture supernatants of WT versus the Δ*qseB* mutant. This residual activity is contributed by another hemolysin we characterized in isolate SSU of *A. hydrophila* (59). These data indicated that the expression of other hemolysin gene was not altered by deletion of the *qseB* gene and that the QseBC-system specifically controlled the hemolytic activity of Act in *A. hydrophila* SSU.

The Δ*qseB* mutant produces lower amounts of protease. Earlier studies have shown that the pathogenic and virulence characteristics of *A. hydrophila* were associated in part with the production of proteases (132, 186). Consequently, we measured protease production and found that Δ*qseB* mutant produced a significantly reduced level of protease as compared to the WT *A. hydrophila* SSU **(Fig. 5.5),** suggesting that QseBC-system also controlled protease production of *A. hydrophila*.

Crystal violet (CV) staining biofilm assay demonstrates more efficient attachment of the Δ*qseB* mutant to polystyrene tubes. To measure the solid surface-associated biofilm formation, we performed a CV staining assay after 24 h growth of WT and its Δ*qseB* mutant in the LB medium. The Δ*qseB* mutant formed a significantly

Figure 5.4. Measurement of hemolytic activity associated with the cytotoxic enterotoxin (Act) of WT *A. hydrophila* SSU and its Δ*qseB* mutant

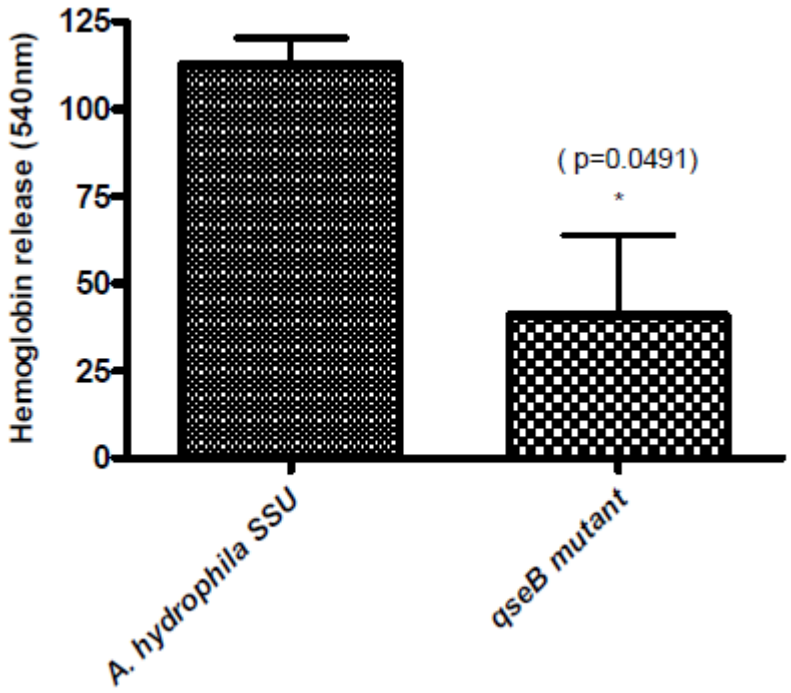

Fig. 5.4. Measurement of hemolytic activity associated with Act of WT *A. hydrophila* SSU and its Δ*qseB* mutant strain by using rabbit RBCs. Hemoglobin release was quantified by measuring absorbance at 540_{nm}, and the hemolytic titers were calculated as the absorbance value of the hemoglobin release multiplied by the dilution of the culture filtrate. The data were normalized to 1 x 10^8 cfu to account for any differences in the growth rates between WT and mutant strains. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. Asterisk (*) indicates statistically significant difference in hemoglobin release between the Δ*qseB* mutant and the WT bacteria by the student's t test ($p \leq 0.05$).

increased solid-surface-associated biofilm in polystyrene tubes**,** with approximately 3-fold increase in the CV staining when compared to that of the WT *A. hydrophila* SSU strain **(Fig.5.6)**. We used Δ*ahyRI* (biofilm deficient) *and* Δ*luxS* mutant (which enhances

biofilm) as controls to compare biofilm formation by different QS mutant strains. These data suggested to us that similar to the Δ*luxS* mutant, QseBC QS system also negatively controlled biofilm formation in *A*. *hydrophila* SSU.

A three-dimensional structured and/or denser biofilm is observed in the Δ*qseB* mutant when examined by SEM. To investigate in detail the surface architecture of bacterial cells aggregated in biofilms formed by the WT and its Δ*qseB* mutant, we performed SEM. We used ruthenium red, which binds strongly to negatively charged polysaccharides, and represents an excellent method for visualization of surface properties of bacteria. We observed very thick intercellular filament bundles of aggregated cells that formed a dense, three-dimensional structure in the Δ*qseB* mutant biofilms **(Fig. 5.7B)** when we compared them to the flattened biofilms formed by the WT bacterium which was less aggregated and connected with fewer filaments **(Fig. 5.7A)**. The SEM observations further confirmed CV staining results that Δ*qseB* mutant produced denser biofilms as compared to the WT strain, indicating that QseBC QS system plays role as a negative regulator of biofilm formation in *A*. *hydrophila* SSU.

Production of AexU, a T3SS effector, and Hcp, a T6SS effector, is not affected in the Δ*qseB* mutant of *A. hydrophila* SSU. Recent studies from our laboratory demonstrated that both T3- and T6- secretion systems, which contribute to host cell cytotoxicity, play crucial role in the pathogenesis of *A*. *hydrophila* SSU (202, 203, 218-220). Moreover, during the infection process, the expression and production of T3SS and T6SS effectors in bacteria are highly regulated (15, 70, 77, 135, 189, 261, 263), and very little is known as to how these T3- and T6- secretion systems are regulated in *A*. *hydrophila*.

To demonstrate any regulation of the QseBC QS system on the T3SS, we

Figure 5.5. Measurement of protease activity of WT *A. hydrophila* SSU and its Δ*qseB* mutant

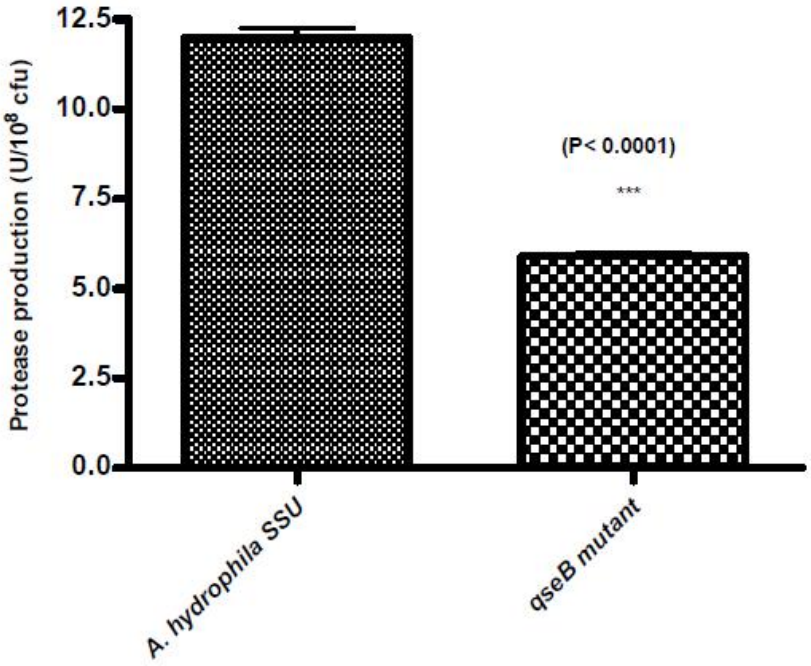

Fig. 5.5. Protease activity in the culture supernatants of WT *A. hydrophila* SSU and its Δ*qseB* mutant strain. The data were normalized to 1 x 10^8 cfu to account for any differences in the growth rates between WT and mutant strains. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. Asterisks (***) indicate statistically significant differences in protease activity between the Δ*qseB* mutant and the WT bacteria by the student's t test ($p<0.0001$).

examined expression and production of AexU in bacterial cell pellets of WT *A. hydrophila* SSU and its Δ*qseB* mutant grown in the LB medium, and, we also infected HeLa cells with WT *A. hydrophila* and its Δ*qseB* mutant in DMEM and monitored the

production of AexU in bacterial cells by Western blot analysis. We found that the Δ*qseB* mutant had similar levels of AexU compared to that of WT bacteria when grown in the LB medium and also during co-culturing with the HeLa cells (data not shown). These data indicated that QseBC QS system had no effect on the production of the T3SS effector, AexU.

The T6SS is newly identified in bacteria and we recently demonstrated that secreted Hcp could bind to the murine RAW 264.7 macrophages from outside and that it could also translocate into host cells, resulting in their apoptosis (220). In this study, to examine the role of QseBC QS system in the regulation of the T6SS, we performed Western blot analysis to determine the production and secretion of Hcp in the WT and Δ*qseB* mutant of *A. hydrophila* SSU.

We found that both the WT and Δ*qseB* mutant produced and secreted Hcp at similar levels, which suggested to us that QseBC QS system had no regulation on the production and secretion of the T6SS effector, Hcp (data not shown).

The Δ*qseB* mutant shows marginally decrease virulence in an animal model. By using *in vitro* experiments, we demonstrated that QseBC QS system positively regulated swimming and swarming motility, protease and Act production, and negatively modulated biofilm formation. To further examine whether these changes in virulence factors regulated by the QseBC QS system had any influence on *in vivo* virulence of *A. hydrophila* SSU, we injected mice intraperitoneally with the Δ*qseB* mutant and the WT strain of *A. hydrophila* at a lethal dose of 5 x 10^7 cfu **(Fig. 5.8).** We noted that 100% of the animals infected with the WT *A. hydrophila* SSU died within 3 days. However, mice infected with the Δ*qseB* mutant strain exhibited 30% lower mortality over a tested period of 16 days, which suggested to us that a marginal bacterial attenuation occurred when we

Figure 5.6. Measurement of biofilm mass by crystal violet (CV) staining formed on polystyrene plastic by the WT *A. hydrophila* SSU and its Δ*qseB* mutant

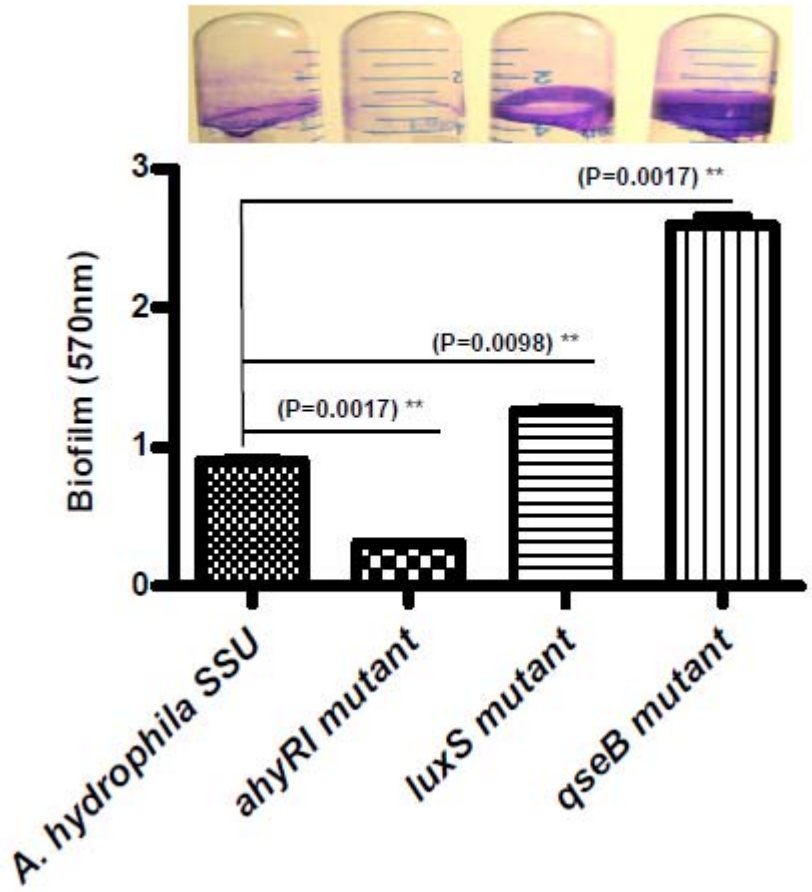

Fig. 5.6. Measurement of biofilm mass by CV staining formed on polystyrene plastic by the WT *A. hydrophila* SSU and its Δ*qseB* mutant. Biofilms were quantified after 24 h of incubation at 37^{o}C. Adherent bacteria were stained with 1% crystal violet and washed with distilled water; the extracted color (with 95% ethanol) was measured at $OD540_{nm}$. The data were normalized to 1 x 10^{9} cfu to account for any differences in the growth rates of the various bacterial strains used. The results were reproduced in three independent experiments, and the error bars represent standard deviations. The Δ*ahyRI* mutant (with reduced biofilm formation) and the Δ*luxS* mutant (with enhanced biofilm formation) were used as controls. Asterisks **, represent statistically significant differences in biofilm formation between the Δ*qseB* mutant and the WT bacteria by the student's t test (p=0.0017).

Figure 5.7. Representative SEM images of biofilm formation by the WT *A. hydrophila* SSU and its Δ*qseB* mutant

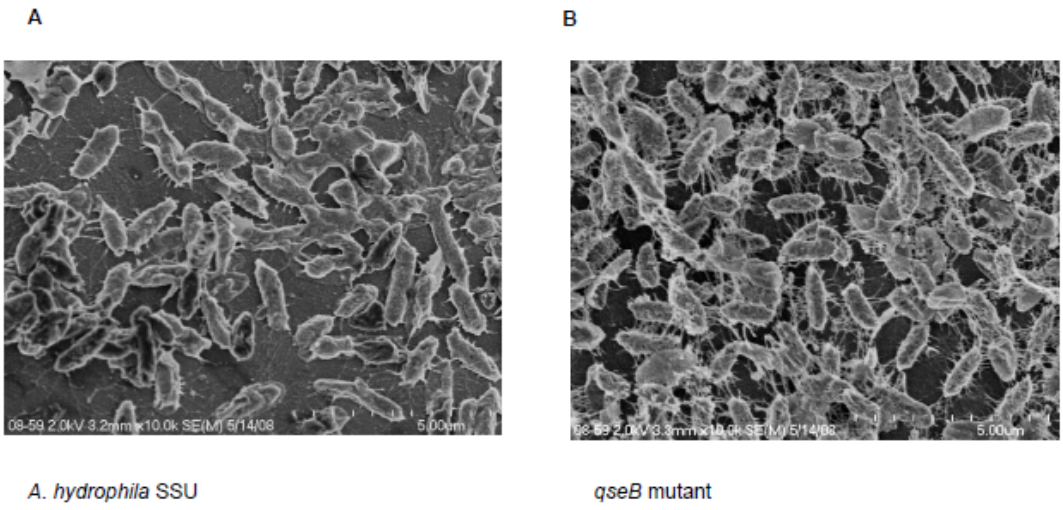

Fig. 5.7. Representative SEM images of biofilm formation by WT *A. hydrophila* SSU and its Δ*qseB* mutant after 48 h of cultivation at 37^{o}C on thermonox cover slips stained with ruthenium red. Compact aggregated cells were well connected with filaments and denser three-dimensional biofilms were formed by the Δ*qseB* mutant (B) as compared to less aggregated cells that were connected with fewer filaments and flattened biofims were produced by the WT bacteria (A).

deleted the *qseB* gene from *A. hydrophila*. Although this difference in survival did not reach statistical significance, it appeared biological relevant as we observed a similar pattern of survival in three independent experiments, with 10 animals/group in each experiment.

DISCUSSION

In this study, we demonstrated the role of QseBC/AI-3 QS system in controlling virulence of *A. hydrophila* SSU. Thus, A. *hydrophila* SSU possesses all three functional QS circuits and might establish a complex QS network to exert its regulatory role during pathogenesis (109, 121). Therefore, *A. hydrophila* SSU represents an excellent model organism to study the role of different QS networks in bacterial pathogenesis.

Flagella are not only important for bacterial movement but also contribute to pathogenesis by aiding in organism's adherence to the target host cells and biofilm formation (114, 116, 117). *Aeromonas* species possess two distinct flagellar systems: a polar flagellum for swimming motility, and several lateral flagella for swarming motility over surfaces (116). We observed that both the swimming and the swarming motility were diminished in *A. hydrophila* SSU when the *qseB* gene was deleted, suggesting to us that *qseB* is a regulator of both polar and lateral flagella. In accordance with our data, recent studies also showed that QseBC regulated swimming motility of several pathogens such as *E. coli* (216), *S.* Typhimurium (11, 148), and *Edwardsiella tarda* (245). However, these investigators only examined the swimming motility. In the present study, we noted that in addition to swimming motility, QseB also regulated swarming motility of *A. hydrophila* SSU. Additionally, Clarke et al. reported that QseBC regulated flagella and motility through the flagellar master regulator; FlhDC (37). They also demonstrated that in order to control motility, QseB directly bound to the *flhDC* promoter both in low and high affinity binding sites (37). Our future study will examine specific mechanism(s) as to how QseBC regulates motility by controlling both the polar and lateral flagella systems in *A. hydrophila* SSU.

Figure 5.8. Decrease *in vivo* virulence of the Δ*qseB* mutant in a septicemic mouse model of *A*. *hydrophila* infection

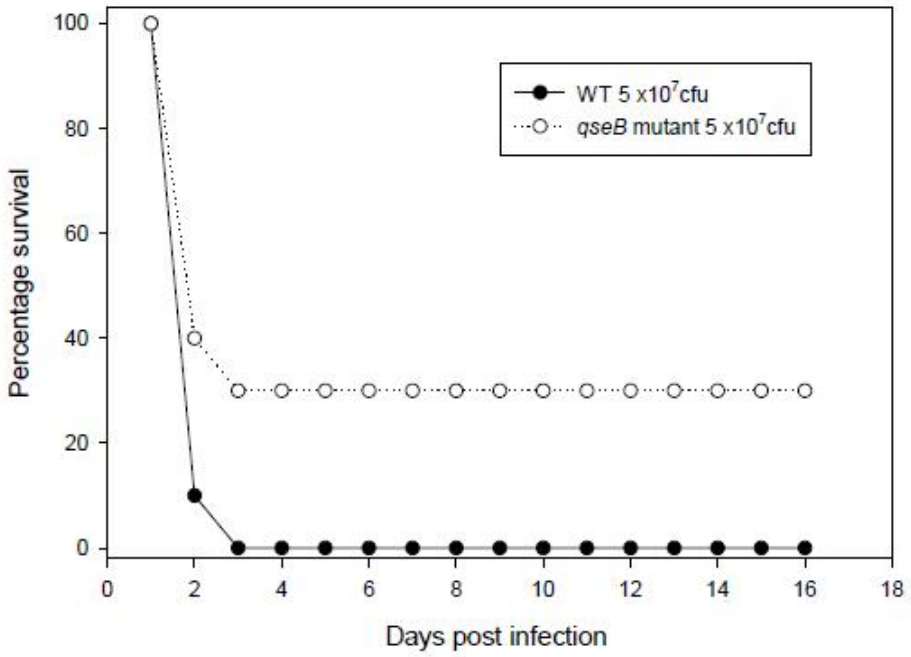

Fig. 5.8. Marginal decrease *in vivo* virulence of the Δ*qseB* mutant in a septicemic mouse model of *A*. *hydrophila* infection. Swiss Webster mice (n=10 per group) were injected intraperitoneally with 5 x 10^7 cfu of WT *A*. *hydrophila* SSU. The same dose was used to infect mice with the Δ*qseB* mutant, and both groups were monitored for death over a 16-day period. Three independent experiments were performed, and percentages of surviving mice over time from a typical experiment are shown. The data were analyzed by using the Kaplan-Meier's survival estimates but difference of animal survival between the Δ*qseB* mutant and the WT *A*. *hydrophila* SSU was not statistically significant (p=0.07).

Interestingly, the *ahyRI*-mediated (AI-1) QS did not regulate either the swimming or the swarming motility (109) but deletion of the *luxS* gene (AI-2 QS) reduced motility of *A. hydrophila* SSU (121). We hypothesize that the *luxS* QS system might regulate motility indirectly through the regulator QseB. However, we still need to delineate a link between the *luxS*- and QseBC- based QS systems in regulating motility of *A. hydrophila* SSU.

We showed that QseB positively regulated protease production in *A. hydrophila* SSU Likewise, in our recent study, we observed that AI-1 QS positively modulated protease activity, particularly metalloprotease (109). Based on these observations, we speculate that regulation of protease production by AI-1 QS system could also be directly or indirectly through QseB. Therefore, it will be intriguing to study the network connection between all these QS systems of *A. hydrophila* SSU in relation to regulation of various virulence mechanisms.

Act is one of the most potent virulence factors of *A. hydrophila* SSU, which possesses several biological activities (33, 66). Further, Act mutant of *A. hydrophila* SSU is significantly attenuated in an animal model, indicating Act contributes to *in vivo* virulence of this pathogen (256). In this study, we further showed that QseB regulated the production of Act, which could be very important for *A. hydrophila* while establishing an infection in the host. In a similar fashion in EHEC, QseC also regulated shiga toxin *via* controlling the transcription of another two component system QseEF (83). It is interesting to note that we also detected a homolog of QseEF two component system in *A. hydrophila* SSU (unpublished data). In future, we intent to study crosstalk between QseBC and QseEF systems in the pathogenesis of *A. hydrophila* and to delineate specific mechanisms that regulate biological activity of Act. Importantly, we observed that AI-1 and AI-2-mediated QS had no influence on the hemolytic activity of Act, suggesting that

different QS systems in *A*. *hydrophila* SSU regulate different sets of virulence factors (109, 121).

Most bacteria in nature are not present as free floating isolated cells; rather they prefer to form surface-associated community, known as biofilm (41, 42). Bacteria present in biofilms are more robust in nature in their ability to withstand chemical and physical stresses and are more resistant to host defenses than the free living or when in the planktonic state (42, 207). Therefore, biofilm formation is considered as one of the most important virulence mechanisms that contribute to human disease transmission and pathogenesis (85, 160). Several studies also pointed out that QS plays a crucial role in controlling biofilm development and establishing efficient infection in both gram-positive (160, 166) and Gram-negative bacteria (47, 265). Earlier, we demonstrated that AhyRI (AI-1) QS positively regulated (109) while LuxS/AI-2-based QS negatively regulated (121) biofilm formation in *A*. *hydrophila* SSU. Similar to the *luxS* system, in this study, we further showed that QseBC also negatively regulated biofilm formation in *A*. *hydrophila*. In agreement with our study, Moreira *et al*., showed that deletion of the *qse*A gene (encoding LysR-type regulator), which is also involved in AI-3 mediated QS circuits (103), from enteropathogenic *E*. *coli* enhanced biofilm formation (148).

However, in contrast to our findings, it was shown that inactivation of the *qseC* gene in *Aggregatibacter actinomycetemcomitans* reduced biofilm growth (158). Likewise, deletion of the *mqsR* gene, a regulator of the QseBC system, in *E*. *coli* also reduced biofilm formation (72). These data indicate that regulation of biofilm formation by the QseBC system is distinct in different bacterial pathogens. Further, these later two studies also showed that alteration in biofilm formation through the QseBC system was dependent on the AI-2-based QS (72, 158). Therefore, it is worth examining in *A*.

hydrophila SSU whether these AI-2 and QseBC QS systems are linked in controlling biofilm formation.

In addition, we also showed that over production of c-di-GMP altered biofilm formation and motility in *A. hydrophila* SSU in a QS dependent manner involving both AI-1 and AI-2 systems (120). The loss in motility by *ggdef*-overexpression in *qseB* mutant, which we described here, may suggest that QseBC QS system is involved in c-di-GMP-dependent regulatory network in *A. hydrophila* (Fig. 5.3).

We and others have shown that bacterial secretion systems, such as T3- and T6- are key virulence factors that contribute to bacterial pathogenesis, particularly in Gram-negative bacteria (19, 137, 202, 203, 218-220). Studies have shown that AI-1 QS regulates T3SS secretion either positively (214) or negatively (15). Additionally, Reading *et al.,* specifically demonstrated that transcription of the T3SS effectors, such as *espFu* in EHEC was regulated by cross talk between QseBC and QseEF systems (178). However, in the present study, the expression and production of a T3SS effector, AexU in *A. hydrophila* SSU was similar between WT and its *qseB* mutant, suggesting that QseBC has no direct role in regulating T3SS effectors. Likewise, we also noted that AI-1 and AI-2-mediated QS had no role in controlling the T3SS (109, 121). In future, we will explore whether QseEF system has any role in regulating T3SS of *A. hydrophia* SSU.

It is largely unknown as to how the effectors secreted by the T6SS are regulated. Recently, we and others showed that QS regulated secretion and function of T6SS effectors, such as Hcp and VgrG (88, 109, 135, 261, 263). However, the level of production and secretion of Hcp between WT and *qseB* mutant were similar, suggesting that QseBC system does not have any significant role in regulating functions of T6SS effectors in *A. hydrophila* SSU. Based on our earlier and this study, we can conclude that in *A. hydrophila*, T6SS is specifically regulated through the AI-1 QS system (109).

We also demonstrated that AI-1 and AI-2 mediated QS systems had opposing effects on the virulence of *A. hydrophila* in a septicemic mouse model (109, 121). While deletion of the *ahyRI* QS genes attenuated the bacterium, the *luxS* mutant of *A. hydrophila* SSU had increased virulence (109, 121). Similar to the *luxS* mutant, deletion of the *qseB* gene from *A. hydrophila* enhanced biofilm formation, however, the *qseB* mutant was less virulent compared to that of WT *A. hydrophila in vivo* model (Fig. 5.8). In an animal model, we expected a marginal decrease in the virulence of the *qseB* mutant as compared to the WT bacteria. This was based on our *in vitro* experiments, where we noted that in addition to serving as a negative regulator of biofilm formation, QseBC also positively regulated some of the important virulence factors, such as Act and protease, which overall balanced the overall pathogenecity of *A. hydrophila*. In agreement with our study, other investigators also showed that the *qseC* mutant and/or interference of QseC signaling in different bacteria resulted in attenuation of bacteria in different animal models tested (11, 119, 158, 176).

In conclusion, we characterized the role of third QS system, QseBC/AI-3, in better understanding of how different QS systems regulate virulence of *A. hydrophila*. We have shown that QseBC has both positive and negative regulations on various virulence factors/mechanisms of *A. hydrophila* SSU that could play an important role in fine tuning the expression of virulence genes at appropriate time to facilitate the pathogen in establishing infection in a highly efficient manner.

CHAPTER 6

Summary and Conclusions

In summary, in the first part of the study, we analyzed and characterized 227 isolates of *Aeromonas* which were obtained from water and clinical specimens by using various phenotypic and genotypic methods as well as some functional assays. Our findings in this study revealed that a subset of *Aeromonas* strains that were present in water samples was responsible for causing disease in humans, suggesting evidence of water-to-human transmission of *Aeromonas* species. This study also provided information on the distribution of virulence factors, including newly identified virulence factors, in a large number of water and stool *Aeromonas* isolates which we believe would be very helpful in differentiating pathogenic and nonpathogenic aeromonads. Another interesting finding of this study was the production of QS signaling molecules, such as N-*acyl* homoserine lactone (AHL), which was greater in clinical isolates than in those from water *Aeromonas* isolates indicating that AHL QS might play a critical role in human infections.

These data intrigued us to further characterize the role of AHL-mediated QS in the regulation of virulence factors of a clinical isolate SSU of *A. hydrophila*. In the second part of this study, we indeed demonstrated that AHL-mediated QS regulated metallo-protease production, secretion of the T6SS effectors (Hcp and VgrGs), biofilm formation, and also controlled *in vivo* virulence in a mouse model of infection with *A. hydrophila*. Further, we noted that *ahyRI* (involved in AHL-mediated QS) mutant abrogated the secretion of Hcp and VgrGs, but the mechanisms as to how AHL molecules or its regulator AhyR control T6SS effectors are not known. Therefore, further

study is necessary to unfold the detail mechanisms as to how *ahyRI* QS system regulates T6SS.

Recent studies showed that AHL is not just a regulator for bacteria but also modulate host cells. However, the mechanisms of immunomodulation of host cells are largely unknown. In order to further dissect the immumodulatory role of different AHLs against bacterial infections, in the third part of the study, we examined immunomodulatory role of different AHLs such as C4-HSL, C6-HSL and 3-oxo-C12-HSL in septicemic mouse model. Our study provided for the first time novel information indicating that pretreatment of mice with AHLs prevented clinical sequelae to enhance survivability of mice after *A. hydrophila* infection, and that they stimulate an innate immune response in mice to clear infection. We further demonastrated specific mechanisms as to how mice clear bacteria form the blood and different organs. Our data showed that AHL pretreatment, particularly with C6-HSL, recruited significantly higher number of neutrophils in the blood compared to the control untreated animals. Additionally, we showed that treatment of murine mcarpophages (RAW 264.7) with C4-HSL and C6-HSL enhanced their phagocytosis rate as compared to the untreated cells. Taken together, our data showed that pretreatment of mice with different HSLs stimulated different host defense/immune cells (e.g., neutrophils, macrophages) and helped them to efficiently clear bacteria from different tissues and the blood. Although one study showed some evidence that peroxisome proliferator-activated receptors (PPARs) could recognize 3-oxo-C12-HSL (89), however, it remains to be identified whether different AHLs are recognized by different pattern receptor (s) in host cells and also what signaling pathways they stimulate to activate different immune cells.

We noted that AHL (AI-1) QS modulated positively and LuxS/AI-2 QS regulated negatively the virulence of *A. hydrophila*. Finally, we demonstrated that the two-

component system QseBC which is involved in AI-3-mediated QS positively regulated swimming and swarming motility, protease production, and functional activity of Act, while this system was a negative regulator of biofilm formation. We speculate that all these three QS systems in *A. hydrophila* SSU are networked to exert their stringent regulation on various virulence factors. Thus particular virulence factors are produced at appropriate time to establish infection in a highly efficient manner.

In conclusion, a detailed understanding of how different QS circuits regulate bacterial virulence and also how QS signaling molecules, such as AHLs, modulate host response would be very crucial in indentifying new treatment modalities, particulary for *Aeromonas*-associated infections, or diseases associated with other Gram-negative bacteria in general.

FUTURE DIRECTIONS

- **To further dissect the virulence mechanisms of those seven clinical and water *A. caviae/media* Group isolates which were involved in water-to-human transmission by using *in vitro* and *in vivo* model of infection in order to better undstand the pathogenesis of *Aeromonas*-associated infections.**
- **To identify specific mechanism(s) as to how AHL-mediated QS regulates function of T6SS effectors such as Hcp and VgrG.** We noted that secretion of both Hcp and VgrG were abrogated in the *ahyRI* mutant, however, it is not known whether AhyR directly interacts with the effector proteins and/or their regulatory genes to control their secretion.
- **To study the influence of AHL treatment on the killing mechanisms of neutrophils such as i) production of myeloperoxidase and elasetase, ii) production of defensins, and iii) formation of neutrophil extracellular trap (NET).** We observed that AHL pretreatment, particularly C6-HSL, significantly recruited higher number of neutrophils in the blood which could be one of the possible reasons that AHL treated animal cleared infection efficiently than that of untreated animals. However, it remains to be determined as to how AHLs influence killing activity of neutrophils during infection.
- **To study the role of macrophages in host resistance during infection of *A. hydrophila*.** We demonstrated that AHL treatment stimulated murine macrophages *in vitro* to increase their phagocytic activity. In future, it would be interesting to investigate *in vivo* whether AHL treatment also recruit macrophages in different tissues during infection and also to examine the role of macrophages during *Aeromonas* infection by specifically depleting macrophages from animals.

- **To examine in detail mechanisms as to how three QS systems and bacterial second messenger c-di-GMP are networked and control virulence of *A. hydrophila*.**

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VITA

Bijay Kumar Khajanchi was born on December 1st, 1978, in Dinajpur, Bangladesh. He attended elementary and high schools in Dinajpur and then moved to Dhaka to obtain his Bachelor of Science and Master of Science degrees in Microbiology from the University of Dhaka, Bangladesh. Subsequently, he worked at the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR, B) for four years, and, in 2005, a travel award from the UNESCO-American Society for Microbiology (ASM) allowed him to visit UTMB and to learn advanced research technologies as a visiting scientist in the laboratory of Dr. Chopra. He matriculated to the Graduate School of Biomedical Sciences at UTMB in 2006. He was awarded James W. McLaughlin Pre-doctoral Fellowship in 2009 and also received several awards from the graduate school at UTMB. During his graduate training, he was also awarded several external travel awards, including the Corporate Activities Student Travel Award from ASM in 2009 and he attended two ASM General Meetings as well as several local meetings where he had oral and poster presentations.

Education

Bachelor of Science, 2000, University of Dhaka, Bangladesh
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Publications

1. **Khajanchi B. K.,** M. L. Kirtley, S. M. Brackman and A. K. Chopra. 2011. Immunomodulatory and protective roles of quorum sensing signaling molecules *N acyl* homoserine lactones during infection of mice with *Aeromonas hydrophila.* Infect Immun. **79:**2646-2657.

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Abstracts and Posters

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