Delineation of Aeromonas hydrophila Pathotypes by Detection of Putative Virulence Factors using Polymerase Chain Reaction and Nematode Challenge Assay

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Delineation of *Aeromonas hydrophila* Pathotypes by Detection of Putative Virulence Factors using Polymerase Chain Reaction and Nematode Challenge Assay

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Submitted in partial fulfillment of the requirements for the Master of Science Degree in Integrative Biology

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Aeromonas hydrophila is a Gram-negative, bacterial pathogen of humans and other vertebrates. Human diseases caused by A. hydrophila range from mild gastroenteritis to soft tissue infections including cellulitis and acute necrotizing fasciitis. When seen in fish it causes dermal ulcers and fatal septicemia, which are detrimental to aquaculture stocks and has major economic impact to the industry. The severity of disease may be dependent on expression of specific virulence factors, which vary among strains called pathotypes. Therefore, it is important to be able to distinguish highly virulent A. hydrophila pathotypes from those less virulent for disease control. To this point, several factors related to A. hydrophila pathogenesis have been identified. To delineate pathotypes, the genomes of twenty-eight Aeromonas isolates were screened by polymerase chain reaction to determine the presence of known virulence factors including: aerolysin (aerA), cytotoxic enterotoxin (act), hemolysin (ahh1), elastase (ahyB), enolase (eno), S-layer protein (ahsA), serine protease (ser), Type IV Aeromonas pilus (tapA), lipase (lip), and Type Three Secretion System (T3SS) components (aopB, ascV). Genes for ahh1, lip, ser, and ahyB were present in all 28 strains tested, but variation was seen from aopB/ascV, ahsA, act, aerA, and eno. The tapA gene encoding a type IV pilus was absent in all 28 isolates screened. After the presence or absence of these genes was determined, corresponding activity such as hemolysis of red blood cells and digestion of elastin was determined using phenotypic assays. Analysis of the data defined 11 different pathotypes based on the genotypic and phenotypic profiles with the largest cluster being ahh1+, ahyB+, lip+, ser+, act+, aerA+, eno+, aopB, ascV, ahsA−, and tapA+. Representatives of the pathotype groups were used in a nematode challenge assay and a cell culture assay to assess the importance of virulence factors in their pathogenicity and their cytotoxicity, respectively. All Aeromonas strains tested, with the exception of A. hydrophila ML09-119, showed significantly greater lethality compared to the E. coli negative control in the nematode challenge, yet only A. hydrophila 1127 was significantly more lethal than the positive control Pseudomonas aeruginosa PAO1. The most lethal strain was A. hydrophila 1127,
which was positive for both S-layer and T3SS. The variation in lethality between *A. hydrophila* strains suggests that *C. elegans* is a suitable model for studying pathogenic mechanisms and elucidating the combination of factors that define highly virulent strains. In the cytotoxicity assay, rounding of cells was observed after 6 hours for *A. hydrophila* strains 1127, 1288, ATCC 7966T, 1280, and ML09-119. Of these strains, only *A. hydrophila* 1127 was positive for the T3SS that delivers the effector protein AexU reported to have ADP-ribosylating activity leading to cell rounding and apoptosis. The data indicate other toxins are likely responsible for the cytotoxic effects. This is supported by the recent descriptions of two toxins, RtxA and VgrG1, in *A. hydrophila* ATCC 7966T that have ADP-ribosylating activity. Additional screening for the *rtxA* and *vgrG1* genes is needed to better understand the cell rounding and cytotoxicity patterns. The study of host-microbe relationships requires an integrative approach using molecular biology, cell culture, and model host systems.
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INTRODUCTION

Infectious diseases, sometimes called communicable diseases, are caused by pathogenic microorganisms. The term pathogen is used to indicate any bacterium, virus, fungus, protozoan or helminth that is the cause of disease. According to the World Health Organization statistics for 2012 (WHO, 2014), infectious diseases consistently rank within the top ten causes of global human death with 3.1 million annual fatalities caused by lower respiratory infections (ranked 3rd), 1.5 million attributed to HIV/AIDS, and another 1.5 million deaths from diarrheal diseases (6th and 7th). Humans are directly at risk from infectious agents and indirectly at risk as infectious diseases affect food supply. The risks associated with food supply include economic losses related to the culling of animals and the unavailability of food.

Regardless of which type of pathogen causes disease, there are common factors in the disease cycle. In order for a pathogen to spread it must be passed from a reservoir to a host organism that is susceptible to the pathogen. A reservoir is the place in nature where a pathogen is maintained in a viable, infectious state. Reservoirs can be living carriers such as humans or other animals, or inanimate materials such as soil, water, air, or food products. A pathogen can be transmitted from a reservoir to a susceptible host by three major types of transmission: 1) contact transmission, 2) vehicle transmission, or 3) vector transmission. In contact transmission, the pathogen moves from an infected host to a susceptible individual through direct or indirect person-to-person interaction. Vehicle transmission is the movement of the pathogen into a host through air-borne, food-borne or water-borne means such as the movement of respiratory pathogens through air handling systems or gastrointestinal pathogens that are spread through consumption of contaminated food or water. Vector transmission is facilitated primarily by a specific arthropod (mosquitos, biting flies, ticks, mites, fleas) that harbors and transmits a pathogen from one host to another. The continued existence of a pathogen requires perpetual transmission to a new host.
Pathogens that cause disease within a host must gain access through a portal of entry. The most common portals of entry are the respiratory system and the gastrointestinal system as breathing, eating, and drinking are absolute necessities of life. A pathogen may access the respiratory system through the inhalation of aerosols or particles containing the infectious microbe. Most diarrheal diseases occur through the fecal-oral route of transmission whereby water or food becomes unsanitary by the introduction of fecal wastes (generally from humans or livestock) and the infectious agents that may be within the feces cause disease in those consuming (oral) the contaminated food or water. Although the skin has the greatest bodily surface area in direct contact with the environment, entry of a pathogen through this barrier usually requires injury such as a laceration or puncture wound (parenteral) or the bite of an insect vector.

Portals of exit are avenues by which pathogens leave the infected host. Often, the same system by which the pathogen gained entrance also is the system with the portal of exit. The pathogen leaves through a portal of exit through expulsion of aerosolized droplets, bodily fluids, feces or wound exudates.

Between entry and exit, if a pathogen is to persist in a host and subsequently cause disease, it must be able to colonize and obtain necessary nutrients for growth while simultaneously evading, suppressing, or eliminating host defenses that work to eliminate the pathogen. The ability of a microorganism to cause disease is referred to as pathogenicity. Pathogenicity is dependent on the genetic make-up of the pathogen and the damage done to the host during the host-pathogen interaction. Much of the damage that occurs from infection is the result of the destruction of tissues and cells as a means of acquiring nutrients for growth, colonization of new areas, and/or destruction of cells and factors associated with physical barriers, innate and adaptive defenses. In any group or species of pathogen, the degree of pathogenicity is determined by the ability of the organism to colonize and invade the tissues and the fatality rates of infected hosts is called virulence. The pathogenicity of an organism and its level of virulence are dependent on its virulence factors that enable it to adhere and colonize, invade and spread, acquire and transport nutrients and evade host defenses.
Within a single species, pathogenic strains can be distinguished from their nonpathogenic counterparts by the presence of genes encoding virulence factors. Even within pathogenic strains the presence or absence of these genes varies. Based on the type of virulence factors present and host clinical symptoms, strains of a species can be identified as a particular pathotype. For example, *Escherichia coli* K-12 is a commensal bacterium that is part of the normal intestinal microbiota of humans and other mammals. But several different pathogenic *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes. Currently, *E. coli* pathotypes are separated into two groups based on clinical illness: diarrheagenic *E. coli* (DEC) strains and extraintestinal *E. coli* (ExPEC). These are divided further on the basis of virulence genes, which are generally organized as large blocks in chromosomes, plasmids, or phages and are often transferrable between *E. coli* strains. For instance uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are pathotypes within the ExPEC cluster, whereas enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) are two of six pathotypes within the DEC group (Kaper, Nataro and Mobley, 2004). To effectively monitor for biological threats such as *E. coli* pathotypes and to ensure public health, it is necessary to define the factors that determine the pathotype and then develop methods to detect and identify specific pathotypes by these factors. Rapid, specific and sensitive methods of detection and identification would permit screening of the food and water supplies, as well as delivering effective treatment in patients with clinical illness due to infection with a specific pathotype.

As with *E. coli*, *Aeromonas hydrophila* is a bacterial pathogen able to cause intestinal and extraintestinal infections. *Aeromonas hydrophila* is one of twenty seven validated species in the genus *Aeromonas* although this may soon change as new species are discovered and modern approaches to taxonomy and defining a bacterial species progress (Beaz-Hidalgo et al., 2013b, 2015; Chen et al., 2014a; Janda and Abbott, 2010). As a group, aeromonads are found worldwide, typically residing in fresh and brackish water environments. Some of the phenotypic traits that characterize aeromonads include Gram-negative, bacillus-shaped bacteria unable to produce endospores. They are facultative anaerobic,
chemoorganoheterotrophs that require organic macromolecules (proteins, carbohydrates, fats) to fulfill their dietary requirements (Martin-Carnahan et al., 2005).

Initially, aeromonads were split into two phenotypic groups based on motility and temperature growth optima. The mesophilic group consists of species that are motile and prefer to grow at 35°C-37°C, and include those species causing disease in humans. *Aeromonas hydrophila*, the most prominent aeromonad in regards to human diseases, falls within this group. The psychrophilic group consists of species that are non-motile, and have an optimal growth temperature between 22°-25°C. *Aeromonas salmonicida* falls into this group and is the cause of furunculosis, a lethal ulcerative disease in cold-water fish (Janda and Abbott, 2010). Grouping aeromonads on the basis of temperature growth optima and motility is no longer widely used as modern molecular approaches, such as multilocus phylogenetic analysis (MLPA) or matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), are used to delineate species and strains, and demonstrate relatedness between these bacteria (Beaz-Hildago et al., 2013b; Chen et al., 2014a; Hossain et al. 2014; Martínez-Murcia et al. 2011).

Although several *Aeromonas* species occasionally have been isolated from intestinal and extraintestinal human clinical samples associated with human illness, the most reported species are *A. hydrophila*, *A. dhakensis*, *A. caviae*, and *A. veronii* biovar *sobria* (Beaz-Hildago et al., 2013b; Chen et al., 2014b, Chen et al., 2014c, Chopra and Houston, 2000; Janda and Abbott, 2010). Other clinically-relevant species include *A. veronii* biovar *veronii*, *A. trota*, *A. schubertii*, *A. jandei*, and *A. popoffii* (Janda and Abbott, 2010). Pathogenic species of *Aeromonas* cause three main disease conditions in humans: gastroenteritis, wound infection, and septicemia although rare conditions such as meningitis and acute necrotizing fasciitis occur (Janda and Abbott, 2010; Parker and Shaw, 2010). Of these three conditions, gastroenteritis is the least severe as it acute yet self-limiting. It generally manifests as watery stool or bloody diarrhea, but has a low probability for severe physical debilitation or development into a chronic condition unless the host is immunocompromised (Wilcox et al., 1992). The route of infection is through
ingestion of an infectious dose from contaminated food or water leading to colonization and subsequent enterotoxin production (Chopra and Houston, 2000; Janda and Abbott, 2010).

Wound infections are more serious and are usually propagated in healthy individuals who become injured and subsequently exposed to an aquatic environment or soil where *Aeromonas* spp. are in high numbers (Voss *et al*., 1992; Wakabongo, 1995). Wounding allows the bacteria to by-pass the skin barrier allowing access to the underlying soft tissues, including adipose, muscle, and connective tissues. As the bacteria move into the tissue layers beneath the dermis they have direct access to the bloodstream where dissemination to other body systems is possible.

Septicemia is the most serious of these infections and usually occurs in immunocompromised individuals, although in rare cases can affect immunocompetent individuals following an extensive traumatic water injury (Chan *et al*. 2000; Duthie *et al*., 1995, Mencacci *et al*., 2003; Janda *et al*., 1996; Riley *et al*., 1996; Shiina, Ii and Iwanaga, 2004). Septicemia involving Gram-negative bacteria can result in dissemination of the pathogen throughout the individual and septic shock. The mortality rate of individuals who go into septic shock is 90%. Compounding this potential condition is the inherent resistance of aeromonads to general antibiotic classes, such as penicillin-derived antibiotics inherent to aeromonads (Janda and Abbott, 2010). Therefore, correct identification of *Aeromonas* infection is vital to delivering effective antimicrobial therapy to control local infection and prevent subsequent dissemination or septic shock. Identifying highly virulent *Aeromonas* strains in the environment could limit the number of infections in human host (Janda *et al*. 1996, Lin *et al*., 1996).

*A. hydrophila* is the most prominent species in regards to human diseases, particularly as a significant cause of soft tissue infections and gastroenteritis. *A. hydrophila* strains possess multiple, strain-specific virulence factors that allow them to invade a host and cause diseases ranging from non-life threatening gastroenteritis, to soft tissue infections leading to cellulitis or life threatening conditions
including severe necrotizing fasciitis and septicemia (Chen et al. 2014c; Janda and Abbott, 2010; Tomás, 2012).

_Aeromonas_ species most commonly reported as the etiological agents of disease in wild and farmed fish and other non-human animals include _A. hydrophila, A. sobria, A. salmonicida, A. bestiarum, A. dhakensis_ and _A. piscicola_ (Hosain et al., 2014; Beaz-Hidalgo and Figueras, 2013a; Soto-Rodriguez et al., 2013). Of these, _A. salmonicida_ and _A. hydrophila_ are the most significant _Aeromonas_ pathogens of the aquaculture industry. For example, an outbreak of fatal motile _Aeromonas_ septicemia in farmed catfish caused by a highly virulent strain of _A. hydrophila_ that began in west Alabama in 2009 has spread to adjacent states resulting in an estimated loss of more than $12 million in catfish aquaculture (Hossain et al., 2014). The multiple, strain-specific virulence factors produced by _Aeromonas_ allow them to cause septicemia, hemorrhage and severe ulcerative diseases in fish and other animals including frogs, salamanders, snakes and fowl (Beaz-Hidalgo and Figueras, 2013a; Carvalho-Castro, _et al._, 2010; Chen _et al._, 2014c; Pridgeon _et al._, 2013; Tomás, 2012; Yu _et al._, 2005; Zhang, Pridgeon and Klesius, 2014).

This thesis research project focused primarily on the multiple virulence factors of _A. hydrophila_. The whole genome of _Aeromonas hydrophila_ ATCC 7966^T_ has been fully sequenced (Seshadri _et al._, 2006). As a result, well-characterized virulence factor genes were mapped to the genome, and several putative virulence factor genes having significant homology to known factors found in other pathogens were discovered. It was also apparent that some reported virulence factors of _A. hydrophila_, including a toxin-delivering type III secretion system and lateral flagella, were absent in this strain. Based on the large amount of research, _A. hydrophila_ and related _Aeromonas_ pathogens cause disease by a multifactorial process employing a complex network of virulence factors (Beaz-Hidalgo and Figueras, 2013a; Janda and Abbott, 2010; Pemberton, Kidd and Schmidt, 1997; Seshadri _et al._, 2006; Tomás, 2012). The variation in these virulence factors from one strain to another likely affects the progression and severity of disease in the host. Defining virulence factors per _A. hydrophila_ pathotypes could lead to developing methods to detect and identify pathogenic from non-pathogenic strains of _A. hydrophila_, as
well as identifying specific pathotypes among the pathogenic strains. Rapid, specific and sensitive methods of detection and identification would permit screening of environmental samples, food and water supplies, as well as those causing disease in human and non-human hosts. The following is a description of the prominent virulence factors reported as major contributors to the disease caused by *A. hydrophila*.

Regardless of disease outcomes, bacterial pathogens must accomplish several objectives before the disease state occurs. First, the bacterium must be able to enter the host, which can occur at multiple points. As mentioned, *A. hydrophila*, entry can occur through ingestion or through a pre-existing surface wound. One of the most common entry methods is accidental wounding and entry of the bacterium into wounds acquired in water-associated activities such as boating accidents. *Aeromonas hydrophila* was among the most common bacterium isolated from soft tissue infections in due to wounds acquired in the 2004 tsunami in Thailand (Hiransuthikul *et al.*, 2005). Although rare, in some cases water used to treat burn victims was contaminated with *Aeromonas* leading to serious deep disease infections and extended hospital stays (Skoll *et al.*, 1998). Many aeromonads have the ability to produce biofilms, allowing them to survive in chlorinated water and retain resistance against antibiotics, reactive oxygen species, and detergents commonly used to for disinfection (Lynch *et al.* 2002). Therefore, even treated water may be a source of wound infections caused by *A. hydrophila*.

After the pathogen enters the host, it must adhere to the host cells and colonize, while evading the host’s innate immune defenses. Several surface-associated structures provide resistance to host immune defenses. For example, the S-layer on the surface of the cell provide resistance to antimicrobial factors present in blood. S-layers are present on many Bacteria and Archaea as an outermost structure composed of repeated protein or glycoprotein monomers assembled outside of the cell into a regularly spaced, two-dimensional array (Sara and Sleytr, 2000). Because S-layer lattices possess pores identical in size and morphology, they work as precise molecular sieves, providing sharp cutoff levels for the bacterial cells. In natural settings, the S-layer provides resistance to infection by parasitic bacteria and bacteriophage. In vertebrate hosts, S-layers on pathogens including *A. hydrophila* provide a selective advantage by
imparting high or intermediate resistance to complement-mediated lysis (Thomas and Trust, 1995; Noonan and Trust, 1997). Furthermore, the S-layer possesses other functions that enhance virulence including adhesion to extracellular matrix proteins of the host, protection against attack by phagocytes, uptake of porphyrins, and anchoring site for hydrolytic exoenzymes (Beveridge et al., 1997; Noonan and Trust, 1997; Sara and Sleytr, 2000). Evidence that the S-layer is a major virulence factor is further supported by vaccine trials in both fish and mice. Purified natural and recombinant S-layer proteins (AhsA) used as vaccines provided immunity against challenge with highly virulent strains of A. hydrophila in carp (Poobalane et al., 2010) and mice (Kokka, Vedros and Janda, 1992), respectively. Additionally, A. hydrophila S-layer negative mutants required cell concentrations 5 to 20 fold higher than the wild-type to reach a lethal dose (LD50) in rainbow trout, Oncorhyncus mykiss (Noonan and Trust, 1997). This evidence demonstrates that the S-layer is a virulence factor, which significantly contributes to the survival of A. hydrophila in a host.

A second characterized cell surface structure is the capsule. Capsules can also provide resistance to phagocytosis by the host’s phagocytic leukocytes and resistance to complement attack. Capsules may also enhance adherence to surfaces. The presence of a capsule on strains of A. hydrophila recovered from diseased fish was reported to enhance adherence to and invasion of fish cells (Merino et al., 1997a). A lack of attachment and invasion occurred when unencapsulated mutant strains or wild-type strains that lacked a capsule due to growth on non-enriched media were examined. In another study Zhang et al. (2003), seventy percent of group II capsule-producing A. hydrophila strains were resistant to non-immune serum (from tilapia, Oreochromis aureus) and phagocyte killing (using leukocytes from the blue gourami, Trichogaster trichopterus), whereas 67% of randomly chosen acapsular strains were sensitive to serum and phagocyte killing. However, two acapsular strains and a non-group II capsule-producing strain, also survived in serum and phagocyte killing, suggesting that the group II capsule may not be the only factor contributing to the resistance. Complicating the role of the capsule as a virulence factor is the reported observation that capsules are produced only when the bacterium is grown on a medium that is highly
enriched with nutrients or glucose, which is unlikely to occur in most natural settings (Martínez et al., 1995).

Simultaneous to evasion of host defenses, the pathogen must attach to host tissue surfaces to colonize a particular area of the host, and to resist the natural flushing and expulsion processes of the host system. In addition to the adherent properties described for the capsule and S-layer, adhesion of *A. hydrophila* to host cells or tissue may be mediated through filamentous structures including pili/fimbriae and sometimes flagella (lateral) that project from the cell envelope of the pathogen, and non-filamentous adhesins such as outer membrane protein A (OmpA) (Barnett et al., 1997; Gavin et al., 2002; Quinn, et al., 1994; Seshadri et al., 2006). In clinical and environmental isolates of *A. hydrophila* (and other pathogenic species), two distinct types of fimbriae have been reported: short, rigid fimbriae (S/R) and long, wavy fimbriae (L/W) (Barnett et al., 1997). The S/R fimbriae are widely distributed (more than 95% of strains) and able to cause autoaggregation, but not hemagglutination or binding to intestinal cells. In contrast, the L/W fimbriae are type IV pili known to hemagglutinate erythrocytes, adhere to epithelial cells and enhance biofilm formation. Two different type IV pili have been described in gastroenteritis-associated *Aeromonas* species: bundle forming pili (Bfp) and Type IV *Aeromonas* pili (Tap) (Kirov et al., 1999; 2000). Bundle forming pili promote colonization by forming bacterium-bacterium linkages, and loss of the gene *bfp* encoding the Bfp protein reduced adhesion of *Aeromonas* to intestinal cells by up to 80% (Chopra et al., 2009). Although Tap pili exhibit high homology to type IV pili of *Pseudomonas* and pathogenic *Neisseria* species, loss of Tap pili in *A. hydrophila* mutants did not alter adherence and colonization (Kirov et al., 2000). Comparatively, genes corresponding to three type IV pili systems including Tap were discovered in *Aeromonas salmonicida* subsp. *salmonicida*, but were not absolutely required for virulence in Atlantic salmon, *Salmo salar* Linnaeus (Boyd, et al., 2008). In an earlier study using *A. hydrophila* (del Corral, Shotts and Brown, 1990), the presence of fimbriae (pili) did not correlate with virulence potential in fish and appeared to be medium dependent; strains grown in liquid media demonstrated enhanced haemagglutination activity.
Also dependent on culture conditions is the expression of lateral flagella. *Aeromonas hydrophila* express a single polar flagellum in all culture conditions and produce peritrichious lateral flagella on solid media. The flagellin protein of the polar flagellum is distinct from lateral flagella; however, synthesis of lateral flagella is dependent upon the presence of polar flagella and the motor proteins of the two flagella are the same (Gavin et al., 2002). Mutation of *Aeromonas hydrophila* lateral flagella genes (*lafB* and *lafS*) resulted in an 84-85% decrease in adherence of the bacterium to human epithelial cells compared to the wild-type control. Biofilm formation was greatly reduced in these mutants as well. While lateral flagella were absent, synthesis of polar flagella was unaffected; however, mutagenesis of polar flagellum components resulted in concomitant loss of lateral flagella (Gavin et al., 2002).

Earlier studies reported the loss of the polar flagellum in *A. hydrophila* non-motile mutants resulted in a 5-6 fold decrease in adherence to and invasion of fish cell lines (Sea Bass larvae cells and epithelioma papulosum of carp cells) compared to the motile, wild-type. Pretreatment of wild-type cells with complement inactivated, anti-flagellin serum resulted in loss of cell invasion, whereas nonimmune serum did not (Merino et al., 1997b). Loss of polar flagella through site-directed mutagenesis of the *flmB* gene involved in flagellar filament assembly resulted in a 49-50% decrease in adherence to human epithelial cells lines HEp-2 and Caco-2, which was fully restored after *flmB* complementation or centrifugation onto the cells (Gryllos et al., 2001; Rabaan et al., 2001). The results suggest that polar flagella contribute adherence properties to *A. hydrophila*, but are not the only adhesins present. Also, these studies did not take into account that loss of genes related to expression of the polar flagellum also result in loss of lateral flagella, or polar and lateral flagella have some shared components. Although both polar and lateral flagella appear to have roles in adherence, lateral flagella would likely contribute to secondary adherence and colonization, as expression requires interaction with a solid surface. Other surface adhesins are more likely to promote the initial attachment. Genes for lateral flagella were absent in the genomic sequence of *A. hydrophila* type strain ATCC 7966T (Seshadri et al., 2006). In an experiment that measured the relative pathogenicity of 80 *Aeromonas* strains by determining the 50%
lethal dose for each isolate in Swiss-Webster mice by intraperitoneal injection, *A. hydrophila* ATCC 7966\textsuperscript{T} was determined to be among the most pathogenic strains (Janda and Kokka, 1991). However, the mode of infection (direct injection) could mask the importance of lateral flagella in pathogenesis, instead of using a natural route of infection such as ingestion.

Among the outer membrane proteins, the porins have been specially described to act like a lectin-type adhesins, binding the bacteria to carbohydrate-rich surfaces like erythrocytes and human intestinal cells. The OmpA family of outer membrane proteins is a group of genetically related porin proteins that are in high-copy number in the outer membrane of mainly Gram-negative bacteria. OmpA proteins have important pathogenic roles including bacterial adhesion, invasion, or intracellular survival as well as evasion of host defenses or stimulators of pro-inflammatory cytokine production (Confer and Ayalew, 2013). An outer membrane protein similar to OmpAI of *A. salmonicida* and 2 additional putative adhesins were identified in virulent strains and the type strain of *A. hydrophila* (Yu *et al*., 2005; Seshadri *et al*.; 2006). However, the specific role of these proteins in pathogenesis has not been reported. Recently, a vaccine trial using poly (lactic-co-glycolic) acid (PLGA) microspheres carrying a divalent DNA vaccine encoding the *Aeromonas veronii* outer membrane protein A (ompA) and *Aeromonas hydrophila* hemolysin (*hly*) protein was performed in mice (Gao *et al*., 2014). One hundred percent of the mice receiving phosphate buffered saline, pET-28a vector or empty microspheres died within 3 to 4 days after challenge with a mixture of *A. veronii* and *A. hydrophila*. Mice administered the divalent DNA (ompA:*hly*) showed sterile protection over 30 days of challenge infection. Mice administered either ompA or *hly* separately displayed a significantly lower levels of protection against challenge.

In 1994, two carbohydrate-reactive outer membrane proteins of 43 kDa and 40 kDa molecular weights were identified from the surface of human isolate of *A. hydrophila* (Quinn *et al*., 1994). Later, the 43 kDa Omp was shown to have hemagglutinating and adhesion properties (Atkinson *et al*., 1987). Cloning and expression of the gene resulted in a recombinant protein that could competitively inhibit *A. hydrophila* from invading fish epithelial cells *in vitro* and confer significant protection to vaccinated fish.
(blue gourami) against experimental *A. hydrophila* challenge (Fang, Ge and Sin, 2003). Amino acid analysis indicated relatedness to other enterobacterial porins, such as the OmpC of *E. coli*, *Salmonella typhi* and *Serratia marcescens* but not OmpA.

*Aeromonas* spp. are able to produce and secrete several exotoxins, which cause damage to host cells. Three toxins and/or effector proteins of *Aeromonas hydrophila*, AexU (*Aeromonas hydrophila* exoenzyme U), VgrG1 (valine-glycine repeat G) and RtxA (repeat in toxin A), are known to disrupt the actin cytoskeleton on HeLa cells resulting in conversion to a rounded phenotype and subsequent induction of apoptosis (Sha *et al.*, 2007; Suarez *et al.*, 2010; Suarez *et al.*, 2012; Vilches *et al.*, 2008). AexU is secreted through the type three secretion system (T3SS or Type III) of Gram-negative bacteria. The T3SS is one of six currently recognized secretion systems described in Gram-negative bacteria. Also called an injectisome, it is composed of proteins that form a needle-like projection on the bacterial surface that inserts into the membrane of a host cell such that effector proteins or toxins can be directly released into the host cells. The T3SS of *A. hydrophila* is composed of 35 genes arranged in 4 transcriptional clusters, (*asc, aop, axs* and *asc*) that encode proteins that form the secretion or translocation components of the injectisome apparatus, regulate translocation or act as chaperones (Vilches *et al.*, 2004). Located outside the T3SS gene clusters is *aexU* (initially called *aexT*-like toxin), which encodes the toxin AexU that is delivered through the T3SS. The protein toxin AexU possesses both ADP-ribosyltransferase (ADPRT) activity and GTPase-activating protein (GAP) activity, which was mainly responsible for disruption of host cell actin filaments leading to apoptosis, similar to the AexT of *A. salmonicida* (Braun *et al.*, 2002; Sierra *et al.*, 2010). AexU inhibits activation of transcriptional factor NF-κB resulting in suppression of cytokine expression and signaling. Repression in the secretion of pro-inflammatory cytokines/chemokines could delay recruitment of phagocytes to the site of infection, thereby allowing the bacteria to multiply and spread to other areas. In studies using *aexU* knock-out mutants, reduced lethality to fish, mice and cells in culture were reported (Sha *et al.*, 2007; Sierra *et al.*, 2010; Vilches *et al.*, 2008). The loss of virulence was more pronounced when genes *ascV* (encodes a secretion apparatus protein), *acrV* (encodes
protective antigen, anti-host factor) or *aopB* (encodes translocation apparatus protein) were inactivated or missing (Burr *et al.*, 2003; Carvalho-Castro *et al.*, 2010; Vilches *et al.*, 2008). Loss of *acrV* or *ascV* was sufficient to prevent the translocation of toxin to the cytosol of target cells resulting in lower cytotoxicity to the fish and human epithelial cells, and greater survival rates in rainbow trout and mice in comparison to the wild type strain.

Chacón *et al.*, (2004) reported that 78% of intestinal and 86% of extraintestinal *A. hydrophila* human clinical isolates were positive for T3SS genes. According to Carvalho-Castro *et al.*, (2008), a higher frequency of *ascV/+aopB+* strains (intact T3SS) were isolated from diseased fish (62.5%) than *ascV/-aopB- or ascV/+aopB- strains. Although strains from the three genetic profiles could produce disease in Nile tilapia (*O. niloticus*), *ascV/+aopB+* isolates promoted sudden disease with widespread hemorrhage whereas skin darkening, apathy and low mortality rates occurred when using the other strains.

Although in some cases T3SS may enhance virulence, *A. hydrophila* strains lacking T3SS still have the capacity to cause cell apoptosis and are lethal. Genomic analysis of the highly virulent strain of *A. hydrophila* ML09-119 causing widespread death in farmed channel catfish showed an absence of the T3SS (Hossain *et al.*, 2013; Pridgeon and Klesius, 2011; Takedar *et al.*, 2013). Genes for the T3SS were absent in the genomic sequence of type *A. hydrophila* ATCC 7966T Seshadri *et al.*, (2006), but as mentioned earlier, this strain was among the most pathogenic strains identified in a mouse challenge (Janda and Kokka, 1991). In contrast, isolates of the related *A. salmonicida* that have lost the plasmid containing the T3SS genes are nearly avirulent (Stuber *et al.*, 2003). Although the T3SS system was absent in *A. hydrophila* ATCC 7966T, genes for a T6SS (type VI secretion system) were present and have been characterized in *A. dhakensis* SSU (Seshadri *et al.*, 2006; Suarez *et al.*, 2008, 2010a, 2010b). Like AexU, the effector protein VgrG1 delivered through the T6SS is an ADP ribosyltransferase that induces HeLa cell toxicity by ADP ribosylation of actin (Suarez *et al.*, 2010a). ADP ribosyltransferase activity prevents the formation of F-actin by the ADP-ribosylation of G-actin leading to apoptosis. A second
A third toxin, RtxA, from *A. hydrophila* is able to cause cell rounding and apoptotic death (Seshadri et al., 2006; Suarez et al. 2012). Suarez et al., (2012) showed the RtxA of ATCC 7966T was exclusively expressed and produced during co-infection of *A. hydrophila* with HeLa cells and was not secreted into the medium, indicating that **rtxA** gene expression required host cell contact. RtxA possesses actin cross-linking activity that resulted in a cell-rounding phenotype and induced host cell apoptosis. The RtxA of *Vibrio cholerae*, which is highly homologous to the RtxA of *A. hydrophila*, is secreted through a Type I secretion system (T1SS) and is likely to be the same secretion apparatus for *A. hydrophila*. It appears that *A. hydrophila* produces several toxins and/or effector proteins (AexU, VgrG1, RtxA) that have redundant functions. What is unknown is if each of these toxins is regulated differently to account for the diverse hosts or host systems they encounter, or what effect the loss of all three toxins have on survival of *A. hydrophila* in a host.

Strains of *A. hydrophila* cause diarrhea by producing enterotoxins (Sha, Kozlova and Chopra, 2002). Three enterotoxins have been identified in *A. hydrophila*. The first is a aerolysin-related cytotoxic enterotoxin (Act) possessing multiple biological activities including the ability to lyse red blood cells,
destroy tissue culture cell lines, evoke a fluid secretory response in ligated intestinal loop models, and induce lethality in mice (Asao et al., 1984, Ferguson et al., 1997; Rose, Houston and Kurosky, 1989; Sha, Kozlova and Chopra, 2002; Xu et al., 1998). Act is a pore-forming toxin similar to the hemolysin aerolysins A (discussed later) that is secreted through a Type II Secretion System and contributes most to intestinal fluid accumulation out of the three toxins. The toxin causes degeneration of crypts and villi in the small intestines, and activates proinflammatory cytokines and arachinoic acid cascades in macrophages and intestinal cells resulting in a fluid secretory response (Chopra et al., 2000). The extent of tissue damage in the host is strongly correlated to the amount of Act secreted.

The other two enterotoxins, Alt and Ast, are cytotonic rather than cytotoxic, causing dysregulation in water absorption in the intestine as a result of increased cAMP levels in enterocytes similar to the action of cholera toxin. Alt is a heat-labile enterotoxin homologous to the Alt enterotoxin in toxigenic strains of E. coli Ast is a heat-stable cytotonic enterotoxin. To further define the role of the three enterotoxins in gastroenteritis, A. hydrophila mutants were generated in which one, two or all three of the enterotoxin genes were knocked out. These mutants and the wild-type strain were tested for their ability to evoke diarrhea in a mouse model (Sha, Kozlova and Chopra, 2002). Fluid accumulation was reduced between 43-64% in the single mutants compared to the wild-type, with the greatest reduction occurring in the Δact mutant. Reduction of fluid accumulation was only 36% for the Δast,Δalt combination, but reached 62 and 73% for the Δact,Δast and Δact,Δalt mutants, respectively. One-hundred percent reduction of fluid accumulation occurred only in the triple mutant A. hydrophila SSU Δact,Δalt,Δast. Colonization of the small intestines of mice by the wild type and mutants was equivalent. The results of this study indicate that Act is the major enterotoxin contributing to the fluid secretory response. Alt and Ast also elicit fluid secretory responses, with Ast having the least significant effect on fluid secretion compared to Alt and Act.

Although Act displays hemolytic activity, it is not the only hemolytic toxin found in A. hydrophila. Early attempts to identify hemolysins from A. hydrophila resulted in partial characterization
of two different hemolysins (Wretlind, Möllby, and Wadström, 1971). The purified hemolysins were independently cytotoxic for HeLa cells and human lung fibroblasts, and lethal to rabbits and mice by intravenous administration. Both hemolysins were dermonecrotic upon intracutaneous injection in rabbit, but did not have any detectable protease activity. This study was the first to demonstrate purified hemolysins as pathogenic factors produced by *A. hydrophila*, and since that time additional toxins and enzymes with hemolytic activity have been identified (Asao *et al*., 1984, Bernheimer, and Avigad, 1974; Erova *et al*., 2007; Heuzenroeder, Wong and Flower, 1999; Hirono and Aoki, 1991; Howard *et al*., 1987, Seshadri *et al*., 2006, Thelestam and Ljungh, 1981; Wang *et al*., 2003; Wong, Heuzenroeder, and Flower, 1998). Two hemolysins receiving most attention are the β-hemolysins Ahhl (Hirono and Aoki, 1991) and aerolysin (Bernheimer and Avigad, 1974; Howard *et al*., 1987). β-pore–forming toxins bind to components in the eukaryotic cell membrane and insert into the bilayer forming a transmembrane β-barrel pore complex causing the cell to swell and lyse (Degiacomi *et al*., 2013). These two hemolysins share little homology (18%) to each other, but show relatedness to well-characterized hemolysins from other bacterial pathogens. Ahhl has 51% amino acid homology to the HlyA hemolysin of *Vibrio cholerae* El Tor strain O17, and falls within the family of hemolysins referred to as HlyA. Aerolysin (AerA) is closely homologous with the cytotoxic enterotoxin Act (Buckley and Howard, 1999, Sha *et al*., 2002); however, comparison of nucleotide and amino acid sequences, biochemistry and interactions with cell membrane components indicate they are different proteins related to the same aerolysin hemolysin family (AerA). Studies in which *ahh1* and *aerA* were detected by PCR amplification indicated these genes are common in *A. hydrophila* isolates recovered from diseased and healthy fish, human patients with diarrhea, extraintestinal infections and various environmental samples including natural waters with *ahh1* occurring more frequently than *aerA* (Aguilera-Arreola *et al*., 2005; Erova, *et al*., 2007; Heuzenroeder, Wong and Flower, 1999; Hussain, *et al*., 2014; Wang *et al*., 2003).

To determine the pathogenic role of these toxins, hemolysin negative mutants were generated using *A. hydrophila* A6, a diarrheal isolate known to be lethal to suckling mice when delivered by gavage
(Wong, Heuzenroeder, and Flower, 1998). Although loss of aerA but not hlyA resulted in some attenuation compared to the wild-type, statistically significant attenuation and complete loss of hemolysis and cytotoxicity to Buffalo Green Monkey (BGM) kidney cell monolayers was achieved when both the hlyA and aerA were inactivated. When assessed in the suckling mouse model, the mean LD$_{50}$ of the wild-type was 6.3 x 10$^7$ and 1.4 x 10$^9$ for the ΔhlyA, ΔaerA double mutant signifying a 20-fold change. In a follow-up study, the group performed a survey to determine the distribution of hlyA and aerA genes in clinical and environmental Aeromonas isolates. For A. hydrophila, hlyA occurred in 96% of the strains, and aerA occurred in 78% of those same strains with 75.4% possessing both genes. All A. hydrophila isolates with the hlyA$^+$aerA$^+$ genotype were β-hemolytic and cytotoxic to Buffalo Green Monkey (BGM) or Green Monkey (Vero) cells (Heuzenroeder, Wong and Flower, 1999). However, A. hydrophila strains that were either hlyA$^-$aerA$^+$ (3.2%) or hlyA$^+$aerA$^-$ (21.3%) were variable in regards to β-hemolysis and cytotoxicity. This evidence indicates that virulence of A. hydrophila is enhanced when both AerA and HlyA hemolysins are present.

In addition to toxins, bacteria release extracellular enzymes promoting destruction of cells and digestion of extracellular matrix constituents of tissues such as collagen, elastin, and glycosaminoglycans. The resultant cell and tissue destruction is accompanied by the release of proteins, nucleic acids, lipids, polysaccharides and other macromolecules such as porphyrins, which are further degraded by additional enzymes to support the nutritional needs of the pathogen. The loss of tissue integrity promotes bacterial spread. Several known and putative determinants of virulence identified as enzymes were found in the genome of A. hydrophila. These included the well-characterized enzymes deoxyribonucleic acid nuclease, elastase, serine protease, chitinase, amylase, phospholipases A$_1$ and C, and enolase as well as new enzymes that may be virulence determinants including collagenase, hyaluronidase, and mucin-desulfating sulfatase (Pemberton, Kidd and Schmidt, 1997; Seshadri et al., 2006).

In a study by Sha et al. (2009), the enzyme enolase was suggested to be required for pathogenesis. Internally this enzyme catalyzes the reversible conversion of 2-phosphoglycerate (2-PGE)
to phosphoenolpyruvate (PEP), but when localized to the cell surface, it is able to bind and promote conversion of human plasminogen to plasmin. The subsequent activation of the fibrinolytic system promotes bacterial penetration by the dissolving of clots leading to the spread of the bacteria. The role of enolase as an important virulence factor was supported when immunization of mice with purified recombinant enolase significantly protected the animals against a lethal dose of the wild-type A. hydrophila. Also, mice immunized with enolase and then challenged with WT bacteria had little histological pathology compared to severe changes found in the infected, nonimmunized group (Sha et al., 2009).

Lipase are produced by many bacterial pathogens. Glycerophospholipid cholesterol acyltransferase (GCAT) is a lipase which catalyzes acyl transfer using phosphatidylcholine and cholesterol to yield 1-acylglycerophosphocholine and cholesteryl ester, a reaction essentially identical to that of the well-known enzyme lecithin:cholesterol acyltransferase of mammalian plasma. The enzyme appears to be present and highly conserved in many species of Aeromonas and has the potential as a genetic marker for the genus Aeromonas (Chacón et al., 2002). The glycerophospholipid-cholesterol acetyltransferase (GCAT) from A. salmonicida, when activated and complexed with lipopolysaccharide, has been shown to be an important hemolytic factor of fish erythrocytes (Røsjø, et al., 1993). Furthermore, a monoclonal antibody directed to A. salmonicida GCAT was capable of neutralizing hemolysis of rainbow trout red blood cells in vitro (Lachmann and Droessler, 2002). However, when fish were challenged with GCAT (ΔsatA) and AspA serine protease (pro-GCAT activator; ΔaspA) deletion mutants, a loss of virulence was not observed thereby questioning the importance of GCAT as a virulence factor (Vipond et al., 1998). The glycerol-phospholipid-cholesterol acyltransferase from A. hydrophila has been purified and described in regards to enzyme structure and activity (Thornton, Howard and Buckley, 1988). In a comparison of GCAT genes between fish isolates of A. hydrophila and A. salmonicida, it was found that the GCAT nucleotide sequences shared 92.9% homology, and the deduced amino acid sequence was 93.7% homologous (Nerland, 1996). Despite the high amino acid similarity,
polyclonal antibodies raised against GCAT of A. hydrophila showed poor reactivity to GCAT of A. salmonicida suggesting major variations in epitopes. Although this enzyme is reported as a virulence factor of A. hydrophila, no study has shown conclusively that GCAT is a virulence factor of A. hydrophila.

Phospholipases catalyze the hydrolysis of phospholipids into fatty acids, thereby breaking down the phospholipid bilayer of host cell membrane leading to cell lysis. Phospholipases A1 (PlA1) and C (Plc) have been reported to occur in A. hydrophila and are encoded in genes pla and plc, respectively (Merino et al., 1999). To study the virulence potential of these phospholipases, mutants lacking phospholipase A1 or C activity were generated and tested for cytotoxicity using Vero cell and EPC (epithelioma papulosum of carp, Cyprinus carpio) monolayers and virulence using an LD50 challenge performed in rainbow trout and mice. Phospholipase C was more cytotoxic, and deletion of the plc (Phospholipase C) gene showed a 10-fold increase in the LD50 for rainbow trout and mouse challenge. When compared to the wild-type strain, cytotoxicity was significantly reduced for the plc- but not for the pla- mutant, suggesting Plc but not PlA1 is a cytotoxic enzyme. For the determination of virulence no difference in the infectious dose was observed between wild-type and pla- mutant; however, the plc- mutant showed a higher LD50 (1 to 2 log units) in both mice and fish, compared to the wild type. This evidence suggested that phospholipase C is an important virulence factor for A. hydrophila (Merino et al., 1999).

Two studies conducted by Cascón et al. (2000a, 2000b), were performed in order to determine the roles of two proteases: a heat-labile serine protease AhpA and a heat-stable metalloprotease AhpB as potential virulence factors produced by A. hydrophila fish strain AG-2. The gene ahpA encoding the serine protease of A. hydrophila AG-2 was cloned into E. coli and a non-proteolytic A. salmonicida subsp. masoucida (Cascòn et al., 2000a). The 68 kDa enzyme was shown to be highly proteolytic to casein, but poorly so to elastin. The predicted amino acid sequence of AhpA shared 88% homology to AspA, the serine protease of A. salmonicida mentioned earlier that is important to the activation of GCAT (Eggset et al., 1994). To further determine the importance of AhpA in virulence, an ahpA- mutant was
generated and tested for enzymatic activity and LD<sub>50</sub> in a rainbow trout challenge. The ahpA mutant had notably less proteolytic activity than the wild type on a casein-supplemented medium; however, intraperitoneal injection of the ahpA mutant into trout resulted in nearly the same LD<sub>50</sub> as the wild type. The data suggested that the AhpA serine protease does not play a direct role in virulence and is in agreement with a similar study using A. salmonicida aspA- mutants (Vipond et al., 1998); however, it might be important to the processing of other protein precursors as well as providing nutrients for the bacteria in a host (Eggset et al., 1994). In contrast, Esteve and Birkbeck (2004) purified a serine protease of 68 kDa with caseinolytic activity that was lethal for eels. The protease was purified from an epizootic strain of A. hydrophila (EO63) isolated from the liver of a European eel (Anguilla anguilla), which was collected during an outbreak of red fin disease in a freshwater farm. Purified enzyme was toxic to eels at an LD<sub>50</sub> of 1.1 µg/fish. The molecular weight and activity of this serine protease was the same for the serine protease reported by Cascòn et al. (2000a). Another serine protease, but one of 22 kDa, was purified by high performance liquid chromatography (HPLC) from the extracellular products of a pathogenic strain of A. hydrophila (B<sub>32</sub>) originally isolated from rainbow trout (Oncorhynchus mykiss) with fatal motile aeromonas hemorrhagic septicemia (Nieto et al., 1991; Rodríguez, Ellis and Nieto, 1992). The enzyme was cytotoxic and had an LD<sub>50</sub> of 150 ng/g fish (rainbow trout fingerlings, intraperitoneal injection); however, further distinction is needed between this serine protease and the enzyme reported by Cascòn et al. (2000a) and Esteve and Birkbeck (2004). A heat-stable α-hemolysin of 68 kDa, which is the same size as the elastolytic serine protease described by others (Cascòn et al., 2000a; Esteve and Birkbeck, 2004), was purified from the same extracellular products of A. hydrophila (B<sub>32</sub>) and had an LD<sub>50</sub> of 2 µg/g fish (Rodríguez, Ellis and Nieto, 1992).

The second protease, a thermostable metalloprotease (ahyB or ahpB), was studied by Cascón et al. (2000b), using a similar approach as described in the previous paragraph. Amino acid sequence alignment demonstrated significant homologies between AhpB and metalloproteases from other pathogens including the elastase from Pseudomonas aeruginosa. Production of the ahpB gene product
resulted in high elastolytic activity but low caseinolytic activity. To determine the role of AhpB protease in the pathogenesis of *A. hydrophila*, the LD<sub>50</sub> was determined for *A. hydrophila* wild-type and *ahpB* mutant by intraperitoneal challenge of rainbow trout. The LD<sub>50</sub> corresponding to the mutant was 3 \times 10^7 CFU, which was 100 times greater than the wild-type. Fish injected with the parental strain died within 3 days, whereas deaths were recorded up to 6 days following injection with *ahpB* mutant. The experimental outcomes support the role of AhpB as a virulence factor (Cascón *et al.*, 2000b). This was further supported in a separate study in which the metalloprotease, purified from *A. hydrophila* EO63 (eel epizootic strain) was toxic to eels at an LD<sub>50</sub> of 3.5 µg/fish (Esteve and Birkbeck, 2004). It is likely that the elastase degrades connective tissue and proteins respectively providing additional nutrients, while the serine protease promotes the activation of enzymes that are produced following colonization of the bacterium. A metalloprotease similar in size to the metalloprotease described by Cascón *et al.* (2000b) and Esteve and Birkbeck (2004) was purified by HPLC from the extracellular products of a pathogenic strain of *A. hydrophila* (B<sub>32</sub>) discussed earlier (Rodríguez, Ellis and Nieto, 1992). Unlike the 22 kDa serine protease purified from this same strain, the metalloprotease was not cytotoxic; however, it was lethal to rainbow trout fingerlings at the same LD<sub>50</sub> (150 ng/g fish) as the serine protease.

Extracellular products (ECPs) secreted from bacteria, such as many of the toxins and enzymes described above, are considered to be essential for pathogenesis. It appears that the virulence associated with *A. hydrophila* infections is highly dependent on the combination of toxins and enzymes present in the ECP. Many of these factors, such as the serine protease and metalloprotease, are under quorum sensing regulation whereas other factors, such as aerolysin and aerolysin-related cytotoxic enterotoxin, are not (Bi, Liu and Lu, 2007; Khajanchi *et al.*, 2009, 2012; Swift *et al.*, 1997, 1999). Because of the relationship between the quorum-sensing dependent expression of some of these virulence factors and the severity of disease, the quorum-sensing regulators AhyR and AhyI are considered virulence factors of *A. hydrophila* and other aeromonads (Khajanchi *et al.*, 2009; Seshadri *et al.*, 2006; Swift *et al.*, 1999).

Alteration of genes encoding these factors greatly affects pathogenicity. For example, significant loss of
protease, nuclease and hemolysin activities, and loss of cytotoxicity of epithelioma papillosum of carp (Cyprinus carpio) cells was reported for an ahyr deletion mutant of the fish pathogen A. hydrophila J-1 when compared to the parent strain (Bi, Liu and Lu, 2007). Loss of the S-layer protein and reduced adherence was also noted in the mutant. Attenuation related to the loss of ahyr was further supported by fish challenge (swordtail, Xiphophorus helleri Hecke). The LD$_{50}$ of A. hydrophila J-1 injected intraperitoneally was 1.8 x 10$^5$ CFU with 100% fish mortality within 3 days, whereas the ahyr mutant had an LD$_{50}$ exceeding 10$^9$ CFU and no signs of illness or death after 1 week post-inoculation. When the same gene (ahyr) and ahyI were deleted in the human diarrheal isolate A. hydrophila SSU (now A. dhakensis), protease production was substantially reduced, and dysregulation of the type 6 secretion system and abnormal biofilm formation occurred (Khajanchi et al., 2009). In an animal challenge, 100% of mice injected intraperitoneally with 3 x 10$^7$ CFU of the wild-type strain dies within 6 days, whereas 50% of the mice injected with the same dose of the mutant survived over a 16 day test period. Death of the other 50% occurred within 4 days post-inoculation. Although the secretion, but not the expression, of effectors Hcp and VgrG1 through the T6SS was interrupted in the ahyrI mutant, the secretion of AexU by the T3SS was unaffected suggesting T6SS but not the T3SS is under quorum sensing regulation (Khajanchi et al., 2009).

In addition to the proteases, lipases, hemolysins and toxins that are common, toxic extracellular products of A. hydrophila, a small 15.5 kDa peptide with acetylcholinesterase activity has been described (Nieto et al., 1991). Acetylcholinesterase (AcChE) hydrolyzes the neurotransmitter acetylcholine, producing choline and an acetate. This bacterial AcChE was purified from the ECPs of A. hydrophila strains associated with fatal motile Aeromonas hemorrhagic septicemia in rainbow trout (Oncorhynchus mykiss) (Nieto, et al. 1991). Injection of rainbow trout with a crude ECP preparation from Aeromonas hydrophila B$_{32}$ (a representative strain from this outbreak, LD$_{50} = 3.2 x 10^4$ CFU) demonstrated that factors within the ECP were responsible for the disease. Among the components of the ECP were a serine protease, metalloprotease, α-hemolysin (discussed earlier; Rodríguez, Ellis and Nieto, 1992) and an acetylcholinesterase (Nieto et al., 1991). Purified forms of the four components were shown to be
independently lethal to trout. Purified AcChE was 300 times more toxic than the crude ECP and was the most toxic component of the four ECP proteins purified from *A. hydrophila* B32, having a very low LD$_{50}$ of 50 ng/g fish. The bacterial AcChE, called ‘ichthyotoxin”, was not cytoytic, proteolytic or hemolytic and gross pathology was absent in injected fish; however, it did produce an observable narcotic effect suggesting that the toxin affected the central nervous system of the fish. The potential neurotoxic role of *A. hydrophila* “ichthyotoxin” was supported in a later study in which *A. hydrophila* AcChE was detected in brain homogenates from challenged fish (IP injection), demonstrating that the toxin did gain access to brain tissue and is produced during *in vivo* infection (Rodríguez, Ellis and Nieto, 1993).

The AcChE-toxin is secreted as a 45 kDa protoxin and subsequently converted into lower molecular weight fragments by the proteolytic action of other components, a serine protease and a metalloprotease, present in the ECP. The smallest, stable and highly active fragment has a MW of 15 kDa and was not immunogenic in fish (Pérez, Rodríguez, Fernández-Briera and Nieto, 1998). The 15 kDa toxin was immunogenic in rabbit, and the lethal effect of the toxin in trout could be neutralized by rabbit anti-15 kDa toxin antiserum. The rabbit antiserum reduced mortality (42% no treatment, 16% antiserum treated) of fish injected with crude ECP, indicating the toxin was a major, but not the sole toxic component (Pérez et al., 2002). Additional studies by this group showed the (neuro) toxin was present in the ECPs of both virulent and avirulent strains of *Aeromonas hydrophila*, other aeromonads and several known fish pathogens within the Vibrionaceae such as *Vibrio anguillarum* and *V. splendidus* (Rodríguez, Fernández and Nieto, 1993; Pérez, et al., 1998). The presence of acetylcholinesterase activity within the ECPs of avirulent strains of *A. hydrophila* indicates a need to investigate why virulence is low even when the AcChE ichthyotoxin is produced. Because activation of AcChE requires action by proteases (serine and metalloproteases) within the ECP, defining virulence may be more dependent on whether or not those activating proteases are present and at levels suitable for activating AcChE.

In the study reported by Pridgeon *et al.* (2013), the ECPs from four *A. hydrophila* isolates (ML-10-51K, ML-10-81K, ML-10-205K, and ML-10-208K) were investigated. The bacteria were recovered from the kidneys of diseased channel catfish taken from fish ponds in West Alabama where a major,
deadly epizootic of *Aeromonas* septicemia had occurred in 2010. *A. hydrophila* strain AL98-C1B was included as it previously was used in virulence studies in 2009 (Pridgeon and Kelsius, 2011). The LD₅₀ values for the 2010 catfish isolates were 1.3 x 10⁵ CFU/fish, whereas the AL89-C1B value was 2.8 x 10⁷ CFU/fish, making the 2010 isolates approximately 200 times more virulent. ECPs from the 5 isolates were used to test toxicity to fish. The results showed that ECPs from the 2009 strain AL89-C1B were unable to kill any catfish fingerlings at a dosage of 50 ug/fish (maximum dose), whereas the LD₅₀ for ECPs from 2010 strains was much lower: 16 ug/fish (*A. hydrophila* ML-10-51K). To determine the toxic components within the ECPs, the components were separated into fractions using cation-exchange chromatography and screened for toxicity to catfish G1B gill cells and channel catfish fingerlings. Nine of 30 fractions were found to be toxic and further analysis by mass spectrometry identified 23 proteins common to the 9 toxic fractions. Well-known virulence factors including hemolysin, aerolysin, elastase, lipase, flagellar proteins, porins and major adhesion Aha1 were part of those proteins identified as well as others with metabolic or unknown functions. A 16 kDa putative uncharacterized protein, which would be in the size range of the 15.5 kDa peptide with acetylcholinesterase activity described by Nieto *et al.* (1991), was among those factors common to all toxic fractions (Pridgeon *et al.*, 2013). Two additional putative uncharacterized proteins each at 23 kDa are similar in size to the 22 kDa serine protease purified from the extracellular products of a pathogenic strain of *A. hydrophila* B₁₂, which was shown to be toxic to fish and a factor necessary for the activation of the AcChE protoxin (Rodríguez, Ellis and Nieto, 1992). However, additional studies would be required to determine if these factors were the same. Finally, hemolytic activity, protease activity, and nuclease activity of the 2010 isolates of *A. hydrophila* were found to be higher than strain AL98-C1B.

In a follow-up study by Zhang *et al.* (2014), immunization with an ECP vaccine was tested to determine if immunity to highly virulent *A. hydrophila* strains could be produced in channel catfish. ECPs were purified from the virulent strain ML-10-51K, emulsified with Freund’s complete adjuvant and injected into catfish. Two weeks post immunization with the ECP emulsion, all experimental fish
survived at a challenge dose of $2 \times 10^7$ CFU/fish, and all sham-immunized fish died within 4-5 hours. The EPC immunized fish were challenged twice more with no mortality seen and increases in serum titers were observed. The immune serum from the fish vaccinated with EPCs conferred partial passive immunity to naïve catfish. The anti-ECP serum allowed for a 48 hour delayed mortality rate, but not full protection against infection with ML-10-51K. This evidence suggests that a passive immunization with ECPs provides protection against infection but anti-ECP serum from previously vaccinated fish are able to slow down the mortality but not completely eliminate it. Since ECPs secreted from A. hydrophila are important to the virulence of the bacterium, research groups are attempting to develop vaccines to protect against the toxicity of ECPs.

Another study, examined the efficacy of four different vaccines, formalized whole cell (WC), an extracellular product (ECP), an outer membrane protein (OMP) and heat-inactivated biofilm (BF) in protecting goldfish (Carassius auratus) against challenge (Viji et al., 2013). These vaccines were developed from a virulent strain of A. hydrophila (AHV1) previously isolated from diseased goldfish. Two preparations of vaccines were made, one with the immunoadjuvant (steroidal saponins extracted from tuber powders of Asparagus racemosus) and one without. Goldfish (Carassius auratus) were inoculated by intraperitoneal injection twice (days 1 and 25) with each of these vaccines. Groups of fish receiving each vaccine treatment were challenged with a live A. hydrophila AHV1 at 25 and 50 days post vaccination and monitored over 10 days. All of the fish that were sham-vaccinated (PBS) died within 6 days. At day 9 post-challenge the percentage of fish surviving challenge were as follows: 60% of fish receiving WC without adjuvant and ECP with adjuvant; 70% of fish receiving ECP, OMP or BF without immunoadjuvant, and 90% of fish receiving BF with adjuvant. Overall OMP and BF preparations with the immunoadjuvant provided greater protection than ECP or WC based vaccines. The vaccine trials further support that virulence associated with A. hydrophila strains is multifactorial rather than dependent on a single major factor, and vaccines that are effective in providing protection against natural infection must be inclusive of multiple virulence factors.
It has become apparent that *A. hydrophila* differ in the pathogenic potential as a result of the presence/absence of virulence factors associated with the disease process and severity. Therefore the term ‘pathotypes’ is now used to describe strain variation and give a comparison of virulent and avirulent strains with their ability to cause disease. In the attempt to further define pathotypes of *A. hydrophila*, two recent studies comparing the genomes of clinical strains of *A. hydrophila* were completed (Grim et al., 2013, 2014). In the first study, the genomes of two strains of *A. hydrophila* (E1 and E2) isolated from a polymicrobial wound infection Shak et al., (2011) were pyrosequenced and compared against the genomes of other aeromonads including the environmental type strain *A. hydrophila* ATCC 7966ᵀ. In addition, these strains were tested for virulence in mice, serum resistance, biofilm formation, hemolytic and protease activities. Strain E1 was highly lethal to mice via intraperitoneal injection producing 100% mortality within 2 days of challenge at a dose of 1 x 10⁷ CFU, while minimal mortality was observed for strain E2, the highly virulent *A. dhakensis* SSU and *A. hydrophila* ATCC 7966ᵀ. The genome of E1 contained a number of virulence factor-encoding genes not found in E2 or ATCC 7966ᵀ including a functional T3SS and toxin AexU, the presence of cytotoxin Act, a lateral flagellar system, exoproteins with putative adhesion and hemagglutinin properties and a number of genomic regions of unknown function. Both E1 and E2 displayed serum resistance, hemolysis of red blood cells, protease activity, motility and no biofilm formation. Serum resistance, swarming motility and hemolytic activity (primarily due to Act) was greater for strain E1, whereas protease activity was comparatively greater for strain E2. Production of toxin AexU and cytotoxicity occurred when E1 was in contact with HeLa cells, but was absent in E2. The results indicated that enhanced virulence of *A. hydrophila* strain E1 pathotype is likely dependent on a combination of factors including serum resistance, the production of toxins Act and AexU and swarming motility, and possibly other undefined factors, but not necessarily protease production (Grim et al., 2013).

*A. hydrophila* wound isolates E1 and E2 were included in a second comparative study analyzing virulence factors in necrotizing-fasciitis causing strains of *A. hydrophila* (Grim et al., 2014). Two strains
of *A. hydrophila*, designated NF1 and NF2, were isolated from an amputated leg and later from the amputation stump of a patient being treated for necrotizing fasciitis. Also included in the study was the *A. hydrophila* type strain ATCC 7966ᵀ, two environmental strains (*A. hydrophila* Riv3 and *A. jandei* Riv2) isolated from river water where the infection was acquired, and other pathogenic aeromonads including *A. caviae* NM22 and *A. dhakensis* SSU isolated from stools of patients with diarrhea. The genomes of all strains were sequenced, and several virulence factor assays were performed to determine if there were pathogenic features unique to these strains. Pairwise average nucleotide identity (ANI) comparisons revealed that *A. hydrophila* NF1 and NF2 were different strains and distinct from all other reference *A. hydrophila* strains including wound isolates E1 and E2. However, comparison of genomic content indicated that the clinical isolates had more genes in common than did environmental and clinical strains.

Mice were challenged by intraperitoneal injection (septicemia-mouse model) and intramuscular injection (necrotizing fasciitis mouse model). When 5 x 10⁷ CFU were delivered by intraperitoneal injection, 100% mortality occurred within 48 hours for *A. dhakensis* SSU and 90% for *A. hydrophila* NF1 (24 hours) and *A. jandei* Riv2 (48 hours). Mortality rates were 35 and 45% for *A. hydrophila* NF1 and Riv3, respectively. A 10% mortality rate occurred among mice challenged with *A. hydrophila* ATCC 7966ᵀ. NF1, SSU and Riv2 displayed significantly higher motility (swimming and swarming) and biofilm formation than the other test strains. However, when the same strains were injected by intramuscular route, NF2 was much more virulent than NF1 and disseminated in high numbers from the muscle to the viscera of mice. While NF1 and NF2 secrete toxin AexU, other virulence-associated factors or activities were variable. For example, protease production was higher in NF2 than in NF1, whereas Act activity was higher in NF1 than NF2. Furthermore, secretion of ExoA (homolog of *P. aeruginosa* Exotoxin A) was detected for NF2, but not NF1. No highly unique virulence factor from those studied was identified in the study, and it is likely that different virulence factors may only be advantageous depending on the mode of infection.

In another recent study, Chen *et al.* (2014c), examined the virulence of four *Aeromonas* species, *A. hydrophila*, *A. veronii*, *A. dhakensis*, and *A. caviae*, isolated from hospital patients with bacteremia.
Sepsis-related and in-hospital mortality rates of patients with bacteremia caused by *A. dhakensis* were significantly greater than bacteremia caused by the other three species. Polymerase chain reaction was used to detect the presence of the genes for heat stable enterotoxin (*ast*), hemolysin (*ahh1*), aerolysin (*aerA*), component of the T3SS (*ascV*), and ADP-ribosylating toxin (*aexT*). These genes were shown previously to encode virulence factors. The hemolysin gene *ahh1* occurred in the majority of *A. dhakensis* and *A. hydrophila* isolates, whereas *aerA* was present in about 33% of these same isolates. Both *ahh1* and *aerA* were absent in all *A. veronii* and *A. caviae* isolates even though 100% of *A. veronii* and 44% of *A. caviae* isolates produced hemolytic patterns on blood agar plates. All *A. hydrophila* were positive for *ast*, and 70% were positive for *ascV* yet the T3SS effector protein gene *aexT* was absent in all *A. hydrophila* (*A. dhakensis* and *A. caviae*) isolates and present in the majority *A. veronii* isolates. Agar plate assays were used to detect exoprotease, amylase, DNase and hemolysin activities. One hundred percent of *A. hydrophila* (n=9) and *A. dhakensis* (n=9) were positive for each enzyme activity, as was nearly all of the 9 *A. veronii* isolates tested. Although the majority of *A. caviae* isolates tested positive for DNase (100%), amylase (89%), and exoprotease (78%) activities, only 44% produced hemolysis on blood agar plates. Isolates were investigated further using a cell cytotoxicity assay, mouse (*BABL/c*) longevity and pathology after intramuscular injection and liquid-toxic (LT) assay of the nematode *Caenorhabditis elegans* infected with aeromonads. Mouse C2C12 fibroblast cells, infected the cells with strains of *Aeromonas* at a multiplicity of infection (MOI) of 25 and toxicity was measured by release of lactate dehydrogenase (LDH). The results showed that on average *A. dhakensis* was significantly more cytotoxic than *A. veronii* or *A. hydrophila* (which were nearly the same). *A. caviae* did not produce significant cytotoxicity. In the mouse challenge, only four of the 18 mice inoculated with *A. dhakensis* survived after 14 days with 14 dying in the first 48 hours. In contrast, 16 of the 18 mice infected with *A. hydrophila* and all of the *A. caviae* or *A. veronii* inoculated mice survived all 14 days. The results in the mouse model correlate with the results of the LT nematode assay as *A. dhakensis* was significantly more lethal to the nematode than the other strains. Ninety percent of individuals were dead in the first day of challenge and nearly all individual worms were dead at day 3. Although not as lethal as
A. dhakensis, survival rates of nematodes challenged with A. hydrophila were significantly lower (20% at day 3) than those for A. veronii (66%) or A. caviae (70%). Animal challenge and cytotoxicity assays clearly demonstrated greater pathogenicity of A. dhakensis compared to the other aeromonads, but there appeared to be no correlation between this trait and the presence/absence of the virulent factor genes or the enzyme activities discussed earlier. However, the study did indicate that C. elegans could be a suitable substitute to using mice to study Aeromonas infections as the two models correlated well in regards to lethal or non-lethal outcomes associated with infections with the four Aeromonas species.

Since the first studies of P. aeruginosa pathogenesis in the nematode C. elegans (Darby et al., 1999; Tan, Mahajan-Miklos and Ausubel, 1999) numerous Gram-positive and Gram-negative bacterial pathogens have been reported to infect and eventually kill this host (Pradel and Ewbank, 2004). The nematode C. elegans has multiple experimental advantages such as short generation time, large numbers of progeny, easy mode of infection, and a simple innate immune system. C. elegans has a generation time of 4 days to go from egg to adult worm, and produce approximately 300 eggs per generation. This short generation time and production of large progeny provides sufficient number of organisms for experiments. The mode of infection of the nematode is simple, a pathogen of choice can be swapped for the normal E. coli feeder strain OP50 and the nematode will simply ingest the bacteria. Many factors of the innate immune system show little to no variation between vertebrate and invertebrate organisms. C. elegans has emerged as an extremely powerful model for the study of innate immunity. The amenability of C. elegans for genetic analysis has resulted in the use of this nematode as a model system for studying host-microbe interactions. Forward and reverse genetic analyses led to detailed characterization of the seven main signaling cascades; however, the correlation between mRNA levels and the proteins they code is generally weak. Furthermore, biological activities including immune response are controlled primarily by proteins which are the main mediators of physiological functions. Because of this, proteomics provides information that cannot be fully understood through transcriptomics of genomics.
A proteomic analysis was developed to study the immune response in *C. elegans* at days 1, 3 and 5 post-infection with *A. hydrophila* ATCC 7966T (Bogaerts et al., 2010). The number of differentially synthesized proteins decreased with time: 47 proteins at day 1, 23 at 3 days and 20 after 5 days. The most strongly induced protein after infection was the galactose binding lectin (galectin) LEC-1. The level of the C-type lectin, CLEC-63, was also increased. Lectins are common effector molecules of the innate system generally binding to carbohydrate moieties on the surface of pathogens resulting in interference between host-pathogen surface interactions, activation of the complement or complement-like cascade leading to lysis of the pathogen, or marking the pathogen for clearance by wandering and resident phagocytes. Infection also induced the synthesis of three lipid binding proteins (LBP-1, LBP-7 and FAR-2). Lipid binding proteins are known to bind to bacterial lipids and mark the pathogen for clearance. Lipid binding proteins also modulate immune responses by controlling the amount of lipid-derived signal molecules such as prostaglandins and other arachidonic acid metabolites. A number of unknown proteins were also isolated and identified, but their roles in immune defense remain unknown. Despite repeated washings of the host, three *Aeromonas* proteins previously identified to be associated with virulence were among the host proteins and were present at each time point. These proteins included a major adhesin Aha1, a TonB–dependent siderophore receptor, and the third was a hypothetical protein believed to be involved with entering the host. This work provides information about the innate immune response produced by the host upon challenge with a pathogen. Although the investigators used *Aeromonas hydrophila* ATCC 7966T with some success, this strain lacks several virulence factors such as the T3SS, lateral flagella and S-layer found in other “highly virulent” strains of *A. hydrophila*. It is likely that the host proteome, and pathogen factors co-purified with the host proteome, will vary according to the strain used in the challenge.

Based on the information presented above concerning virulence factors, one may consider if there is a single critical factor required for virulence, or if virulence is multifactorial thereby requiring the presence of multiple factors for successful colonization and subsequent disease. Using a genetic subtraction approach, Yu *et al.* (2005), identified 19 putative virulence factors in a highly virulent strain
of *A. hydrophila* that were not present in avirulent strains. Gene deletion mutants were constructed and tested in a blue gourami fish model to determine if the putative virulence genes played a significant role in pathogenesis. A variety of single virulence gene knockout mutants were generated for highly virulent strains AH-1 and AH-3, but failed to show significant change in the LD\textsubscript{50}, except for the single knockout in the T3SS (*ascN*), which resulted in a one-log increase in the LD\textsubscript{50} value was observed. Additionally, double deletion mutants were created in AH-1 resulting in a small and insignificant change to the LD\textsubscript{50} value when compared to the wild type. However, a triple mutant in which the genes for the S-layer, metalloprotease, and serine protease (*AhsA, MepA, SerA*) were deleted resulted in a significant 1 log increase in the LD\textsubscript{50}, which provided support for the idea that *A. hydrophila* pathogenicity is multifactorial. Taken as a whole, disease caused by *A. hydrophila* is complex with bacterial strains, infection route, and animal model all being important variables.

The overall objective of this work was to determine the presence of specific virulence factors in strains of the bacterial pathogen *A. hydrophila*, and test these strains in a cell-culture assay to test for cell cytotoxicity and an animal challenge assay for delineating pathotypes. First, defining a pathotype would require identifying virulence factors inherent to each strain uses. *Aeromonas* isolates from human, fish, other animals and environment sources were used to detect the presence or absence of selected virulence factor genes. To date, many virulence-associated factors encoded in genes have been reported in *A. hydrophila*. Some of these factors were selected based on previous studies as described earlier that provided evidence supporting their role in virulence. The factors vary between different strains, therefore screening for these factors will allow for the identification of pathotypes based on presence of the selected genes. The virulence factors selected for this study included: aerolysin (*aer = aerA*), hemolysin (*ahh1*), cytotoxic enterotoxin (*act*), phospholipases A1 and C (*lip*), an S-layer protein (*ahsA*), enolase (*eno*), elastase (*ahyB*), serine protease (*ser*), a Type IV *Aeromonas* pilus (*tapA*), and two genes that make up a T3SS (*aopB* and *ascV*). Once the presence of virulence factor genes was confirmed, phenotypic assays were used to confirm the expression and activity of these factors. Pathotypes were determined according
to the pattern of virulence factor genes and expression. The virulence factor profiles of each strain were
determined and representative pathotypes were used to challenge rainbow trout (Oncorhynchus mykiss)
gonad cells (RTG-2; ATCC CCL-55) in a cell culture assay and Caenorhabditis elegans in an animal
challenge assay. It was hypothesized that survival rates of C. elegans challenged with various pathotypes
of A. hydrophila, as well as A. bestiarum and A. salmonicida, would differ such that the nematode could
be used a suitable animal model for investigating host-pathogen interactions varying virulent strains of
Aeromonas.
MATERIALS AND METHODS

**Bacterial strains and culture specifications.** A total of 28 *Aeromonas* strains were selected for this study, including 9 human clinical strains, 14 strains from fish, leech and reptile origins and 5 from environmental or food sources (Table 1). Included in this study were the type strains for *Aeromonas hydrophila* (ATCC 7966<sup>T</sup>), *A. bestiarum* (ATCC 51108<sup>T</sup>), and *A. salmonicida* subsp. *salmonicida* (ATCC 33658<sup>T</sup>). All strains except Ahy ML09-119 were previously used in studies at the University of South Florida (USF) in Tampa (McGarey, et al., 1990; McGarey et al., 1991; McGarey, D.J., 1991), preserved frozen at -80°C and transferred to Kennesaw State University. *A. hydrophila* ML09-119 is a highly virulent strain causing widespread mortality in farmed catfish and was provided by Dr. Mark Liles of Auburn University (Hossain et al., 2014). Each isolate was grown on Ryan’s *Aeromonas* medium (Oxoid Ltd, Hampshire, UK); a formulation for the selective growth of *Aeromonas* species. Flat, dark green isolated colonies were sub-cultured onto nutrient agar (Difco, Franklin Lakes, NJ) for use in diagnostic tests. All strains were verified as aeromonads by observing the characteristic colony traits on nutrient agar (convex, smooth, entire, round, opaque), Gram-negative bacilli in a single arrangement, and oxidase positive reaction.

Once confirmed, each strain was inoculated into a buffered, semi-solid maintenance medium containing a base of Minimal Broth Davis without Dextrose (K₂HPO₄, KH₂PO₄, C₆H₇NaO₇, MgSO₄ and (NH₄)₂SO₄; Difco, Franklin Lakes, NJ) to which 0.1% yeast extract (w/v), 1.0% peptone (w/v), 0.5% NaCl (w/v) and 0.8% agar (w/v) were added. This medium allowed the isolates to remain viable for up to 2 months. In addition to the maintenance medium, each strain was frozen in Luria-Bertani (LB) broth (Difco, Franklin Lakes, NJ) with 10% glycerol and placed at -80°C for long term storage and later use.
Table 1. A list of the twenty-eight strains of *Aeromonas* used in this study included *Aeromonas hydrophila* (Ahy), *Aeromonas bestiarum* (Abe) and *Aeromonas salmonicida* (Asa). Strains were acquired from the American Type Culture Collection (ATCC), University of South Florida (USF, Tampa, FL), National Collection of Marine Bacteria (NCMB) and Auburn University (Auburn, AL).

<table>
<thead>
<tr>
<th><em>Aeromonas</em> strain</th>
<th>Collection Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahy 7966&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>Tin of milk with a fishy odor, Type strain</td>
</tr>
<tr>
<td>Ahy 35654</td>
<td>ATCC</td>
<td>Unknown source; QC strain for API</td>
</tr>
<tr>
<td>Ahy 43408</td>
<td>ATCC</td>
<td>Nasal ulcer of Leopard frog, <em>Rana pipiens</em>, CA, US</td>
</tr>
<tr>
<td>Ahy 43409</td>
<td>ATCC</td>
<td>Skin ulcer of California red-legged frog, <em>Rana aurorus dragtoni</em>, CA, US</td>
</tr>
<tr>
<td>Ahy 43414</td>
<td>ATCC</td>
<td>Ulcerated stomach of a boa constrictor, <em>Constrictor constrictor</em>, US</td>
</tr>
<tr>
<td>Ahy 49140</td>
<td>ATCC</td>
<td>Clinical isolate; QC strain for Microscan</td>
</tr>
<tr>
<td>Ahy 49847</td>
<td>ATCC</td>
<td>Clinical specimen, John’s Hopkins; USQC strain for Becton Dickinson</td>
</tr>
<tr>
<td>Ahy 51307</td>
<td>ATCC</td>
<td>Leech intestine, US</td>
</tr>
<tr>
<td>Ahy 1127</td>
<td>USF</td>
<td>Skin lesion of a gizzard shad, <em>Dorosoma cepedianum</em>, Lake George, FL, US</td>
</tr>
<tr>
<td>Ahy 1135</td>
<td>USF</td>
<td>Skin lesion of a gizzard shad, <em>Dorosoma cepedianum</em>, FL, US</td>
</tr>
<tr>
<td>Ahy 1165&lt;sup&gt;*&lt;/sup&gt;</td>
<td>USF</td>
<td>Blood from gizzard shad, <em>Dorosoma cepedianum</em>, FL, US</td>
</tr>
<tr>
<td>Ahy 1275</td>
<td>USF</td>
<td>Human stool, US</td>
</tr>
<tr>
<td>Ahy 1277</td>
<td>USF</td>
<td>Human wound, US</td>
</tr>
<tr>
<td>Ahy 1279</td>
<td>USF</td>
<td>Human urine, US</td>
</tr>
<tr>
<td>Ahy 1280</td>
<td>USF</td>
<td>Human clinical isolate, US</td>
</tr>
<tr>
<td>Ahy 1282</td>
<td>USF</td>
<td>Human clinical isolate, US</td>
</tr>
<tr>
<td>Ahy 1283</td>
<td>USF</td>
<td>Human feces, US</td>
</tr>
<tr>
<td>Ahy 1284</td>
<td>USF</td>
<td>Human feces, US</td>
</tr>
<tr>
<td>Ahy 1288</td>
<td>USF</td>
<td>Skin of normal yellow mouth trout, <em>Cynoscion regalis</em>, FL, US</td>
</tr>
<tr>
<td>Ahy Y9</td>
<td>USF</td>
<td>Sediment isolate, Hillsborough River, FL, US</td>
</tr>
<tr>
<td>Ahy ML09-119</td>
<td>Auburn</td>
<td>Channel catfish (<em>Ictalurus punctatus</em>) with motile <em>Aeromonas</em> septicemia, AL, US</td>
</tr>
<tr>
<td>Abe 51108&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>Infected fish, France</td>
</tr>
<tr>
<td>Abe 14715</td>
<td>ATCC</td>
<td>Juvenile silver salmon intestines, <em>Oncorhynchus kisutch</em>, France</td>
</tr>
<tr>
<td>Abe BAA-231</td>
<td>ATCC</td>
<td>Cake, April 1995</td>
</tr>
<tr>
<td>Abe 1286</td>
<td>NCMB</td>
<td>Mouth lesion, rainbow trout, <em>Salmo gairdneri</em>, US</td>
</tr>
<tr>
<td>Asa 33658&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>Atlantic salmon, <em>Salmo salar</em>; United Kingdom</td>
</tr>
<tr>
<td>Asa 1287</td>
<td>USF</td>
<td>Sewage effluent, US</td>
</tr>
<tr>
<td>Asa 1300</td>
<td>USF</td>
<td>Skin lesion of Atlantic croaker, <em>Micropogonias undulates</em>, St. John’s River, FL, US</td>
</tr>
</tbody>
</table>

<sup>*</sup>Phenotype consistent with *Aeromonas dhakensis*
**Isolation of genomic DNA.** Genomic DNA (gDNA) was isolated from each isolate using the Wizard® Genomic DNA purification kit (Promega, Madison, WI). One colony from a primary culture plate was aseptically transferred to 5 mL of LB broth and grown overnight at 30°C. One-milliliter of broth culture was used to isolate the gDNA following the manufacturer’s instructions for Gram-negative bacteria: *Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria*, Wizard® Genomic DNA Purification Kit (www.promega.com/protocols/). Briefly, the protocol required concentrating bacterial cells by centrifugation (10,000 x g, 2 min), followed by resuspension of the cell pellet in lysis solution and heat treatment (80°C) to lyse the cells. RNAse was added and after a 20-minute incubation (37°C) protein precipitation and removal, and then DNA precipitation in alcohol were performed. Once the DNA was washed and dried, it was dissolved in a rehydration buffer solution. The DNA preparation was stored at 4°C as needed. The concentration and purity of each gDNA preparation were measured using the ThermoScientific® NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Inc, Waltham, MA) at 260/280 nm. The concentration of DNA was determined using the formula 1 OD = 50 μg/mL DNA.

**PCR screening for virulence factor genes.** The purified gDNA from each *Aeromonas* strain was tested for the presence of 10 different virulence factor genes (Table 2) using endpoint polymerase chain reaction (PCR). Each reaction was place into 0.2 mL thin-walled PCR tubes using a total reaction volume of 25μl containing 12.5μl of 2X GoTaq® Hot Start Master Mix (Promega, Madison, WI), 2μl of primers for a 1 μM final concentration, 1-2μl of gDNA template (100ng amount of DNA), and nuclease free water to complete the 25 μl reaction. Designed primers and expected amplicon sizes were reported from previous studies for the virulence factor genes *aerA* and *ahh1, aopB, ascV, lip, ahyB, ser, act, eno,* and *tapA* as listed on Table 2. Primers for the S-layer gene *ahsA* were designed for use in this study. The published nucleotide sequence for the S-layer protein from *A. hydrophila* TF7 (highly virulent fish isolate) was used as a template reference for primer design (Thomas and Trust, 1995). Primers were designed by using the Primer-BLAST tool supported by the The National Center for Biotechnology
The Primer 3 software was designed to generate primers that are specific to intended PCR target (Ye et al., 2012). The forward and reverse primer pair used to amplify a 254 bp fragment of ahsA was 5’- CCTGTTCGAGACTGCTCTGG- 3’ and 5’-
CGGGAGTACCGTTAGCCAAA - 3’.

Table 2. List of virulence factors included in this study with references and expected amplicon lengths in number of base pairs (bp). The 16s rRNA gene primers specific to Aeromonas were used to verify PCR amplification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Primer Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s rRNA (+ control)</td>
<td>953</td>
<td>Lee et al., 2002</td>
</tr>
<tr>
<td>aerA (aerolysin)</td>
<td>309</td>
<td>Wang et al., 2006</td>
</tr>
<tr>
<td>aopB, ascV (T3SS)</td>
<td>951, 891</td>
<td>Carvalho-Castro et al., 2010</td>
</tr>
<tr>
<td>lip (phospholipases A1 and C)</td>
<td>382</td>
<td>Sen and Rogers, 2004</td>
</tr>
<tr>
<td>ahsA (S-layer)</td>
<td>254</td>
<td>This study</td>
</tr>
<tr>
<td>ahyB (elastase)</td>
<td>593</td>
<td>Wilms and McGarey, 2011</td>
</tr>
<tr>
<td>ser (serine protease)</td>
<td>211</td>
<td>Nam and Joh, 2007</td>
</tr>
<tr>
<td>ahhl (hemolysin)</td>
<td>130</td>
<td>Wang et al., 2006</td>
</tr>
<tr>
<td>act (cytotoxic enterotoxin)</td>
<td>232</td>
<td>Kingombe et. al., 1999</td>
</tr>
<tr>
<td>eno (enolase)</td>
<td>250</td>
<td>Sha et. al., 2009</td>
</tr>
<tr>
<td>tapA (Type IV Aeromonas pilus)</td>
<td>650</td>
<td>Kirov et al., 2000</td>
</tr>
</tbody>
</table>

The conditions for the PCR reactions (Table 3) were the same for all the factors except for the S-layer protein (ahsA), as it required a lower annealing temperature. The conditions for PCR were a 5-minute initial denaturation at 95°C followed by 34 cycles of a 30-second denaturation at 95°C, 45-second annealing at 63°C, and a 1-min elongation at 72°C. The final elongation was 5-minutes at 72°C after which the reaction was held at 4°C until the reaction could be analyzed by gel electrophoresis. To confirm
amplification, primers specific to the 16s ribosomal RNA gene of *Aeromonas* were used for a known positive amplification control. The negative control consisted of a reaction mixture without template gDNA to ensure that non-specific amplification and contamination did not occur. The pattern of gene presence and absence was used to group the 28 strains into pathotypes by their virulence genes profile.

**Table 3.** PCR reaction conditions for the amplification of virulence-associated genes in *Aeromonas*.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>5 min.</td>
</tr>
<tr>
<td><strong>34 Cycles</strong></td>
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</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Primer Annealing</td>
<td>63°C (57°C)*</td>
<td>45 sec.</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>1 min.</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72°C</td>
<td>5 min.</td>
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</table>

*Annealing temperature for ahsA (S-layer protein).

PCR reactions were analyzed by agarose gel electrophoresis. A 2% agarose gel was used for separation and visualization of PCR amplicons. The gel was made by weighing 1.0 g of analytical grade agarose (Promega, Madison, WI) and adding it to a microwavable flask containing 50 mL of 1X Tris-Acetate-EDTA buffer (TAE; 40mM Tris-EDTA, 1mM EDTA; Promega, Madison, WI). The mixture was microwaved at 30-45 second intervals, stopped and swirled to keep the agarose suspended. This continued for 2-3 min total time until the boiling point was reached and the agarose was completely dissolved. The agarose solution was cooled at room temperature for 5 min. One microliter of ethidium bromide solution (EtBr) was added to the liquefied agarose gel, swirled to mix and poured into a gel casting tray. The tray comb was put in place and the gel allowed to set at room temperature for 20-30 minutes until it had completely solidified.
Five microliters of 6X blue/orange DNA loading dye (Life Technologies, Grand Island, NY) was added to the 25 uL PCR reactions prior to running the agarose gel. A 100 base pair (bp) DNA ladder (Promega, Madison, WI) was used to provide standard bp size markers to estimate the bp sizes of the amplicons. Once solidified, the agarose gel was placed into the electrophoresis unit. The unit was filled with 1X TAE until the gel was covered. The comb was removed and the samples and molecular weight ladder were loaded into the sample wells. The gel was run at 100V until the orange dye line was approximately 75-80% of the way down the gel. Gels were observed under ultraviolet light illumination using the ChemiDoc™ XRS+ with ImageLab™ software (Bio-Rad, Hercules, CA) and the results digitally recorded.

The DNA ladder was used as a size guide (manufacturer's package insert provided the size of each band in the ladder) to interpret the bp size of an amplicon, which was used to determine if the resulting amplicon size for each gene matched the reported size.

**Phenotypic assays to determine activity of aerA, act, ahh1, lip, ser, ahyB and eno associated virulence factors.** Several phenotypic assays were used to demonstrate the activity or expression of the selected virulence factors. For the aerA, act, and ahh1 hemolytic factors, blood agar plates were used to detect activity of these factors. Strains were taken from the maintenance medium, inoculated onto BBL™ Columbia Agar with 5% Sheep Blood (Becton, Dickinson and Company, Sparks, MD) and incubated at 30°C for 24 to 48 hours after which the presence or absence hemolytic activity was determined. A β-hemolytic *Staphylococcus aureus* was used as a positive control, and non-hemolytic *Escherichia coli* as the negative control.

To test for lipase activity (*lip* gene), strains were inoculated onto Spirit Blue Agar Plates containing a lipid substrate (Difco, Franklin Lakes, NJ), which allowed for the detection of lipolysis. Strains were incubated at 30°C for 24 and 48 hours and checked for areas of clearing around the colony.
growth. The lipase positive control was *Staphylococcus aureus* and *Escherichia coli* served as the negative control as it lacks lipase activity.

Activity of the serine protease (ser) was determined by the hydrolysis of the protein casein when the bacteria were grown on skim milk agar plates. Casein imparts an opaque characteristic to the medium. Enzyme activity was noted when a transparent zone occurred around the bacterial growth indicating degradation of casein. Lack of a transparent zone was indicative of no activity. Strains of *Aeromonas* were inoculated onto skim milk plates and incubated at 30°C for 24 and 48 hours and zones of clearing were determined to be a positive reaction. The protease positive control was *Staphylococcus aureus* and *Escherichia coli* served as the negative control as it lacks caseinolytic activity.

All strains were previously tested for elastase activity by Hans Wilms and Donald McGarey using an elastin-orcein bilayer agar medium originally developed by Hasan *et al.* (1992) and further modified by the investigators (Wilms and McGarey, 2011).

The presence of pili (Type I-*fim* and Type IV *Aeromonas* pilus *tapA*) on the surface of the *Aeromonas* strains was previously investigated by undergraduate Directed Study students Joshua Roepke (Spring 2013) and John Neville (Fall 2012) under the direction of Donald McGarey, Ph.D. They used agglutination assays to detect surface expression of pili/fimbriae. It was reported that *E. coli* type I pili/fimbriae hemagglutinate guinea pig erythrocytes (and the yeast *Saccharomyces cerevisiae*) and type IV pili hemagglutinate rabbit erythrocytes (Xicohtencatl-Cortes *et al.*, 2009). Human erythrocytes (ABO blood types O and AB), guinea pig erythrocytes, rabbit erythrocytes, sheep erythrocytes and formalin-treated *S. cerevisiae* cells were used to test for agglutination against all *Aeromonas* strains. Positive agglutination controls included *Escherichia coli* (EHEC) O157:H7 (type IV pili) and *E. coli* K-12 (type I pili). Evidence for agglutination was performed by macroscopic and microscopic observation, and in different formats (slide and tube assays). Because expression of Type I pili/fimbriae (from *fim*) was reported to be negatively influenced by CRP-cAMP in *E. coli* (Müller, *et al.*, 2009), all bacterial strains
were grown in both nutrient rich (brain heart infusion) and nutrient limited (nutrient broth) media and
tested in early log, late log and stationary phases of growth for agglutination.

Phenotypic detection of enolase activity, determined by plasminogen binding to the surface of
bacterial cells (Sha et al., 2009), was not performed.

**Phenotypic assays to determine expression of S-layer protein.** In order to detect expression of
the S-layer protein, three assays were used on each *Aeromonas* strain. Brilliant blue agar plates have been
used to determine expression of the S-layer protein a previous study (Cipriano and Bertolini, 1988).
These plates were made by adding 100 μg/mL of Coomassie Brilliant Blue G250 to Tryptic Soy Agar.
Strains of *A. hydrophila* were incubated for 48 hours at 30°C and observed for the presence of blue or
white colonies. Blue colonies are positive for the S-layer, while the white colonies are negative.

Congo red agar has been used to detect the presence of the S-layer in strains of *A. hydrophila*
(Ishiguro et al., 1985). Nutrient agar containing 30μg of Congo red per mL were used to differentiate S-
(white) and S+ (red) strains. Strains were grown on Congo red agar, incubated for 24 hours at 23°C, a
temperature, which allows for the growth of S- and S+ strains.

To further test the expression of the S-layer protein, a clumping assay was used to visualize the
autoagglutination of the strains (Paula et al., 1988). All 28 strains of *A. hydrophila* were inoculated into
Brain Heart Infusion broth, incubated for 24 hours at 30°C, and observed for the presence or absence of
turbidity. A strain was positive for S-layer if cells dropped out of suspension by auto-agglutination as
seen at the concentration of the cells in a defined pellet at the bottom of the tube.

**Fish cell culture assay for activity of AopB and AscV.** To determine the expression of the
*aopB* and *ascV* genes (T3SS) in *Aeromonas* strains, a cell culture assay was performed according to
Braun et al. (2002), with some minor modifications. The experimental strains used in this experiment
included: Ahy 7966ᵀ, Ahy 35654, Ahy 43409, Ahy 49847, Ahy 1127, Ahy 1288, Ahy 1280, Asa 1300,
Rainbow trout (Oncorhynchus mykiss) gonad cells (RTG-2; ATCC CCL-55) were grown in 75 cm² flasks at 22°C in minimal essential medium supplemented with 25 mM HEPES buffer, 1% penicillin, and 10% FetalFlex serum substitute. Three days prior to infection with Aeromonas test strains, fish cells were seeded into 24-well plates at 1.0 x 10⁴ cells per well. Each experimental well was infected by 2:1 or 20:1 multiplicity of infection (MOI). A bacterial growth curve for Aeromonas hydrophila generated by KSU Directed Study undergraduate student Parris Tanksley (Spring 2012), was used to determine cell concentration in the mid-log phase of growth based on spectrophotometric optical density at 420 nm to calculate the correct inoculum size for the assay.

Infections were carried out at 22°C and checked after 6 hours for the presence of dead cells and cell rounding. The negative controls consisted of wells with only fish cells and wells containing fish cells and an avirulent E. coli OP50 (nematode feeder strain) to establish a baseline count of the number of dead and rounded cells for comparison to the number of rounded and dead cells in the experimental wells containing Aeromonas.

In addition to observing cell rounding as a potential cause for actin-remodeling, the percentage of dead cells were calculated by using scanning laser confocal microscopy (Zeiss LSM confocal 700 microscope) and a co-localization program from ImageJ. Using two distinct fluorescent dyes allowed for the differentiation of dead cells from living. Hoechst 33342 dye (trihydrochloride trihydrate, Invitrogen™) is able to penetrate living and dead cells and stain the nuclei blue, while the propidium iodide (Life Technologies™) is only able to stain the nuclei if the cell membrane has been disrupted (dead cells). The confocal image of the cells can be split into two channels, one that shows the Hoechst 33342 dye (total cells), and one that shows the propidium iodide channel (the dead cells). The co-localization process allows the comparison of the channels and any overlap from the Hoechst 33342 channel into the propidium iodide channel equated to the percentage of dead cells in that image.

**Nematode challenge Assay.** To investigate the level of virulence of the pathotypes, a nematode challenge assay was used to determine differences in lethality among the pathotypes (Bogaerts et al.,
2010; Chen et al., 2014b). *C. elegans* N2 worms were grown on Nematode Growth Media-lite (NGM) with *E. coli* OP50 as a food source. Worms were flipped every four days to a fresh NGM-OP50 plate to ensure the health of the worms. Adult worms were selected four days after flipping and added to a fresh NGM-OP50 plate containing a bleaching solution. This was used to synchronize the life cycles of the worms in the assay. Four days after bleaching 30 worms were selected and distributed onto fresh NGM plates. Included in each trial was a negative control NGM plate inoculated with the normal food source *E. coli* OP50. Few worms should die under this condition. *Pseudomonas aeruginosa* (PA01) had been previously studied in a nematode challenge assay and was shown to be detrimental to the worms and was included as a positive control. *Aeromonas* strains representing a pathotype group were each plated onto the NGM plates and worms added to the plate. The pathotype representatives that were tested by this assay included Ahy 7966T, Ahy 1127, Asa 1287, Ahy 43409, Ahy ML09-119, Ahy 1288, Asa 1300, and Abe BAA-231. Plates were examined for the presence of dead worms at 24, 48, 72, and 96 hours. Dead worms were counted and removed from the plate, while living worms were transferred onto a new plate containing the same treatment. Worms that died by transfer or desiccation were removed but not included in the study.

**Statistical Analyses.** Analysis of variance (ANOVA) was used to test for differences in *Aeromonas* strains in relation cell cytotoxicity and cell rounding. Shapiro-Wilk tests were performed on the residuals from the fitted models to determine if the error terms were normally distributed. In the case of cell cytotoxicity study, the assumption was violated so an angular transformation was required to normalize the data (p > 0.05). Cell rounding did not meet normality assumptions. For these data, Kruskal-Wallis tests were conducted and ranked transformation with Tukey post hoc tests were used to analyze the rounding data. All analyses were performed using Statistical Analysis Systems version 9.2 (SAS Institute, Inc.).
Z-stat tests were used to test for significant differences in pathogenicity for the nematode challenge assay. For further confirmation Chi squared tests were performed to compare OP50 and PA01 to the *Aeromonas* test strains. All analyses were performed using Microsoft Excel.
RESULTS

Diagnostic tests for verification of *A. hydrophila* strains. For quality control, each strain was established from frozen stock and cultured on *Aeromonas* selective media (Ryan’s formulation). An isolated colony was then sub-cultured onto nutrient agar to observe colony morphology, Gram stain, and oxidase reaction. All strains tested were confirmed to be in pure culture and displayed characteristic colony traits on both the *Aeromonas* selective and nutrient agars. All isolates were confirmed to be Gram-negative, bacilli in single arrangement and oxidase-positive.

PCR amplification of virulence genes. To delineate pathotypes of *A. hydrophila*, virulence genes were first detected by PCR using genomic DNA isolated from each strain and primer sets designed to amplify each gene. A gene was considered present if an amplicon of the expected size was visualized on an agarose gel. Figures 1 and 2 show the expected amplicons for *act, ahh1, ahsA, ahyB, aerA, aopB, ascV, lip, ser* and the positive control. No amplification occurred for *tapA* and the negative control.

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<td>Lane 1: 100 bp ladder, Lane 2: 16s rRNA (+) control (953 bp), Lane 3 (-) control no template, Lane 4: <em>ahh1</em> (130 bp), Lane 5: <em>lip</em> (382 bp), Lane 6: <em>ahyB</em> (593 bp), Lane 7 <em>ser</em> (211 bp), Lane 8: <em>tapA</em> (not amplified).</td>
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*Figure 1.* Agarose gel of amplified virulence genes of *Aeromonas hydrophila* ATCC 7966T.
The results of PCR amplification of all virulence factor genes for all 28 Aeromonas strains are summarized on Table 4. PCR reactions were considered reliable by the presence of the genus specific 953 bp amplicon targeting the Aeromonas 16srRNA gene and no amplicon in the negative control reaction. All 28 strains tested positive for the hemolysin (aahI), the elastase (ahyB), the serine protease (ser), and the lipase (lip). None of the strains tested positive for the type IV Aeromonas pilus (tapA). PCR confirmation of the cytotoxic enterotoxin (act) gene occurred in 25 (of 28) strains, with aerolysin (aerA) detected in 24 and enolase (eno) in 21 of the strains. The T3SS components aopB and ascV and the S-layer protein ahsA occurred in only 5 of the 21 A. hydrophila strains tested (Ahy 35654, Ahy 43408, Ahy 1127, Ahy 1283 and Ahy 1284), but not in the A. bestiarum or A. salmonicida isolates. Amplification of aopB and ascV only occurred in tandem as both proteins are required for a functioning T3SS. The amplification of aopB and ascV genes in Ahy ML09-119 and the type strain Ahy 7966T agreed with the genomic sequencing of these strains in which the T3SS was absent (Seshadri et al., 2006; Tekedar et al., 2013).

Figure 2. Agarose gel of amplified virulence genes of Aeromonas hydrophila. Lane 1: 100 bp ladder, Lane 2: act (232 bp), Lane 3: (-1) control, Lane 4: aerA (309 bp), Lane 5 ashA (254 bp), Lane 6 aopB (951 bp), Lane 7 ascV (891 bp).
**Table 4.** Detection of virulence factor genes by end-point PCR and agarose gel electrophoresis 28 strains of *A. hydrophila* (Ahy), *A. bestiarum* (Abe), and *A. salmonicida* (Asa). Presence of the gene (positive amplification) is noted by + whereas absence (or negative amplification) was denoted by -.

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<tr>
<th>Strains</th>
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<th>lip</th>
<th>ser</th>
<th>act</th>
<th>aerA</th>
<th>eno</th>
<th>aopB</th>
<th>ascV</th>
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</table>
Phenotypic assays to determine expression of virulence factors Type IV pilus (TapA) and elastase (AhyB). After PCR strains were tested for verification of gene expression into a functional protein product. This information would allow for the delineation of strains into pathotypes based on the results of the phenotypic assays. Different assays were selected based on the protein or enzyme that was tested. Phenotypic reactions for Type IV Aeromonas pilus (TapA) and elastase (AhyB), were previously determined by other investigators. In the hemagglutination assays, the positive control E. coli strains agglutinated red blood cells as expected; however, all Aeromonas strains were negative for agglutination for all red blood cell types and yeast cells, and for both (nutrient rich and nutrient limited) growth conditions. The lack of hemagglutination agreed with the lack of amplification of tapA in all 28 Aeromonas strains tested. All A. hydrophila, A. bestiarum and A. salmonicida strains used in this study exhibited elastolytic activity (Wilms and McGarey, 2011). This was in agreement with the results of ahyB amplification, which was positive for all 28 aeromonads.

Determination of Aeromonas pathotypes based on presence and activity of virulence genes. After determination of the genotypic and phenotypic profile the data was combined to create pathotypes based on the presence of the virulence gene and the activity of the gene product (Table 5). The results delineated 28 strains of Aeromonas spp. into 11 distinct pathotypes. The pathotypes were numbered from 1-11 based on the percentage of members in the group starting with the highest percentage and ending with the lowest. The most common pathotype (P1) included 11/28 strains (39%) had the genotype ahh1+, ahyB+, lip+, ser+, act+, aerA+, eno+, aopB+, ascV+, ahsA+, and tapA-. The majority of isolates were A. hydrophila, with two strains of A. bestiarum falling into this grouping. The next most common pathotype (P2) included 4/28 strains (14%) had this genotype ahh1 ahyB+, lip+, ser+, act+, aerA+, eno-, aopB+, ascV-, ahsA+, and tapA-, showing a loss of the enolase (eno) gene. Pathotype group 3 (P3) contained 3/28 members with the genotype of ahh1+ ahyB+, lip+, ser+, act+, aerA+, eno+, aopB+, ascV+, ahsA+, and tapA-, this grouping possesses all the virulence factors in P1, with the addition of the S-layer (ahsA) gene. All three strains in the P3 grouping were A. salmonicida. The P4 pathotype group contained 2/28 strains (7%)
with the profile \( ahh^1 \) \( ahy^B \), \( lip^+ \), \( ser^+ \), \( act^+ \), \( aer^A \), \( eno^+ \), \( aopB^+ \), \( ascV^+ \), \( ahsA^+ \), and \( tapA^+ \). P4 had all the same genes as the pathotype 1 group with addition of the TTSS genes (\( aopB \), \( ascV \)). The remaining 7 pathotypes (P5-P11) were genotypically unique and each fell into separate pathotype groups.

**Table 5.** Pathotype grouping of *Aeromonas* based on amplification and activity of 11 different virulence factor genes.

<table>
<thead>
<tr>
<th>Strains of <em>Aeromonas</em></th>
<th>( ahh^1 )</th>
<th>( ahy^B )</th>
<th>( lip^+ )</th>
<th>( ser^+ )</th>
<th>( act^+ )</th>
<th>( aer^A )</th>
<th>( eno^+ )</th>
<th>( aopB^+ )</th>
<th>( ascV^+ )</th>
<th>( ahsA^+ )</th>
<th>( tapA^+ )</th>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Ahy 7966(^i), Ahy 43409, Ahy 43414, Ahy 51307, Ahy 1275, Ahy 1279, Ahy 1280, Ahy 1282, Ahy Y9</td>
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<tr>
<td>Abe BAA-231, Abe 1286</td>
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Phenotypic assay for aerolysin (AerA), hemolysin (Ahh1) and cytotoxic enterotoxin (Act).

After the strains were incubated on the blood agar media, the results were read after 24 and 48 hours. The most common result after 24 hours was a beta hemolytic reaction (β) on the RBCs in the blood agar plate. Strains that did not possess all three hemolysis factors produced a delayed beta hemolytic reaction at 24 hours (partial hemolysis observed), which was complete at 48 hours. Beta hemolytic reactions verified the action of the Act, Ahh1, and/or Aer (Table 6). The results from the blood agar plate assay confirm the presence of the known hemolysins.

Phenotypic assay for lipase and serine protease. The results of lipase and serine protease, as determined by casein hydrolysis, activities are summarized on Table 6. After 24 hours all strains tested showed a zone of clearing around the bacterial growth confirming lipolytic activity. Therefore all strains tested expressed a functional lipase capable of lipid degradation. The serine protease activity was confirmed by a zone of clearing around the colony due to the digestion of casein, which was detected in all the strains after 24 hours. Two isolates, *A. bestiarum* BAA-231 and *A. salmonicida* 33658, exhibited partial clearing at 24 hours and complete clearing after 48 hours.

Assay for the confirmation of S-layer (AhsA) protein. To test for the activity of the S-layer protein Congo red agar, Coomassie Brilliant Blue agar and autoagglutination in brain heart infusion broth were used. It was reported that strains possessing an S-layer would appear red on Congo red agar and blue on Coomassie Brilliant Blue agar, whereas strains without the S-layer would appear white. Despite repeated adjustments to the formulations, the distinction between blue and white or red and white colonies could not be determined. All colonies had some degree of red or blue coloration on these media. Therefore, autoagglutination in brain heart infusion (BHI) broth was used as the definitive test to indicate the presence of the S-layer strains positive for S-layer (Paula et al., 1988). Autoagglutination was indicated by precipitation of cells at the bottom of the test tube without turbidity in the top broth portion of the tube, whereas strains not expressing an S-layer displayed even turbidity (growth) throughout the tube. All strains were confirmed motile by previous experiments, so precipitation did not result due to a
Table 6. Results of hemolysis, lipase and serine protease (casein proteolysis) plate assays, and autoagglutination in Brain Heart Infusion Broth (BHI). For hemolysis and proteolysis, the term delayed indicates partial reaction at 24 hours and complete at 48 hours. For agglutination, ++ indicates positive clumping, + indicates a weak reaction and – indicates no clumping in 24 hours.

<table>
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<tr>
<th>Aeromonas strain</th>
<th>Hemolysis</th>
<th>Lipase</th>
<th>Casein Proteolysis</th>
<th>Agglutination in BHI</th>
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<td>Asa 1300</td>
<td>Beta</td>
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lack of motility (McGarey, 1991). Nineteen out of the twenty eight strains were negative for the S-layer by autoagglutination. A. hydrophila strains ML09-119 and Ahy Y9 showed weak autoagglutination reactions. All A. salmonicida strains (33658, 1287, 1300), two of the four A. bestiarum (BAA-231, 1286) and two of the twenty one A. hydrophila strains (1127 and 1288) produced reactions indicative of the presence of the S-layer (Table 6). The genomic sequence for the S-layer protein is extremely variable so the activity detected in the phenotypic assay was a strong indicator of the presence of the S-layer protein.

Cell culture assay to detect cytotoxicity related to the T3SS. AexU is delivered through a functional T3SS leading to cell rounding and subsequent apoptosis. A cell culture cytotoxicity assay was performed using rainbow trout (Oncorhynchus mykiss) gonad cells (RTG-2) and the results summarized in Figures 3-5. The two negative controls, fish cells without bacteria and fish cells with avirulent E. coli OP50, had an average rounding percentage of 3% and 5%, respectively, and were not significantly different. Infection of the trout cells with selected strains of Aeromonas was performed at multiplicity of infections of 2:1 (bacterial to fish cells) and 20:1, with the most cell rounding and cytotoxicity to the trout cells and occurring at the 20:1 MOI. At an MOI of 2:1, cell rounding at 6 hours for all strains was not significantly different from the E. coli OP50 negative control. For the 20:1 MOI at 6 hours, 5 out of the 11 experimental Aeromonas strains had rounded 100% of the RTG-2 cells. These included A. hydrophila strains ATCC 7966T, 1127, 1280, 1288, and ML09-119. Rounding was predicted to occur for Ahy 1127 as it possessed T3SS genes aopB and ascV, but not for the other 4 strains as they lacked these same T3SS genes. The data indicate other toxin genes and secretion systems that were not included in the original genetic screening could be causing the cell rounding and cytotoxicity.

The remaining experimental strains had low percentages of rounding at a MOI 20:1. For example, rounding caused by A. hydrophila strains 35654 and 49847 were nearly equal (5.4% rounding and 5% rounding, respectively) to the negative controls. The lack of cell rounding for Ahy 35654 was unexpected as it was found to have genes associates with the T3SS. It is possible that the effector protein AexU is absent in this strain despite the T3SS being present.
Figure 3. Light and confocal microscope images from experimental infection of rainbow trout gonad cells (RTG-2) with *Aeromonas*. (A.) Negative result for rounding of cells infected with *A. hydrophila* 35654. Cells exhibit a normal elongated phenotype. (B.) Rounding of cells as a result of infection with *A. hydrophila* ML09-119. (C.) Nuclei of cells stained with Hoechst 33342 and (D.) propidium iodide 6 hours post-inoculation with *E. coli* OP50 indicative of low cytotoxicity to cells. (E.) Nuclei of cells stained with Hoechst 33342 and (F.) propidium iodide 6 hours post-inoculation with *A. hydrophila* 1288 indicating significant cytotoxicity to cells.
Cell Culture Assay to detect cytotoxicity of selected *A. hydrophila* strains. The same strains used to determine cell rounding were used to determine cytotoxicity. Using confocal microscopy, the percentage of dead cells were calculated using a colocalization macro in Image J. The results from this experiment are shown in Figure 5. The strains that showed the highest percentage of dead cells, and therefore higher cytotoxicity, at the MOI of (20:1) were Ahy ML09-119 (73.9%), Ahy 1288 (72.6%), Ahy 7966\(^T\) (60.4%), Ahy 1127 (57.4%), Ahy 1280 (52.9%) and Asa 1300 (40.6%). Those strains exhibiting significant rounding were the same as those producing significant cytotoxicity. Only one strain, Asa 1300 caused a low percentage of cell rounding, but a moderate level (though not statistically significant) of cytotoxicity. Strains showing intermediate cytotoxicity included Abe BAA-231 (14%) and Ahy 43409.
The remaining experimental strains showed only marginal or no differences in cytotoxicity compared to the *E. coli* OP50 negative control.

**Figure 5.** Cytotoxicity of RTG-2 Cells. Six pathotypes were tested for cytotoxicity with RTG-2 cells infected with various strains of *Aeromonas*. Strain Ahy 1280, Ahy 7966\(^T\), Ahy ML09-119, Ahy 1127, and Ahy 1288 were significantly different from the OP50 negative control (** all p-values < 0.005).

**Nematode challenge assay.** To examine the relative pathogenicity of the pathogens, 8 strains of *Aeromonas* representing 5 pathotypes were used in the nematode challenge assay. Three strains from P1 (Ahy 7966\(^T\), Ahy 43409, and Abe BAA-231), one strain from P2 (Ahy ML09-119), two strains from P3 (Asa 1287, Asa 1300), one strain from P6 (Ahy 1127), and one strain from P7 (Ahy 1288) were used in the trial. To indicate the level of pathogenicity, percentage survival of the nematodes was calculated by dividing the number of living worms at the end of the trial by the total worms. The negative control *E. coli* OP50 had a high percent survival (98%), while the positive control *Pseudomonas aeruginosa* (PA01)
had a survival percentage of 65%. Pathotype 6 (Ahy 1127) had the lowest percent survival at 48%, followed by Asa 1287 (57%), Ahy 79667 (61%), Abe BAA-231 (69%), Asa 1300 (70%), Ahy 1288 (75%), and Ahy ML09-119 (75%). All of the results (Figure 6) were significantly different from the OP50 negative control (All p-values < 0.05) except for Ahy ML09-119, while only Ahy 1127 was significantly more lethal than the positive control PA01 (p-value < 0.05). In comparison to other strains, the two most lethal strains, A. hydrophila 1127 and A. salmonicida 1287 were positive for the S-layer, but differed in other factors. Whereas the T3SS is present in A. hydrophila 1127 it is absent in A. salmonicida 1287, or there is enough variation in the T3SS of A. salmonicida that the primers used to amplify the T3SS genes failed to anneal at the PCR condition. A. salmonicida was positive for the hemolysins aerolysin and aerolyisin-related cytotoxic enterotoxin, and enolase. The least virulent strains, Ahy ML09-119 and Ahy 1288, were the only two used in this assay that were negative for the enolase gene. What is even more striking is the inverse results of the cytotoxicity and nematode challenge assays: the two strains most cytotoxic to cells (RTG-2) in culture have the lowest lethality percentages in the nematode challenge assay.

Figure 6. Nematode Challenge Assay to determine the level of pathogenicity of selected Aeromonas pathotypes. * indicates significantly different from OP50 (-control) all p-values < 0.005. ★ indicates significantly different from PA01 (+ control) all p-values < 0.05.
DISCUSSION

It is known that *Aeromonas* species are capable of causing disease in humans, fish, and other vertebrates. Within a species, the type and severity of disease varies depending on the expression of virulence factors possessed by the infecting strain, site of infection and host factors. Identifying pathotypes of *Aeromonas* based on their virulence factor profile using rapid genetic screening techniques such as multiplex PCR will provide a means for distinguishing highly virulent strains from less virulent strains, and allow for control of these organisms in environmental and clinical scenarios. To accomplish this objective, it is necessary to define those factors that impart the high virulence characteristics from those that do not. Because virulence in *Aeromonas* pathogens is likely multifactorial requiring different factors for adherence and colonization, evasion of the host immune defenses and acquisition of nutrients from the host, defining pathotypes is a complex task. To complicate matters, specific factors may only contribute toward causing disease when in a specific host or by route of infection. The first objective of this research project was to screen 28 isolates representing three species of *Aeromonas* for different 10 virulence factors. These factors have been tested by others in fish or mouse and cell culture models to confirm each factor’s role in pathogenicity and conferring virulence. These isolates were grouped as pathotypes based on the presence/absence and activity pattern of these factors, and selected representatives from the groups were tested by cell culture and nematode challenge. It was hypothesized that survival rates of *C. elegans* challenged with various pathotypes of *A. hydrophila* would differ such that the nematode will be a suitable animal model for investigating host-pathogen interactions using varying virulent strains of *A. hydrophila*. In this study, the nematode challenge assay was used to determine the level of pathogenicity by lethality of selected pathotypes. In previous research *Pseudomonas aeruginosa* and *Aeromonas* spp. have been shown to be pathogenic to the nematode *C. elegans* (Bogaerts et al., 2010; Chen et al., 2014b; Darby et al., 1999; Tan, Mahajan-Miklos and Ausubel, 2012). In this study *P. aeruginosa* PAO1 was used as a positive control and to determine if selected
strains of *Aeromonas* were more pathogenic to the nematode. The feeder strain *E. coli* OP50 was included as the negative control.

Historically, virulence factors have been identified using animal, human, and fish cell culture lines (salmon, trout, catfish, goldfish, zebrafish and blue gourami). While cell culture has its benefits, the response to infection by cells in culture versus a host organism vary. In this study, the two least virulent strains in the nematode challenge assay, *Aeromonas hydrophila* ML09-119 and 1288, were the most cytotoxic for rainbow trout gonad cells. Undoubtedly, host response to infection likely play an important role in affecting the outcome. What host responses were triggered by infection and how they affected strains Ahy ML09-119 and Ahy1288 differently than other test strains deserves further investigation. In a study using microarray hybridization and analysis, *A. hydrophila* infection cause up-regulation of more than 200 genes in a neonatal mouse host and about 50 genes in cells derived from mouse small intestine crypt epithelial cells. Of these, 26 were common to the two models and primarily associated with innate immunity. Also up-regulated gene expression varied between *A. hydrophila* strains. However, one common feature among the most virulent strains was the up-regulation of *jun* in both models. Jun is a central transcriptional factor in the apoptosis pathways and an integral part (with c-Fos) of AP-1 transcription factor complexes for pro-inflammatory cytokine transcription. Jun was not up-regulated in avirulent infections, thereby supporting the use of Jun as a predictor of virulence (Hayes *et al.*, 2007). This study demonstrated that major differences with some common responses occur depending on the system used to evaluate virulence.

Although fish and mice have been excellent models for assessing LD$_{50}$ of various *Aeromonas* species, these models are generally too complex (genetically and physiologically) for studying host-pathogen interactions at the molecular level (Pradel and Ewbank, 2004). Other complications using these models to evaluate bacterial virulence include difficulty in controlling diverse responses between individuals, expenses associated with maintaining facilities and animals, ethics and generation time. To overcome these obstacles, a number of genetically defined invertebrate model systems are being used to
investigate host-pathogen relationships. Easy-to-handle, simple host organisms such as *Dictyostelium discoideum* (amoeba/slime mold), *Tetrahymena thermophila* (amoeba), *Caenorhabditis elegans* (nematode), and *Drosophila melanogaster* (fruit fly) can be helpful in identifying universal virulence factors of a pathogen that are required in other infection models and in deciphering their function. Recently it was reported that virulence assessments using a *Tetrahymena thermophila*-Aeromonas coculture model correlated well with virulence assessments performed in zebrafish, demonstrating the usefulness of *Tetrahymena* as a host model system for investigating virulence determinants in *Aeromonas* species (Pang *et al*., 2012; Li *et al*., 2011). It is reasonable to assume that the mechanisms used by *A. hydrophila* to protect itself from predation/parasitism by protozoa and bacteriophages in the aquatic environment are also useful in resisting antimicrobial proteins and phagocytes that are early responders in the host innate defense. Applying the *Aeromonas* strains used in this study to *Tetrahymena thermophila* would be useful in determining if the survival outcome is the same or not. From there, additional questions could be asked including expression of host factors that are most likely to affect the outcome of infection.

Upon screening for genes in this research, it became apparent that some factors were common to all strains. The virulence factors that were detected in 100% of strains included the hemolysin (*ahhl*), the elastase (*ahyB*), the serine protease (*ser*), and the lipase (*lip*). These virulence factors have been shown to be critical in virulence of *Aeromonas* spp. and aid in tissue destruction in the host organism. However, because these factors were common to all strains used in this study, their presence would not explain the differences seen in the cell cytotoxicity and nematode challenge assays. They may contribute to the overall level of virulence as most strains produced 30-40% nematode mortality, but other factors seem to be at play. For example, the most lethal strain in the nematode challenge assay was *A. hydrophila* 1127. This strain was determined to have both the T3SS apparatus (*aopB, ascV*) and the S-layer protein (*ahsA*). The S-layer is known to protect against phagocytosis and the lytic effect of antimicrobial peptides such as complement. In nature, the S-layer protects the bacterium from bacteriophage attachment and subsequent infection. In the nematode, the S-layer may protect against digestive enzymes and inhibit binding of
galectins or C-type lectins that are expressed during infection (Bogaerts et al., 2010). Even if these lectins bind to the surface and activate complement-like proteins in the nematode, the S-layer is likely to prevent deposition of active complement factors on the surface of the bacterium thereby protecting it from opsonization and lysis. While the S-layer provides a means of evasion, the T3SS and its effector protein AexU cause damage through induction of apoptosis. Confirmation of this inferred mechanism could be investigated further using gene deletion mutants in combination with assays targeting specific gene expression in the host.

The second most lethal strain in the nematode challenge, *A. salmonicida* 1287, also had an S-layer but no T3SS. It did possess genes for additional hemolysins aerolysin and the aerolysin-related cytotoxic enterotoxin. As discussed in the introduction, the presence of both *ahhl* and *aerA* are associated with higher virulence as determined by LD$_{50}$ values compared to strains possessing one or the other gene alone. The combination of S-layer with these other component may explain the greater virulence of *A. salmonicida* 1287 compared to the less virulent pathotype 1 strains (*A. hydrophila* ATCC 7966T, *A. hydrophila* ATCC 43409, *A. bestiarum* BAA-231) that share the same virulence factor profile except lacking an S-layer. However, *A. salmonicida* 1300 falls within the same pathotype group, as *A. salmonicida* 1287, yet was not as virulent. It appears that other undetected factors are involved within the pathotype 3 group.

The least virulent strains, *A. hydrophila* strains ML09-119 and 1288, were the only two used in this assay that were negative for the enolase gene. Surface expressed enolase is known to bind plasminogen and increase its susceptibility to proteolytic cleavage by tissue plasminogen activating factor thereby converting plasminogen into plasmin. The surface bound plasmin was shown to be more resistant to the action of its specific physiological inhibitor, the antiprotease α$_2$-antiplasmin (Sha et al., 2009). This would result in greater fibrinolytic activity around the bacteria and thereby enhance the dissolution of fibrin clots enabling greater spread of the bacteria from the initial infection location. However, a lack of enolase may be one factor affecting the outcome as *A. hydrophila* 1127 also lacks enolase, but is highly
virulent. An assay for surface activity of enolase was not performed due to complexity and a lack of equipment to perform the assay, but the data indicate this must be done to better understand the role it may play in the nematode infection.

Further analysis of the cell culture assay determined that cell rounding and cell cytotoxicity were related as strains that experienced extreme rounding, also showed a high level of cytotoxicity. Of the five strains that caused 100% cell rounding, the percentage of cell death for Ahy ATCC 7966T, Ahy 1288, Ahy 1127, Ahy 1280, and Ahy ML09-119 was 60%, 73%, 57%, 53% and 74%, respectively, after the 6 hour infection at a MOI of 20. Of these, only A. hydrophila 1127 was positive for the T3SS; however other toxins inducing apoptosis including AexT, VgrG1 and RxtA, have been described. (Sha et al., 2007; Suarez et al., 2010; Suarez et al., 2012; Vilches et al., 2008). A. hydrophila ATCC 7966T does contain the genes for a T6SS that could be used to secrete the ADP-ribosylating toxin VgrG1 and another ADP-ribosylating toxin, RtxA, secreted through a more common T1SS, thereby producing the cell rounding and cell cytotoxicity associated with T3SS and effectors (Seshadri et al., 2006). Contrary to these results, A. hydrophila ATCC 35654 possesses the genes for a functional T3SS, yet no significant rounding is observed. Potentially this strain lacks the gene for the effector AexU toxin or expression of this effector is not occurring. Presence of the T3SS in Aeromonas strains without AexU has been reported (Grim et al., 2013, 2014). Using the data generated in this study, screening the genomes for the toxin genes corresponding to effectors aexU, aexT, vgrG1 and rtxA may provide a better perspective as to why strains without T3SS are able to induce cell rounding and apoptosis.

If the pathotype grouping is correct, then it would be reasonable to expect similar results for for cell rounding and cytotoxicity using genetically identical cells in culture. Cell culture minimizes differences that might occur between individuals even with genetically identical organisms such as BALB/c mice. This was not the case as variation within pathotype groups was significantly different. Within pathotype 1, five strains were used in the cell culture assay (Ahy 7966T, Ahy 43409, Ahy 51307, Abe BAA-231 and Ahy 1280). A. hydrophila 1280 and ATCC 7966T had similar results for cell rounding
and percentage of cell dead, but in A. *hydrophila* ATCC 35654 and ATCC 51307, and *A. bestiarum* BAA-231 caused very little cell rounding and cell cytotoxicity. This same scenario was seen in comparison of *A. hydrophila* strains ML09-119 and ATCC 43409 of pathotype group 2 as the latter did not show significant cell rounding or cell cytotoxicity. The rest of the strains tested were from unique pathotypes so no comparisons could be drawn from them. For all strains tested in this research, only known virulence factors with reported nucleotide sequences were selected. Now that the genomes of several strains of *A. hydrophila* isolated from diseased fish and humans have been sequenced, annotated, and are openly available, it will be possible to design primers to conserved regions of virulence factors common to these isolates. Furthermore, genomic sequencing of the strains used in this study such as *A. hydrophila* 1127 and ATCC 35654 will allow for a more comprehensive analysis of virulence factor genes that would be useful to explaining the experimental observations of this study.

The results of the nematode challenge showed that *A. hydrophila* 1127 and 1287 are both more pathogenic than the positive control *Pseudomoans aeruginosa* PA01 as the percent survival was significantly less in the *Aeromonas* strains. Both of these strains are different species belonging to different pathotype groups with *A. hydrophila* 1127 being the single representative of pathotype 6, while *A. salmonicida* 1287 belongs to pathotype group 3. As mentioned, Ahy 1127 was the most lethal in the nematode trial, and it displayed significant cell rounding and cell cytotoxicity in the cell culture assay.

The lethal catfish strain *A. hydrophila* ML09-119 has the highest percent survival of any strain tested in the nematode challenge, and was not significantly different from the negative control. It lacks the genes for the enolase, the T3SS components and the S-layer, so its level of pathogenicity corresponds to its profile, but this strain has been thoroughly studied and is extremely lethal to farmed channel catfish in Alabama (Hossain *et al.*, 2014; Pridgeon and Klesius, 2011; Pridgeon *et al*., 2013; Tekedar *et al*., 2013). In this study the model organism *C. elegans* was not affected by this highly virulent strain. It is unknown whether the differences in the host factors are responsible for this difference in virulence, or the differences in environmental factors (*in situ* versus *in vitro*) under which the bacteria were raised.
influenced the expression of genes related to virulence. It is known that availability of specific nutrients including calcium and iron, temperature, pH and cell density are cues for transcriptional regulation of genes associated with virulence such as siderophores, enzymes and toxins.

This study has provided information on the presence and absence of virulence factors in *Aeromonas* spp. and allowed for the delineation of these strains into pathotypes. These pathotypes were then tested for cell cytotoxicity and pathogenicity in rainbow trout (*Oncorhynchus mykiss*) gonad cells (RTG-2) and the nematode (*C. elegans*). The data suggest that cell culture and nematode challenge are useful for defining pathotypes, but additional work is needed. It is proposed that additional screening be performed for the major surface adhesion protein *aha*1, the toxins *rtxA*, *vgrG1*, *aexU* and *aexT*, components of the type 6 secretion system, and acetylcholine esterase. Screening the strains used in this study for these additional genes that are now in the literature may provide better insight into the data from the cell culture assay and nematode assays. Greater perspective would be provided by whole genomic sequencing of these strains and analysis of known virulence factors as well as identifying genes demonstrating homology to virulence factors described for other bacteria. And while putative virulence genes may be detected, it will be necessary to develop or use other assays to determine if expression occurs or not under specific conditions. While there is still much work to do, being able to identify critical virulence in extremely virulent strains of *A. hydrophila* and other aeromonads will allow for faster recognition and better treatment regimens in hospitals, while at the same time help save millions in lost revenue in the aquaculture industry due to major outbreaks of motile aeromonas septicemia.
INTEGRATION OF THE THESIS RESEARCH

This study has utilized several disciples of biology to comprehensively study various strains of *A. hydrophila* and test virulence using a model organism. The main foundation on the research was based in microbiology as the organisms studied are classified as microorganisms. This study included strains isolated from clinical specimens, the environment, and isolates from fish, humans and other vertebrates and invertebrates. Furthermore, this research integrated multiple areas of biology including molecular biology, bacteriology, cell biology, organismal biology, and the disciplines of chemistry and statistics. The information can be used to differentiate more virulent strains from less virulent strains. Diseases caused by *A. hydrophila* have a large impact in human disease and massive aquaculture loss, and this research provided an integrative approach to mitigate the impact of *A. hydrophila* infections.

The organisms used in this study, various species of *Aeromonas, Pseudomonas aeruginosa* and *Escherichia coli* are bacteria. The culturing and maintenance of these organisms fall within the sub-discipline of microbiology called bacteriology.

In order for pathotypes to be determined, selected virulence genes need to be positively detected by genotype and phenotype. To determine if a gene is present a molecular biological tool PCR of the strains was conducted. Primers were identified or designed to match template DNA in the genomic DNA of the strains tested. These are tools of molecular biology. In order to obtain these data phenotypic assays must be used to detect the presence of a protein.

Phenotypic assays are various biochemical tests that determine if a protein is actively being expressed by the bacterium. *A. hydrophila* possesses toxins, enzymes that breakdown lipids (lipase), erythrocytes (aerolysin, hemolysin), proteins (serine protease), elastin (elastase), and other tissues. These enzymes or toxins can be detected by plating them on media with erythrocytes or lipids. When the
enzyme or toxin comes into contact with the substrate/target cell, it will produce an observable reaction. Understanding the basis of these enzymatic reactions requires the knowledge of biochemistry.

Cell culture assay was used to detect apoptosis induced through an injectisome (T3SS) mechanism. Rainbow trout gonad cells (RTG-2) were grown and transferred in a 24 well plate, and then after 3 days were infected by selected pathotypes of A. hydrophila. Information from this experiment provided valuable data on the presence or absence of T3SS and potential other toxins at play. Using a confocal microscope and fluorescent dyes, it was possible to distinguish dead cells from living cells. Strains with significantly more dead cells than the control were considered to be virulent. These are experimental approaches that combine aspects of cell biology with advanced microscopy.

This research project is focused on the virulence of selected strains of A. hydrophila. To test for the level of virulence, organismal biology was needed to further classify pathotypes based on pathogenicity. The model organism Caenorhabditis elegans was utilized to accomplish this goal. C. elegans has been a good model to study host-pathogen relationships. The use of the nematode to study microbial pathogenesis branches into the area of organismal biology.

This research has demonstrated that virulence tends to vary in strains of A. hydrophila. Furthermore this research can be used to determine highly pathogenic strains from lower pathogenic strains. This information can prove helpful in controlling the massive fish-die offs and mitigate loses of wild and farmed fish. Therefore, socioeconomic issues such as maintaining a healthy, sustained food source while reducing economic loss due to diease are factors that help drive the research. Because aeromonads cause debilitating human diseases, improvement to human health and a better outcome if infection occurs also drives this research.
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