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IMPORTANT ROLE OF THE TYPE VI SECRETION SYSTEM IN THE VIRULENCE OF AN EMERGENT HUMAN PATHOGEN *Aeromonas hydrophila*: DELINEATING THE MECHANISM OF ACTION OF ITS EFFECTORS

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IMPORTANT ROLE OF THE TYPE VI SECRETION SYSTEM IN THE VIRULENCE OF AN EMERGENT HUMAN PATHOGEN *Aeromonas hydrophila*: DELINEATING THE MECHANISM OF ACTION OF ITS EFFECTORS

By

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Dissertation
Presented to the Faculty of the University of Texas Medical Branch Graduate School of Biomedical Sciences at Galveston in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Keywords: *Aeromonas hydrophila*, Type VI secretion system (T6SS), Virulence factors, Hemolysin coregulated protein (Hcp), Valine-glycine protein (VgrG)

August 2010
Galveston, Texas

In memory of my mother, Amanda Real (1947-2008)

Acknowledgments

- Dr. Ashok K Chopra. Ph.D., C.Sc.
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- Protein Chemistry Core
- Mass Spectometry Core
- Department of Microbiology and Immunology
- McLaughlin Endowment Fellowship

IMPORTANT ROLE OF THE TYPE VI SECRETION SYSTEM IN THE VIRULENCE OF AN EMERGENT HUMAN PATHOGEN *Aeromonas hydrophila*: DELINEATING THE MECHANISM OF ACTION OF ITS EFFECTORS

Publication No. ____
Giovanni Suarez, Bs., M.Sc., Ph.D.
The University of Texas Medical Branch at Galveston, August 2010

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Aeromonas hydrophila causes various human diseases, including gastroenteritis, wound infections and septicemia. In this study, we characterized the new type 6 secretion system (T6SS) from isolate SSU of *A. hydrophila* and demonstrated its role in bacterial virulence. We have provided evidence that the T6SS is independent of other secretion systems and that the *vasH* gene is essential for the expression of the genes encoding the T6SS gene cluster. We demonstrated T6SS's ability to translocate effector proteins into the host cells and also showed that T6SS mutants were less toxic to human and murine cell lines, and more efficiently phagocytosed by macrophages. Importantly, bacterial-host cell interaction was needed for the T6SS to induce cytotoxicity in eukaryotic cells. Based on 2-dimensional gel electrophoresis and mass spectrometric analyses of bacterial supernatants, we identified a member of the VgrG protein family, (VgrG1), containing a vegetative insecticidal protein (VIP-2) domain at its carboxyl-terminal end, as well as the hemolysin co-regulated protein (Hcp). We provided evidence that VgrG1 showed ADP-ribosyltransferase activity associated with its VIP-2 domain. Our data indicated that episomal expression of the *vgrG1* gene in HeLa Tet-Off cells disrupted the actin cytoskeleton, followed by a decrease in cell viability and an increase in apoptosis. We also provided evidence that the expression of the *hcp* gene in HeLa cells resulted in apoptosis of the host cells without changes in their morphology. In addition, we showed that the addition of exogenous recombinant Hcp (rHcp) reduced bacterial uptake by macrophages. These results were substantiated by the *in vivo* analysis of cytokine profiling and activation of host immune cells after infection with the *ΔvasH* mutant supplemented with rHcp indicating that this T6SS effector inhibited production of pro-inflammatory cytokines and induced production of immunosuppressive cytokines, such as IL-10 and TGF-β. In summary, we showed that the T6SS represent an important virulence determinant of *A. hydrophila*.

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Introduction

Aeromonas species are associated with a variety of human and animal diseases. The genus *Aeromonas* inhabits fresh water sources, although the organisms are also found in estuarine and salt water (57, 94). The presence of *Aeromonas* species in water distribution systems and different foods indicates its potential as a food-borne pathogen, and, consequently, this organism represents a public health concern. More recently, studies have shown increased resistance of *Aeromonas* to various antibiotics and water chlorination. The organism has the ability to form biofilms in potable water pipes and reservoirs and is isolated from water distribution systems and produces a wide panel of virulence factors. Consecuently, *Aeromonas* was included on the "Contaminant Candidate list" published by the Enviromental Protection Agency (27, 151).

Among the different species of *Aeromonas*, *A. hydrophila* is most commonly associated with a wide variety of human diseases, which include skin and wound infections, gastroenteritis, and septicemia, which are often fatal (30, 64, 134). Gastroenteritis is the most common manifestation associated with *Aeromonas*, and although it is most often associated with young, elderly, or immunocompromised people, several cases of intestinal and extraintestinal infections in immunocompetent individuals have led to the suggestion that the virulence of this pathogen is not entirely dependent upon the immune status of the host (33, 202). As in the case of others forms of bacterial gastroenteritis, underlying factors such as liver and gastrointestinal diseases, as well as recent ineffective antimicrobial therapy against *Aeromonas* have been reported as relevant for the development of *Aeromonas*-associated diseases (33, 210).

The evidence of *Aeromonas*-associated infections was also noted after natural disasters such as the tsunami in Thailand, the hurricane Katrina in New Orleans, and the earthquake in Sichuan, China. In these locations, *Aeromonas* was the predominant bacteria isolated from wounds and soft tissue infections as well as in floodwater samples (83, 161, 197).

Our laboratory has characterized two of the most potent virulence factors from a diarrheal isolate SSU of *A. hydrophila*, namely *Aeromonas* cytotoxic enterotoxin, Act, and the type 3 secretion system (T3SS) effector AexU (32, 180). Act is secreted via the T2SS and possesses hemolytic, cytotoxic, and enterotoxic activity. In addition, Act inhibits phagocytosis and evokes the production of prostaglandins and proinflammatory cytokines from murine and human macrophages as well as from human epithelial cells (63, 65). On the other hand, AexU is a T3SS effector, which is translocated directly into the eukaryotic host cell cytoplasm. AexU has ADP-ribosyltransferase (ADPRT) and GTPase activating protein (GAP) activity and is able to induce changes in the eukaryotic host cell morphology by altering actin polymerization patterns leading to apoptosis. Also, AexU inhibits the activation of NF-κβ in intoxicated eukaryotic cells (184, 185).

Protein secretion in gram-negative bacteria presents a challenge because the secreted proteins must pass through at least two membranes before they can reach the extracellular milieu. To date, five secretion systems have been molecularly well characterized in gram-negative bacteria. These systems are highly conserved across different bacteria with unique characteristics, which permit their differentiation from one another (47). Recently, a new protein secretion mechanism called virulence-associated secretion (VAS) or the type VI

secretion system (T6SS) was described in *Vibrio cholerae* (165). Previously, a gene cluster encompassing the T6SS was identified by bioinformatics analysis as being highly conserved among several gram-negative pathogens, and yet it exhibited differences in its organization in various bacteria (44). It is believed that the primary function of the T6SS is to mediate the extracellular export of virulence factors (163) and also to the translocate them directly into the host cell cytoplasm (122, 191, 193). This mechanism of secretion is different from T3- and T4-secretion systems because the T6SS represents an assembly of genes with a novel linkage that secretes proteins lacking the classical Sec-dependent signal sequences (217). Several studies indicated participation of this cluster in the pathogenicity of different bacteria, such as *Pseudomonas aeruginosa* in which the role of the T6SS-associated effector, hemolysin coregulated protein (Hcp), has been demonstrated in cystic fibrosis (145). Likewise, in *Francisella tularensis* and *Salmonella enterica*, these gene clusters are necessary for intracellular growth in eukaryotic cells (45, 153). In *Burkholderia mallei*, it was found that the T6SS is required for virulence in the hamster model of glanders infection (171). Most important, however, is that the role of this secretion system in the virulence of bacterial pathogens in general is still largely unknown.

In the first part of this study, I report for the first time the presence of a functional T6SS gene cluster in a clinical isolate SSU of *A. hydrophila*. Our characterization of the T6SS showed that this cluster is able to secrete and translocate Hcp into eukaryotic host cells leading to apoptosis mediated by caspase 3 activation. Additionally, we showed that mice immunized with a secreted component of this system (Hcp) were protected from a lethal challenge dose of the wild-type (WT) bacterium (193). In the second part, by the proteomics

analysis of secreted proteins via T6SS, I report the existence of a member of the VgrG family, which has a COOH-terminal extension containing a vegetative insecticidal protein-2 (VIP-2) domain, known for its actin ADP-ribosylating activity. This gene was localized outside the T6SS gene cluster, and I called it *vgrG1*. Furthermore, I noted that the expression of the gene encoding either the full-length VgrG1 or its COOH-terminal domain that contained only the VIP-2 domain induced caspase 9 mediated apoptosis in HeLa Tet-Off cells (191). Overall, we demonstrated that VgrG1 is an important virulence factor of *A. hydrophila* that is secreted and also translocated via the T6SS. This is the first characterization of VgrG with actin ADP-ribosylating activity from any bacteria.

In the third part of this study, I showed that Hcp plays a role in modulating innate immunity by inhibiting phagocytosis of *A. hydrophila*, thus allowing its multiplication and spread to different organs of the host. My results show that Hcp is able to bind macrophages and induce the production of interleukin (IL)-10 and transforming growth factor (TGF)-β, affecting the activation and maturation of macrophages and consequently the recruitment of other cellular immune components needed to clear the bacterial infection (192).

In summary, the results obtained in this study highlight the importance of T6SS in pathogenesis and virulence associated with *A. hydrophila* SSU.

Chapter 1: Literature Review

Aeromonas hydrophila

Taxonomy

Superkingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Aeromonadales

Family: Aeromonadaceae

Genus: *Aeromonas*

Description

The genus *Aeromonas* was created in 1943 when it was separated from other bacteria with polar flagellum (189). It was formerly placed in the family Vibrionaceae; however, later it was placed it in its own order and family, the Aeromonadales and Aeromonadaceae, respectively. To date based on DNA hybridization and sequence analysis techniques; there are 27 species of *Aeromonas* reported, although some of them are still under validation due to their similarities with other species (Table 1.1). Species of *Aeromonas* are gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that

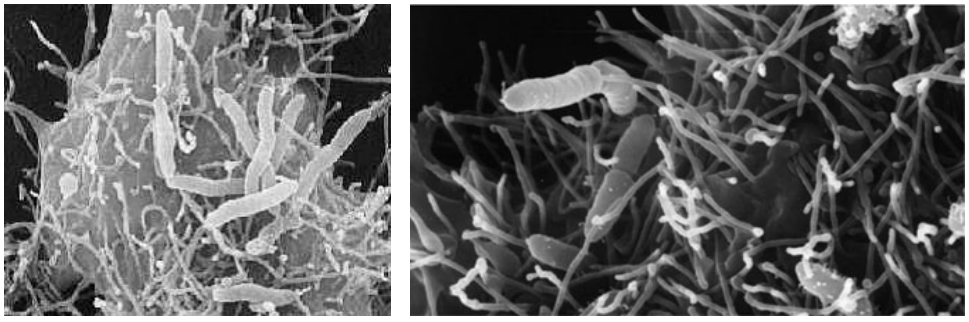

Figure 1.1: Scanning electron micrographs of *A. hydrophila* adhering to human epithelial cells.
Downloaded from Google images; search parameter "*Aeromonas hydrophila*".

occur ubiquitously in aquatic environments. The family Aeromonadaceae shares many biochemical characteristics with members of the Enterobacteriaceae family, from which they are primarily differentiated by being oxidase-positive. The genus *Aeromonas* was divided into two major groups based on growth and biochemical characteristics (97). The first group or mesophilic group, typified by *A. hydrophila, A. caviae, A. sobria, A. veronii,* and *A. schubertii,* is associated with a variety of human infections and consists of motile forms that grow well at 35-37ºC. The second group or psychrophilic group is mostly a fish pathogen. These strains are non-motile and their optimal growth temperature is around 22-25ºC. Isolated strains from this group reside in the species *A. salmonicida* (96).

Table 1.1: Species of the Genus *Aeromonas*.

Species	Subspecies	Similar to
A. allosaccharophila		*A. veronii/A. Ichthiosmia/A. culicicola*
A. aquariorum		*A. hydrophila* sub *dhakensis*
A. bestiarum		
A. bivalvium		
A. caviae		*A. eucrenophila/A. encheleia/A. punctata*
A. culicicola		*A. veronii*
A. diversa		
A. encheleia		*A. eucrenophila/A. caviae/A. punctata*
A. enteropelogenes		*A. trota*
A. eucrenophila		*A. caviae/A. encheleia/A. punctata*
A. fluvialis		
A. hydrophila		
	anaerogenes	
	dhakensis	*A. aquariorum*
	hydrophila	
	proteolytic	
	ranae	
A. ichthiosmia		*A. veronii/A. allosaccharophila*
A. jandaei		
A. media		
A. molluscorum		
A. piscicola		
A. popoffii		
A. punctata		*A. caviae/A. encheleia/A. eucrenophila*
	caviae	
	punctata	
A. salmonicida		
	achromogenes	
	masoucida	
	pectinolytica	
	salmonicida	
	smithia	
A. schubertii		
A. sharmana		
A. simiae		
A. sobria		
A. tecta		
A. trota		
A. veronii		*A. ichthiosmia/A. culicicola/A. allosaccharophila*

Animal diseases caused by *Aeromonas*

Aeromonas has been associated with fish diseases long before it was known to cause infections in humans. *Aeromonas* species are linked to major die-offs and fish kill around the world causing major economic losses. Two groups of fish diseases have been recognized; first, fish furuncolosis, which is associated with infections caused by *A. salmonicida*, particularly in salmonids. This disease ranges from an acute form characterized by septicemia, hemorrhages at the base of the fins, inappetence, and melanosis to a chronic form which is more common in older fish and is characterized by lethargy, slight exophthalmia and hemorrhages in muscles and internal organs (96). The second group of diseases is caused by mesophilic species, mainly *A. hydrophila* and *A. veronii,* and includes hemorrhagic septicemia in carp, tilapia, perch, catfish, and salmon and red sore disease in bass and carp, and ulcerative infections in

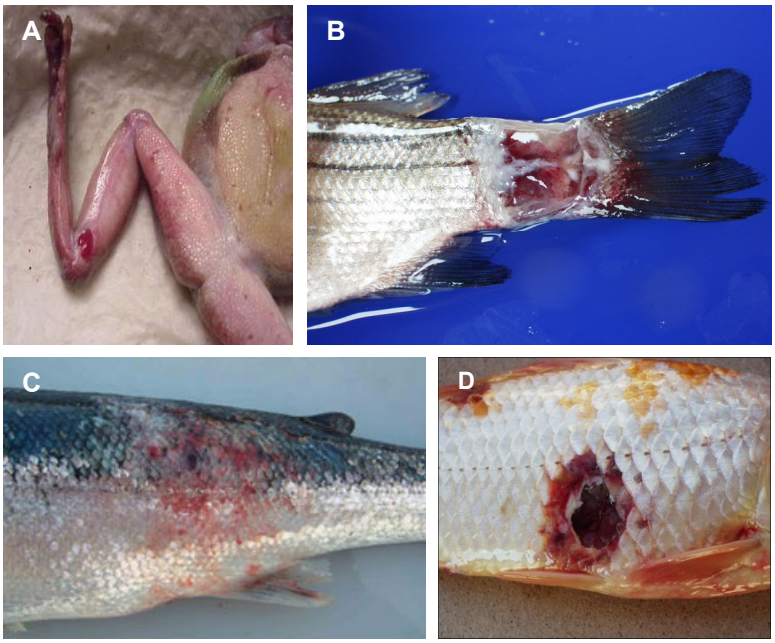

Figure 1.2: Animal infections caused by *Aeromonas* spp.
A. Red leg with ulcerations in frogs; B. Striped Bass. C. Atlantic salmon; D. Goldfish. Downloaded from Google images; search parameter "*Aeromonas* fish infections".

catfish, cod, carp, and goby (96) (Figure 1.2).

In addition, *Aeromonas* species are able to cause a variety of diseases in cold- as well as in warm-blooded animals. These diseases include ulcerative stomatitis in snakes and lizards, red leg disease in frogs, septicemia in dogs, septic arthritis in calves, infections in seals, and seminal vesiculitis in bulls (96, 141, 201).

Human diseases caused by *Aeromonas*

The *Aeromonas* species mainly associated with human infections are *A. caviae*, *A. hydrophila*, and *A. veronii* biovar sobria (96), and these species of *Aeromonas* are responsible for around 85% of all clinical infections (95).

The mesophilic *Aeromonas* species have been commonly isolated from patients with gastroenteritis although their roles in disease causation remain unclear, and their role as an etiological agent of the disease is still under discussion (96). This is due to the inability to distinguish between infecting and colonizing strains in the gastrointestinal tract, which makes it difficult to establish a clear distinction between enteropathogenic and non-enteropathogenic strains (96). Evidence supporting the role of *Aeromonas* in gastroenteritis came from detailed case reports, recovery of bacteria from diarrheal samples, epidemiologic-controlled studies, and by the fact that *Aeromonas* shows low colonization rates in asymptomatic patients (8, 83, 95, 97). However, evidence about diarrheal outbreaks caused by *Aeromonas* species is controversial, and most of the clinical cases reported came from food-poisoning intoxication caused by consumption of infected fish (96, 200, 210).

There has been considerable debate as to whether the mesophilic *Aeromonas* are primary enteropathogens, prompted largely by the failure to establish significant infection in volunteer studies (96). Understanding the clinical significance of enteric isolates of *Aeromonas* has been complicated by the fact that some studies have demonstrated similar isolation frequencies from symptomatic and asymptomatic adults (8), while others have shown significant correlations between diarrhea and enterotoxin-producing *Aeromonas* species (18, 73). In addition, there is available evidence indicating that people are generally unaffected by enteric *Aeromonas*; thus, *Aeromonas* may be a natural part of the gut flora, either transiently or on along-term basis. A number of factors, including age, immunocompetence, infectious dose, underlying illness, and production of sufficient virulence factors by the infecting organism, affect the ability of *Aeromonas* species to cause disease (96).

Aeromonas species are also associated with sepsis, wounds, eye, respiratory tract, and other systemic infections which arise following contamination of lacerations and fractures with *Aeromonas*-rich waters (97). In past decades, several reports about septicemia involving the genus *Aeromonas* have been documented mostly in immunocompromised patients who are in the middle-aged group (53-62 years old) (37, 46, 96, 211). Among immunocomprimised patients, *Aeromonas* bacteremia is more frequent in people with hepatic cirrhosis (54%), other chronic liver disease (26%), neoplasia (33%), and biliary disease (24%) (109, 119, 203). These patients account for ~80% of the total cases of *Aeromonas*-associated sepsis (109, 113, 119), and three species, namely *A. hydrophila, A. caviae,* and *A. veronii*, account for ~95% of the reports on *Aeromonas*-asssociated septicemia (98).

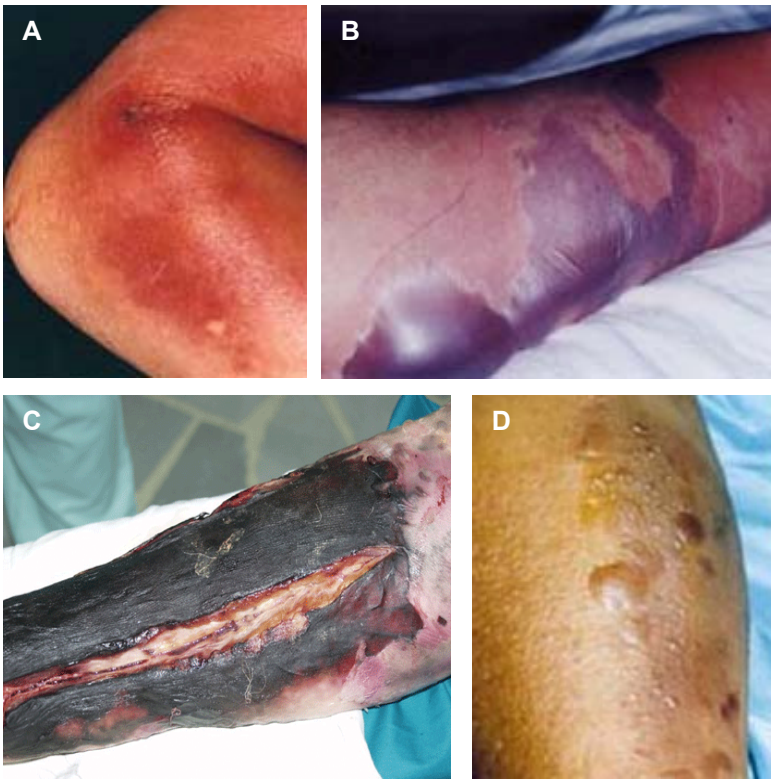

Figure 1.3: Human infections caused by *Aeromonas hydrophila*
A. Cellulitis; B. Ecthyma gangrenosum. C. Necrotizing Fasciitis; D. Myonecrosis. Downloaded from Google images; search parameter "*Aeromonas hydrophila* human infections".

Other common but less frequent subset of cases associated with *Aeromonas* bacteremia involves people with history of traumatic events with septic episodes. Thus, necrotizing fasciitis, myonecrosis, or cellulitis caused by *Aeromonas* infections could lead to amputation of limbs, or even in the worst scenario to *Aeromonas* sepsis (2, 115, 136) (Figure 1.3).

The less frequent group of *Aeromonas* septicemia is in healthy people who have developed sepsis associated with *Aeromonas* without any recognized risk factor for infection (100, 101, 112, 174). Although it is difficult to estimate the mortality rate for this group of people, it is lower than mortality rates observed in immunocompromised and traumatized people infected with *Aeromonas*. This low mortality rate could be due to better immune status of the healthy individuals.

Aeromonas species can be associated with skin and soft tissues infections ranging from mild problems, such as pustular lesions to serious subcutaneous infections (cellulitis), and to deep layer and subcutaneous tissue infections (necrotizing fasciitis and myonecrosis). After the gastrointestinal tract, skin layers are the second most common site from which *Aeromonas* species are recovered. As mentioned earlier, open wound lesions represent a gateway for *Aeromonas* infections; however, the immune status of the patient determines how far the infection may progress. Therefore, healthy patients with non-severe wounds involving only superficial skin layers can resolve *Aeromonas* infections. On the other hand, necrotizing fasciitis and myonecrosis occur more often in immunocompromised patients with liver diseases and malignancy (96). Secondary effects of *Aeromonas* infection can result in serious wound infections, septic arthritis, and septic shock (53, 111).

Aeromonas species have played a major role as important pathogens after natural disasters. Water samples taken after hurricane Katrina from the New Orleans Superdome and the Charity Hospital showed high levels of *Aeromonas* species (10^6-10^7 cfu/mL) (161). In addition, after Thailand's tsunami in 2004, *Aeromonas* species were the most common pathogen identified in skin and soft tissue lesions, accounting for 22.6% of all isolates recovered from 396 patients (83). Additionally, *Aeromonas* was the ninth most common bacterial pathogen recovered from wound lesions in Wenchuan survivors after the 8.0 earthquake in Sichuan, China (197).

Peritonitis caused by *Aeromonas* species could be an extension of gastrointestinal or biliary tract infection, or result from bacterial spread from blood or lymph to the peritoneal cavity (220). In a retrospective study (87), *Aeromonas*

peritonitis caused by bacterial blood spread was most common in people with liver diseases (97%). This form of peritonitis was accompanied by monomicrobic bacteremia (50%). In contrast, peritonitis as a result of gastrointestinal or biliary tract infections has shown polymicrobic cultures, typically involving *E. coli* and *K. pneumoniae* together with *Aeromonas* species (87). Other forms of peritoneal infections with *Aeromonas* involve spontaneous bacterial peritonitis and ambulatory peritoneal dialysis-associated peritonitis. Both of these forms of infection are associated with patients having liver diseases (26, 28, 220). A clinical study from Korea and Taiwan showed that *Aeromonas* was the third most common gram-negative bacteria that caused spontaneous bacterial peritonitis (28). In addition, *A. hydrophila* and *A. veronii* are the most common species associated with bacterial peritonitis in Southern Asia, causing a gross mortality rate of ~56% of all infected cases associated with *Aeromonas* species (28, 87, 220).

Aeromonas species have also been found in the respiratory tract causing infections that range from epiglottitis to empyema, lung abscesses, and pneumonia, with the latter representing the most often reported respiratory complication associated with *Aeromonas* species. Other less common infections caused by *Aeromonas* affect the urogenital tract (86, 87) and the eyes, resulting in endophthalmitis, keratitis, and corneal ulcerations (105, 156, 167, 188).

Virulence factors

Virulence is defined as the ability to incite disease at the specific end point. Thus, it can be measured in different ways, such as lethality, grade of invasion, production of toxins, and so on (24). On the other hand, pathogenicity is

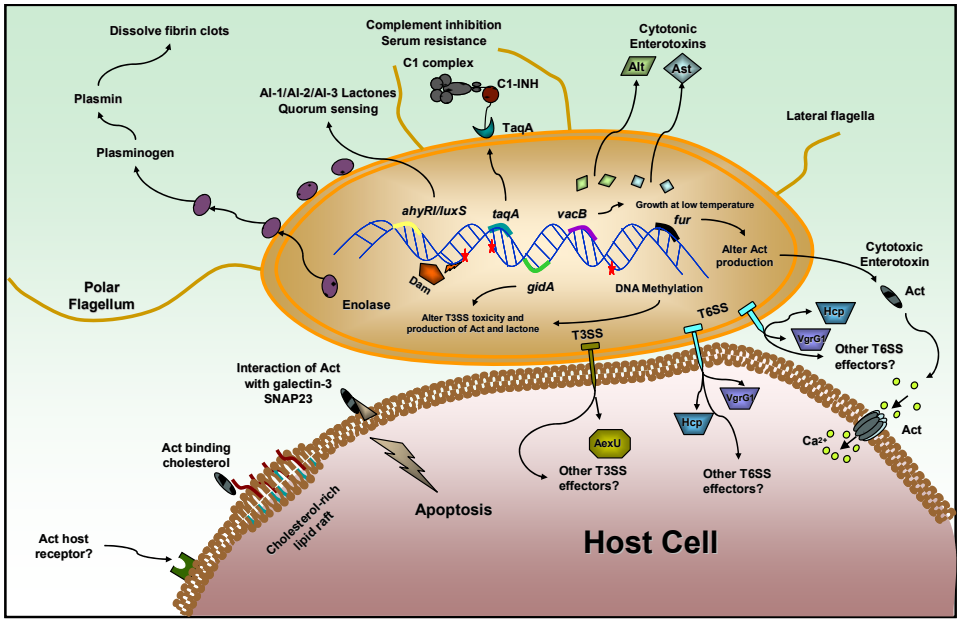

Figure 1.4: Virulence factors associated with *Aeromonas hydrophila*

defined as the capacity to cause disease in a particular host (24, 58). Multiple features such as inoculum, route of infection, susceptibility, etc., help to define infection and pathogenicity (58).

Gastroenteritis and wound infections are the only two *Aeromonas* infections predominant in healthy humans. Other pathologies involving *Aeromonas* species include people with underlying illnesses, such as liver diseases, or people who are immunocompromised.

Mesophilic *Aeromonas* species can produce a range of virulence factors, including attachment mechanisms and production of a number of toxins (Figure 1.4). Several studies have demonstrated that strains of *A. hydrophila* produce lectins and adhesins, which allow adherence to epithelial surfaces and gut mucosa (29, 96). *Aeromonas* species produce two types of flagella, the polar flagellum, Pof, which is needed for motility in liquid enviroments and this flagellar gene is constitutively expressed. The lateral flagellum, Laf, produces swarming

motility on solid surfaces, and the *laf* gene is inducible (96, 108, 126). Similarly, adherence and invasion of Hep-2 cells by faecally derived *A. hydrophila* have been reported (23, 114). Two types of pili have been characterized (23), one is short and rigid similar to the type I pili of *E. coli*, and the other is long and wavy, similar to the type IV pili (96, 126). The latter have two sub-types. One is related to the bundle-forming pili (Bfp) which is involved in adherence to enterocytes; and the other, called type IV *Aeromonas* pilus (Tap), and has unknown function (96, 106, 107, 126). One study has shown that Tap pili from *A. salmonicida* have moderate importance in virulence in Atlantic salmon (20).

Species of *Aeromonas* are capable of producing a number of extracellular toxins and enzymes. The primary toxins produced are hemolysins, of which the most significant is aerolysin, produced by many strains of *A. hydrophila* and *A. sobria* (94). This is a pore-forming cytolysin, heat-labile β-hemolysin, with phospholipase A and C activity. This toxin is able to insert into the host cell membrane bilayer causing leakage of cytoplasmic contents. Also, hemolytic enterotoxins have been reported by some authors (31). Accordingly, a weak hemolysin, glycerophospholipid:cholesterol acyltransferase (GCAT), was characterized from *A. hydrophila* and *A. salmonicida* (155). In addition, at least one cytotonic enterotoxin with similar activity to cholera toxin was demonstrated (96, 118). Another toxin with hemolytic activity is the *Aeromonas* cytotoxic enterotoxin (Act). This toxin is a pore-forming toxin secreted via the T2SS (179). It has been demonstrated that Act is able to induce fluid accumulation in murine ligated intestinal loops and to stimulate the production of proinflammatory cytokines, such as TNFα, IL-1β, and IL-6 (32). Other toxins that play a role in gastroenteritis are the *Aeromonas* heat-labile cytotonic enterotoxin (Alt) and the

Aeromonas heat-stable cytotonic enterotoxin (Ast) (179). Indeed, mutants of *A. hydrophila* SSU devoid of *act, alt,* and *ast* genes caused no fluid secretion in the ligated intestinal loops of mice (179). A vacuolating toxin has been found in some strains of *Aeromonas veronii*. This toxin has serine protease activity, is non-hemolytic, and is able to cause apoptosis in Vero cells (128). Evidence for plasmid-encoded production by *A. hydrophila* and *A. caviae* of a cytotoxin similar to Shiga-like toxin 1 has been reported (79).

Invasins have also been reported, although limited studies suggest that *Aeromonas* species are invasive (40). In these studies, the degree of invasion is very low compared to the classical invasive enteropathogens such as *E. coli, Shigella*, or *Y. enterocolitica* (66).

Species of *Aeromonas* also produce a range of cell-surface and secreted proteases, such as metalloproteases, serine proteases, and aminopeptidases, which probably enhance virulence by degradation of proteins present in the extracellular matrix and connective tissue, including albumin, fibrinogen, elastin, and collagen (76, 90, 93, 96). Another important factor associated with *Aeromonas* virulence is the lipopolysaccharide (LPS). Studies show that *Aeromonas* belonging to the serogroups O:11, O:16, O:18, and O:34 are associated with most cases of bacteremia (98).

The role of quorum sensing in *Aeromonas* virulence has been reported (104, 196). Quorum sensing can drive several virulence factors of *Aeromonas*, including formation of biofilms (196). It is known that bacterial biofilms show characteristic expression patterns, which include more virulence factor production as a result of gene activation due to bacterial communication (29). Bacteria forming biofilms are more resistant to antimicrobial agents, such as

chlorine and antibiotics, and to host defense systems. Mutant strains of *Aeromonas* for *ahyI* gene, which do not produce N-butanoyl-L-homoserine lactone (C4-HSL), fail to form mature biofilms and show low production of serine proteases and metalloproteases when compared to their parental strain (195, 196). However, the levels of these proteases could be restored by the addition of exogenous C4-HSL into the medium (196). Contrary, mutant strains for the gene *ahyR* showed no effect on the biofilm formation (121), but they have no protease activity even after addition of exogenous C4-HSL into the medium (29). Recent studies showed a relationship between T3SS, T6SS, Act, and the production of lactones in *A. hydrophila*, indicating the importance of quorum sensing system in the regulation of bacterial virulence (104, 180).

Recently, the role of enolase in pathogenesis of *A. hydrophila* SSU was described (178). *A. hydrophila* surface enolase is able to bind plasminogen/plasmin which promotes bacterial penetration, colonization, and dissemination.

Production of virulence factors, including hemolysins and proteases, by *Aeromonas* is influenced by environmental temperature (129). In that respect, the role of the *vacB* gene from *A. hydrophila* in bacterial growth at 4°C was reported. *A. hydrophila vacB* mutant strain showed reduction in growth rates at 4°C (56), thus, this mechanism could be an important virulence determinant in food intoxication associated with *A. hydrophila*.

Several studies have reported the expression in *Aeromonas* of a protein highly related to the T3SS protein exoenzyme S (ExoS) from *Pseudomonas aeruginosa*. The homolog of ExoS has been reported in *A. salmonicida* (AexT), which is secreted after contact with the fish cells, inducing cytotoxic effects in

gonad cells of rainbow trout (29). The homolog of ExoS from *A. hydrophila*, called AexU, is able to induce rounded phenotype followed by apoptosis of HeLa cells (185). This cytotoxic effect is mediated by ADP-ribosylating activity and GAP activity mediated by known conserved domains of AexU (184, 185). Differences in the T3SS gene cluster between *A. salmonicida* and *A. hydrophila* have been reported. The gene cluster for *A. salmonicida* possesses 19 open reading frames (ORFs) which are encoded on a plasmid (21). On the contrary, the T3SS gene cluster of *A. hydrophila* SSU, a human diarrheal isolate, has 35 ORFs which are encoded on the bacterial chromosome (180). However, another T3SS gene cluster from a fish isolate of *A. hydrophila* has only 25 ORFs (227). Although the number of ORFs in different isolates of *Aeromonas* is different, they maintain similarities between them as well as with the T3SS gene cluster from other bacteria, such as *Yersinia* species. Thus, *Aeromonas* strains mutated for genes which are homologs of genes with known functions in the T3SS of *Yersinia,* such as *yscV, yopB, yopD,* and *lcrV,* (called *ascV, aopB, aopD*, and *acrV* in *Aeromonas*), showed alteration in translocation of AexT in *A. salmonicida* (21) and AexU in *A. hydrophila* SSU, leading to reduced bacterial virulence (57, 180, 185, 227). In addition, site-directed mutagenesis of known catalytic motifs for ADP-ribosylation and GAP activity of AexU reduced cytotoxic effects on eukaryotic cells (184).

SECRETION SYSTEMS IN GRAM NEGATIVE BACTERIA

The cell wall of gram-negative bacteria is quite different from that of gram-positive bacteria and molecules exported from the bacterial cell must cross the periplasmic space sandwiched between two layers of membranes that comprise

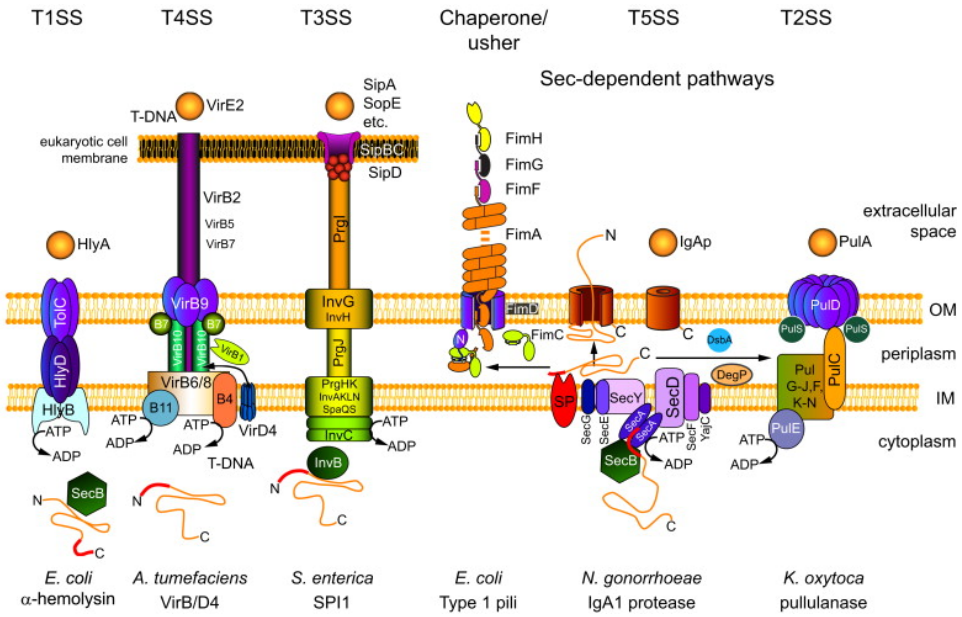

Figure 1.5: Schematic representation of the Gram-negative bacteria secretion systems. Gerlach RG. *et al* (2007)(67). ©Copyright. Reproduced with Elsevier permission.

the gram-negative cell wall. So far, most information concerning bacterial protein secretion mechanisms has been elucidated from studies of pathogenic bacteria in relation to their capacity to infect, multiply, and to survive when in contact with the host cell. Most of these survival strategies are based on the capacity to produce and secrete virulence factors such as adhesins, toxins, enzymes and mediators of motility (47). Molecular analysis of the protein secretion pathways of gram-negative bacteria has revealed the existence of at least six major mechanisms of protein secretion, which are highly conserved in the gram-negative bacteria and are functionally independent with respect to the outer membrane transport mechanism, but with similarities in the inner membrane transport (Figure 1.5).

The secretion systems are distinguished by conserved structural components, as well as secreted effectors and the mechanisms they use. The T2- and T5- secretion systems mediate the secretion of proteins through the

outer membrane in a contiguous step after they have been imported into the periplasm via the Sec- or Tat- system. On the contrary, T1-, T3-, and T4-secretion systems transport effectors in a single step mechanism from the cytoplasm to the extracellular media using multimeric apparatus. It has been reported that at least three of these systems (type 3, 4, and 6) involve multiprotein complexes, which are able to secrete and translocate protein effectors or nucleic acids directly into the eukaryotic cell cytoplasm. In this document, I will focus my review on the recently reported and poorly understood T6SS.

The type six secretion system

The T6SS gene cluster is widely distributed along Proteobacteria, although some similar sequences appear in two other phyla, the Planctomicetes and the Acidobacteria. However, phylogenetic analysis suggests that these events could be the result of horizontal gene transfer from Proteobacteria (15) (Figure 1.6).

The T6SS was initially designated as IAHP cluster (IcmF-associated homologous protein) due to one of the genes that is highly homologous to *icmF* of *L. pneumophila* T4SS (43, 175). However, the majority of the proteins encoded in the T6SS did not show homology with other T4SS components (15). On an average, the T6SS gene cluster contains around 15-25 genes, with some of them isolated based on their secretion potential. Proteins secreted or translocated via the T6SS do not have any signal peptide or secretion sequence, which indicate that they are transported through the bacterial membranes in a Sec- or Tat-independent manner (44, 52, 150, 217). The other genes represent structural

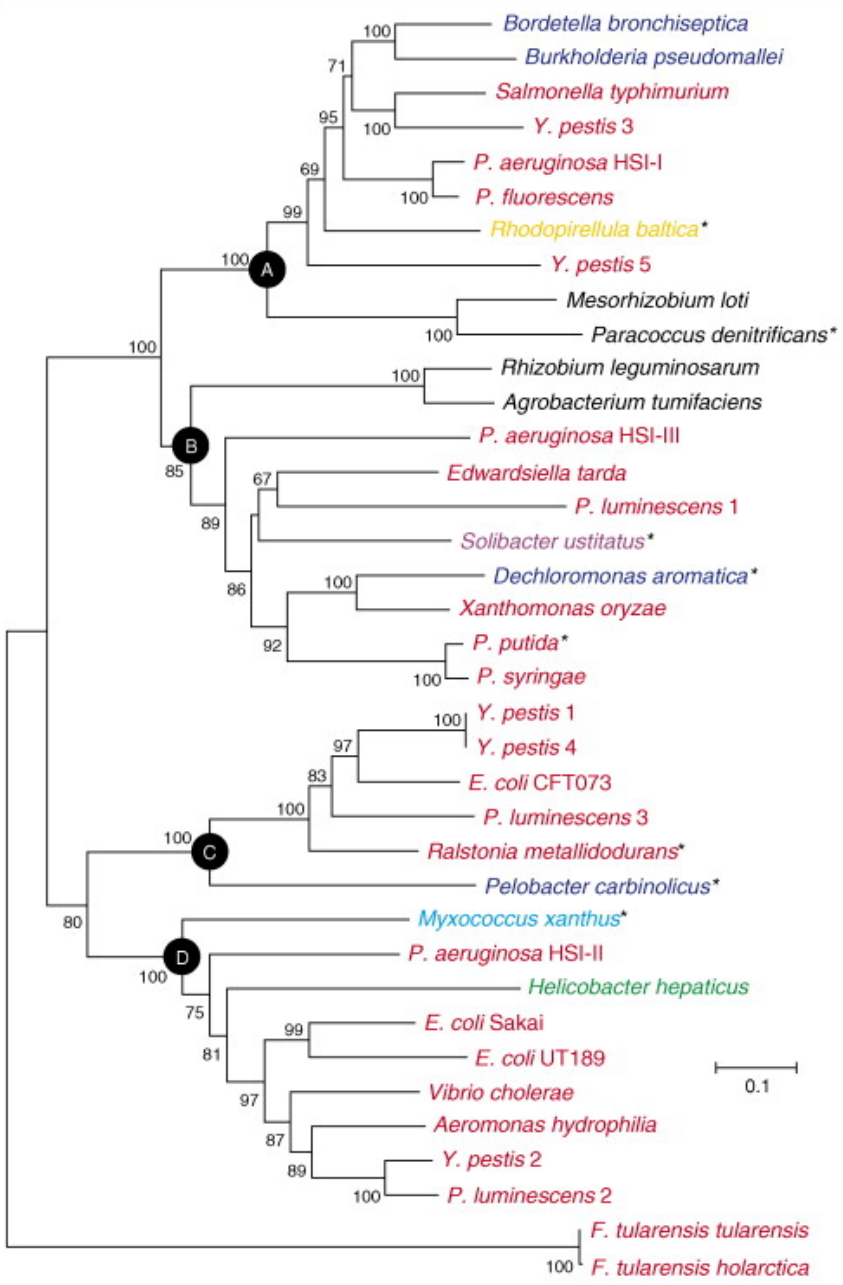

Figure 1.6: Evolutionary relationships of type VI secretion systems.
Distances were calculated based on the protein motifs DUF770 and DUF877 from all classes of bacteria possessing T6SS gene cluster. Bacterial taxons are indicated by font color: Alphaproteobacteria, black; Betaproteobacteria, blue; Gammaproteobacteria, red; Deltaproteobacteria, turquoise; Epsilonproteobacteria, light green; Acidobacteria, purple; Planctomycetales, yellow. Asterisk indicates species that are not considered to be pathogens or symbionts. The scale bar indicates 0.1 substitutions per site. Bingle L. *et al* (2008) (15)

components, or proteins involved to assist in protein translocation. However, there are still a considerable number of T6SS gene cluster genes which remain uncharacterized with unknown functions (9, 17, 45, 52, 120, 130, 153, 165, 171, 193, 221, 230).

Based on bioinformatics and proteomics analyses, several T6SS effector candidates have been reported. The hemolysin coregulated protein (Hcp) and members of the valine-glycine repeat protein family (VgrG) are the common secreted proteins among different bacteria (166).

All bacteria with a functional T6SS secrete Hcp, and it has become an indicator of the functionality of the T6SS gene cluster. Crystalographic studies showed that Hcp is able to form 40 Å hexameric rings, which polymerize in tubes which are up to 100 nm long (145). Thus, proteins could be transported out of the bacteria through the tube structure formed by Hcp (145, 166) (Figure 1.7). In some bacteria, a *vgrG* gene is linked to the *hcp* gene, and the expression of the *vgrG* gene is required for the secretion of Hcp. Likewise, expression of the *hcp* gene is required for the secretion of VgrG (60, 164, 165). In addition, structural analysis of Hcp and VgrG showed that these proteins independently formed channel-like structures, which could be used to transport macromolecules (116, 145, 164). These data suggested that VgrG and Hcp proteins could be part of the secretion apparatus and as well as play a role as an effector, although the latter role for these proteins is controversial (52, 145, 165, 171, 182, 193).

The VrgG protein family was initially associated with the Rhs (Recombination hot spot) family, and although VgrG and RhsG proteins are not homologous, they share some common characteristics, such as their hydrophilic nature, large size and regularly repeated peptide motifs (60, 212).

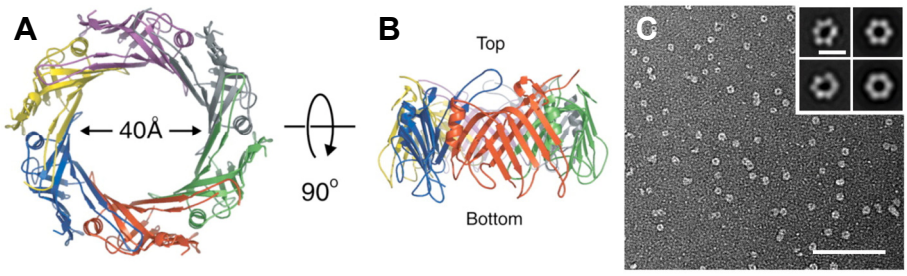

Figure 1.7: Schematic representation of Hcp hexameric rings.
(A) Top view of a ribbon representation of the crystallographic Hcp1 hexamer. (B) Edge-on view of the Hcp1 hexamer shown in (A). (C) Electron microscopy and single-particle analysis of Hcp1. Scale bar, 100 nm. (Inset) (Left) Representative class averages and (right) the same averages after six-fold symmetrization. Inset scale bar, 10 nm. Mougous JD. *et al* (2006)(145). ©Copyright. Reproduced with the American Association of the Advancement of Science permission.

Sequence analyses of VgrG proteins from different bacteria showed that all of them were highly conserved in their NH_2-terminal domains. The domain called VgrG or COG3501 (an uncharacterized protein which is conserved in bacteria) carried a region belonging to the phage GPD superfamily (c|01392) and a domain belonging to the DUF586 superfamily (pfam04524). By structural analysis, VgrG proteins share features of the bacteriophage T4 (15, 164), specifically with the gp5 and gp27 proteins of the bacteriophage T4 tail spike (60, 116, 164). During phage infection, the tail spike is able to insert into the bacterial outer membrane in the same way VgrG could insert into the host membranes and deliver effector molecules (Figure 1.8).

Some VgrG proteins have different COOH-terminal extensions with different activities; these domains, based on similarity between the RhsG and VgrG family proteins, could have been acquired by horizontal transfer (15, 60). Examples of these domains are *Yersinia intermedia* carrying a mannose-binding domain and *Y. pestis* VgrGs harboring tropomyosin-like, YadA-like, and pertactin-like domains. Likewise, VgrG from *P. aeruginosa* carried a zinc-

metalloprotease domain, while VgrG1 and VgrG3 from *V. cholerae* contained a repeat in structural toxin A (RtxA) and peptidoglycan binding domains, respectively (164). However, only recently, the translocation of VgrG1 from *V. cholerae* and VgrG1 from *A. hydrophila*, which has actin-ADP ribosylation activity, into eukaryotic host cells with deleterious effects was reported (122, 191).

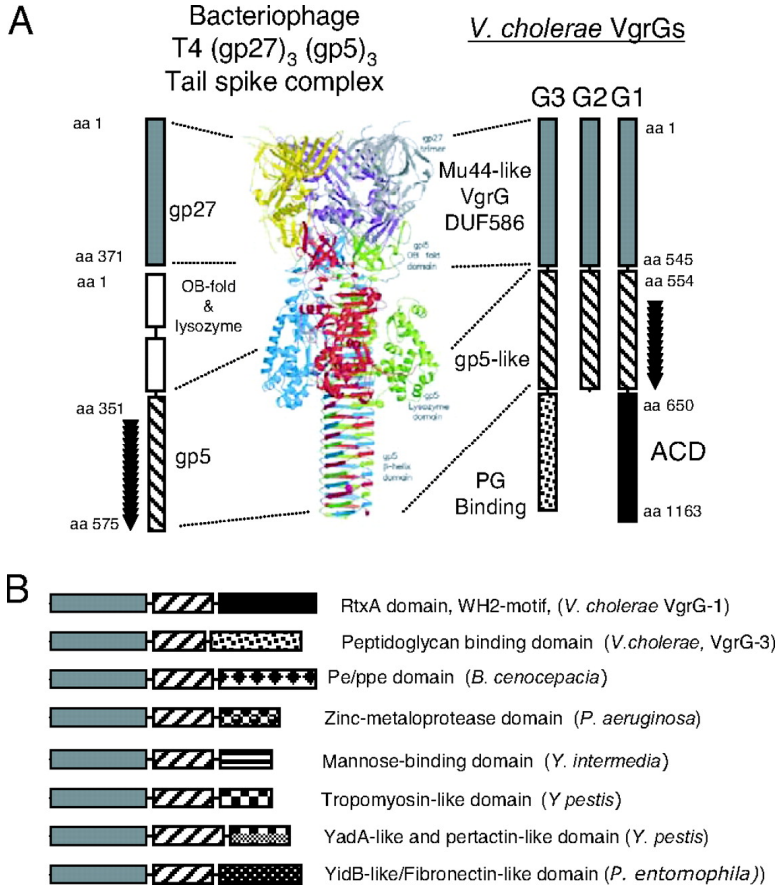

Figure 1.8: VgrGs structural features.
(A) V. cholera VgrGs share similarities with the phage tail proteins gp27 and gp5, omitting the OB-fold and lysozyme domains of the phage gp5 protein. The gp5 domain forms the cell-puncturing "needle" of the phage tail. VgrG-1 and VgrG-3 contain a C-terminal actin-cross-linking domain (ACD) and peptidoglycan (PG)-binding domain, respectively. Amino acid numbers are designated for gp27, gp5, and VgrG-1. (B) Graphical representation of a subset of the VgrG-family members with extended C-terminal domains. These proteins consist of a gp27-like domain (gray), gp5-like domain (hatched) and different C-terminal extensions (bars with various patterns). Pukatzki S, *et al* (2007) (164). ©Copyright. Reproduced with the Nature Publishing Group (A), and the National Academy of Sciences of the USA (B) permissions.

Other proteins secreted via the T6SS have been identified, which are unique for specific bacteria. These proteins are EvpP from *E. tarda* (230); RbsB, a ribose binding protein, from *R. leguminosarum* (17, 92); and TssM, a deubiquitinase, from *B. Mallei* (171). Homologous proteins to RbsB have been found in many gram-negative bacteria that carry T6SS; this includes *A. tumefaciens, A. hydrophila,* and some species of *Vibrio*. These proteins have been implicated in nitrogen fixation, ribose uptake, and chemotaxis (16, 17). The RbsB protein also shares similarities with the autoinducer-2 (AI-2)-binding protein LuxB from *V. harveyi*, that together with its ability to bind AI-2 from *A. actinomycetemcomitans,* suggest a role in quorum sensing (16, 17).

Other components of the T6SS gene cluster are represented by proteins with specific functions such as ATPases, phosphatases, kinases, and structural proteins. The T6SS gene cluster encodes ClpB homolog protein. The ClpB belongs to the family of cytosolic ring forming ATPases associated with a variety of cellular activities (AAA+), which have proteolytic activity and play a key role in maintaining protein quality by unfolding proteins to be degraded by the proteosome (60, 148). In contrast to other members of the ClpB family of proteins, the T6SS ClpB protein, called ClpV, fails to solubilize protein aggregates; however, maintains its ATPase activity which could be important in remodeling (unfold/fold) peptides to be transported through the T6SS apparatus (78, 224). The importance of ClpV as a T6SS energy provider became evident after it was shown that deletion of the *clpV* gene in *V. cholerae* induces alterations in the secretion of Hcp and VgrG (19).

Two sensor proteins have been identified in the T6SS gene cluster; PpkA, a serine/threonine kinase, and PppA, a serine/threonine phosphatase. These

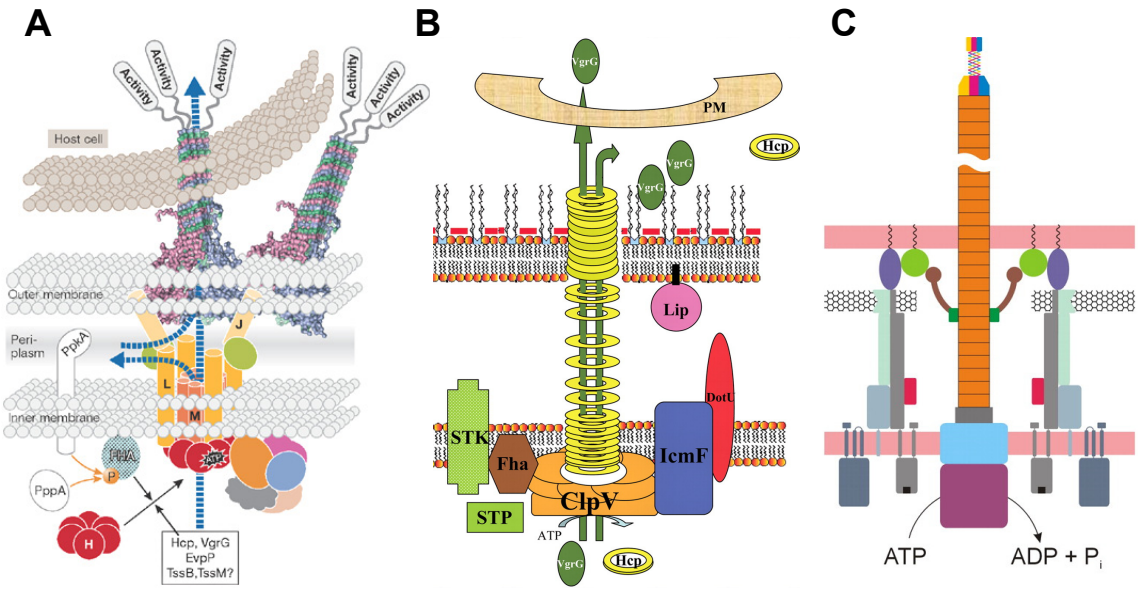

Figure 1.9: Models of the T6SS assembly and function.
A. Cascales, E., 2008(25). ©Copyright. Reproduced with the European Molecular Biology Organization permission; B. Filloux, A., *et al.,* 2008 (60). ©Copyright. Reproduced with the Society of General Microbiology permission; C. Leiman, PG, *et al*., 2009 (116) ©Copyright. Reproduced with the National Academy of Siences of the USA permission.

proteins sense environmental signals controlling the correct assembly of the apparatus. Thus, when the signal is present, PpkA induces phosphorylation of the forkhead-associated protein (FHA), which acts as a scaffold protein promoting the clustering of the ClpV ring and inducing the secretion of Hcp. Contrary to PpkA, when the sensing signal is missing, PppA inhibits Hcp secretion by dephosphorylation of FHA and dissociation of the ClpV complex (146). In the absence of PpkA, Hcp is accumulated in the periplasm suggesting a two step secretion mechanism where FHA phosphorylation controls the secretion through the outer membrane (25, 146). However, other findings such as lack of a canonical Sec- or Tat- sequence and absence of cleavage during the transport suggest that T6SS is a one step mechanism of secretion (217, 221).

Another gene frequently conserved in the T6SS gene cluster is *vasH*, which is the sigma-54 activator homolog (165, 193). Mutation of this gene attenuates the virulence of *V. cholerae* in the *D. discoideum* model (165). Further, the *vasH* gene deletion prevents production of Hcp and VgrG proteins in *A. hydrophila* (193). These results indicate that *vasH* has a role in the regulation of the T6SS in *V. cholerae* and *A. hydrophila*. However, the VasH-dependent regulation in *P. aeruginosa* has not yet been shown. In contrast, the T6SS gene cluster II in *P. aeruginosa* is regulated by the LasR/RhlR quorum sensing system (173).

Some models of the T6SS apparatus have been proposed based on the available data (Figure 1.9), however, although in the recent years the information regarding the T6SS has increased considerably, the full mechanism of secretion and translocation of effectors through the T6SS are still unknown.

Most bacteria carrying functional T6SS have pathogenic or symbiotic interactions with eukaryotic organisms (44). Some of these pathogens are human health threats, including category A or B biowarfare agents, such as *V. cholerae*, *Y. pestis*, *F. tularensis, B. mallei, S. typhimurium, E. coli, P. aeruginosa, B. cenocepacia, A. hydrophila, E. tarda*, so on (9, 44, 45, 52, 61, 74, 145-147, 153, 168, 171, 182, 193, 223, 230). Defects in virulence determinants, such as defect in adherence, cytotoxicity, intracellular growth, host-cell invasion, survival and/or persistence in the host have been associated with mutations in the T6SS genes or in genes controlling its network of regulation (25). Thus, a decrease in the ability to invade HEp-2 cells has been noted after overproduction of ClpV dominant-negative mutant in *S. typhimurium* and *Y. pseudotuberculosis* and after deletion of the T6SS gene cluster in *S. enterica* (61, 172). *E. tarda* T6SS mutants

have lower rates of replication in fish phagocytes resulting in non-fatal infections (168), Likewise, the T6SS in *S. enterica,* specifically the SciS protein, limits intracellular bacterial growth in macrophages, thereby limiting bacterial toxic effects and favoring bacterial persistence in the host. The expression of the *sciS* gene is negatively regulated by SsrB wich induces the expression of important genes required for intracellular multiplication. However, later during infection, the levels of SsrB decrease with concomitant increases in the expression of the *sciS* gene which results in preventing bacterial overgrowth (153). In *F. tularensis*, T6SS is essential for phagosome formation and disruption, escape to the cytoplasm and intracellular replication (11, 45). In *V. cholerae* and *B. cenocepacia*, the T6SS is responsible for resisting predation by the amoeba *D. discoideum* and to affect the morphology of macrophages (9, 164). In *P. aeruginosa,* mutations in the T6SS decrease virulence in the rat model of chronic pulmonary infection (160). Further, antibodies against Hcp have been detected in serum of patient with cystic fibrosis and chronic infections with *P. aeruginosa* (145). Finally, alterations in the T6SS of *B. cenocepacia, V. cholerae, V. parahaemolyticus*, and *V. anguillarum* induce defects in biofilm formation (9, 54, 91, 213).

INNATE IMMUNITY AGAINST BACTERIA

Innate immunity is the first line of protection against challenging organisms such as bacteria, viruses, and parasites. Innate immunity needs to be fast, non-specific, and effective in detecting microbial invaders. This process involves a series of wide spectrum receptors expressed by the host cells, known as pattern recognition receptors (PRR), such as toll like receptors (TLR), scavenger

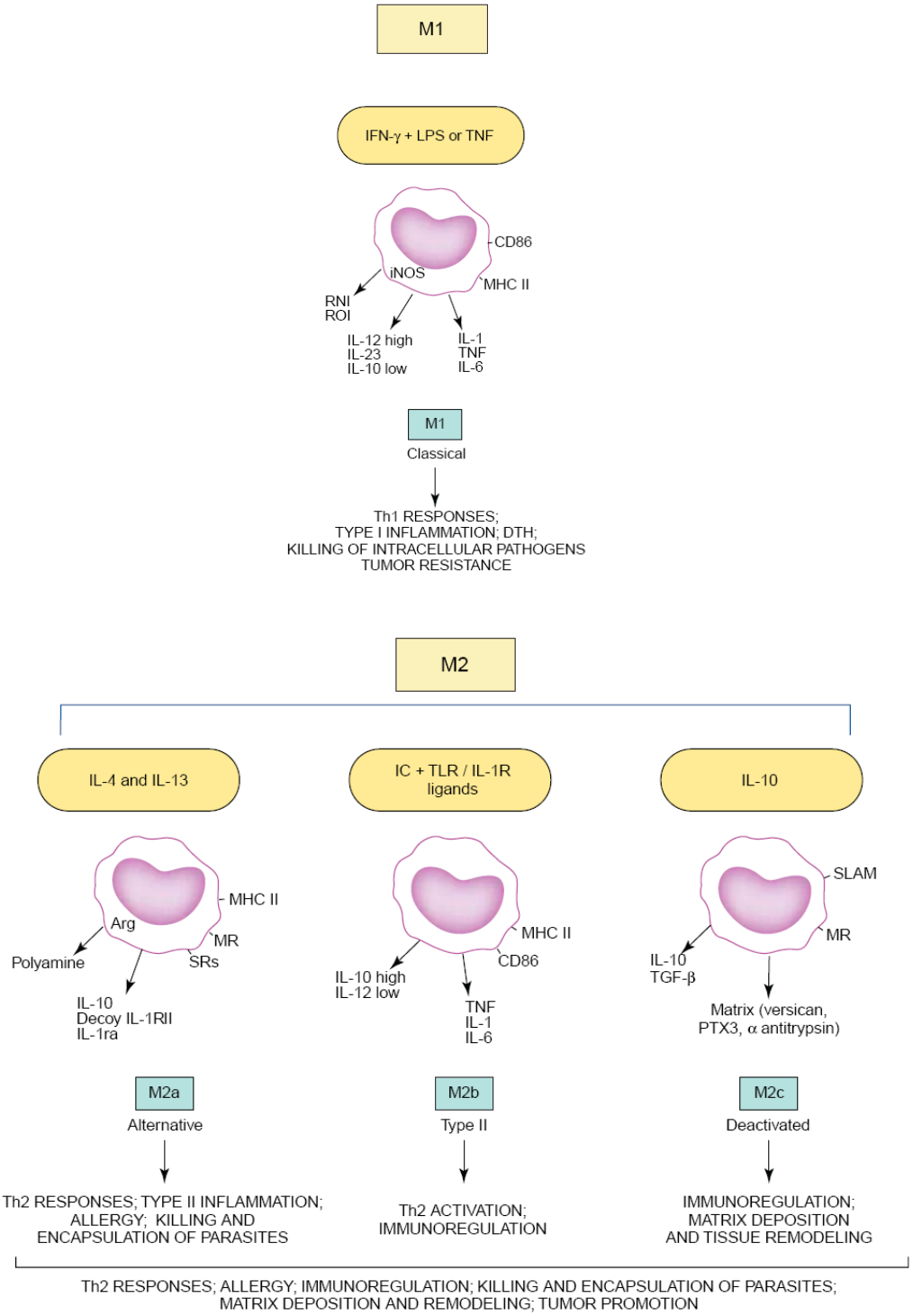

Figure 1.10: Inducers and selected functional properties of different polarized macrophages populations.
Mantovani A. *et al* (2004)(124). ©Copyright. Reproduced with Elsevier permission.

receptors, lectins, Fc receptors, complement receptor, etc. (198). These receptors are able to sense different microbial ligands (known as pathogen-associated molecular patterns or PAMPs) which include lipopolysaccharide (LPS), unmethylated DNA, RNA, flagellin, peptidoglycans (PGN), and so on (13, 99, 132, 198). Signaling through the PRR trigger a signal cascade that determines the immune response and direct maturation, activation, secretion of pro-inflammatory cytokines/chemokines, recruitment of cellular effectors (i.e., neutrophils, macrophages, dendritic cells (DCs), natural killer cells (NK)), as well as in phagocytosis and microbial degradation (81, 157) in order to control dissemination and removal of the invading microorganisms (89, 132).

Activation of macrophages is an important step in the modulation of immune responses; two forms of macrophage activation have been proposed: the classical activation of macrophages (M1) which is characterized by the induction of Th1-like responses mediated principally by IFN-γ and TNF-α, while the alternative activation of macrophages (M2) induces a Th2- like immune response that is mainly mediated by the production of IL-4 and IL-13 (124). However, a subdivision of the alternative pathway of activation called “deactivation” has also been proposed (124). This “deactivation” is mediated by the production of immunosuppressive cytokines IL-10 and TGF-β, down regulation of MHC–class II molecules and pro-inflammatory cytokines/chemokines, and low levels of IL-4 and IL-13 and low level production of reactive oxygen and nitrogen intermediates (71, 85, 137). This kind of activation could predispose the host to infection by the induction of regulatory cells which are inhibitors of inflammation even in the presence of inflammatory cytokines/chemokines (144) (Figure 1.10).

Phagocytosis is a mechanism important for innate immunity as well as for adaptive immunity because this process helps to clear invading organisms and dead cells from injured tissue and also is necessary for processing and presentation of microbial antigens to T-cells in order to develop a specific adaptive immune response (38, 81, 132).

Bacteria have developed different mechanisms to avoid innate immunity and thus, facilitate proliferation and spread in the host tissues. Hence, *P. aeruginosa* and *S. enterica* modified their LPS to avoid recognition by TLR4 (55, 75, 103), and *Neisseria* spp, altered the antigenicity of several surface molecules to avoid phagocytosis through Fcγ receptors (176). Other pathogens such as *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Bacillus anthracis* target mitogen-activated protein kinase (MAPK) signaling cascades interfering with the activation of NF-$\kappa\beta$ and with the production of pro-inflammatory cytokines (152, 194, 199). *P. aeruginosa* and *A. hydrophila* SSU target the Rho family GTPases which are essential for the control of actin polymerization and consequently for phagocytosis (41, 70, 184). *S. enterica* and *Shigella flexneri* deregulate activation of macrophages by inducing apoptosis through the T3SS effector proteins which activate caspase-1 signaling (1, 82, 232) in the same way *Y. enterocolitica* and *Y. pseudotuberculosis* inhibit anti-apoptotic signals through repression of NF-$\kappa\beta$ activation (162, 170, 209, 225). Other bacteria subvert immune system by living in the macrophage vacuolar system; however, they have to avoid their degradation in the phagosome. Hence, *Listeria monocytogenes* escapes into the host cytoplasm (51), and *S. enterica* and *Mycobacterium tuberculosis* manipulate the phagosome trafficking and maturation avoiding their degradation (190, 206). Another mechanism of evasion of innate immunity is exemplified by components

of *Mycobacterium vaccae*, *Bordetalla pertussis*, and *Y. enterocolitica* which manipulate the expression and secretion of pro-inflammatory cytokines by inducing the production of immunosuppressive cytokines, particularly IL-10. This mechanism avoids the activation of macrophages, maturation of DCs, and recruitment of granulocytes (131, 187, 231).

It has been reported that several virulence factors of *A. hydrophila* are able to impair innate immunity, thus, mutants for the T3SS of *A. hydrophila* SSU are more easily phagocytosed than the wild type bacteria. Also, the T3SS effector protein AexU, which has ADP-ribosylation (185) and Rho-GAP activity, is able to induce the rounded phenotype by targeting the actin cytoskeleton of the host and also inhibits activation of NF-κβ when produced in HeLa cells (184, 185). Another toxin is Act, which can induce the expression and secretion of proinflammatory cytokines and activation of the mitogen-activated protein kinase (MAPK) signaling and apoptosis in murine macrophages and epithelial cells, as well as to inhibit phagocytosis (63). Overall, *A. hydrophila* has developed multiple mechanisms to circumvent innate immunity to establish an infection.

Chapter 2: Materials and Methods

CELL LINES AND TRANSFECTIONS

HeLa, a human cervical epithelial cell line; HT-29, a human colonic intestinal epithelial cell line, and RAW 264.7, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA). These cells were grown in DMEM-high glucose supplemented with 10% fetal bovine serum (FBS) under normal cell culture conditions at 37^{o}C, 5% CO_2 and humid atmosphere (185). HeLa Tet-Off cells, was obtained from Clontech (Mountain View, CA). These cells were cultured in Dulbecco's modified eagle medium (DMEM) with high glucose (Invitrogen-Gibco, Carlsbad, CA), supplemented with 10% tetracycline free FBS (Clontech) and 100 μg/mL G-418 (Cellgro, Herndon, VA) as described above. The HeLa Tet-off cell line was stably transfected with the tetracycline-controlled transactivator (rTA) that regulated the expression of the gene of interest cloned in the pBI-EGFP (enhanced green fluorescent protein) vector, which contained a tetracycline response element (TRE) (Clontech).

The HeLa Tet-Off cells were transfected by electroporation with the recombinant pBI-EGFP plasmids containing different encoding regions of genes of interest. Before electroporation, HeLa Tet-Off cells were grown in T75 flasks to ~80% confluence and then trypsinized and washed with DMEM without serum. Single cell suspensions (~5x10^{6} cells/mL) were transfected with different plasmid constructs in 4-mm cuvettes (Bio-Rad, Hercules, CA) by using an exponential protocol (300 V, 950 μF, and ∞Ω) in a Gene Pulser Xcell (Bio-Rad). The cells were then recovered in complete medium, plated, and grown under standard

tissue culture conditions. HeLa Tet-Off cells transfected with the pBI-EGFP vector alone (without any insert) were used as a negative control in different assays. Western blot analysis and flow cytometry, after intracellular staining using specific antibodies, were also performed to examine the production of recombinant proteins in HeLa Tet-Off cells.

BACTERIAL CULTURES

Escherichia coli HMS174-DE3 cells, obtained from Novagen (Madison, WI), were grown after transformation with appropriate recombinant plasmid DNA in Luria Bertani (LB) medium supplemented with appropriate antibiotics (185). *E. coli* DH5α cells were used to propagate and maintain different recombinant plasmids.

Wild-type (WT) *A. hydrophila* SSU and its various mutant strains were grown in LB medium at 37^{o}C with continuous shaking (180 rpm). Deletion mutants of *A. hydrophila* SSU, namely Δ*act* and Δ*act*/Δ*vasH*, were grown in LB medium supplemented with 100 μg/mL kanamycin (Sigma St. Louis, MO) and streptomycin plus spectinomycin (100 μg/mL each; Sigma), respectively. The *act* gene encodes a cytotoxic enterotoxin which is secreted through the type 2 secretion system (T2SS) (32), while the *vasH* gene encodes a transcription activator and represents an important component of the T6SS (193).

VECTORS

The pET-30a vector (Novagen Madison, WI) was used for hyperexpression and purification purposes of the recombinant proteins. The DNA fragments were cloned into the *Bgl*II and *Xho*I or *Sal*I restriction enzyme sites of the vector. The recombinant proteins contained at the NH_2-terminal end a

histidine tag for nickel affinity chromatography. The pBI-EGFP vector that separately controls expression of the gene encoding EGFP and the gene of interest was used to express and produce recombinant proteins in a HeLa Tet-Off cell system (Clontech). The DNA fragments encoding different proteins or their various domains were cloned into the *Mlu*I and *Nhe*I restriction enzyme sites.

For VgrG1 translocation studies, the gene encoding either the full-length VgrG1 or the NH_2-terminal domain of VgrG1 from *A. hydrophila* ATCC 7966 without the stop codon was cloned in the pGEN222 vector with an *ompC* promoter inducible by 75 mM NaCl (59), between the *Cla*I and *Mlu*I restriction enzyme sites. Downstream of the above-mentioned *vgrG1* genes, the β-lactamase gene (*blaM*, without the region encoding the signal peptide) was cloned between *Mlu*I and *Sal*I restriction enzyme sites to produce in-frame fusions. Consequently, we had VgrG1 (full-length and its NH_2-terminal domain) fused with Bla at their COOH-terminal ends. These recombinant plasmids were electroporated into *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutant strains. The production of fusion proteins from these bacterial strains was tested after 2 h of cultivation at 37°C in DMEM in the presence of 75 mM NaCl by Western blot analysis using antibodies against Bla (Abcam, Cambridge, MA) as well as antibodies against VIP-2 domain of VgrG1 and VgrG2 (191).

GENERATION OF Δ*vasK* AND Δ*act*/Δ*vasK* KNOCKOUT MUTANTS

The upstream and downstream flanking DNA sequences to the *vasK* gene were amplified by PCR from the genomic DNA (gDNA) of *A. hydrophila* SSU. After the PCR reactions, the resulting up- and down- stream flanking DNA

fragments (1472- and 1198-bp, respectively) were ligated together through the introduced common *Bgl*II enzyme site and cloned into the pBluescript vector at *Xba*II/*Kpn*I restriction enzyme sites, generating a recombinant plasmid pBlue*vasK*UD. Subsequently, a streptomycin/spectinomycin (Sm^r/Sp^r) gene cassette flanked by the *BamH*I site was removed from plasmid pHP45Ω and inserted at the *Bgl*II site (compatible with the *BamH*I site) of pBlue*vasK*UD which generated the recombinant plasmid pBlue*vasK*UDSm^r/Sp^r. After digestion with *Xba*II/*Kpn*I restriction enzymes, the DNA fragment containing the up- and down-*vasK* flanking DNA sequences, as well as the Sm^r/Sp^r gene cassette, was removed from the above plasmid and ligated into the pDMS197 suicide vector (harbors tetracycline resistance [Tc^r] and the *sacB* gene [encodes levansucrase, which is lethal to bacteria when the *sacB* gene is induced with sucrose]) at the compatible restriction enzyme sites, and the resulting plasmid (pDMS197*vasK*UDSm/Sp) was transformed into *E. coli* SM10 (λ*pir*). The recombinant *E. coli* [pDMS197*vasK*UDSm/Sp] clone was conjugated with either the WT *A. hydrophila* SSU-R (rifampin [Rif] resistant) or its Δ*act* mutant to generate Δ*vasK* single- and Δ*act*/Δ*vasK* double- knockout mutants of *A. hydrophila* SSU, respectively. The transconjugants were selected based on their resistance to appropriate antibiotics and sucrose and subjected to further analyses. Briefly, transconjugants were plated onto LB agar plates with Rif (200 μg/ml), Sm^r/Sp^r (50 μg/mL each) and 15% sucrose. Single colonies that replicated on plates with Sm and Sp antibiotics, but were sensitive to Tc, were verified by Southern blot analysis (179) using the *vasK* gene probe.

GENERATION OF Δ*vasH* AND Δ*act*/Δ*vasH* KNOCKOUT MUTANTS

To generate the Δ*vasH* knockout mutant of *A. hydrophila* SSU in the WT background strain, first the recombinant plasmid pDMS197*vasH* was constructed. To clone the *vasH* gene in the pDMS197 vector, this gene was PCR amplified using gDNA of *A. hydrophila* SSU. The PCR product and vector were digested with *Xba*I/*Xma*I enzymes, ligated and electroporated into the *E. coli* SM10 (λ*pir*) strain. To clone the Sm/Sp^r gene cassette into the *vasH* gene of pDMS197*vasH* plasmid, the cassette was PCR amplified using the pHP45Ω plasmid and N-*Kpn*I and C-*Kpn*I primers (193). Then *E. coli* SM10 strain containing the final recombinant plasmid pDMS197*vasH*Sm/Sp was conjugated with WT *A. hydrophila* and its Δ*act* mutant strain (SSU-R). Transconjugants were plated as described above, and the correct identity of the clone verified by Southern blot analysis using the *vasH* gene probe.

COMPLEMENTATION OF *A. hydrophila* SSU Δ*vasH* AND Δ*vasK* KNOCKOUT MUTANTS

To complement the *vasH* gene, the latter was PCR amplified using gDNA of *A. hydrophila* SSU**.** This DNA fragment (1.5 kb) was cloned in the pBR322 vector (Tc^r and Ap^r) at *BamH*I-*EcoR*I sites and transformed into the *E. coli* TOPO 10 strain. The pBR322-*vasH* (Tc^s and Ap^r) recombinant plasmid was isolated from the *E. coli* strain and electroporated in the *A. hydrophila* Δ*vasH* mutant. To complement the *vasK* gene in the *A. hydrophila* mutant, pCR2.1-*vasK* plasmid was digested with *Eco*RI restriction enzyme and the gene (3.5-kb fragment) was excised from gel, ligated with pBR322 vector and transformed into *E. coli* TOPO 10 cells. The pBR322-*vasK* recombinant plasmid was isolated from the *E. coli* strain and electroporated into the *A. hydrophila* mutant strain. We then generated as a control the WT *A. hydrophila* strain containing the pBR322 vector alone. To

test the presence of the pBR322-*vasH* and pBR322-*vasK* recombinant plasmids and of pBR322 vector in *A. hydrophila*, the plasmid DNA was isolated from all of the above-mentioned strains and digested with appropriate restriction endonucleases.

RECOMBINANT PROTEIN PRODUCTION

E. coli HMS174-DE3 cells containing pET-30a recombinant plasmids were grown in 10 mL of LB medium supplemented with kanamycin (100 μg/mL) overnight. On the next day, the bacterial culture was diluted 1:10 and grown for 1 h and then induced with 1 mM of IPTG (Sigma) for 4 h at 37°C. Recombinant proteins were purified by using the ProBond purification system (Invitrogen) and following the non-denaturing protocol as described by the manufacturer. The proteins from the nickel column were eluted with 250 mM of imidazole. One-mL fractions were collected and subjected to sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining, to identify fractions containing the protein of interest. Then these fractions were mixed and dialyzed overnight against phosphate-buffered saline (PBS) at 4°C. Protein concentration was measured by using a Bradford assay (Bio-Rad).

ANTIBODY PRODUCTION

Female Swiss Webster mice (n=5; Taconic Farms, Germantown, NY) were immunized *via* the intraperitoneal route with 10 μg of purified recombinant protein mixed with complete Freund's adjuvant (Sigma) or a synthetic adjuvant derived from *Salmonella minnesota* monophosphoryl-lipid A (MPL)/ trehalose dicorynomycolate (TDM)/ tubercule bacillus cell wall skeleton (CWS) in 2%

Tween 80 (Sigma). The animals were boosted with the respective antigen on day 15 by using incomplete Freund's adjuvant (Sigma). Sera were obtained from mice after bleeding them at weeks 2 and 4 after immunization. The antibody specificity was determined by Western blot analysis by using whole *E. coli* lysates containing recombinant proteins, as well as the respective purified recombinant protein as the source of antigen.

WESTERN BLOT ANALYSIS

For assessment of antibody reactivity in sera, recombinant proteins (2 μg/lane) were subjected to SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare) following the standard Western blot procedure (36). Membranes were cut into strips corresponding to the lanes of the gel, blocked with 1% bovine serum albumin [BSA]/5% skim milk, and were subsequently incubated with sera taken from individual mice (1:1000) and diluted in Tris-buffered saline (TBS), pH 7.6, and 0.5% skim milk for 1 hr with constant shaking at room temperature. The strips were then incubated for 1 hr with secondary antibody (Goat α-mouse IgG [diluted 1:10000] conjugated with horse-radish peroxidase ([HRP] [Southern Biotechnology Associates, Inc., Birmingham, AL]). Five washes of strips were performed between various steps using TBS/0.05% Tween 20 for 10 min each. The blots were developed with Super Signal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL) followed by X-ray film exposure.

Likewise, HeLa Tet-Off cells were lysed in SDS-Tris-Glycine buffer (36) after 24 h of transfection and subjected to electrophoresis and Western blot analysis as described above.

INTRACELLULAR STAINING

HeLa Tet-Off cells after 24 h of transfection with various recombinant plasmids were permeabilized using CytoFix™/CytoPerm™ (Becton Dickinson, San Diego, CA). The antibodies contained in the hyper-immune serum (diluted 1:100), as well as the pre-immune serum (diluted 1:100), was used as the source of primary antibodies. Then, the recombinant proteins were visualized using phycoerythrin (PE)-conjugated α-mouse IgG antibody (Santa Cruz, Santa Cruz, CA), as previously described (185). The samples were acquired in a FACScan™ (Becton Dickinson) and analyzed using CellQuest™ (Becton Dickinson) software and WinMDI©.

HOST CELL MORPHOLOGY

HeLa cells plated in 6-well plates were co-cultured with different mutant strains of *A. hydrophila* SSU at an MOI of 5 in DMEM without FBS. Changes in morphology were evaluated by phase contrast microscopy over a 90-120 min period. In parallel, to test the importance of bacterial-host cell contact in morphological changes of the HeLa cells, we performed co-cultures of bacteria and host cells by using transwell inserts having a 0.4-μm pore size (Costar, Corning, NY). This strategy prevented direct contact of bacteria with the host cells, but allowed the diffusion of soluble factors produced by bacteria onto the host cells.

HeLa Tet-Off cells were electroporated with *hcp, vgrG2* and various *vgrG1* constructs and, after 24 h of incubation, were stained with Alexa-fluor 568-conjugated phalloidin (Invitrogen-Gibco) following the manufacturer's instructions. Briefly, the cells were fixed and permeabilized *in situ* with Cytofix/Cytoperm (Becton Dickinson, San Jose, CA) for 20 min at 4^{o}C. Then, the

cells were scraped and stained with Alexa fluor 568-conjugated phalloidin for 1 h at room temperature and washed with Perm/Wash solution (Becton Dickinson). The cells were acquired in a FACScan flow cytometer and analyzed with FACSDiva software (Becton Dickinson). Additionally, the stained cells were placed on glass slides by using a Shandon cytospin cytocentrifuge (Thermo Scientific Waltham, MA) at 1500 rpm for 5 min. The cover slips were mounted with medium containing DAPI (Vector, Burlingame CA), and the images were then acquired and analyzed under fluorescence microscopy (Olympus BX51/DPManager v.1.2.1.107/DPController v.1.2.1.108, Olympus Optical CO. LTD).

QUANTIFICATION OF G- AND F-ACTIN

The G-actin/F-actin In Vivo Assay Kit (Cytoskeleton, Denver, CO) was used to quantify the amounts of G- and F- actin present in HeLa cells transfected with the pBI-EGFP vector expressing and producing various domains of VgrG1. Likewise, G-actin/F-actin ratios were determined in HeLa cells co-cultured with different mutant strains of *A. hydrophila* SSU, following the manufacturer's instructions. Briefly, HeLa cells plated in 12-well plates were lysed with 300 μL of lysis buffer previously warmed to 37^{o}C. The cells were scraped, and the mixture passed through a 25-gauge needle. The total cell lysates were centrifuged at 100,000 *xg* for 1 h at 37^{o}C and the supernatants collected in 1.5-mL microfuge tubes. The pellets were resuspended in the same volume (300 μL) of the SDS-PAGE loading buffer. Supernatants containing G-actin and pellets containing F-actin were subjected to SDS-10% PAGE and the proteins transferred to nitrocellulose membranes. The Western blot analysis was performed by using an anti-actin

antibody (Cytoskeleton) diluted 1:1000 followed by anti-rabbit-HRP conjugated secondary antibody. The density of each band was calculated by using AlphaEasyFC software (Alpha Innotech, San Leandro, CA). The results were reported as a percentage of F- and G-actin per sample. The sum total of G- and F- actin corresponded to 100% (133, 229).

TRANSLOCATION ASSAYS

Bacterial strains grown to log phase were washed and re-suspended in PBS, and their turbidity measured at OD_{600} nm. Human colonic epithelial cells, HT-29, were grown in 6 well plates to ~80% confluence in DMEM medium supplemented with 10% FBS before culturing with bacteria, and the infection was performed at an MOI of 5 in DMEM/0.5% FBS medium. The bacteria and host cells were co-cultured for 2 h at 37ºC in 5% of CO_2. Subsequently, four fractions were collected: i) supernatant fraction, ii) cytoplasmic fraction, iii) eukaryotic host membrane fraction, and iv) whole bacterial lysates (181, 185, 193). The supernatant fraction was collected by removing the medium and centrifuging it at 1000 *x g* for 10 min. The supernatants were separated from the pellet and filtered through a 0.22-μm membrane filter. Proteins present in the supernatant fraction were precipitated with trichloroacetic acid (TCA) (10% final concentration) and pelleted by high- speed centrifugation at 14000 *x g* for 15 min at 4ºC. The pellet was re-suspended in 1X Lammeli loading buffer (36).

The cytoplasmic fraction was collected following lysis of the host cells with 500 μl of sterile water. The cells were disrupted by gentle pipetting and then centrifuged at 6000 *x g* for 10 min at 4ºC. The eukaryotic membrane fraction was obtained by extraction of the proteins from the above pellet with 500 μl of the cell

lysis buffer (200 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 0.1% Triton X-100, 10 mM tris-HCl pH 7.0). Only eukaryotic cell membranes were affected by this method, while the bacterial membrane integrity was maintained. After centrifugation, the remaining pellet was re-suspended directly in 1X loading buffer. This fraction was considered as the whole bacterial lysate fraction. Samples as obtained above were separated by 4-20% gradient SDS-PAGE, and then proteins were electro-transferred to nitrocellulose membranes for performing Western blot analysis as described earlier. As controls of contamination between fractions, antibodies to actin (eukaryotic cytoplasmic protein), calnexin (eukaryotic membrane protein), and DnaK (bacterial cytosolic protein) were run in parallel for Western blot analysis. Although less likely, the cytoplasmic fraction could contain bacterial secreted proteins that are present in endocytic vesicles.

For studying translocation of VgrG1::Bla fusion proteins in HeLa cells, we used CCF4-AM as a substrate for Bla. CCF4-AM, a fluorescence resonance energy transfer (FRET)-based substrate for β-lactamase, contains a cephalosporin core linked to coumarin and fluorescein. CCF4-AM is hydrophobic and permeable to mammalian cell membranes. Once in the cytoplasm, this substrate is cleaved by esterases allowing its retention. CCF4 requires an excitation of 408 nm and produces emissions of 460 nm (blue from coumarin) and 530 nm (green from fluorescein). In the presence of β-lactamase activity, the substrate is cleaved, resulting in the breakage of FRET and loss of 530 nm emission (green) (122, 186).

For translocation proposes, the full-length VgrG1::Bla or the NH_2-terminal VgrG1::Bla from the pGEN222 vector was produced from *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutant strains, as described in the earlier section. These mutant

strains harboring only the vector pGEN222 were used as a negative control. HeLa cells that were previously detached by using a non-enzymatic solution (Sigma) were placed in flow cytometry tubes in DMEM medium without serum at a concentration of $1x10^6$cells/mL. These cells were infected at an MOI of 10 with *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutant strains producing different forms of VgrG1::Bla fusion proteins. After 15 min of infection, CCF4-AM working solution (6X) (Invitrogen) was added and the tubes incubated for 45 min. The cells were then acquired in a Becton Dickinson FACSAria flow cytometer in which a violet laser diode (408 nm) was used for excitation and which detected emissions at 450/40 (blue) and 530/30 (green). Ratios between percentages of blue HeLa cells and percentages of green cells were calculated for analysis (191).

HCP BINDING TO RAW 264.7 MACROPHAGES

To perform this experiment, supernatants of *A. hydrophila* SSU Δ*act* and double knockout mutant Δ*act*/Δ*vasH* were used to avoid the cytotoxic effects mediated by Act. Briefly, bacteria were grown overnight in the LB medium containing appropriate antibiotics. The next day, the cells were washed 3X with PBS, quantified by optical density measurements at 600 nm and grown in DMEM supplemented with 1% FBS at a concentration of 5 x 10^6 cfu/mL. After 2 h, the medium was centrifuged and filtered through a 0.22-μm filter. Subsequently, 2 x 10^6 RAW 264.7 cells/well were grown in 6-well plates. After the cells were attached, the medium was exchanged with 2 mL of conditioned medium used to grow bacteria (see above) and incubated for 2 h at 37ºC. Then, the host cells were washed 3X with PBS and lysed with 500 μL of water. The supernatant was

collected as a cytoplasmic fraction, and the pellet as a membrane fraction. These samples were subjected to SDS-4-20%-PAGE, transferred to nitrocellulose membranes and tested by Western blot analysis for the presence of Hcp using specific antibodies. Similar assays using rHcp were preformed. For these assays, DMEM supplemented with 1% FBS containing rHcp (5 μg/mL) was used instead of bacteria-grown conditioned DMEM (193).

PHAGOCYTOSIS

RAW 264.7 murine macrophages were plated and grown to a density of $1x10^5$ cell/well in a 96-well plate in DMEM with 10% FBS under normal tissue culture conditions for 2 h. Subsequently, 5 x 10^5 bacteria that were previously washed and re-suspended in PBS were added. The plate was centrifuged at 300 *x g* for 5 min to facilitate contact between macrophages and bacteria and then incubated for 30 min at 37ºC. Next, gentamicin was added at a final concentration of 100 μg/mL for 1 h to kill extracellular bacteria. Subsequently, the cells were washed twice with PBS, and lysated in 200 μL of water. Different dilutions were plated on LB agar plates and incubated overnight at 37ºC. The colony formation units (cfu) were calculated based on the number of bacteria used for infection by determining the number of colonies inside the macrophages multiplied by the dilution factor (192).

HOST CELL APOPTOSIS

We evaluated the extent of apoptosis of HeLa Tet-Off cells expressing and producing different recombinant proteins by detection of cytoplasmic nucleosomes and measurement of caspase 3 and 9 activation. Cytoplasmic nucleosomes were detected by the cell death ELISA kit (Roche, Indianapolis, IN)

following the manufacturer's instructions. The HeLa Tet-Off cell lysates, anti-DNA peroxidase, and anti-histone biotinylated antibodies were incubated in streptavidin-coated, 96-well plates for 2 h at room temperature. Subsequently, the plates were washed three times and incubated with ABTS (2,2"-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) as the substrate. The color reaction was measured in a microplate reader at a 405 nm wavelength. Colorimetric caspase 3 and 9 activity assays (Biovision Inc., Mountain View, CA) were performed as follows: 200 μg of protein (whole-cell lysate from HeLa Tet-Off cells expressing different *vgrG1* fragments), 50 μL of 2X reaction buffer and 5 μL of peptide substrate (N-acetyl-Leu-Glu-His-Asp-p-nitroaniline [LEHD-pNA] for caspase 9 assays, or N-acetyl-Asp-Glu-Val-Asp-7-amino-4-p-nitroanilide [DEVD-pNA] for caspase 3 assays) were incubated at 37^{o}C between 2 and 24 h. The color reaction was measured every hour at a 405 nm wavelength in a microplate reader (185, 193).

HOST CELL VIABILITY

To determine host cell viability, we performed the incorporation of 7-amino actinomycin D (7-AAD, Becton Dickinson) and the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] assay for cell survival as we previously described (185). HeLa Tet-Off cells expressing and producing different recombinant proteins were detached from the tissue culture plate with 0.25% trypsin-EDTA, washed and then incubated for 10 min with 7-AAD (5 μL per tube). Immediately after staining, the cells were examined in a FACScan flow cytometer to determine the percentage of EGFP and 7-AAD double-positive cells. For MTT assays, HeLa cells at a concentration of 3 x 10^5 cells/mL were

plated in 96-well plates, after transfection with pBI-EGFP alone and containing *hcp* gene, and grown under normal tissue culture conditions. After 24 h, 50 μg of MTT (Chemicon-Millipore, Billerica, MA) was added and the plates incubated for 3 h at 37ºC. Any crystals that formed were dissolved by adding 100 μL of 0.04 N HCl in isopropanol. The color reaction was acquired with a microplate reader at 570 nm (191, 193).

LDH RELEASE ASSAY

RAW 264.7 murine macrophages and HeLa cells were infected at an MOI of 0.5 with WT *A. hydrophila*, and Δ*act*, Δ*act*/Δ*vasH* and Δ*act*/Δ*vasK* mutants. During infection, cell morphology was monitored, and at various time points after infection, host cell cytotoxicity was measured by the release of lactate dehydrogenase (LDH) enzyme using CytoTox 96® kit (Promega, Madison, WI) in the tissue culture supernatant (180). The percentage of cytotoxicity was calculated as recommended by the manufacturer using the following formula: [(OD_{490} Sample – OD_{490} Spontaneous)/ (OD_{490} Maximum Release – OD_{490} Spontaneous)] x 100. OD_{490} spontaneous indicated LDH release from uninfected cells into the culture supernatant and maximum release denoted LDH release obtained by lysis of the uninfected cells. Three independent experiments were performed in duplicate wells (193).

DETECTION OF SPECIFIC HCP ANTIBODIES FROM SERA OF MICE INFECTED WITH WT *A. hydrophila* SSU

A group of 10 Swiss Webster mice was infected i.p. with WT *A. hydrophila* at a dose of approximately 1 LD_{50}. After 2 weeks of infection, sera from the surviving mice were pooled and used as the source of primary antibodies in the

Western blot analysis with purified rHcp as an antigen (181). As a negative control, pre-immune sera were used.

ANIMAL SURVIVAL EXPERIMENTS

Groups of 10 Swiss Webster mice were infected *via* the i.p. route with WT *A. hydrophila*, ∆*vasH*, and ∆*vasK* mutants as well as their complemented strains in accordance with an approved IACUC protocol. We also used WT *A. hydrophila* with pBR322 vector alone as a control in these experiments. Deaths were recorded for 16 days post-infection. The bacterial doses used represented approximately 2 LD_{50} of WT *A. hydrophila* (222). In another experiment, animals were immunized with purified rHcp (as described for the antibody production experiment) and then challenged with the WT *A. hydrophila* at a dose of 3 LD_{50} by the i.p. route after 1 month of immunization. Control animals included those that were given the adjuvant alone (without the antigen) and then infected with the WT bacterium. Deaths were recorded for 16 days post-infection (193).

2-DIMENSIONAL (2-D) GEL ELECTROPHORESIS AND MASS SPECTROMETRY

The LB cultures of the *A. hydrophila* SSU mutants, Δ*act* and Δ*act*/Δ*vasH,* were grown overnight and then re-grown for 2 h to a cell density of 5 x 10^6 cells/mL in 10 mL of DMEM with high glucose (Invitrogen/Gibco) and without the FBS at 37^{o}C. The supernatants obtained after centrifugation at 6000 *x g* for 10 min were filtered through 0.2-µm filters. Next, the supernatants were precipitated with trichloroacetic acid (TCA) (Sigma; 10% v/v final concentration), and the pellet was collected by high speed centrifugation at 14000 *x g* for 20 min at 4^{o}C. The protein pellets were washed 3 times with cold acetone (Sigma), dried and resuspended in 200 µL of DeStreak rehydration solution (GE Healthcare). For the

first dimension, the proteins were separated in 13 cm, 3-10 pH non-linear strips (GE Healthcare) using the following protocol: rehydration 50V, 10 h; 250V, 1 h; 500V, 1 h; 1000V, 2 h; and 8000V, 7 h, in a IPphor apparatus (GE Healthcare) at 75 μA/strip and 20°C. Subsequently, the strips were incubated in equilibrium buffer (GE Healthcare) for 10 min followed by SDS-(4-20%) PAGE. The gels were stained with Sypro-Ruby (Bio-Rad), and images for analysis were acquired in a Gel-Doc system (Bio-Rad).

As an analysis strategy for the 2-D gels, we sought for those proteins that were secreted *via* the T6SS. The proteins present in supernatants of the Δ*act* mutant of *A. hydrophila* SSU, but not in the supernatants of the Δ*act*/Δ*vasH* mutant, were considered prime candidates for future study. For this analysis, we used 3 gels per bacterial mutant strain, and the differential spots from the 2-D gels were identified by using Progenesis Samespot v.2.0.2733.19819 software (Nonlinear Dynamics, Durham, NC). Different spots were manually picked, trypsin digested, and analyzed by mass spectrometry (MALDI-TOF) at the Biomolecular Resource Facility Core at the University of Texas Medical Branch at Galveston. The peptide sequences then were matched against the proteobacteria database, and results with high homologies (low expectation numbers [**Table 4.1**]) were examined (191).

ADP-RIBOSYLATION ASSAY

HeLa cells were detached from the tissue culture flask with trypsin-EDTA, washed, re-suspended, and lysed by sonication in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and protease inhibitors. The whole lysate was centrifuged at 14000 *xg* for 10 min at 4°C, and the

supernatant was used for the ADP-ribosylation assay (80). Briefly, 50 μg of normal HeLa cell lysates [as a source of host protein(s) that are ADP-ribosylated] were incubated with 1 μg of different purified recombinant VgrG1 proteins and 10 μM of NAD conjugated with Biotin (R&D Systems, Minneapolis, MN) for 30 min at 37^{o}C. The reaction was stopped by adding SDS-sample buffer and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes (GE Healthcare) and incubated with streptavidin-HRP after blocking of non-specific sites with 1% BSA. Super signal west pico chemiluminescence substrate was used to develop the blot, and this was followed by X-ray film exposure.

In order to confirm the ADP-ribosylation of target protein for VgrG1, an ADP-ribosylation assay as described above was followed but instead of HeLa cell lysates, 1 μg of recombinant non-muscle actin (Cytoskeleton) was used as the target protein for ADP-ribosylation (191).

BACTERIAL SPREADING

Mice were infected via the i.p route with sub-lethal doses of *A. hydrophila ΔactΔvasH* mutant alone or in combination with the rHcp protein. The animals were euthanized after 48 h, and sections of liver, spleen, and lungs were homogenized and used to determine bacterial burden (3, 192).

INTRAPERITONEAL LAVAGE

After 4 h of challenge of mice via the i.p. route with a sub-lethal dose of *A. hydrophila ΔactΔvasH* mutant alone or in combination with the rHcp, the animals were euthanized, and the peritoneal cavity was flushed with 1.5 mL of sterile Hanks solution (Invitrogen). The lavage was collected and centrifuged at 1500 rpm for 5 min. The supernatant was used for evaluating various cytokines by the

multiplex beads array, and the pellets containing host cells were used for determining their phenotypes by flow cytometry (see later sections) (192).

FLOW CYTOMETRY Hcp-BINDING ASSAY

The whole cell population from the intraperitoneal lavage of mice was incubated with rHcp (10 μg/mL) for 1 h. Then, the cells were incubated for 30 min with anti- mouse CD16/CD32 antibodies (Becton Dickinson). Next, the cells were dispensed into different tubes and incubated with pre-immune mouse serum (1:100) as an isotype control, or with the mouse anti-Hcp serum (1:100) for 1 h. Subsequently, the host cells were incubated with Alexa Fluor 488 conjugated goat anti-mouse antibodies (Invitrogen) for 45 min, followed by incubations with PE-Cy5 conjugated, anti-mouse F4/80 and PE conjugated, anti-mouse Gr-1 antibodies and their respective isotype controls. Between steps, the cells were washed twice with chilled Hanks solution, and all the incubations were performed on ice. The cells were acquired in a FACScanto (Becton Dickinson) flow cytometer and analyzed by using FACSDiva software (Becton Dickinson) (192).

INTRAPERITONEAL CYTOKINES

The supernatants obtained after i.p. lavages of mice (see under section intraperitoneal lavage) were tested for cytokine/chemokine levels by a multiplex beads array (Millipore, Bedford, MA) following the manufacturer's instructions. Briefly, 25 μL of the supernatant was mixed with the bead suspension for 2 h and followed by 1 h incubation with biotin-conjugated secondary antibodies and 30 min incubation with PE-streptavidin. The data were acquired and analyzed in a Bioplex 200 system which uses a Bioplex manager software V.5.0 (Bio-Rad) (192).

We measured levels of TGF-β by using an ELISA (eBiosciences, San Diego, CA) and following the manufacturer's instructions. Briefly, 100 μL of intraperitoneal lavage supernatant samples was activated with 20 μL 1N HCl for 10 min, which was then neutralized by adding 20 μL 1N NaOH. An aliquot (100 μL) of the activated samples was then incubated in an anti-TGF-β, pre-coated plate for 2 h, followed by 1 h incubation with biotin-conjugated detection TGF-β antibody, and 30 min incubation with avidin conjugated-HRP. Between steps, five washes with 200 μL of PBS-0.05% Tween 20 were performed. Tetramethylbenzidine (TMB) was used as the HRP substrate, and the plate was read in a microplate reader Versa-max (Molecular Devices, Sunnyvale, CA) (192).

FLOW CYTOMETRY

Cell pellets collected after i.p. lavages of mice (see under section intraperitoneal lavage) were stained with a panel of antibodies conjugated with FITC (anti-CD11c, anti-CD69), PE (anti-Gr-1, anti-MHC-class II), and PE-Cy5 (anti-F4/80) for flow cytometry (eBiosciences, San Diego, CA). The cells were incubated with antibodies for 1 h and then washed twice with 1 mL of PBS and fixed with 2% paraformaldehyde. The cells were acquired in a FACScan and analyzed by FACSdiva software (Becton Dickinson). Prior to incubation with antibodies, receptors Fcγ III/II were blocked using anti-CD16/CD32 antibodies (BD Pharmingen, Palo Alto, CA) for 30 min (192).

STATISTICAL ANALYSIS

Two-way ANOVA and Bonferroni posttests were used for statistical analysis of the data using GraphPad Prism version 4.02 for windows (Software

MacKiev, San Diego, CA). The animal mortality data were analyzed by the Fisher exact, and Logrank tests.

Chapter 3: Molecular Characterization of a Functional Type VI Secretion System from a Clinical Isolate of *Aeromonas hydrophila* [1]

INTRODUCTION

Protein secretion in Gram-negative bacteria presents a challenge because the secreted proteins must pass through at least two membranes before they can reach the extracellular milieu. To date, five secretion systems have been molecularly well-characterized in Gram-negative bacteria. These systems are highly conserved across different bacteria with unique characteristics which permit their differentiation from one another (47). Recently, a new protein secretion mechanism called virulence-associated secretion (VAS), or type VI secretion system (T6SS), was described in *Vibrio cholerae* (165). Previously, a gene cluster encompassing the T6SS was identified by bioinformatics analysis as being highly conserved among several gram-negative pathogens, and yet it exhibited differences in its organization in various bacteria (44). It is believed that the primary function of the T6SS is to mediate the extracellular export of virulence factors (165). This mechanism of secretion is different from T3- and T4-secretion systems because the T6SS represents an assembly of genes with a novel linkage that secretes proteins lacking the classical Sec-dependent signal sequences (217). Several studies indicated participation of this cluster in the pathogenicity of different bacteria, such as *Pseudomonas aeruginosa* in which the role of T6SS-associated effector, hemolysin co-regulated protein (Hcp), was

[1] 193. **Suarez, G., J. C. Sierra, J. Sha, S. Wang, T. E. Erova, A. A. Fadl, S. M. Foltz, A. J. Horneman, and A. K. Chopra.** 2008. Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. Microb Pathog **44:**344-361.

demonstrated in cystic fibrosis (145). Likewise, in *Francisella tularensis* and *Salmonella enterica*, these gene clusters were necessary for intracellular growth in eukaryotic cells (45, 153). In *Burkholderia mallei*, it was found that the T6SS was required for virulence in the hamster model of glanders infection (171). Most important by the role of this secretion system in the virulence of bacterial pathogens in general is still largely unknown.

In this study, we reported, for the first time, the presence of a functional T6SS gene cluster in a clinical isolate SSU of *A. hydrophila*. Our characterization of the T6SS showed that this cluster is able to secrete and translocate effector proteins into eukaryotic host cells and that mice immunized with a secreted component of this system (Hcp) were protected from a lethal challenge dose of the WT bacterium. Thus, the components of the T6SS in *A. hydrophila* SSU constitute exciting candidates for the potential development of preventative or therapeutic vaccines, as well as targets for antimicrobial drug development.

RESULTS

Type VI secretion system gene cluster is present in *Aeromonas hydrophila* SSU

DNA sequence analysis demonstrated the presence of a T6SS gene cluster in the genome of diarrheal isolate SSU of *A. hydrophila* (DQ667172) (Figure 3.1A). We first noted the possible presence of the T6SS in this isolate by polymerase chain reaction (PCR)-amplification of the portion of *vasH*-, *vasK*-, and *hcp*-encoding genes based on the sequence of *V. cholerae* T6SS (165). Our subsequent genome sequencing and annotation of the sequence from *A. hydrophila* ATCC 7966 (177) further provided evidence for the presence of T6SS

in *A. hydrophila* SSU. We then designed primers based on the sequences of the corresponding T6SS gene cluster from *A. hydrophila* ATCC 7966 and *V. cholerae* to cover the entire T6SS gene cluster in isolate SSU of *A. hydrophila*. Most interesting are our findings that although the T6SS cluster is present in both *A. hydrophila* SSU and ATCC 7966 strains, the T3SS encoding genes are found only in the clinical isolate SSU, but not in the environmental isolate ATCC 7966 (177).

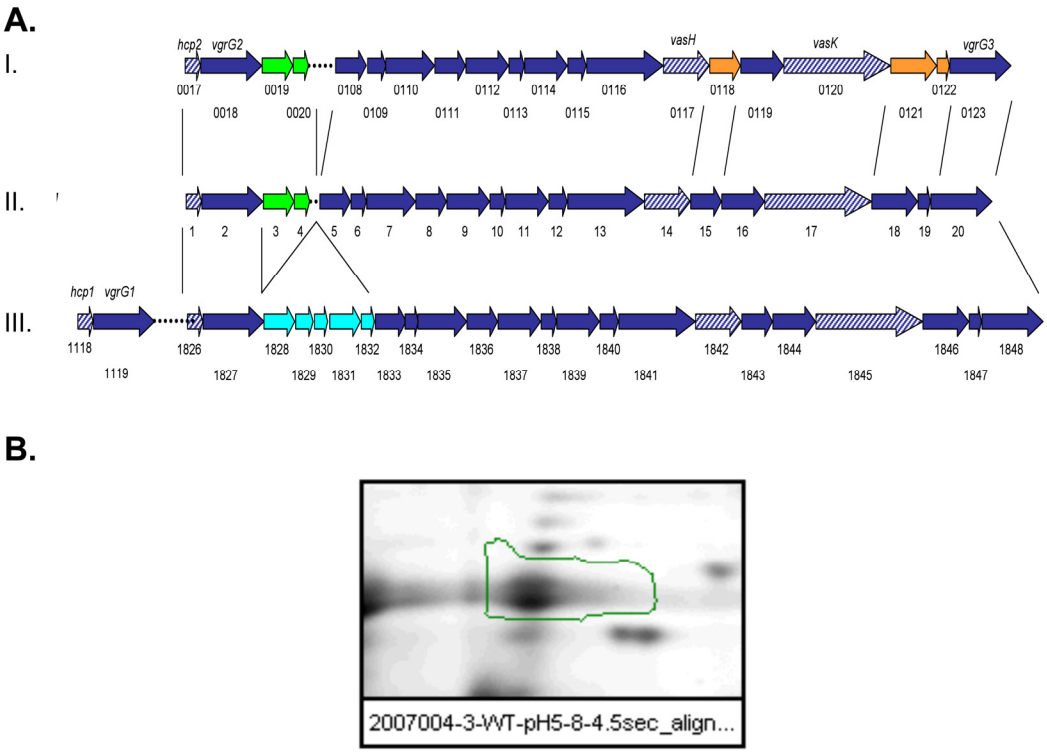

Figure 3.1: T6SS gene cluster of *A. hydrophila*.
A. Diagram showing the genetic organization of the T6SS gene cluster of *A. hydrophila* SSU (II) in comparison with the similar cluster presents in *Vibrio cholerae* N16691 (I) and *A. hydrophila* ATCC 7966 (III). Shown in green are the genes present only in *V. cholerae* and *A. hydrophila* SSU T6SS gene clusters. Hatched blue arrows are genes that were pursued during this study. Genes in cyan color are those that are present only in *A. hydrophila* ATCC 7966 strain (III). Genes in orange exhibit low identity (<25%) between *V. cholerae* and *A. hydrophila* SSU T6SS gene cluster. **B.** Two-dimensional gel electrophoresis of supernatants from *A. on of VgrG1* into HeLa cell cytoplasm.
was confirmed by mass-spec analysis.

This T6SS gene cluster in *A. hydrophila* SSU contained 20 open reading frames (ORFs), out of which 18 genes had high identity (>80%) with genes present in a similar cluster that was identified in *A. hydrophila* ATCC 7966 (177). We also noted that 10 ORFs had considerable identity (>50%) with genes present in the *V. cholerae* N16961 strain T6SS gene cluster (Figure 3.1A), with the remaining 10 genes showing significant diversity in the sequence. Table 1 depicts the list of genes linked to the T6SS gene cluster present in both *A. hydrophila* strains (SSU and ATCC 7966) and in *V. cholerae,* the identity/homology between common genes/proteins, and the gene product description based on *A. hydrophila* SSU conserved domain analysis.

Previous studies in several Gram-negative bacteria have demonstrated the secretion of effector proteins via the T6SS (52, 145, 165, 171). For example, *V. cholerae* secretes four proteins via the T6SS; one is a homolog of hemolysin-coregulated protein (Hcp) and the other three are members of the Vgr family of proteins (165). *A. hydrophila* SSU T6SS gene cluster contains ORFs with a high identity to *hcp* (ORF 1, 71% identity), as well as to two members of the *vgr* family (COG3501), VgrG2 (ORF 2; 51% identity with VCA0018) and VgrG3 (ORF 20; 48% identity with VCA0123) from *V. cholerae* (Table 3.1).

The *clpB* gene (VCA0116 [ORF 13]), present in the T6SS gene cluster of *A. hydrophila* SSU, is a member of the AAA+ (ATPase associated with diverse cellular activities) protein family and is a chaperon protein associated with thermotolerance and translocation of aggregated proteins in an energy-dependent manner (214). Other genes in the T6SS gene cluster of *A. hydrophila* include homologs of the genes in *V. cholerae*, such as *vasA*, *vasF*, *vasK* and *vasH* (ORFs 7, 12, 17 and 14, respectively).

Table 3.1: Comparison of T6SS gene cluster of *A. hydrophila* SSU with *V. cholerae* N16961 and *A. hydrophila* ATCC 7966

A. hydrophila SSU[1]	*V. cholerae*[2]	Identity/homology (%) [1] vs. [2]	*A. hydrophila* ATCC 7966[3]	Identity/homology (%) [1] vs. [3]	Gene product name	Conserved Domains
1	VCA0017	71/79	AHA_1826	95/98	Hemolysin coregulated protein Hcp	COG3157
2	VCA0018	51/52	AHA_1827	80/85	VgrG protein	COG3501, DUF586
3	VCA0019	52/38			hypothetical protein	
4	VCA0020	25/37			hypothetical protein	
			AHA_1828		hypothetical protein	
			AHA_1829		hypothetical protein	
			AHA_1830		hypothetical protein	
			AHA_1831		hypothetical protein	TRP repeat, Sel1
			AHA_1832		hypothetical protein	ImpG homolog
5	VCA0108	74/82	AHA_1833	92/97	hypothetical protein	COG3517
6	VCA0109	24/31	AHA_1834	94/95	hypothetical protein	COG3518, GPW/gp25
7	VCA0110	58/52	AHA_1835	96/98	VasA	COG3519, DUF879
8	VCA0111	51/43	AHA_1836	93/97	hypothetical protein	COG3520, Acyl-CoA dehydrogenase/oxidase C-term
9	VCA0112	18/34	AHA_1837	92/94	hypothetical protein	COG3456, SMAD/FHA
10	VCA0113	40/39	AHA_1838	95/98	Putative Lipoprotein	COG3521
11	VCA0114	61/60	AHA_1839	94/98	hypothetical protein	COG3522
12	VCA0115	41/45	AHA_1840	9497	VasF/IcmH	COG3455
13	VCA0116	63/60	AHA_1841	94/98	ClpA/ClpB family protein	COG0542, AAA, AAA_2, AAA+, chaperone
14	VCA0117	27/40	AHA_1842	94/96	Sigma-54 dependent transcriptional regulator/VasH	COG3829, AAA, Interaction RNA pol sigma factor 54, Fis-type Helix-turn-helix, core AAA+ ATPase, Homeodomain-like.
15	VCA0118	7/27	AHA_1843	90/91	hypothetical protein	
16	VCA0119	26/32	AHA_1844	95/97	ImpA related domain protein	COG3515, ImpA-related N-ter
17	VCA0120	53/41	AHA_1845	96/99	ImcF family protein/VasK	COG3523, DUF1215, IcmF-related, conserved hypothetical ATP binding protein
18	VCA0121	1/17	AHA_1846	96/98	ImpA related domain protein	COG3515, ImpA-related N-ter
19			AHA_1847	96/97	paar motif protein	COG4104, PAAR
20	VCA0123	48/52	AHA_1848	87/93	Rhs element Vgr family protein	COG3501, DUF586, Phage_GPD

COG: Cluster of Orthologous Groups of proteins; Most of these analysis data were obtained from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez/) and the Integrated Microbial Genomes web page (http://img.jgi.doe.gov). The alignments were performed using the Clustalw software program (http://clustalw.genome.jp).

Recently, the function of 3 genes present in the *P. aeruginosa* T6SS gene cluster was described. These genes include *ppkA*, which has kinase activity; *pppA,* which has phosphatase activity; and *fha1*, a scaffold protein with a forkhead-associated (FHA) domain (146). The T6SS gene cluster of *A. hydrophila* SSU has an ORF, which contains an FHA domain (ORF 9) and could have a function similar to that of its homolog in *P. aeruginosa*. However, neither significant similarities nor kinase- or phosphatase-conserved domains were found after sequence alignments of *ppkA* and *pppA* genes from *P. aeruginosa,* with any of the 20 genes found in *A. hydrophila* SSU T6SS gene cluster (Table 3.1).

Other members of this T6SS gene cluster in *A. hydrophila* SSU include two ImpA N-terminal-related domain proteins (ORFs 16 and 18 [COG3515]) which could be associated with the export of proteins (135); a putative lipoprotein (ORF 10 [COG3521]), and a PAAR motif protein (ORF 19 [COG4104]).

Hcp is secreted and translocated into human colonic epithelial cells *via* the T6SS in *A. hydrophila* SSU

Previous reports have shown that inactivation of the *vasK* gene in *V. cholerae* or its homologs in other bacteria blocks secretion of Hcp or other proteins associated with the T6SS gene cluster (52, 145, 165). In a similar way, *vasH*, a sigma-54 dependent transcriptional activator, in *V. cholerae* controls the expression of the *hcp* gene (165). Mass spectrometry analysis, after 2-dimensional (2D)-gel electrophoresis, of culture supernatants from Luria-Bertani (LB)-grown WT *A. hydrophila* SSU revealed the presence of a protein which is highly homologous to Hcp from *V. cholerae* (Figure 3.1B). These findings indicated the functionality of the T6SS gene cluster of *A. hydrophila* SSU under normal culture conditions, which consisted of overnight incubation in LB medium at 37^{o}C with shaking (150 rpm).

Based on above-mentioned studies, we decided to generate deletion mutants for *vasH* and *vasK* genes in *A. hydrophila* SSU to evaluate the effects of these mutations on the expression and secretion of Hcp. For these experiments, HT-29 human colonic epithelial cells were infected with the WT *A. hydrophila* SSU and its corresponding mutants, and Hcp was detected in various cellular fractions by Western blot analysis using Hcp-specific antibodies. As shown in Figure 3.2A, deletion of the *vasH* gene abrogated the expression and secretion of Hcp (lane 4), whereas deletion of the *vasK* gene eliminated the secretion

without affecting the expression and translocation of Hcp (lane 6). After complementation of both of these genes, *vasH* and *vasK*, in their respective mutants, the secretion and expression was restored, although the secretion was not fully complemented (lanes 3 versus 5 and 7). We also demonstrated complementation of these mutants *in vivo,* indicating no potential polar effects of the mutation.

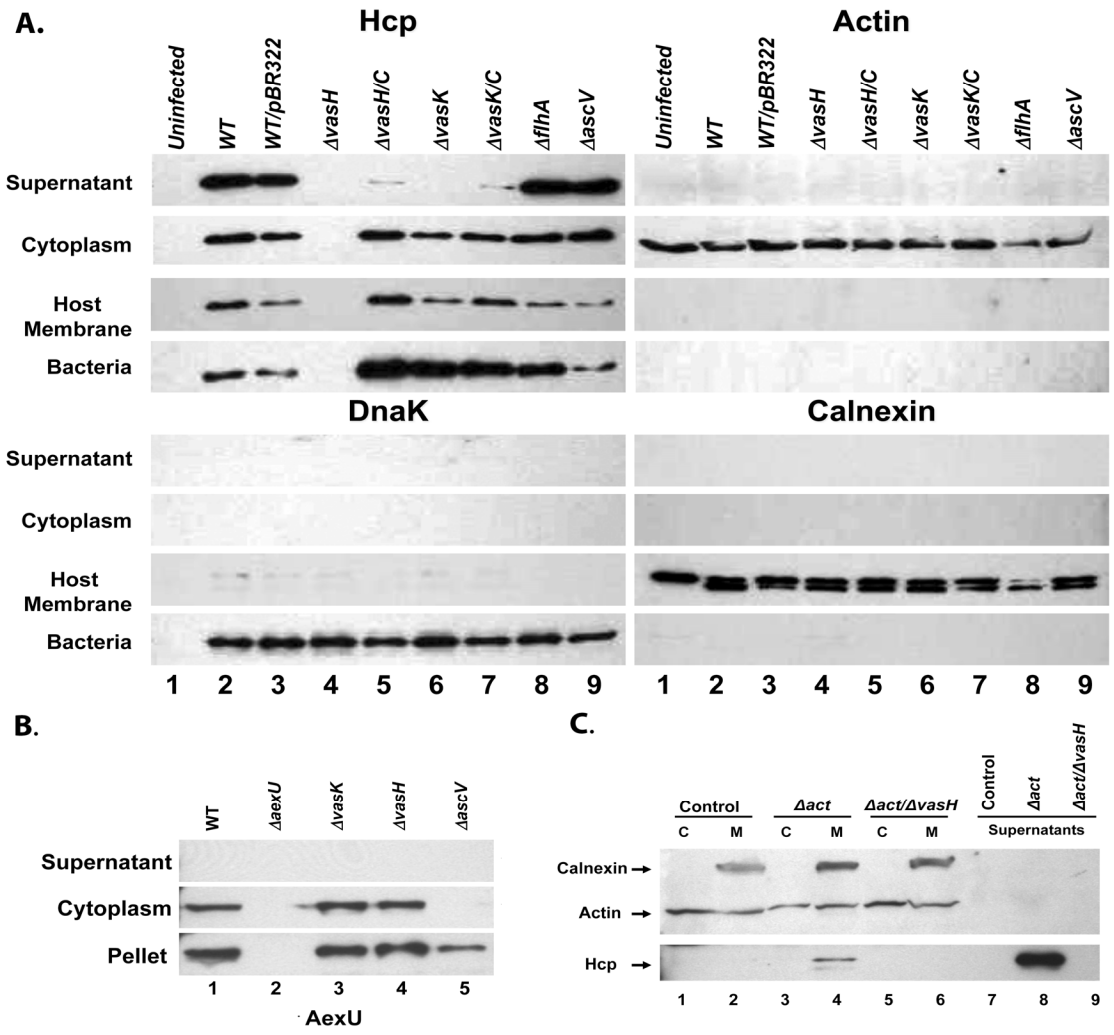

Figure 3.2: The T6SS of *A. hydrophila* is functional and independent.
A. Production of Hcp from *A. hydrophila* SSU and the translocation of Hcp into host cells. HT-29 human colonic epithelial cells were infected with different *A. hydrophila* strains (MOI of 5) for 2 hr at 37°C in DMEM/0.5% FBS medium. The culture supernatants were TCA precipitated (supernatant fraction). The infected host cells were osmotically lysed and centrifuged to obtain soluble (cytoplasmic fraction representing translocated effectors) and insoluble fractions. The insoluble fraction was resuspended in cell lysis buffer containing 0.1% Triton X-100 and centrifuged to obtain soluble (host cell membrane fraction) and insoluble (intact bacterial pellet) fractions. The samples were run on a gradient SDS-4-20% PAGE and subjected to Western blot analyses using anti-Hcp, anti-actin, anti-DnaK and anti-calnexin antibodies. Lane 1: Untreated HT-29 cells; Lane 2: *A. hydrophila* WT; Lane 3: WT bacteria containing pBR322; Lane 4: Δ*vasH* mutant; Lane 5: Δ*vasH* complemented strain; Lane 6: Δ*vasK* mutant; Lane 7: Δ*vasK* complemented strain; Lane 8: Δ*flhA* mutant, and lane 9: Δ*ascV* mutant. **B**. Production and translocation of AexU are not affected by mutations in the T6SS components. HT-29 cells were infected with the WT *A. hydrophila* SSU (lane 1), Δ*aexU* mutant (lane 2), Δ*vasK* mutant (lane 3), Δ*vasH* mutant (lane 4), and Δ*ascV* mutant (lane 5). The culture supernatants were TCA precipitated (supernatant fraction). The infected host cells were osmotically lysed and centrifuged to obtain soluble (cytoplasmic fraction) and insoluble fractions (pellet). The samples were run on a gradient SDS-4-20% PAGE and subjected to Western blot analyses using anti-AexU antibodies. **C**. Hcp binds to the cell membrane of RAW 264.7 murine macrophages. Supernatants from *A. hydrophila* Δ*act* and ∆*act*/∆*vasH* mutants were added to RAW 264.7 cells and incubated for 2 h at 37°C. The host cells were washed, osmotically lysed, and centrifuged to obtain soluble (cytoplasmic) and insoluble (membrane) fractions. Samples were run on a gradient SDS-4-20% PAGE and subjected to Western blot analyses using the following antibodies: anti-calnexin, anti-actin and anti-Hcp. Control: Macrophages incubated with 1% FBS-DMEM (lanes 1 and 2); Δ*act:* Macrophages incubated with supernatants from *A. hydrophila* Δ*act* mutant (lanes 3 and 4); Δ*act*/Δ*vasH*: Macrophages incubated with supernatants from *A. hydrophila* Δ*act*/Δ*vasH* mutant (lanes 5 and 6). The supernatants from the Δ*act* (lane 8) and Δ*act*/Δ*vasH* (lane 9) mutants of *A. hydrophila* SSU were used as a control for the presence of Hcp. C=Cytoplasmic fraction from RAW 264.7 macrophages. M=Membrane fraction from RAW 264.7 macrophages.

Since it has been reported that cytotoxicity induced by *V. cholerae* in murine J774.1 macrophages requires direct cell-cell contact, and deletion of the *hcp* gene abrogates these morphological changes (165), we decided to evaluate the ability of WT *A. hydrophila* SSU and its various mutants to translocate Hcp into HT-29 cells. The above-mentioned bacteria were co-cultured with HT-29 cells, and, after 2 h of infection, the supernatants were removed, and the cells were fractionated to obtain cytoplasmic proteins, eukaryotic host membrane proteins, and the remaining pellet containing the bacterial cells (181). To ensure

that there was no cross-contamination of various cellular fractions, each one of the fractions was tested for the presence of actin, calnexin and DnaK as markers for host cytoplasmic proteins, host membrane proteins and bacterial intactness, respectively. We were able to detect the translocation of Hcp into the host cell cytoplasm by Western blot analysis of the HT-29 cells after infection with WT *A. hydrophila* SSU (Figure 3.2A, lane 2). However, as expected, no translocated Hcp was seen in HT-29 cells infected with the Δ*vasH* strain (lane 4). In addition, although the Δ*vasK* mutant was unable to secrete Hcp into the extracellular media, translocation of Hcp was not affected (lane 6). We did not observe any cross-contamination of cellular fractions, and the bacterial integrity was intact, when antibodies to actin, calnexin, and DnaK were used for Western blot analysis (Figure 3.2A).

T6SS in *A. hydrophila* SSU is independent of the Type III Secretion System (T3SS) and the flagellar system

The WT *A. hydrophila* SSU harbors different secretion systems, including the T3SS (180) and the flagellar system (225, 226), which are involved in the secretion as well as in the direct translocation of proteins into host eukaryotic cells. Therefore, we decided to test if shutting down these secretion systems would impact the secretion of Hcp. For these experiments, cellular fractions of HT-29 cells after infection with WT *A. hydrophila* SSU and its mutants for the T3SS (Δ*ascV*) and for the flagellar system (Δ*flhA*) were used. The Δ*ascV* mutant is able to express the *aexU* gene (encoding a T3SS effector protein) but unable to secrete the expressed protein (181), and the *flhA* gene codes for a protein that is believed to be part of the export apparatus for lateral flagellar assembly, as the

flhA mutant of *A. hydrophila* showed reduced adherence and biofilm formation (22).

Our data indicated that translocation and secretion of Hcp was not affected in the Δ*ascV* and Δ*flhA* mutants, indicating that, indeed, the T6SS is independent of the T3SS and the flagellar secretion system in mediating the secretion and translocation of Hcp (Figure 3.2A, lanes 8 and 9). Furthermore, translocation of AexU (T3SS effector) was not affected in the Δ*vasK* and Δ*vasH* mutants, as shown in Figure 3.2B (lanes 3 and 4). As positive controls, we infected HT-29 cells with Δ*aexU* and Δ*ascV* mutants, and no translocation of AexU was noted (lanes 2 and 5). As expected, we noted the expression of the *aexU* gene in WT, *vasH*, *vasK*, and *ascV* mutants of *A. hydrophila* SSU (pellet fraction, lanes 1, 3, 4, and 5).

Binding of secreted Hcp to murine macrophages

Previous studies in *P. aeruginosa* indicated the ability of Hcp to form hexameric rings after secretion, and the findings suggested that these rings could be inserted into the eukaryotic membrane as a part of the "translocon" (145). Since Hcp was detected in the culture supernatant of WT *A. hydrophila* SSU in abundant amounts (Figure 3.1B), we decided to confirm if secreted Hcp was able to bind the cell membrane of RAW 264.7 murine macrophages to possibly initiate cell signaling from outside, in addition to the ability of Hcp to affect host cell signaling as a result of its translocation. For this experiment, a double mutant, Δ*act*/Δ*vasH*, was generated to avoid the cytotoxic effects induced by Act present in the culture supernatants. Culture supernatants of *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutants were collected after 2 h of inoculation, filtered, and added to

RAW 264.7 cells for 2 h. After washing the host cells, cytoplasmic and membrane fractions were obtained. As shown in Figure 3.2C, Western blot analysis demonstrated that Hcp present in the supernatant of the Δ*act A. hydrophila* SSU mutant was able to bind to the cell membrane of RAW 264.7 cells (lane 4). In contrast, Hcp was not detected in the cytoplasmic fraction of these cells (lane 3), indicating no cross-contamination of the cytosolic and membrane fractions of the host cells. As expected, we did not observe Hcp binding in uninfected RAW 264.7 cells (lanes 1 and 2) and in host cells infected with the Δ*act*/Δ*vasH* mutant (lanes 5 and 6). The latter mutant is unable to express the *hcp* gene. Lanes 7-9 show the presence or absence of Hcp in the culture medium of Δ*act* and Δ*act*/Δ*vasH* mutants of *A. hydrophila* SSU. Similar assays were performed using recombinant Hcp (rHcp) obtaining essentially the same results (data not shown).

A. hydrophila T6SS inhibits phagocytic activity and mediates cytotoxicity

Since infection by *A. hydrophila* SSU is not intracellular, and mutations in the *vasH* and *vasK* genes are able to alter expression and secretion of proteins (e.g., Hcp) associated with the T6SS, we decided to evaluate the effects on phagocytosis caused by mutation in those two genes, namely *vasH* and *vasK* of *A. hydrophila* SSU.

To test for phagocytic activity, RAW 264.7 macrophages were infected with WT *A. hydrophila* SSU and Δ*vasH* and Δ*vasK* mutants, and the intracellular colony forming units (cfu) were estimated as an indicator of phagocytosis. As noted from Figure 3.3A, phagocytosis was significantly increased in RAW 264.7

macrophages infected with Δ*vasH* and Δ*vasK* mutants when compared to host cells infected with the WT *A. hydrophila.*

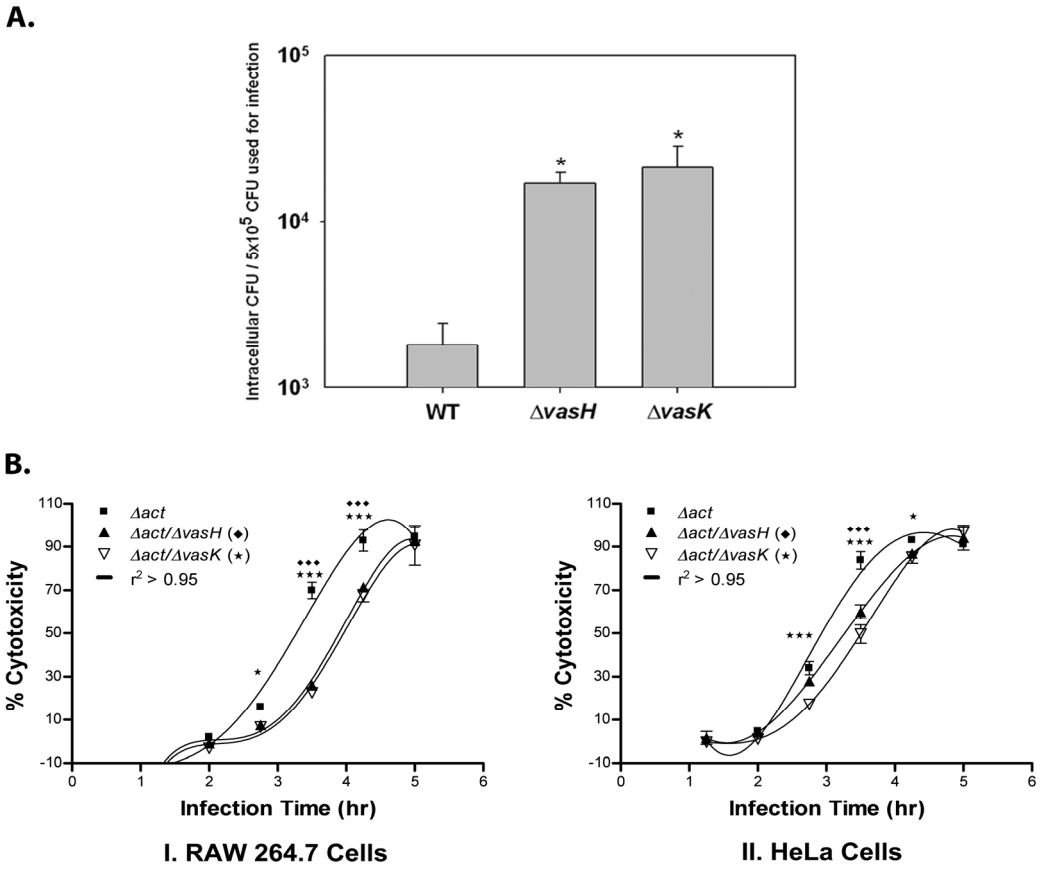

Figure 3.3: The T6SS gene cluster mutants of *A. hydrophila* are less virulent that the parental strain.

A. Phagocytosis is enhanced in *A. hydrophila* Δ*vasH* and Δ*vasK* mutants. RAW 264.7 murine macrophages were infected at an MOI of 5 with *A. hydrophila* strains, namely, WT, Δ*vasH* and Δ*vasK* mutants. Thirty minutes after infection, the cells were washed and treated with 100 μg/mL of gentamicin for 1 h. Then, RAW 264.7 cells were washed and lysed with water. The bacteria were plated at different dilutions and the colony forming units were determined. ∗ Denotes statistically significant values ($p<0.01$) compared to the parental strain (WT). **B**. Cytotoxicity associated with the T6SS. RAW 264.7 cells (Panel I) and HeLa cells (Panel II) were infected with Δ*act*/Δ*vasH* mutant (solid triangle) and Δ*act*/Δ*vasK* mutant (open triangle) double-knockout mutants and their parental strain Δ*act* mutant (solid square) at an MOI 0.5. At different time points, cytotoxicity was measured by the lactate dehydrogenase (LDH) enzyme release assay. ∗∗∗ or ♦♦♦ denotes statistically significant values ($p<0.001$) compared to the parental strain *Δact*. ∗ Denotes statistically significant values ($p<0.05$) compared to the parental strain. Three independent experiments in duplicate wells were performed.

The cytotoxic effect of *A. hydrophila* SSU Δ*vasH* and Δ*vasK* mutants was then tested in RAW 264.7 macrophages and HeLa cells by measuring the release of lactate dehydrogenase (LDH) enzyme. RAW 264.7 and HeLa cells were incubated with either the *A. hydrophila* mutant deleted for the act gene (as a control) or the Δ*vasH* and Δ*vasK* mutants at an MOI of 0.5 for different time points, and supernatants were collected for measuring the LDH release. Significant differences in cytotoxicity between the Δ*act* and Δ*act/*Δ*vasK* or Δ*act/*Δ*vasH* strains were detected in RAW 264.7 and HeLa cells after 3-4.5 h of infection (Figure 3.3B). Although the Δ*act* background strain was used in this experiment as a control to remove the strong cytotoxic effects associated with this protein, five hours after infection, the percentage of cytotoxicity induced by the different bacterial mutant strains was similar to that of the control strain (Δ*act* of *A. hydrophila* SSU) as a consequence of other virulence factors produced (i.e., AexU). These data indicated that mutations in the *vasH* and *vasK* genes were able to alter the biological effects associated with the T6SS of *A. hydrophila*. Although differences in cytotoxicity induced by the parental versus mutant strains may appear small, we believe these are biologically meaningful, as there are other *A. hydrophila* virulence factors that also lead to cell toxicity.

Expression of the *hcp* gene in HeLa Tet-Off cells and induction of apoptosis

Since we showed that Hcp is translocated into eukaryotic cells, we decided to express the *hcp* gene into HeLa cells from pBI-EGFP vector using the HeLa cell Tet-Off system. HeLa Tet-Off cells were transfected by electroporation with the pBI-EGFP vector containing the *hcp* gene (519 bp), and the transfection efficiency was measured by flow cytometry as the percentage of EGFP

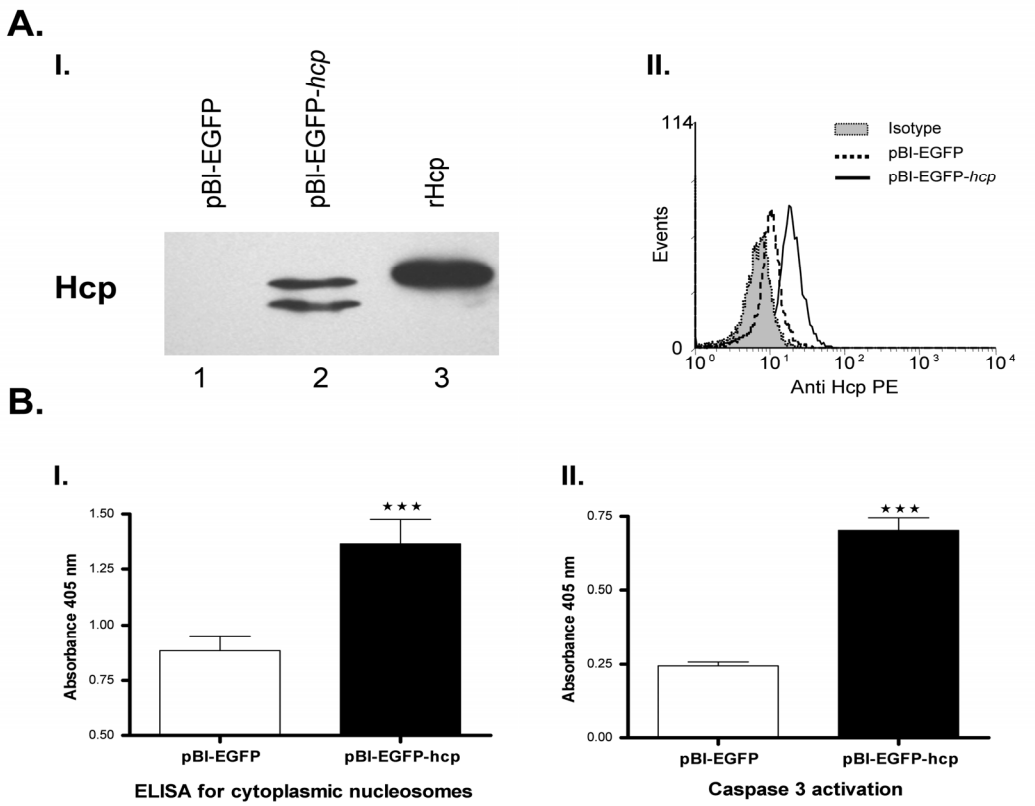

Figure 3.4: Induction of apoptosis by Hcp in HeLa Tet-off cells.
A. Expression and production of Hcp in transfected HeLa Tet-Off cells. Panel I. Western blot analysis showing production of Hcp in whole cell lysates of HeLa Tet-Off cells after 24 h of transfection with pBI-EGFP-*hcp* (lane 2) or pBI-EGFP (empty vector) (lane 1) plasmid. Recombinant Hcp was used as a positive control (lane 3). Panel II. Expression of the *hcp* gene in HeLa Tet-Off cells after permeabilization and intracellular staining using anti-Hcp antibodies. Mouse pre-immune serum was used as an isotype control. The cells were acquired using a FACScan flow cytometer and analyzed using WinMDI software, gated on EGFP-positive cells. **B.** Induction of apoptosis in HeLa Tet-Off cells transfected with the *hcp* gene. Panel I. Detection by ELISA of cytoplasmic nucleosomes in HeLa Tet-Off cells transfected with hcp after 24 h. *** denotes statistically significant values ($p<0.001$) compared to those in cells transfected with the pBI-EGFP (empty vector) plasmid. Standard deviations were calculated from duplicate samples from one representative experiment. A minimum of three experiments was performed with similar results. Panel II. Colorimetric caspase 3 detection in total lysates of HeLa Tet-Off cells transfected with the *hcp* gene after 24 h. Figures are representative of three independent experiments. *** denotes statistically significant values ($p<0.001$) compared to those cells transfected with the pBI-EGFP (empty vector) plasmid. Standard deviations were calculated from duplicate assays from one experiment. A minimum of three experiments was performed with similar results.

(enhanced green fluorescent protein)-positive cells. Expression and production of Hcp were evaluated by Western blot and flow cytometry analyses. A band of ~20

kDa was observed by Western blot analysis in the HeLa Tet-Off cells transfected with pBI-EGFP-*hcp* plasmid (Figure 3.4A, panel I, lane 2). The lower band probably represented a degradation product of Hcp. These results were confirmed by flow cytometry by performing intracellular staining of cells transfected with pBI-EGFP-*hcp* plasmid (Figure 3.4A, panel II). As negative controls, we used host cells transfected with pBI-EGFP vector alone and isotype antibody.

We then evaluated cytotoxicity and mitochondrial activity by colorimetric MTT assays in the HeLa cells transfected with the pBI-EGFP-*hcp* plasmid, and there was no significant difference between the cells transfected with the vector alone (pBI-EGFP) and the cells transfected with the vector containing the *hcp* gene after 24 h of transfection (data not shown). Likewise, incorporation of 7-amino actinomycin D (7-ADD), which permeates the membranes of dead and dying cells and stains their DNA, was not significantly different between vector alone and the *hcp* transfected HeLa cells after 24 hr of infection (data not shown). These studies led us to examine any apoptosis of the host cells that might be associated with Hcp.

To evaluate the apoptotic rate in the HeLa Tet-Off cells transfected with the *hcp* gene for 24 h, we measured the cytoplasmic histone-associated DNA fragments (nucleosomes) by ELISA. As shown in Figure 3.4B (panel I), cells producing Hcp had a significantly higher rate of apoptosis compared to HeLa cells transfected with the vector alone ($p<0.001$). To confirm these results, we assessed caspase 3 activity in these cells by a colorimetric activity assay. The activation of caspase 3 was significantly increased in the HeLa cells producing Hcp ($p<0.001$) when compared to cells transfected with the vector alone (Figure

3.4B, panel II). We could not perform similar experiments in which host cells were treated with rHcp as most of the protein produced from *E. coli* was membrane bound, thus requiring harsh conditions for its solubilization, which resulted in a loss in biological activity.

T6SS is important for the virulence of *A. hydrophila* SSU in the mouse model

To evaluate the importance of the *hcp* gene during *in vivo* infection, we challenged mice with WT *A. hydrophila* at a sub-lethal dose, and collected the sera from the surviving mice. Subsequently, the sera were probed by Western blot analysis against the purified rHcp. As shown in Figure 3.5A, the surviving

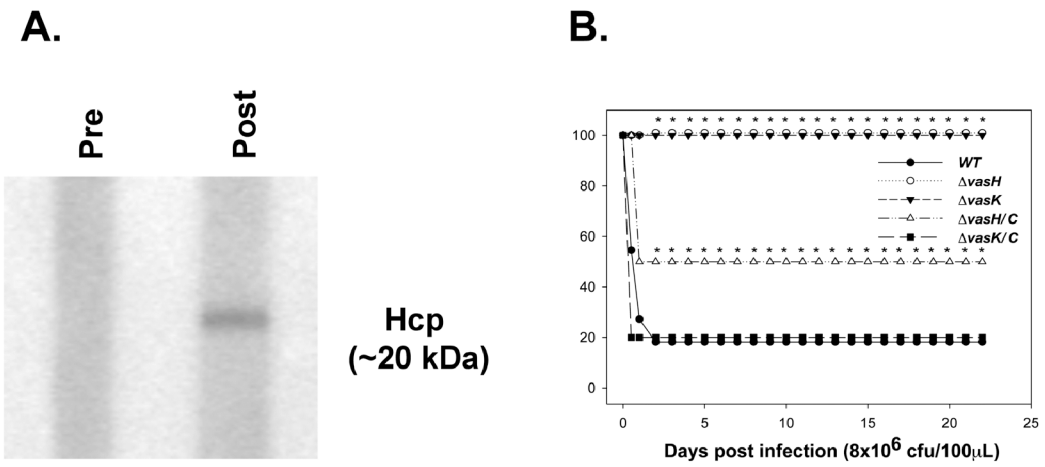

Figure 3.5: Role of T6SS during *A. hydrophila* infection in a mouse model.
A. Swiss Webster mice were infected i.p., with WT *A. hydrophila* at a dose of 1 LD_{50} (1 x 10^5 cfu/100 μL). After 2 weeks of infection, sera from the surviving mice were collected and pooled. The serum was diluted 1:100 and used as a source of primary antibodies in Western blot analysis against rHcp. Pre-immune serum was used as a negative control. **B**. Groups of 10 Swiss Webster mice were infected i.p. (8 x 10^6 cfu) with *A. hydrophila*: WT (solid circles), Δ*vasH* mutant (open circle) and Δ*vasK* mutant (solid triangle). Same doses of the complemented strains were also used, Δ*vasH*/C (open triangle) and Δ*vasK*/C (solid square). Deaths were recorded for 2 weeks post-infection. The bacterial doses represented approximately 2 LD_{50} of WT *A. hydrophila*. ∗ Denotes statistically significant values ($p<0.001$) of the mutants (Δ*vasH* and Δ*vasK*) compared to the WT bacterium and of Δ*vasH* mutant compared to the Δ*vasH*/C strain ($p<0.05$) using the Fisher exact test. The death curve for WT *A. hydrophila* with pBR322 vector alone was similar to that of the WT bacterium (without the vector) (not shown).

mice developed specific antibodies against Hcp. As a control, sera obtained before infection were also tested. The presence of anti-Hcp specific antibodies indicated that Hcp was produced during *in vivo* infection and that it highlighted the immunogenic potential of this protein. Therefore, we decided to test the ability of Hcp to protect mice from challenge with ~3 LD_{50} (5 x 10^7 cfu) of WT *A. hydrophila* SSU. Mice were immunized with 10 μg of rHcp, boosted after 3 weeks, and challenged with WT bacteria. Then, their survival rates were monitored for 2 weeks. All of the mice immunized with rHcp survived after challenge, whereas all of the control (non-immunized) animals died within 48 h (Table 3.2). We used only 3 LD_{50} dose initially, as in addition to the T6SS, other toxins produced by *A. hydrophila* are lethal to mice. Therefore, it is important that other virulence-associated genes should also be deleted to develop a safer vaccine.

Finally, to evaluate the role of T6SS during infection, we infected mice via the i.p route with WT, as well as with the Δ*vasH*, Δ*vasK*, and their respective complemented mutant strains (Δ*vasH*/C and Δ*vasK*/C) of *A. hydrophila* SSU (8 x 10^6 cfu), and the survival rate of the animals was recorded. After 48 hr, 80% of the animals infected with the WT and Δ*vasK*/C, and 50% of the animals infected with Δ*vasH*/C strain of *A. hydrophila* died (Figure 3.5B). In contrast, 100% of the animals infected with Δ*vasH* and Δ*vasK* mutant strains survived 16 days

Table 3.2: Protection of mice immunized with rHcp against lethal challenge of WT *A. hydrophila* SSU

	Survivals
Immunized	5/5
Non Immunized	0/5

Animals were challenged with 3 LD_{50} ($5x10^7$ cfu/ 100 µL)

postinfection (Figure 3.5B). Importantly, at higher doses of the WT bacterium (5 x 10^7 cfu), no difference in the survival rate of mice was noted when compared to the Δ*vasH* and Δ*vasK* mutant strains. Although the Δ*vasK* mutant is still able to translocate Hcp, its virulence was attenuated in the mouse model similar to that of the Δ*vasH* mutant, which does not produce Hcp (Figure 3.2A, lanes 4 and 6).

DISCUSSION

Although the presence of a T6SS was recently described in *V. cholerae* (165), bioinformatic analysis demonstrated that many gram-negative bacteria harbor the T6SS gene cluster (44). Some of these bacteria that carry the T6SS gene cluster, which has been variously named in different pathogens, include *Yersinia pestis*, *Legionella pneumophila* (the T6SS gene cluster named as IAHP [IcmF-associated homologous protein]), *Pseudomonas aeruginosa* (the gene cluster known as HSI [Hcp secretion Island]), Enteroaggregative *Escherichia coli* (EAEC) O42 (the gene cluster known as aai [AggR-Activaded Island]), *Salmonella enterica* (the gene cluster known as Sci), *Agrobacterium tumefaciens, Edwardsiella ictaluri, Edwardsiella tarda, Rhizobium leguminosarum, Burkholderia mallei* (17, 44, 52, 138, 153, 169, 171), and recently in *A. hydrophila* SSU (GenBank accession number DQ667172) and *A. hydrophila* ATCC 7966 (177). The latter is an environmental isolate (type strain ATCC 7966 of *A. hydrophila* subspecies hydrophila), and we recently annotated its genome sequence in collaboration with The Institute for Genome Research (TIGR). However, the role of this secretion system in the virulence of bacterial pathogens, in general, is largely unknown.

Hcp and VgrG family of proteins are known effectors of T6SS (52, 145, 165, 171). It has been reported that a member (VgrG1) of the Vgr family from *V. cholerae* shares with RtxA toxin a subdomain that mediates actin crosslinking and cytotoxicity when it is expressed in the cytoplasm of eukaryotic cells. However, this activity has been associated with a second domain of this protein which is not present in VgrG2 and VgrG3 found in *A. hydrophila* (Table 3.1) (183).

Further, studies have shown that mutations affecting the ATPase domain of ClpV1 (a member of the *clpB* family) in *P. aeruginosa* abrogates Hcp secretion (145). These results suggest that the *clpB* gene in *A. hydrophila* T6SS gene cluster (Table 1) could play a similar role in unfolding proteins to be secreted, as well as in providing energy for this process.

The T6SS gene cluster in *A. hydrophila* SSU contains homologs of *vasA*, *vasF, vasK,* and *vasH* genes found in *V. cholerae* (ORFs 7, 12, 17, and 14 respectively) (Table 3.1). For the first three genes, their functions remain poorly characterized (COG3519, COG3455, and COG3523, respectively). The *vasH* gene encodes a sigma-54 factor-dependent transcriptional activator carrying a factor for inversion stimulation (Fis)-type helix-turn-helix and homeodomain-like motifs, which are involved in DNA-binding and ATPase activity (AAA+ core). Approximately one-half of the proteins that interact with RNA polymerase sigma factor-54 domain can be phosphorylated by a sensor kinase. The ATPase activity in most of these proteins has been associated with conformational changes that promote interaction with Sigma-54 factor, and the Fis-type domain and the homeodomain are directly associated with DNA-binding (10, 139, 140). Thus, *vasH* in *A. hydrophila* SSU could be a key component necessary for the

expression of components of T6SS, which mediates the association between RNA polymerase and DNA.

Hcp and VgrG proteins are proposed effectors associated with the T6SS gene cluster as they are associated with cytotoxicity in some *in vitro* models, such as the rounded phenotype in the J774.1 murine macrophages, and killing of *Dictyostelium* amoeba in *V. cholerae* (165). However, it has also been reported that expression of the *hcp* gene is required for VgrG secretion (165). This, together with the ability of Hcp to form hexameric rings after secretion, suggest that Hcp could be part of the translocon and that VgrG then passes through the Hcp channel (145, 165, 214).

In Figure 3.2A, we showed importance of *vasH* and *vasK* genes in the T6SS gene cluster of *A. hydrophila* SSU. Likewise, studies conducted by *Mougous et al.* (2006) demonstrated by fluorescence microscopy that IcmF (homolog to VasK) in *P. aeruginosa* could be required (but not totally essential) for the efficient assembly of the T6- secretion apparatus. Therefore, we consider that VasK may constitute a structural protein of the T6SS in *A. hydrophila* SSU that provides the stability necessary to secrete Hcp into the extracellular milieu in the absence of cell- to- cell contact. However, when the T6SS apparatus makes a cell- to-cell contact with eukaryotic membranes, its (VasK) function could be redundant due to the stability that the translocon (e.g., Hcp) could render, thus still allowing Hcp to be translocated into the host cell (Figure 3.2A). Finally, the ability of *A. hydrophila* SSU Hcp to bind to murine macrophages (Figure 3.2C) supported the contention proposed for Hcp from *P. aeruginosa* that Hcp alone could be able to attach to the cellular membrane (145).

Previous reports indicated that a homolog of *vasK*, called *icmF* and *sciS* in *L. pneumophila* and *S. enterica*, respectively, is involved in the intracellular survival and replication of these bacteria (153, 175). Similarly, our findings indicated that mutations in components of the T6SS gene cluster (e.g., *vasH* and *vasK*) could alter the virulence of *A. hydrophila* by altering the ability of bacteria to be phagocytosed. Our future studies will delineate how deletion of *vasH* and *vasK* genes leads to increased phagocytosis of the bacteria.

Finally, we provided evidence that expression of the *hcp* gene in HeLa cells led to host cell apoptosis (Figure 3.4). It has been proposed that Hcp from *P. aeruginosa* could be able to form a channel allowing the release of ions as well as the transport of macromolecules (145). Bacterial pore-forming proteins like alpha toxin from *Staphylococcus aureus*, listeriolysin O from *Listeria monocytogenes* and alpha-hemolysin from *E. coli* are able to induce apoptosis by the selective release of ions leading to DNA fragmentation (215). However, alpha toxin can induce apoptosis or necrosis depending upon its concentration (215). The induction of apoptosis by Hcp in *A. hydrophila* might be related to the formation of pores in the cell membrane, and, since the amount of Hcp produced by the HeLa cells is not high because the pBI-EGFP vector possesses a minimal cytomegalovirus promoter which lacks the enhancer, the effects that we are seeing are consistent with the induction of apoptosis rather than necrosis. Our future studies will be targeted at studying Hcp-induced apoptosis in details.

In Figure 3.5A, we demonstrated circulating antibodies to Hcp in mice after infection with the WT *A. hydrophila* SSU. Following infections with *P. aeruginosa,* similar results were reported in patients with chronic respiratory infections in that they showed a high titer of antibodies against Hcp (145). In

addition, animals of different species showed, following infection with glanders disease, the presence of anti-Hcp antibodies (171). Since anti-Hcp antibodies were present in the sera of mice infected with the sub-lethal dose of *A. hydrophila* and these antibodies were protective against subsequent challenge of animals with the WT bacterium (Table 3.2), we speculate that Hcp could be a potential target for vaccine development.

Importantly, our data reported in Figures 3.2A and 3.5B indicated that although Δ*vasK* mutant could translocate Hcp, it was attenuated in a mouse model of infection. These results raise the question whether the secreted Hcp plays a more important role in bacterial virulence than the translocated one and will be studied in our future studies. The susceptibility of the Δ*vasH* and Δ*vasK* mutants to be phagocytosed, compared to that of the WT *A. hydrophila*, could explain the increased survival of the mice infected with these mutants. Thus, Δ*vasH* and Δ*vasK* mutants could be cleared from the intraperitoneal space before infection becomes systemic. Further, since these mutants killed mice at higher doses (5×10^7 cfu compared to 8×10^6 cfu) , these data implied that the presence of T3SS effectors, as well as Act could be contributing to animal lethality, which confirms our previous studies (180, 181).

It is also interesting to note that both Δ*vasH* and Δ*vasK* mutants showed increased phagocytosis (Figure 3.3A) in spite of the fact that the Δ*vasK* mutant could translocate Hcp efficiently (Figure 3.2A). These data could indicate: i) secreted Hcp might play an important role in phagocytosis, and ii) the translocated Hcp might have been altered in its function in the Δ*vasK* mutant. Our future studies will be focused on these aspects as it relates to Hcp.

In summary, we reported the presence of a functional T6SS gene cluster in *A. hydrophila* SSU and showed, for the first time, the ability of this secretion system to translocate Hcp into the eukaryotic cells and to induce apoptosis that was mediated by caspase 3 activation. Additionally, we showed that immunization of mice with Hcp protected animals from subsequent challenge with the lethal dose of the WT bacterium.

Chapter 4: A type VI secretion system effector protein VgrG1 from *Aeromonas hydrophila* that induces host cell toxicity by ADP-ribosylation of actin [2]

INTRODUCTION

Sequence analyses of VgrG proteins from different bacteria showed that all of them were highly conserved in their NH_2-terminal domains. The domain called VgrG or COG3501 (an uncharacterized protein which is conserved in bacteria) carried a region belonging to the phage GPD superfamily (c|01392) and a domain belonging to the DUF586 superfamily (pfam04524). The VgrG domain also shared similarities to the gp5 and gp27 proteins of the bacteriophage T4 tail spike (60, 164). Some VgrG proteins had different COOH-terminal extensions, which contained domains having different activities. For example, *Yersinia intermedia* carried a mannose-binding domain, and *Y. pestis* VgrGs harbored tropomyosin-like, YadA-like, and pertactin-like domains. Likewise, VgrG from *P. aeruginosa* carried a zinc-metalloprotease domain while VgrG1 and VgrG3 from *V. cholerae* contained a repeat in structural toxin A (RtxA) and peptidoglycan binding domains, respectively (164). Only recently reported was the translocation of VgrG1 from *V. cholerae* into eukaryotic host cells with deleterious effects (122), suggesting that VgrG proteins with extended COOH-termini could have a role as T6SS effectors.

Our recent analysis of the complete genome of *A. hydrophila* ATCC 7966, an environmental isolate, indicated the presence of two copies of the gene

[2] 191. **Suarez, G., J. C. Sierra, T. E. Erova, J. Sha, A. J. Horneman, and A. K. Chopra.** 2010. A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* that induces host cell toxicity by ADP ribosylation of actin. J Bacteriol **192:**155-168.

encoding VgrG2 and VgrG3 within the T6SS gene cluster, whereas one gene that encoded VgrG1 was located outside of the T6SS gene cluster (193).

Adenosine-diphosphate (ADP)-ribosylation proteins or toxins are able to catalyze the transfer of ADP-ribose from nicotinamide-adenine dinucleotide (NAD) to a target protein. This mechanism is used by some bacterial toxins to alter eukaryotic cell functions (84, 204). Proteins with ADP-ribosylating activity are grouped into four different families based on their respective targets (84, 205). Type I proteins, such as cholera and pertussis toxins, target heteromeric guanosine-triphosphate (GTP)-binding proteins (68, 102); type II proteins, like diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, modify elongation factor 2 (EF2) (207, 218); type III proteins (i.e., *Clostridium botulinum* C3 exoenzyme) target small GTP-binding proteins (6); and type IV proteins are specific for ADP-ribosylation of G-actin. The last family of proteins includes: *C. botulinum* C2 toxin, *C. perfringens* iota-toxin, *C. spiroforme* toxin, *C. difficile*, and *Bacillus cereus* vegetative insecticidal protein (VIP) (4, 5, 12, 77, 158, 159, 208).

The *A. hydrophila* SSU genome has not been sequenced; however, the proteomics analysis of the data in the present study indicated the existence of a member of the VgrG family of proteins, VgrG1, with its gene localized out of the T6SS gene cluster. We also reported in the current paper the expression and secretion of VgrG1 from *A. hydrophila* SSU following 2-dimensional gel electrophoresis and mass spectrometry. By sequence alignment analysis, we found that this protein has a COOH-terminal extension containing a vegetative insecticidal protein-2 (VIP-2) domain, known for its actin-ADP-ribosylating activity (77). We noted that the expression of the gene encoding either the full-length VgrG1 or its COOH-terminal domain (amino acid residues 702-927) that

contained only the VIP-2 domain induced a rounded phenotype in HeLa Tet-Off cervical epithelial cells. In contrast, expression of the gene encoding the NH_2-terminal domain (amino acid residues 1-701) of VgrG1 or full-length VgrG2 did not have any effect on HeLa cell morphology. We also observed increased apoptosis in the HeLa Tet-Off cells expressing the full-length or the COOH-terminal domain of VgrG1. Overall, we demonstrated that VgrG1 is an important virulence factor of *A. hydrophila,* which is secreted and also translocated via the T6SS. Further, this is the first characterization of VgrG with actin-ADP ribosylating activity.

RESULTS

A. hydrophila SSU secretes VrgG1 *via* the T6SS

We analyzed supernatants from *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutant strains by 2-dimensional gel electrophoresis and identified differentially expressed and produced protein spots by mass spectrometry (MALDI-TOF). Subsequently, the peptide sequences were matched with the proteobacteria database. We chose to use *A. hydrophila* SSU Δ*act* mutant as the parental strain to avoid any interference of T6SS-associated cytotoxicity with that contributed by Act. It has been shown that deletion of *vasH*, encoding a sigma 54-activator, prevented the transcription, translocation and secretion of effector proteins via the T6SS (e.g., Hcp) (165, 193). The approach used in this analysis was to identify protein spots present in the Δ*act* mutant strain, but not in the Δ*act*/Δ*vasH* mutant, thus allowing us to specifically target T6SS-secreted proteins. Of the 32 differentially expressed and produced spots (Table 4.1), we detected homology to the known T6SS proteins, such as Hcp-1, Hcp-2, VgrG1, VgrG2, and VgrG3,

which were recently identified in other gram-negative bacteria. These differentially produced proteins related to the T6SS gene cluster showed low expectation scores (high homology) compared to those proteins not related to the T6SS gene cluster. However, these proteins with high expectation scores (low homology) need to be analyzed carefully (Table 4.1), and such studies are currently underway in our laboratory.

We identified well-defined clusters by 2-dimensional gel electrophoresis in the culture supernatant of the Δ*act* mutant strain that were absent from the culture supernatant of the Δ*act*/Δ*vasH* mutant strain. One of these clusters contained various isoforms of Hcp, while the other contained VgrG homologs.

Table 4.1: Mass spectrometric alignment of differentially produced proteins spots from supernatants of *A. hydrophila* SSU (Δ*act* versus Δ*act*/Δ*vasH* strains) based on 2-D gel electrophoresis.

Spot	Rank	Protein Name	Protein Expectation Score
5_4804	1	VgrG-3 protein [Aeromonas hydrophila]	9.97631E-70
5_5573	1	VgrG-3 protein [Aeromonas hydrophila]	2.50594E-65
5_5562	1	VgrG-2 protein [Aeromonas hydrophila]	9.97631E-53
5_4798	1	VgrG-3 protein [Aeromonas hydrophila]	6.29463E-46
5_6426	1	VgrG-3 protein [Aeromonas hydrophila]	3.15479E-43
5_4785	1	VgrG-3 protein [Aeromonas hydrophila]	6.29463E-39
3_3026	1	putative hemolysin co-regulated protein [Aeromonas hydrophila]	1.99054E-35
5_4809	1	Rhs element Vgr family protein [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	3.97164E-34
5_5996	1	VgrG-3 protein [Aeromonas hydrophila]	3.97164E-33
5_6503	1	putative vgrG protein [Aeromonas hydrophila]	9.97631E-26
5_5611	1	phosphatidylcholine-sterol acyltransferase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	9.97631E-20
5_6093	1	putative vgrG protein [Aeromonas hydrophila]	7.92447E-14
3_5936	1	Rhs element Vgr family protein [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	1.25594E-13
5_5992	1	VgrG-3 protein [Aeromonas hydrophila]	9.97631E-10
3_4252	1	HcpA homolog [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	9.97631E-09
3_6571	1	HcpA homolog [Aeromonas hydrophila subsp.hydrophila ATCC 7966]	1.99054E-08
5_5746	1	hypothetical protein AHA_3948 [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	1.25594E-05
5_6471	1	inorganic pyrophosphatase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	0.007924466
5_4403	1	vgrG protein [Aeromonas salmonicida subsp. salmonicida A449]	0.019905359
5_539	1	type III restriction protein res subunit [Xanthobacter autotrophicus Py2]	0.019905359
5_260	1	ribosomal large subunit pseudouridine synthase B [Aeromonas salmonicida subsp. salmonicida A449]	0.039716412
5_4807	1	transcriptional regulator, GntR family [Mesorhizobium sp. BNC1]	0.199053585
3_6711	1	hypothetical protein ECA4275 [Erwinia carotovora subsp. atroseptica SCRI1043]	0.629462706
3_6570	1	hypothetical protein Acry_2197 [Acidiphilium cryptum JF-5]	1.255943216
5_5609	1	flagellar hook-associated 2 domain protein [Shewanella baltica OS155]	1.255943216
5_6215	1	hypothetical protein msr9585 [Mesorhizobium loti MAFF303099]	1.255943216
5_4784	1	hypothetical protein RPC_0976 [Rhodopseudomonas palustris BisB18]	2.505936168
5_5356	1	regulatory protein [Oceanicola granulosus HTCC2516]	6.294627059
5_548	1	hypothetical protein HI0688 [Haemophilus influenzae Rd KW20]	6.294627059
5_6707	1	peptidyl-prolyl cis-trans isomerase C [Yersinia pestis KIM]	6.294627059
5_6113	1	putative ABC-type multidrug transport system, ATPase component [Photobacterium profundum 3TCK]	7.924465962
5_6708	1	general secretion pathway protein L [Vibrio cholerae MZO-3]	15.8113883

Highlighted areas represent protein spots matching with VgrG1 from *A. hydrophila* ATCC 7966

From the VgrG cluster (Figure 4.1A, highlighted in green), we identified proteins (isoforms) with high homology (expected score: 1.2e-032) to VgrG1 (gi:117619461) of the environmental isolate ATCC 7966 of *A. hydrophila*, whose genome sequence was recently annotated (177). Figure 4.1B depicts the alignment of sequences of 12 peptides (highlighted in red) obtained by mass spectrometric analysis of VgrG1 from *A. hydrophila* SSU with that of the sequence of *A. hydrophila* ATCC 7966 VgrG1. These peptide sequences obtained from *A. hydrophila* SSU exhibited 100% homology with the sequence of VgrG1 from *A. hydrophila* ATCC 7966 (177). Since the genome of *A. hydrophila* SSU is not totally sequenced yet, and we only have sequenced T6SS and T3SS gene clusters (180, 193), we were unable to detect, based on computer alignment programs, matches of VgrG1 of *A. hydrophila* SSU with that of VgrG1 of *A. hydrophila* ATCC 7966. This is because the *vgrG1* gene is located on the bacterial chromosome outside of the T6SS gene cluster in contrast to *vgrG2* and *vgrG3* genes which reside within the T6SS gene cluster.

Importantly, we identified the presence of a VIP-2 domain in the COOH-terminal portion (amino acid residues 702-927) of VgrG1 of *A. hydrophila* (both SSU and ATCC 7966 strains) (E-value 5e-10) by using a specialized BLAST search of conserved domains (Figure 4.1B [indicated by underlining]). The VIP-2 domain belongs to the family of ADP-ribosyltransferase toxins which possess an actin ADP-ribosylating activity and share significant homology with multiple bacterial toxin proteins, such as C2 toxin of *C. botulinum*, iota toxin from *C. perfringens*, *C. piriforme* toxin, and *C. difficile* binary toxin A (4, 5, 12, 77, 158, 159, 208).

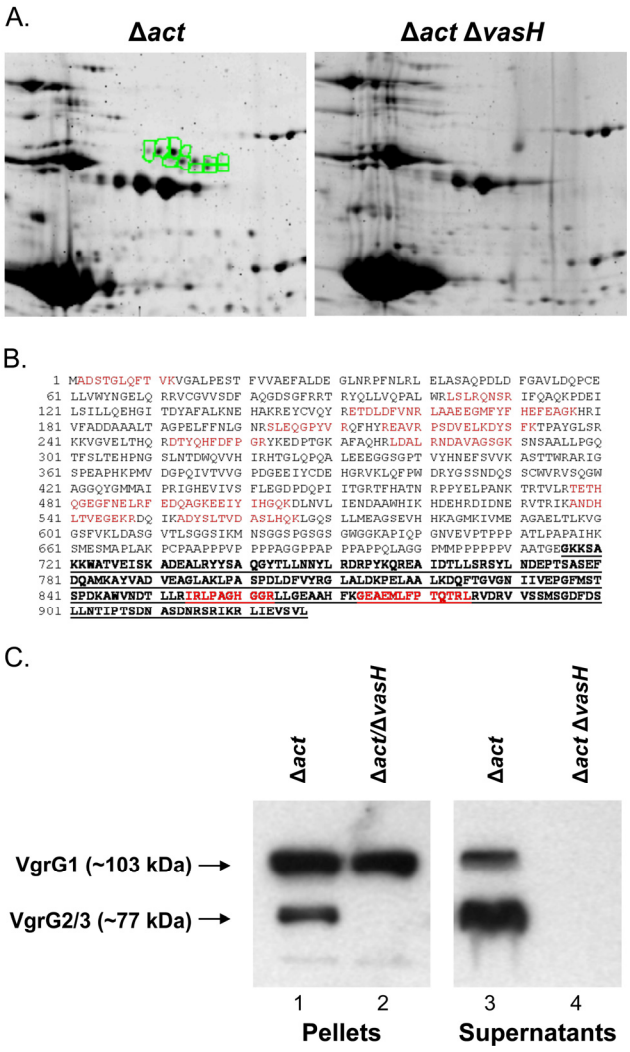

Figure 4.1: Identification of proteins secreted via T6SS in the supernatant of *A. hydrophila* SSU.
A. Comparison of 2-D gels containing proteins from the supernatants of the *A. hydrophila* SSU Δ*act* mutant (left) and the Δ*act*/Δ*vasH* mutant strain (right). Highlighted spots (in green) represent a cluster of proteins secreted via the T6SS, which were identified by mass spectrometric analysis. **B**. Alignment of VgrG1 from *A. hydrophila* ATCC 7966 (gi|117619461) with peptides identified via mass spectrometry (red) on one of the secreted proteins (VgrG1) from *A. hydrophila* SSU. Bold-underlined sequence represents VIP-2 domain. **C**. Western blot analysis on pellet and supernatant fractions of *A. hydrophila* SSU Δ*act* (lanes 1 and 3) and Δ*act*/Δ*vasH* (lanes 2 and 4) mutant strains using specific antibodies to the VIP-2 domain of VgrG1 in combination with specific antibodies against VgrG2.

Figure 4.2A depicts amino acid residues in red that are conserved between the reference sequence (cd00233) and the VIP-2 domain of *A.*

hydrophila ATCC 7966 (125). In order to confirm the secretion of VgrG proteins by *A. hydrophila* SSU, we performed Westerns blot analysis on supernatants from both Δ*act* and Δ*act*/Δ*vasH* mutant strains of *A. hydrophila* SSU by using antibodies specific to the VIP-2 domain of VgrG1 as well as antibodies against VgrG2 (which recognize all members of the VgrG family) (Figure 4.1C).

As we demonstrated by 2-dimensional gel electrophoresis/mass spectrometric analysis, we were able to detect VgrG1 (~103 kDa) and VgrG2/3 (exhibiting similar molecular masses; ~77 kDa) in the supernatant and pellet fractions of *A. hydrophila* SSU Δ*act* mutant (Figure 4.1C, lanes 1 and 3). However, the Δ*act*/Δ*vasH* mutant of *A. hydrophila* SSU was unable to secrete any member of the VgrG family of proteins (Figure 4.1C, lane 4). Importantly, we could detect the presence of VgrG1 but not of VgrG2/3 in the pellet fraction of *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant (Figure 4.1C, lane 2), imply that since the *vgrG1* gene is out of the T6SS gene cluster, its regulation is independent of VasH. However, the inability of VgrG1 to be secreted by the Δ*act*/Δ*vasH* mutant (Figure 4.1C, lane 4) indicated that a T6SS apparatus was needed for the secretion of VgrG1.

We noted that the NH_2-terminal domain of VgrG1 (amino acid residues 1-701) harbored the VgrG domain (COG3501) present in all the members of VgrG proteins (Figure 4.2B). This domain remains as an uncharacterized conserved domain of an unknown function. Interestingly, within the VgrG domain, there is a phage GPD superfamily region (c|01392), which is present in the bacteriophage T4 tail spike, and the DUF586 (pfam04524) region, which has no known function (Figure 4.2B). As noted in Figure 4.2C, the COOH-terminal extension of *V. cholerae* VgrG1 has an RtxA domain, unlike the VIP-2 domain

(cd00233/pfam01129) found in VgrG1 of *A. hydrophila* SSU and ATCC 7966 strains (Figure 4.2B). Recent reports have shown structural similarities between the VgrG domain and gp27 and gp5, as well as with Hcp-hexameric rings and gp25 of bacteriophage T4 (116, 164) (Figure 4.2C). These findings highlighted the similarity of the T6SS apparatus to the bacteriophage T4 needle-like structure

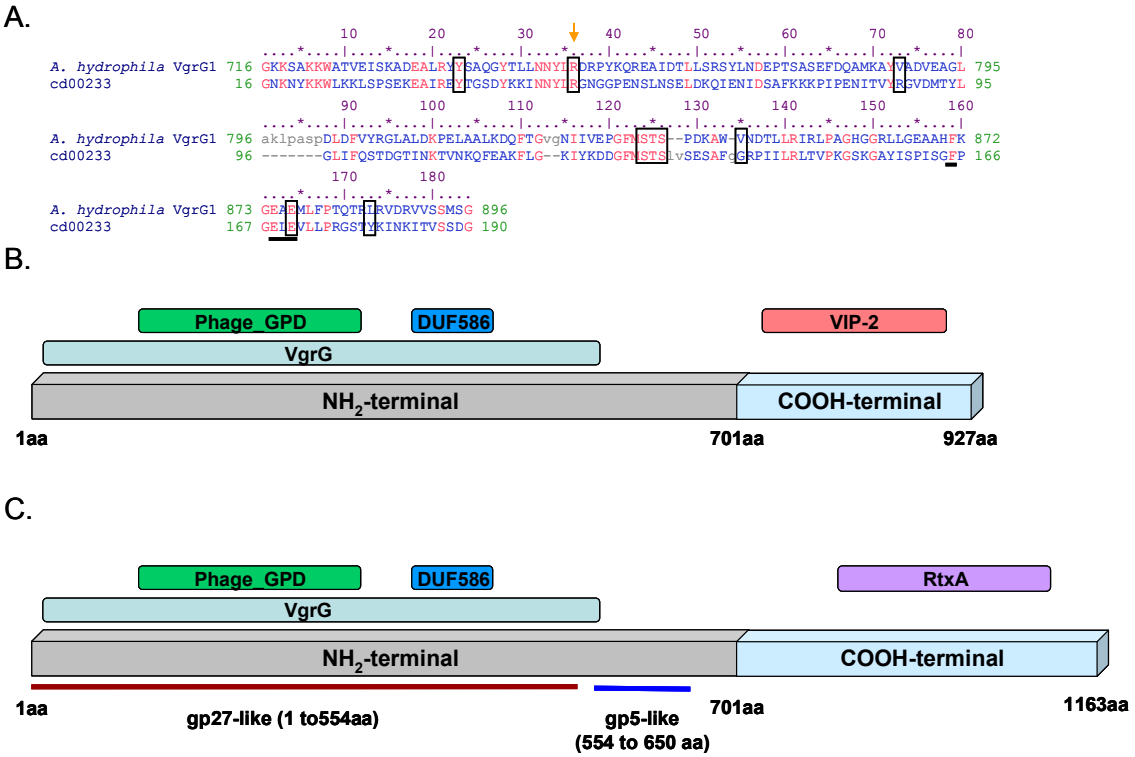

Figure 4.2: Alignment and schematic representation of VgrG1 proteins.
A. Alignment of the VIP-2 reference sequence (cd00233) with the sequence present in the COOH-terminal fragment of *A. hydrophila* ATCC 7966 VgrG1. In red are conserved amino-acid residues. CD Length: 201; Bit Score: 62.27; E-value: 5e-10 (taken from BLAST-Conserved Domains report). Amino acid residues in open boxes constitute a conserved Ser-Thr-Ser motif, which plays an important role in catalysis and structure as well as in NAD binding. Underlined sequences denote ADP-ribosylating toxin turn-turn motif, and an orange arrow shows conformational flexibility of ligand binding pocket. **B**. Schematic representation of the conserved domains present in VgrG1 *A. hydrophila* ATCC 7966 (gi|117619461) and **C**. *V. cholerae* N16961 (gi|15641427). The gp27- and gp5-like motifs are represented by red and blue lines, respectively (modified from Pukatzki, et al 2007) (38). The encoding fragments representing the NH_2-terminal (gray), COOH-terminal (cyan) and the full-length (gray and cyan) of VgrG1 from *A. hydrophila* ATCC 7966 were cloned into a pET-30a vector to produce recombinant proteins, and into pBI-EGFP vector for expression in the HeLa Tet-off cells. The positions of VIP-2 and RtxA domains in VgrG1 of *A. hydrophila* and *V. cholerae* are also shown.

and suggested that there are similar functions of T6SS and bacteriophage in the translocation of proteins through host cell membranes.

VgrG1 from *A. hydrophila* possesses actin-ADP-ribosyltransferase (ADPRT) activity

Since the COOH-terminal domains of VgrG1 were highly conserved between *A. hydrophila* ATCC 7966 and *A. hydrophila* SSU and because the NH_2-terminal domains of all known VgrGs have significant homologies, we tested the ADPRT activity associated with the VgrG1 of *A. hydrophila* ATCC 7966. We cloned the full-length *vgrG1* gene and its NH_2-terminal domain-encoding DNA fragment (1-2106 bp [1-701 amino acid residues], which does not contain the region encoding VIP-2 domain) and the COOH-terminal end-encoding DNA fragment (2107-2784 bp [702-927 amino acid residues], which harbors only the VIP-2 domain) in pET-30a vector and produced the recombinant proteins in *E. coli.* The purified rVgrG1 full-length and rVgrG1 COOH-terminal domain proteins were able to catalyze the incorporation of Biotin-ADP (from 6-Biotin-17-NAD) into

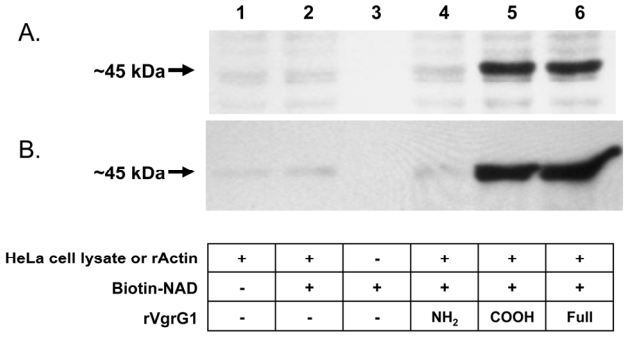

Figure 4.3: The VgrG1 protein has ADP-ribosyltransferase activity.
VgrG1 has ADP-ribosyltransferase (ADPRT) activity, which is associated with the presence of VIP-2 domain. ADPRT assays were performed by using 6-Biotin-17-NAD, purified rVgrGs proteins, and HeLa cell lysates (**A**) or recombinant non-muscle actin (**B**) as a source of target protein. The reaction mixtures were separated on SDS-12%PAGE, electro-blotted to nitrocellulose membranes, and the incorporation of Biotin-ADP by target protein was detected by using streptavidin-HRP. The nature of samples loaded in each lane of the gel is depicted in a table underneath panel B.

an approximately 45-kDa target protein when lysates of Hela cells (Figure 4.3A, lanes 5 and 6) or recombinant non-muscle actin (Figure 4.3B, lanes 5 and 6) were used as the source of target protein. In contrast, purified rVgrG1 NH_2-terminal domain protein was not able to catalyze this reaction (Figures 4.3A and 3B, lane 4). The appropriate negative controls, which included HeLa cell lysate or rActin alone (Figures 4.3A and 4.3B, lane 1), or in conjunction with NAD (lane 2) as well as NAD alone (lane 3), did not exhibit any ADPRT activity.

A. hydrophila SSU Δ*act* mutant strain induces a rounded phenotype in HeLa cells which is cell contact-dependent

In order to test the cytotoxic effect of T6SS effector proteins of *A. hydrophila* on eukaryotic cells, changes in the morphology of the latter were evaluated in co-cultures of normal HeLa cells infected with either *A. hydrophila* SSU Δ*act* mutant (with functional T6SS) or the Δ*act*/Δ*vasH* mutant strain (with non-functional T6SS). We noted that HeLa cells co-cultured with *A. hydrophila* SSU Δ*act* mutant showed a host-bacterial, contact-dependent, rounded morphology after 90 min of co-culture (Figure 4.4A-II, column 1). In contrast, this phenotype was minimally observed in co-cultures of HeLa cells with the *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant (Figure 4.4A-II, column 2). Importantly, we could demonstrate reversal of this rounding phenotype of HeLa cells when the *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant was complemented with the *vasH* gene *in trans* by using the pBR322 vector (Figure 4.5A).

In parallel, co-cultures using transwell inserts of *A. hydrophila* SSU strains with HeLa cells were employed to test whether host-bacterial cell contact was, indeed, needed for the induction of the cell-rounding phenotype. Consequently,

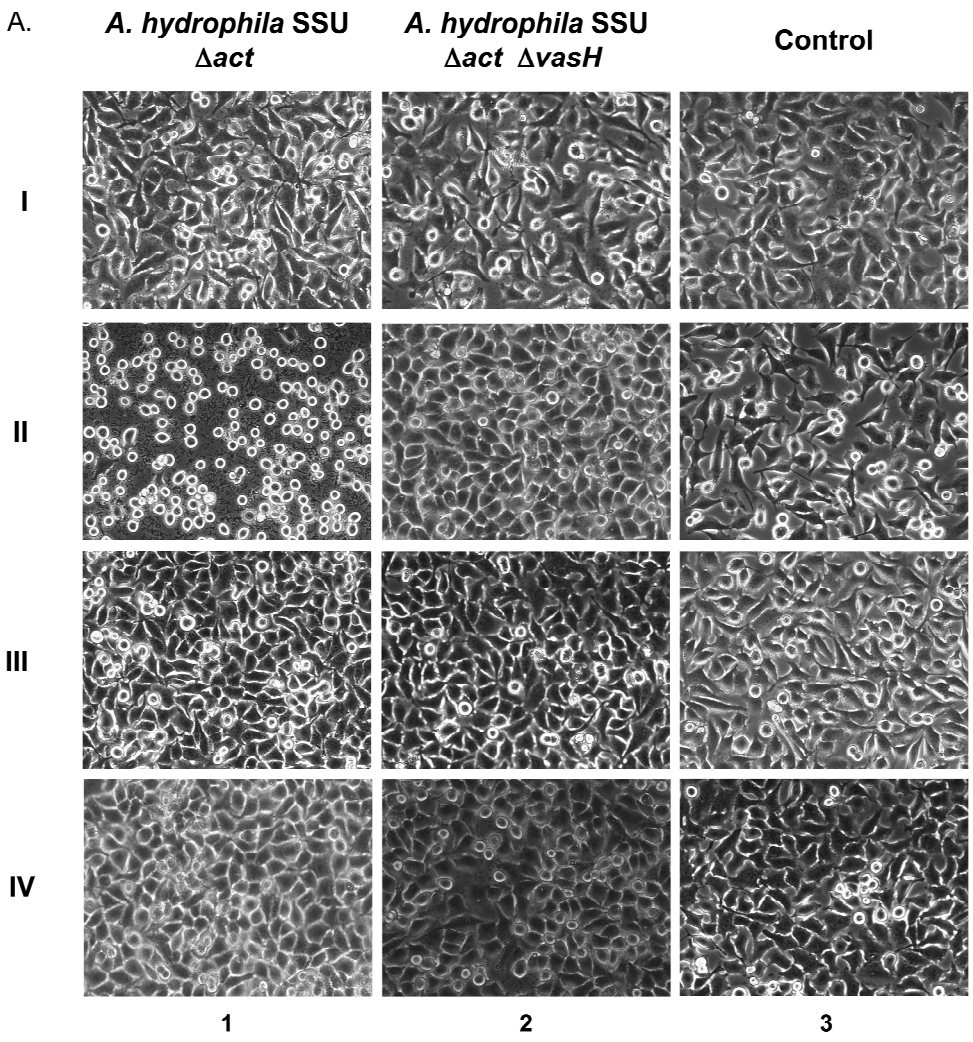
A.
A. hydrophila SSU Δact
A. hydrophila SSU Δact ΔvasH
Control
I
II
III
IV
1
2
3
B.
*
80
70
60
50
40
30
20
10
0
% total actin
actin
Δact
Δact ΔvasH
Control
F - actin
G - actin
* p<0.001

Figure 4.4: Morphological changes on HeLa Tet-off cells induced by VgrG1 of *A. hydrophila*.
A. Induction of HeLa cell-rounded phenotype in co-cultures with different strains of *A. hydrophila* SSU. HeLa cells were co-cultured for 90 min with *A. hydrophila* SSU Δ*act* mutant (column 1) and Δ*act*/Δ*vasH* mutant (column 2), as well as uninfected HeLa cells (column 3), in direct bacterial-host contact (panel II) or by using trans-well inserts (panel III). Supernatants from co-cultures in direct cell-to-cell contact were collected after 90 min, and used as pre-conditioned media on fresh HeLa cells cultures (panel IV). Initial morphology of HeLa cells at 0 min is shown in panel I and non-infected HeLa cells (control) in column 3. Magnification 40X. **B**. Quantification of G- and F-actin by Western Blot and densitometric analyses. HeLa cells in direct contact with different strains of *A. hydrophila* SSU were harvested after 90 min of co-culture. Cells were lysed and processed as is indicated in the materials and methods section. The bar graph represents data on percentages of G- and F- actin of HeLa cells infected with different mutant strains from three independent experiments, and the Western blot image is representative of all of them. * Indicates statistically significant difference.

bacterial strains were placed in the upper chamber, while the HeLa cells were placed in the bottom chamber to avoid direct host-bacterial cell contact. Importantly, in contrast to direct-contact co-cultures, HeLa cells co-cultured by using transwell inserts with different *A. hydrophila* SSU strains had cell morphology similar to that of the control cells without the bacteria (Figure 4.4A-III, columns 1 and 2 versus 3).

In order to confirm that induction of cell-rounded morphology required direct bacterial-host cell contact instead of an additive/synergistic effect of different soluble factors produced by bacteria, supernatants of co-cultures of HeLa cells with different strains of *A. hydrophila* SSU were taken after 90 min, filtered through a 0.2 μm membrane, and used as pre-conditioned media on fresh HeLa cells. Host cells exposed to those supernatants did not show any changes in their morphology even after 2 h of exposure (Figure 4.4A-IV, columns 1 and 2 versus control HeLa cells, column 3). These results indicated that induction of the cell-rounding phenotype of HeLa cells required direct bacterial-host cell contact and that the protein effector (s) was translocated directly into the eukaryotic cell

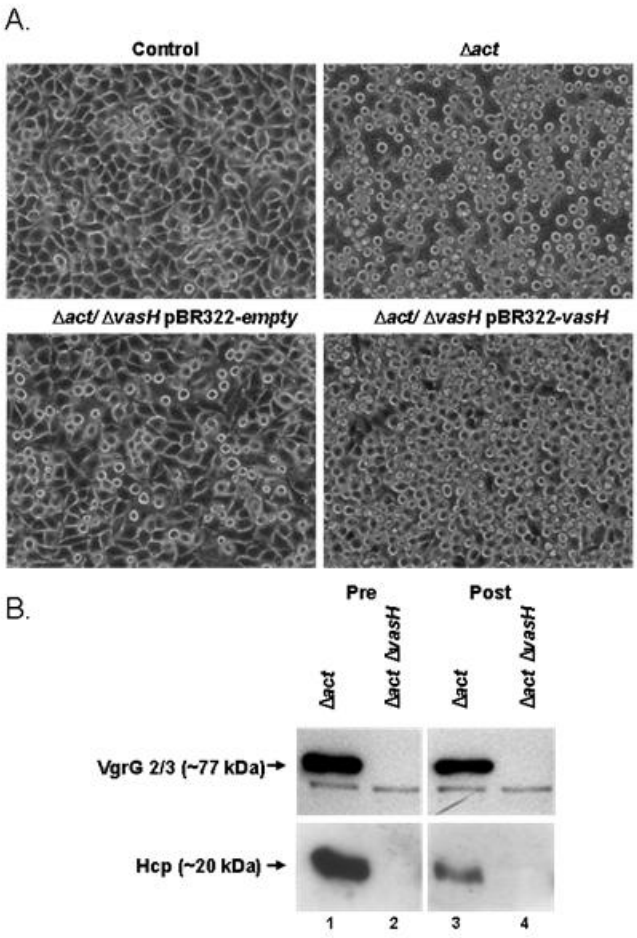

Figure 4.5: Morphological changes on HeLa Tet-off cells induced by *A. hydrophila* Δact/ΔvasH complemented with *vasH* gene.
A. Induction of HeLa cell-rounded phenotype in co-cultures with different strains of *A. hydrophila* SSU. HeLa cells were co-cultured in direct bacterial-host cell contact for 90 min with *A. hydrophila* SSU Δ*act* mutant (Top right), Δ*act*/Δ*vasH* pBR322-empty (with vector alone) (Bottom left), and Δ*act*/Δ*vasH* pBR322-*vasH* in which the Δ*act*/Δ*vasH* mutant of *A. hydrophila* SSU was complemented with the *vasH* gene using the pBR322 vector *in trans* (Bottom right). Normal morphology of HeLa cells is shown in Top left panel (control). Magnification 40X. **B**. Detection of Hcp2 and VgrG 2/3 in tissue culture supernatants after co-culturing of HeLa cells with different strains of *A. hydrophila* SSU. Pre (lanes 1-2): filtered tissue culture supernatants after 90 min of co-culture of HeLa cells with bacteria and before they were used as pre-conditioned media on fresh HeLa cells. Post (lanes 3-4): same conditioned tissue culture supernatants after 120 min on HeLa cell cultures.

cytoplasm via the T6SS. Figure 4.5B shows the presence of Hcp and VgrG2/3 in conditioned medium before (Pre) and after (Post) incubation (for 90 min) with fresh HeLa cells based on Western blot analysis. It is unclear as to why we detected less amount of Hcp and VgrG2/3 in the post conditioned media, the

decrease in Hcp was more prominent than VgrG2/3, it could possibly be related to either degradation of the proteins or their binding to the host cells (193).

To evaluate a relationship between rounded phenotype and actin ADP-ribosylation, we quantified ratios of G- and F- actin by Western blot analysis in HeLa cells co-cultured with different strains of *A. hydrophila* SSU. As seen in Figure 4.4B, HeLa cells co-cultured with *A. hydrophila* SSU Δ*act* mutant showed a significant change in the ratio of G/F-actin (70%/30%) compared to HeLa cells co-cultured with *A. hydrophila* Δ*act*/Δ*vasH* mutant (52%/48%) and control HeLa cells (51%/49%). These results correlated with findings regarding the rounded phenotype of HeLa cells co-cultured with the parental (Δ*act*) *A. hydrophila* SSU strain (Figure 4.4A-II, column 1).

A. hydrophila SSU is able to translocate VgrG1 into the eukaryotic host cell cytoplasm via the T6SS

We examined the importance of cell-to-cell contact for the induction of the rounded phenotype of HeLa cells by evaluating the translocation of VgrG1 into the host cell cytoplasm. For these studies, we expressed full-length *vgrG1::bla* and *vgrG1-NH$_2$::bla* gene fusions in *A. hydrophila* SSU ∆*act* and ∆*act*/∆*vasH* mutant strains. We showed by Western blot analysis using antibodies to β-lactamase, the presence of fusions proteins in the bacterial pellet as well as in the supernatant of the parental *A. hydrophila* SSU ∆*act* strain (Figure 4.6C, lanes 2 and 3). On the contrary, using the ∆*act*/∆*vasH* mutant strain, we could detect the fusion proteins in the bacterial pellet but not in the supernatant (Figure 4.6C, lanes 5 and 6). Appropriate negative controls did not show the presence of fusion proteins either in the pellet or the supernatant fraction of the above-mentioned mutant bacteria (Figure 4.6C, lanes 1 and 4). Similar size fusion proteins were

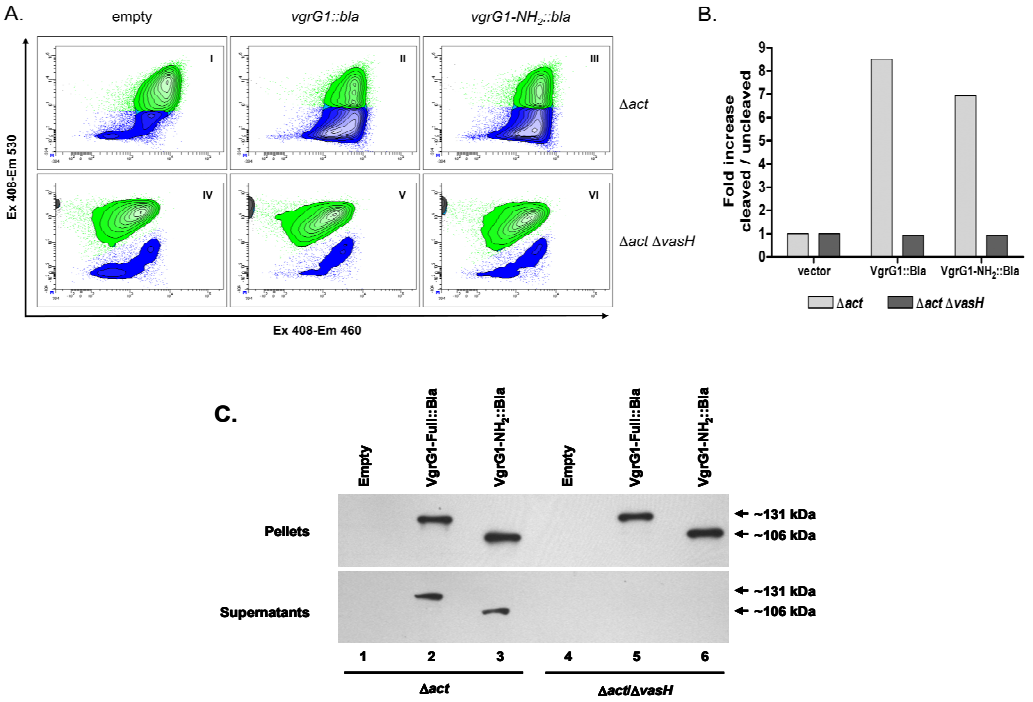

Figure 4.6: Translocation of VgrG1 into HeLa cell cytoplasm.
HeLa cells were infected with *A. hydrophila* SSU Δ*act* or Δ*act*/Δ*vasH* mutant strain expressing and producing full-length VgrG1::Bla (referred to as VgrG1::Bla) or VgrG1-NH_2::Bla. As a control, HeLa cells were infected with bacteria containing the empty vector. **A**. Flow cytometric density plots showing disruption of CCF4 FRET (from green to blue) due to translocation of Bla into HeLa cell cytoplasm infected with *A. hydrophila* Δ*act* parental strain (panel II and III) compared to host cells infected with *A. hydrophila* SSU containing the empty vector (panels I and IV). HeLa cells infected with *A. hydrophila* Δ*act*/Δ*vasH* mutant expressing and producing the fusion proteins were not able to translocate Bla and did not disrupt the CCF4 FRET (panels V and VI). For analysis, $2x10^5$ HeLa cells were acquired and gated in side forward/side scatter to avoid aggregates. **B**. Fold increase in percentage of blue HeLa cells against percentage of green HeLa cells (after infection with *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutant strains) compared to percentage of HeLa cells infected with bacteria carrying the empty vector. The graph shows data from a representative experiment. **C**. Western blot analysis of *A. hydrophila* SSU Δ*act* (lanes 1 to 3) and Δ*act*/Δ*vasH* (lanes 4 to 6) mutant bacterial pellets (top panel) and supernatants (bottom panel) using antibodies to Bla. The production of full-length VgrG1 (VgrG1-Full::Bla) (lane 2) and VgrG1-NH_2::Bla (lane 3) was detected in both bacterial pellet and supernatant of *A. hydrophila* SSU Δ*act* parental strain. In the Δ*act*/Δ*vasH* mutant strain, fusion proteins were detected in the pellets (lanes 5 and 6) but not in the supernatants. Bacterial strains transformed with the empty vector were used as a control (lanes 1 and 4).

detected on Western blots when antibodies to VgrG2 and VIP-2 domain of VgrG1 were used (data not shown). Importantly, the latter antibodies did not detect the

VgrG1-NH_2::Bla fusion protein.

These bacterial strains were then used to infect HeLa cells and we tracked the β-lactamase activity associated with VgrG1 fusion proteins in the host cell cytoplasm by using the CCF4 FRET-based substrate. By flow cytometry, we showed that the *A. hydrophila* SSU Δ*act* mutant strain expressing and producing either the full-length VgrG1::Bla or the VgrG1-NH_2::Bla was able to cleave the substrate present in the HeLa cell cytoplasm, thus turning the cells from green to blue (Figure 4.6A, panel I [15% blue/85% green] versus Panel II [60% blue/40% green] and panel III [55% blue/45% green]). Panel I shows HeLa cells transfected with the *A. hydrophila* SSU Δ*act* mutant strain with vector alone, which served as a negative control. On the contrary, when we compared it to the controls, *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant strains expressing and producing the same fusion proteins were found to not have induced any significant changes in the proportion of blue/green cells (Figure 4.6A, panel IV [16% blue/84% green] versus panel V [17% blue/83% green] and panel VI [15% blue/85% green]). Figure 4.6B shows the fold increases in blue cells (with cleaved substrate) over green cells (with uncleaved substrate) in a representative experiment from 5 independent experiments.

Expression of the *vgrG1* gene in HeLa Tet-Off cells induces a rounded phenotype

To determine the functionality of the COOH-terminal VIP-2 domain present in VgrG1, the full-length gene and its NH_2- and the COOH-terminal encoding gene segments from *A. hydrophila* ATCC 7966 were cloned into a pBI-EGFP vector and then expressed in HeLa Tet-Off cells. The pBI-EGFP vector allowed co-expression of the gene of interest together with the expression of the

gene encoding EGFP to differentiate the transfected from untransfected host cells. The HeLa Tet-Off cells were transfected with the pBI-EGFP vector that contained different fragments of VgrG1 (full-length versus its NH_2- or COOH-terminal end domain) and the expression of various versions of the *vgrG1* gene was evaluated by Western blot analysis by using antibodies specific for VgrG2 and the VIP-2 domain of VgrG1 (Figure 4.7A).

VgrG2 is highly homologous to its family members, and within the NH_2-terminal domain of VgrG1, it shares ~84% identity and ~90% homology. Consequently, antibodies to VgrG2 recognized the full-length (~103 kDa) and the NH_2-terminal domain (~77 kDa) of VgrG1 in HeLa Tet-Off cell lysates (Figure 4.5A, lanes 2 and 3). However, the COOH-terminal domain (~25 kDa) of VgrG1 was not recognized by these antibodies because VgrG2 does not possess the VIP-2 domain (Figure 4.7A, lane 1). On the other hand, VIP-2 domain is present only in VgrG1, and we do not expect any cross-reaction of anti-VIP-2 antibodies with other members of the VgrG protein family. As expected, antibodies to the VIP-2 domain of VgrG1 reacted with only the full-length and the COOH-terminal domain of VgrG1 (Figure 4.7A, lanes 5 and 7) but not with the NH_2-terminal domain of VgrG1 (Figure 4.7A, lane 6). Lanes 4 and 8 in Figure 4.7 represented the cell lysates from HeLa Tef-Off cells expressing only the pBI-EGFP vector, which served as a negative control.

After 24 h of transfection of the HeLa-Tet-Off cells expressing either the gene-encoding, full-length VgrG1 (Figure 4.7B-III) or its COOH-terminal domain (Figure 4.7B -IV) showed a rounded morphology, in contrast to HeLa Tet-Off cells expressing the gene encoding only the NH_2-terminal domain of VgrG1 (Figure 4.7B -II), the vector alone (pBI-EGFP) (Figure 4.7B -I), or full-length

VgrG2 (Figure 4.8) that maintained an elongated morphology which is characteristic of this cell line. In order to evaluate alterations in the actin cytoskeleton of the HeLa Tet-Off cells expressing and producing different forms of the VgrG1, we evaluated ratios of G/F-actin by Western blot analysis. In addition, the host cells were also stained with Alexa fluor-568-Phalloidin (red stain) and analyzed by fluorescent microscopy and flow cytometry.

As shown in Figures 4.7B-III and 4.7B-IV, HeLa Tet-Off cells expressing and producing full-length VgrG1 and the COOH-terminal domain (EGFP positive cells) showed a rounded phenotype and the actin cytoskeleton was severely disrupted, as evidenced by little or no phalloidin staining. On the other hand, HeLa Tet-Off cells expressing/producing only the vector (Figure 4.7B -I) or the VgrG1 NH_2-terminal domain (Figure 4.7B -II) showed a normal phenotype with an

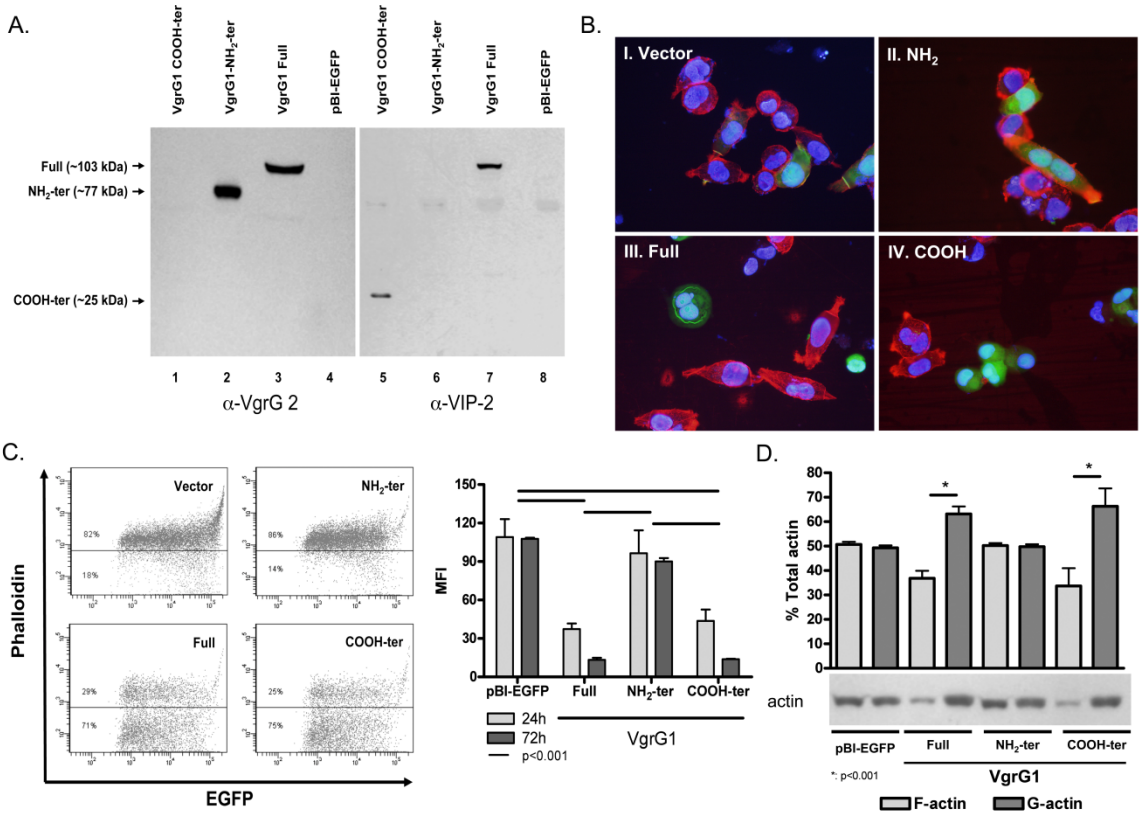

Figure 4.7: Induction of rounded phenotype of HeLa Tet-off cells by episomal expression of VgrG1.
A. Western blot analysis of HeLa Tet-off cell lysates expressing and producing different fragments of VgrG1. The various forms of VgrG1 were detected by using two types of sera. We used sera from mice immunized with rVgrG2 of *A. hydrophila* SSU which cross-reacted with the NH_2-terminal portion of VgrG1 (left panel) and from mice immunized with the rVIP-2 domain of VgrG1 of *A. hydrophila* ATCC 7966 which recognized only VgrG1 and its COOH-terminal domain. The samples loaded in each of the lanes 1-8 are depicted on the top. **B**. Morphological changes of HeLa Tet-off cells induced by the expression of different VgrG1-encoding fragments of *A. hydrophila* ATCC 7966. Host cells were stained for actin-cytoskeleton by using Alexa fluor 568-phalloidin (red), and expression of enhanced fluorescent green protein (EGFP) encoding gene was detected in cells successfully transfected with the pBI-EGFP vector alone (I) or by those containing genes encoding (II) an NH_2-terminal, full-length (III), and COOH-terminal (IV) fragments of VgrG1. Magnification 40X. **C**. Quantification of actin-cytoskeleton (F-actin) as measured by fluorescent phalloidin staining of HeLa Tet-off expressing and producing different VgrG1 fragments. Flow cytometry dot plots showing HeLa Tet-off cells stained with Alexa fluor 568-phalloidin and expressing different encoding fragments of VgrG1. The analysis was performed on EGFP-positives cells. Percentage of positives cells from a representative experiment (72 h) is shown in the plotted quadrant (Left panel), and mean fluorescent intensity values (MFI) from three different assays at 24 h and 72 h were potted (Right panel). Statistical differences at 24 h ($p<0.01$) and 72 h ($p<0.001$) were noted between cells expressing vector alone (pBI-EGFP) versus cells expressing and producing full-length VgrG1 and the COOH-terminal fragment and between cells expressing and producing NH_2-terminal fragment versus cells expressing full-length VgrG1 and COOH-terminal fragment. **D**. Quantification of F-actin and G-actin present in HeLa Tet-off cells expressing and producing different VgrG1 fragments. The percentages of F- and G-actin per sample (30 μL) were analyzed by Western blot analysis and by using antibodies to actin followed by densitometric scanning of the blots. A densitometric quantification from three different assays was plotted and a representative Western blot image is shown. Statistical differences at 24 h ($p<0.001$) were noted between fractions containing F- and G-actin in HeLa cells expressing full-length VgrG1 and the COOH-terminal encoding fragments. Asterisks indicate statistically significant differences. The designation ter refers to NH_2- or COOH-terminal domains.

intact actin cytoskeleton (red staining) having morphology similar to that of adjacent un-transfected cells (no EGFP, but phalloidin-positive cells).

Changes in the actin cytoskeleton were quantified by flow cytometry after phalloidin staining of the transfected cells expressing and producing EGFP. We found a lower percentage of phalloidin-positive HeLa Tet-Off cells producing full-length VgrG1 (29%) and its COOH-terminal domain (25%), when these were compared to HeLa Tet-Off cells that produced the VgrG1 NH_2-terminal domain (86%) or expressed the vector alone (82%) (Figure 4.7C, left panel). In addition,

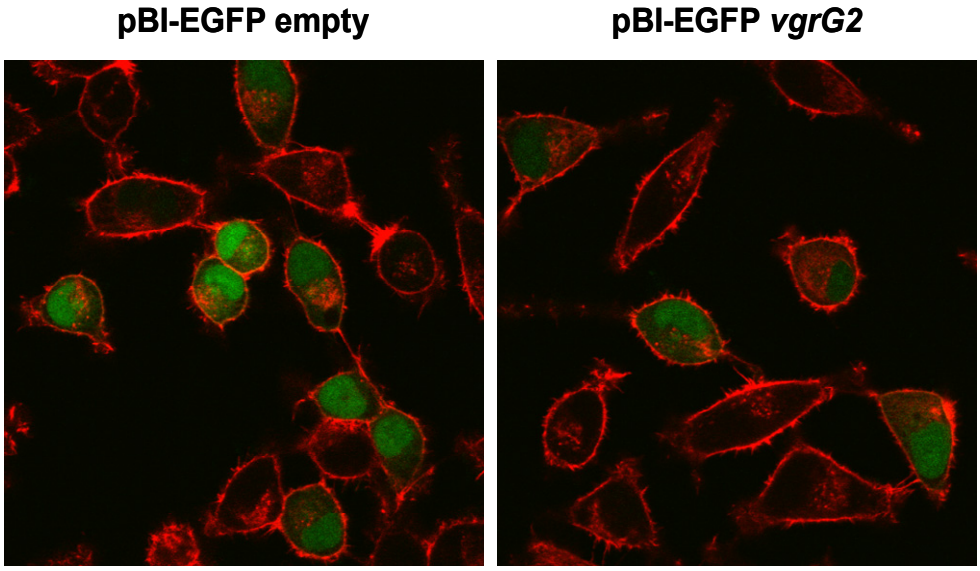

Figure 4.8: Morphological changes of HeLa Tet-off cells induced by expression of *vgrG2* gene of *A. hydrophila* SSU.
Cells were stained for actin-cytoskeleton by using Alexa fluor 568-phalloidin (red), and expression of the gene encoding EGFP was detected in HeLa cells successfully transfected with the pBI-EGFP vector alone (Left) or containing the *vgrG2* gene (Right). Magnification 40X.

we analyzed the mean fluorescence intensity (MFI) of the actin cytoskeleton staining in the transfected HeLa Tet-Off cells. As shown in Figure 4.7C, right panel, after 24 and 72 h of transfection, the MFI of HeLa Tet-Off cells expressing and producing the full-length VgrG1 and the VgrG1 COOH-terminal domain was significantly lower than that of HeLa Tet-Off cells producing the VgrG1 NH_2-terminal domain or expressing the vector alone ($p<0.001$).

Since HeLa Tet-Off cells expressing and producing full-length VgrG1 or its COOH-terminal domain showed low fluorescence intensity when stained with phalloidin, we quantified the amount of G- and F-actin present in these samples by Western blot analysis and densitometric scanning of the blots (Figure 4.7D). HeLa cells expressing genes encoding either the full-length VgrG1 or its COOH-terminal domain showed increased amounts of G-actin (63% and 67%,

respectively) in contrast to cells expressing the gene encoding the NH_2 terminal domain or HeLa cells transfected with the vector alone (49% and 48%, respectively). Together, these results showed the importance of the VIP-2 domain of VgrG1 in the induction of the rounded phenotype of host cells via ADP-ribosylation of actin.

Expression of the *vgrG1* gene in HeLa Tet Off cells induces apoptosis

The viability of the HeLa cells expressing and producing VgrG1 was evaluated by the incorporation of 7-AAD. This compound permeates the membrane of dead and dying cells and binds to the DNA. After 24 and 72 h of transfection of the HeLa Tet-Off cells producing different forms of VgrG1 (EGFP-positive cells), we analyzed and quantified by flow cytometry the percentage of 7-AAD-positive cells. At 72 h, the percentage of 7-AAD-positive cells was significantly higher in HeLa Tet-Off cells producing the full-length (44.7 ± 7.7%) and the COOH-terminal domain (46.3 ± 5.5%) of VgrG1 compared to that of HeLa Tet-Off cells expressing and producing the NH_2-terminal domain of VgrG1 (14 ± 4.2%) ($p<0.01$) or the vector alone (13.6 ± 5.7%) ($p<0.001$) (Figure 4.9A).

Using data on the differences in the viability of host cells demonstrated by 7-AAD incorporation, we decided to evaluate the rate of apoptosis induced by *vgrG1* expression in HeLa Tet-Off cells. Apoptosis was evaluated by quantification of the cytoplasmic nucleosomes and activation of caspases 3 and 9. We found a significant increase ($p<0.001$) in the cytoplasmic nucleosomes of the HeLa Tet-Off cells expressing and producing the full-length and the COOH-terminal domain of VgrG1 which we compared to HeLa Tet-Off cells producing the NH_2-terminal domain of VgrG1 or expressing the vector alone after 24 h of

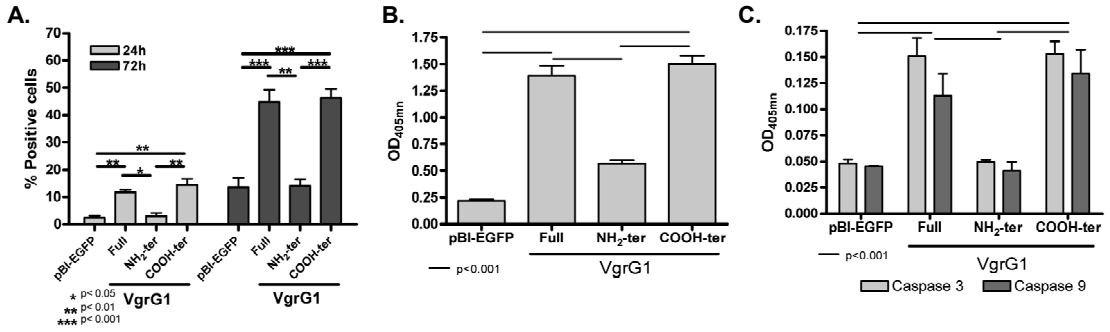

Figure 4.9: Cytotoxic effects of VgrG1 on HeLa Tet off cells.
A. Viability of HeLa Tet-off cells expressing genes encoding different fragments of VgrG1 from *A. hydrophila* ATCC 7966. Percentage of dead and/or dying cells were quantified by incorporation of 7-AAD and flow cytometry on HeLa Tet-off cells expressing vector alone (pBI-EGFP) or producing different fragments of VgrG1 (NH_2- and COOH- terminal, and full-length) after 24 h and 72 h of transfection. Results from three different assays were plotted, and statistical significance is indicated by upper horizontal lines. Apoptosis of HeLa Tet-off cells expressing genes encoding different fragments of VgrG1. **B**. Apoptosis rates were measured by quantification of cytoplasmic nucleosomes and; **C**. caspase 3 and caspase 9 activity in lysates of HeLa Tet-off cells expressing vector alone (pBI-EGFP), or expressing genes encoding NH_2- and COOH- terminal fragments, and the full-length VgrG1 from *A. hydrophila* ATCC 7966. Results from three different assays were plotted and statistical significance ($p<0.001$) is indicated by upper horizontal lines. The designation ter refers to NH_2- or COOH-terminal domains.

transfection (Figure 4.9B). Accordingly, the activation of caspase 3 and 9 was significantly higher ($p<0.001$) in the HeLa Tet-Off cells producing the full-length VgrG1 and its COOH-terminal domain than in cells producing the NH_2-terminal domain of VgrG1 or expressing the vector alone (Figure 4.9C).

DISCUSSION

In this study, by 2-dimensional gel electrophoresis of culture supernatants of Δ*act* and Δ*act*/Δ*vasH* mutants of *A. hydrophila* SSU followed by mass-spectrometry, we identified a member of the VgrG family of proteins that possessed a COOH-terminal extension with an ADP ribosyltransferase domain characteristic of VIP-2. Since we do not have the full genome sequence of *A. hydrophila* SSU, the precise location of the *vgrG1* gene on the chromosome of *A.*

hydrophila SSU is therefore currently unknown. Consequently, we preferred to conduct studies with rVgrG1 of *A. hydrophila* ATCC 7966, as this protein is highly conserved in the SSU and 7966 strains. Further, based on our earlier studies, the virulence signatures of a clinical isolate SSU are better defined and characterized compared to that of an environmental isolate ATCC 7966. The VrgG protein family was initially associated with the Rhs (Recombination hot spot) family, and although VgrG and RhsG proteins are not homologous, they do share some common characteristics, such as their hydrophilic nature, large size and regularly repeated peptide motifs (60, 212). Based on the similarity between the RhsG and VgrG family proteins, it was hypothesized that VgrG could have acquired the multiple COOH-terminal extensions by horizontal transfer (15, 60).

It has been reported that several Gram-negative bacteria encode VgrG proteins with different COOH-terminal extensions that have a variety of functions. Hence, *V. cholerae* VgrG1 has an RtxA motif, while *V. cholerae* VgrG3 has a peptidoglycan-binding domain. Also, a *P. aeruginosa* VgrG has a zinc metalloprotease domain and *Y. pestis* has two VgrGs with COOH extensions, one with a tropomyosin-like domain, and the other, a YadA-like and pertactin-like domain (164). In this study, we reported that the VgrG1 protein from *A. hydrophila* carries a VIP-2 domain, which has ADPRT activity with deleterious effects on the host cells. Thus far, this is the only VgrG member of the family known that has the VIP-2 domain and is present only in tested *A. hydrophila* strains, namely SSU and ATCC 7966.

It is important to note that the presence of VgrG1 was detected in the bacterial culture supernatants by Western blot analysis only when antibodies against VgrG2 were combined with antibodies against the VIP-2 domain of

VgrG1 and by using a highly sensitive, west femto chemiluminescence substrate (Figure 4.1C). These data might suggest the availability of a limited number of immuno-reactive epitopes in the VIP-2 domain to which antibodies could be generated. This, in combination with the low concentration of VgrG1 present in the bacterial culture supernatant, might cause difficulties in its detection.

Interestingly, VgrG1 could be detected and identified in the concentrated supernatants of bacterial cultures through highly sensitive fluorescent Sypro-Ruby staining used for 2-dimensional gels and mass spectrometric analysis (Figure 4.1A). On the other hand, VgrG2/3 could be easily detected in bacterial culture supernatants (Figure 4.1C, lane 3) and in tissue culture medium of bacterial-host cell co-cultures by Western blot analysis using only antibodies against VgrG2 (Figure 4.5). We believe that this difference in detection of VgrG2/3 and VgrG1 by Western blot analysis could be due to varying amounts of VgrGs present in the culture supernatants. We could easily detect VgrG2 and -3 in Western blots because of possible higher expression of their genes and more secretion of the corresponding proteins and or their possible less degradation. Further, accumulation of both of these proteins in a single band due to their similar molecular sizes could result in a band which is much stronger in intensity. In HeLa Tet-Off cell lysates, we could also easily detect the presence of VgrG1 on Western blots by employing either VgrG2 or VIP2-specific antibodies (Figure 4.7A) because highly concentrated protein preparations were used for the assay and the gene was hyperexpressed using the pBI-EGFP vector.

By bioinformatic and structural analyses, VgrG proteins were shown to share structural features of a bacteriophage T4 tail spike. Thus, VgrG's amino- and central- parts are similar to those of gp27 protein, and the carboxyl part

similar to that of the gp5 protein of a T4 phage (25, 164). During phage infection, the tail spike is inserted into the bacterial outer membrane, and, hence, VgrG could act similarly, i.e., as a needle tip that, in conjunction with the tube-like structure formed by Hcp, could puncture the host cellular membrane and translocate effector protein(s) (25, 60, 116, 122, 154). In these models, the COOH terminal extensions from some VgrG proteins, such as VgrG1 from *V. cholerae* with actin cross-linking activity (Figure 4.2C) and VgrG1 from *A. hydrophila* with actin-ADPRT activity (Figure 4.2B) are introduced directly into the host cellular cytoplasm where they exhibit their action.

Currently, VgrG1 from *V. cholerae* is the only protein with a COOH-terminal extension that has been characterized (39, 165, 183). This protein carries an RtxA motif (Figure 4.2C) that induces covalent actin cross-linking, leading to cell rounding of the eukaryotic cells (39, 122, 164, 165, 183). Sequence alignment of VgrG1 from *V. cholerae* and *A. hydrophila* ATCC 7966 showed a high homology in the VgrG domains (NH_2-terminal) (55%), but a low homology (8%) in the COOH- terminal regions where the RtxA or the VIP-2 domain is respectively located (Figures 4.2B and C). Although VgrG1 from *V. cholerae* and VgrG1 from *A. hydrophila* were able to induce a cell-rounded phenotype, its induction was by a different mechanism. While VgrG1 from *V. cholerae* covalently cross-links actin to alter the cytoskeleton architecture (122, 164), VgrG1 from *A. hydrophila* ADP-ribosylates actin to impede its polymerization.

In our earlier study, we characterized a T3SS effector protein AexU, which also has ADPRT activity. This effector induced a rounded phenotype when its corresponding gene was expressed in HeLa Tet-off cells (185). We are currently

exploring in detail the mechanism of action of AexU. In addition, our previous study indicated that the production and translocation of AexU was not altered when the *vasH* gene was deleted from the parental *A. hydrophila* strain, however, this *vasH* mutant showed delayed cytotoxicity in HeLa cells as well as in RAW 264.7 murine macrophages (193). Together, these data suggested that even though AexU and VgrG1 could induce rounded phenotype in the host cell via the ADPRT activity, their mechanisms of regulation could be different and their corresponding genes might be activated or repressed under different stimuli.

Recently published data showed the translocation of VgrG1 from *V. cholerae* into the eukaryotic cell cytoplasm (122). In addition, these investigators showed that tagging VgrG proteins at their COOH-terminal ends did not affect their translocation (122). Since VgrG1s from *V. cholerae* and *A. hydrophila* share similar VgrG cores (COG3501) at their NH_2-terminal ends, we used a similar approach to examine the translocation of *A. hydrophila* VgrG1 into HeLa cells with successful results. We showed translocation of both the full-length VgrG1 and its NH_2-terminal domain into the cytoplasm of HeLa cells when this effector was tagged at its COOH-terminal end with Bla protein in the *A. hydrophila* SSU Δ*act* mutant strain. Importantly, an isogenic mutant deleted for the *vasH* gene (with a non-funtional T6SS apparatus) was not able to translocate VgrG1 into HeLa cells, indicating an absolute need for the T6SS to translocate this effector.

The VIP-2 motif present in VgrG1 of *A. hydrophila* was previously characterized in iota toxin of *C. perfringens* among others (4, 5, 12, 77, 158, 159, 208). The VIP-2 domain possesses actin ADPRT activity that prevents actin polymerization and leads to rounding of the eukaryotic cells (204, 205). Our results showed the functionality of the VIP-2 motif present in VgrG1 of *A.*

hydrophila. As we expected, the rVgrG1 showed actin ADPRT activity associated with the presence of VIP-2 motif in *in vitro* assays, and this activity induced cell-rounding morphology after episomal expression of the *vgrG1* gene in eukaryotic cells. Our results corroborated with the findings of others (204, 205).

Yu et al. (228) reported the presence of a sequence containing *vgrG, vsdC,* and a type III secretion-related protein gene (AY376445) in *A. hydrophila* strain PPD134/91. Blast conserved domain analysis of this sequence showed that VgrG (GI:60328264) corresponded to a protein without the COOH-terminal extension, and that VsdC (GI:60328266) has a VIP-2 motif. Alignment of a similar sequence found in *A. hydrophila* ATCC 7966 with that of *A. hydrophila* PPD134/91 showed high identity/homology with two open reading frames corresponding to VgrG1 (GI:117619461) and a type III secretion-related protein (GI:117621298). Although *vgrG* and *vsdC* represented two, independent, open reading frames in *A. hydrophila* strain PPD134/91, a similar sequence in *A. hydrophila* ATCC 7966 was represented by only one *vgrG1* gene (GI:117619461). Likewise, in *A. hydrophila* SSU, based on our proteomics and Western blot analysis data, it is apparent that the VIP-2 domain is linked to the VgrG core in a single open reading frame. In contrast, the type III secretion-related protein encoding open reading frame was present in both *A. hydrophila* ATCC 7966 and in *A. hydrophila* PPD134/91.

Eukaryotic cells treated with actin ADPRT activity bacterial toxins, like C2 from *C. botulinum* and iota toxin from *C. perfringens*, die after disruption of the cytoskeleton architecture (205). We found that HeLa Tet-Off cells expressing the *vgrG1*-encoding fragments containing the VIP-2 domain had less viability than did cells expressing and producing the VgrG1 NH_2-terminal domain or the mock

control. These differences in viability corresponded to higher rates of apoptosis in cells expressing and producing the VIP-2 domain.

It is known that surface epithelial cells become apoptotic after detaching from their underlying basement membrane by a process called anoikis (62), which is characterized by the loss of mitochondrial membrane potential through the action of Bax/Baf that results in caspase activation and DNA fragmentation (69). The actin cytoskeleton plays an important role in mitochondrial function (110, 149). After depolymerization of actin filaments induced by latrunculin-A or cytochalasin-D, Bax protein is localized in the outer mitochondrial membrane, where it interacts and keeps open the voltage-dependent anion channels, and/or forming oligomeric pores in the outer membrane which allow the loss of membrane potential, and the release of both cytochrome c and other pro-apoptotic factors (127, 216, 219). Apoptosis induced by VgrG1 of *A. hydrophila* could be mediated by perturbation of the mitochondrial function. Thus, after the alteration of the actin cytoskeleton by VgrG1-ADPRT activity, release of cytochrome c could activate caspase 9 and, subsequently, caspase 3, as our results indicated.

It has been shown in certain diseases, such as inflammatory bowel disease and infectious enterocolitis, that disruption of the intestinal epithelial barrier could lead to watery diarrhea due to the loss of water and electrolytes (34, 35, 142). Gastrointestinal infections with *A. hydrophila* induce severe diarrhea, and we previously reported the participation of three different toxins produced by *A. hydrophila* SSU, namely Act, and the heat-labile (Alt) and heat-stable (Ast) enterotoxins as players in inducing bloody and non-bloody diarrhea (7, 179). The studies described in this paper have introduced another possible player which, by

inducing cell- rounding morphology, could disrupt the epithelial intestinal integrity barrier, thus allowing entry of other virulence factors associated with *A. hydrophila* SSU pathogenesis. Thus, VgrG1 actin-ADPRT activity could trigger actin depolymerization with fatal effects on the intestinal epithelial barrier.

Chapter 5: Role of a type 6 secretion system effector Hcp of *Aeromonas hydrophila* in modulating activation of host immune cells[3]

INTRODUCTION

We reported that the *ΔvasH* mutant of *A. hydrophila* SSU was unable to express genes encoding hemolysin-coregulated protein (Hcp) and the valine-glycine repeat family of proteins VgrG2 and VgrG3, which constitute part of the T6SS gene cluster (193). However, this mutant was able to express, but unable to secrete VgrG1 that resides outside of the T6SS gene cluster (191). Further, deletion of one prevents the secretion of the other, thereby demonstrating their dual roles as structural components of the T6SS apparatus and as effector proteins (193). Importantly, both Hcp and VgrGs represent a hallmark of the T6SS secreted proteins in all of the bacteria that possess this system (15, 25, 60). Although the role of VgrG1 in bacterial virulence was convincingly been demonstrated recently by us in *A. hydrophila* (191) and in *V. cholerae* (122), the mechanism of Hcp in modulating the organism's virulence is poorly understood.

Innate immunity is the first line of host defense against challenged organisms, and pattern recognition receptors (PRR) sense different microbial ligands (known as pathogen-associated molecular patterns or PAMPs) (13, 99, 132, 198), resulting in the triggering of signaling cascades which determine the host immune response by modulating maturation, activation, and recruitment of

[3] 192. **Suarez, G., J. C. Sierra, M. L. Kirtley, and A. K. Chopra.** 2010. Role of a type 6 secretion system effector Hcp of *Aeromonas hydrophila* in modulating activation of host immune cells. Microbiology ***In Revision***.

cellular effectors (e.g., neutrophils, macrophages, dendritic cells [DCs], and natural killer [NK] cells) (81, 89, 132, 157).

Phagocytosis is crucial for both innate and adaptive immunity (38, 81, 132), and macrophages and DCs are professional antigen-presenting cells (APCs) which act as tissue sentinels and are able to present antigens to naïve T-cells (72, 88, 89). Bacteria have developed different mechanisms to avoid innate immunity ranging from their ability to avoid recognition by toll-like receptor (TLR)-4 (55, 75, 103), altering antigenicity of surface molecules to avoid phagocytosis (176), interfering with mitogen-activated, protein kinase (MAPK)-signaling cascades (152, 194, 199), modulating actin polymerization and apoptosis (1, 41, 70, 82, 162, 170, 209, 225, 232), manipulating phagosome trafficking and maturation (51, 190, 206), and inducing the production of immunosuppressive cytokines, particularly, IL-10. The latter mechanism avoids activation of macrophages, maturation of DCs, and recruitment of granulocytes (131, 187, 231).

In this study, we showed that Hcp plays a role in modulating the innate immunity by inhibiting the phagocytosis of *A. hydrophila*, thus allowing its multiplication and spread to different organs of the host. Our results show that Hcp is able to bind to macrophages and induce the production of IL-10 and transforming growth factor (TGF)-β, affecting the activation and maturation of macrophages, and, consequently, the recruitment of other cellular immune components needed to clear bacterial infection.

RESULTS

Hcp inhibits phagocytosis of *A. hydrophila* SSU *Δact/ΔvasH* mutant *in vitro*

Previously, we reported that *A. hydrophila* carrying a deletion of the *vasH* or *vasK* gene was more susceptible to being phagocytosed by RAW 264.7 murine macrophages (193). These mutant strains exhibited different phenotypes in terms of Hcp secretion and translocation (193). For example, deletion of the *vasH* gene abrogated the expression of the T6SS gene cluster, including the *hcp* gene, while deletion of the *vasK* gene affected only the secretion of Hcp to the extracellular milieu without any effect on its expression or translocation (193). We confirmed that the difference in phagocytosis between the parental *Δac*t strain of *A. hydrophila* and that of the T6SS gene cluster knock out strain, *A. hydrophila Δact/ΔvasH*, was not due to differences in their growth rates or to toxic effects of rHcp on RAW 264.7 cells for at least up to 6 h, as determined by the MTT and 7-AAD assays.

Since the secretion of Hcp was affected in both the *ΔvasH* and *ΔvasK* mutant strains, we hypothesized that the secreted form of Hcp could be playing a role in the bacterial inhibition of phagocytosis. To test this, we used conditioned medium from the *Δact* parental strain of *A. hydrophila*, which contained Hcp (Fig. 5.1A) in the phagocytosis assay. Consequently, RAW 264.7 macrophages were infected with the *Δact/ΔvasH* mutant in the above-mentioned conditioned medium, thus allowing complementation of the mutant with exogenous Hcp. We noted that the ability of the *A. hydrophila Δact/ΔvasH* mutant to be phagocytosed by macrophages was reduced in the presence of Hcp (Fig. 5.1B). To confirm that this effect was indeed contributed by Hcp and not by other secreted bacterial

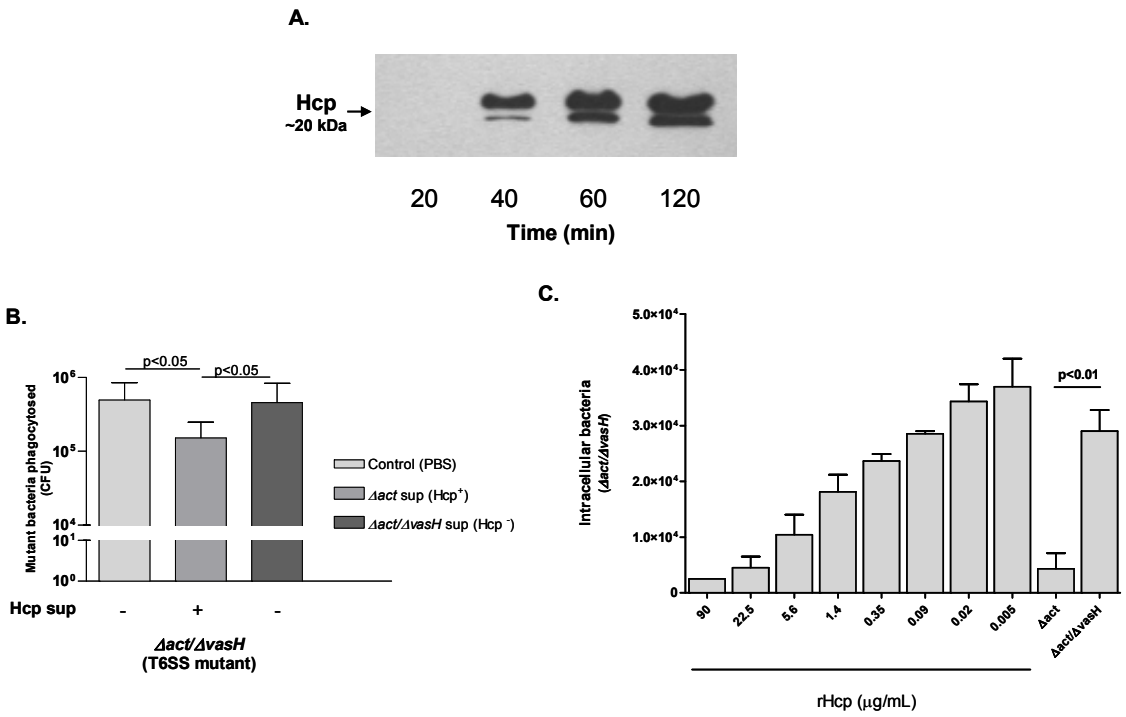

Figure 5.1: Hcp inhibit phagocytosis of *A. hydrophila* SSU *Δact/ΔvasH* mutant.
A. Western blot analysis of the tissue culture medium used for the growth of the parental *A. hydrophila* SSU Δ*act* strain by using antibodies to Hcp. An aliquot (1 x 10^7 cfu) of bacterial culture was grown in 5 mL of DMEM supplemented with 0.5% FBS at 37ºC. An aliquot (500 μL) was taken at different time points up to 2 h, centrifuged, filtered through a 0.2-μm membrane, and proteins in the samples separated by SDS-15% PAGE. The proteins were then transferred to the nitrocellulose membrane, and the presence of Hcp was detected by using Hcp-specific antibodies previously developed in the laboratory. As a control, we used tissue culture medium in which the Δ*act*/Δ*vasH* mutant was grown for the same periods of time and no reaction for Hcp was detected (data not shown). The presence of a doublet on the Western blot may represent the degradation product of Hcp. **B**. Conditioned medium containing Hcp inhibits phagocytosis of the Δ*act*/Δ*vasH* mutant. Phagocytosis assay using the *A. hydrophila* Δ*act*/Δ*vasH* mutant and RAW 264.7 cells was performed in conditioned medium from *A. hydrophila* SSU Δ*act* parental strain as the exogenous source of Hcp. We also used conditioned medium from *A. hydrophila* Δ*act*/Δ*vasH* mutant as a control for any other secreted proteins/factors independent of the T6SS that could affect phagocytosis. Another control included fresh DMEM supplemented with 0.5% FBS. **C**. rHcp inhibits phagocytosis of the *A. hydrophila* Δ*act*/Δ*vasH* mutant. A phagocytosis assay employing the Δ*act*/Δ*vasH* mutant of *A. hydrophila* by RAW 264.7 cells in the presence of different concentrations of Hcp was performed to show the role of Hcp in the inhibition of phagocytosis. As a control, we included *A. hydrophila* Δ*act*/Δ*vasH* mutant without rHcp, as well as the *A. hydrophila* Δ*act* parental strain. The graph represents the mean of three independent experiments ± standard deviations. The statistical difference was calculated by a one-way ANOVA test.

proteins, we performed a phagocytosis assay by using the *ΔactΔvasH* mutant in conjunction with different concentrations of purified rHcp. We found that

phagocytosis of the mutant by macrophages was inhibited by rHcp in a dose-dependent fashion (Fig. 5.1C). Our positive and negative controls in this assay included macrophages infected with the Δ*act* and Δ*act*/Δ*vasH* strains of *A. hydrophila*, respectively. As expected, the former showed minimal phagocytosis, while the latter exhibited much higher levels of phagocytosis by macrophages. These data clearly indicated that the secreted Hcp played a role in bacterial phagocytosis.

Hcp decreases the survival rate of mice infected with the *Δact/ΔvasH* mutant of *A. hydrophila* SSU

Since the secreted form of Hcp decreased bacterial phagocytosis, and we showed earlier that mice infected via the i.p. route with the *Δact/ΔvasH* mutant had a better survival rate when compared to mice infected with its parental *Δact* strain (193), we hypothesized that bacteria producing Hcp would have a better chance to evade innate immunity and cause systemic effects. Hence, we challenged mice with a sub-lethal dose of the *Δact/ΔvasH* mutant together with rHcp at different concentrations and recorded deaths for 16 days. We found that the addition of rHcp decreased the survival rates of mice in a dose-dependent manner after infection with the *Δact/ΔvasH* mutant, with 100% of the mice dying with 5 and 10 μg/mL of rHcp (Fig. 5.2A). In addition, we challenged mice with two different doses of the *Δact/ΔvasH* mutant in the presence of one selected dose (10 μg/mL) of rHcp (Fig. 5.2B). Our data indicated that the groups of mice infected with the mutant in combination with rHcp died in greater numbers. Thus, this increase in bacterial virulence could be associated with inhibition of phagocytosis in the peritoneal cavity, which allowed bacteria to multiply and cause systemic infection.

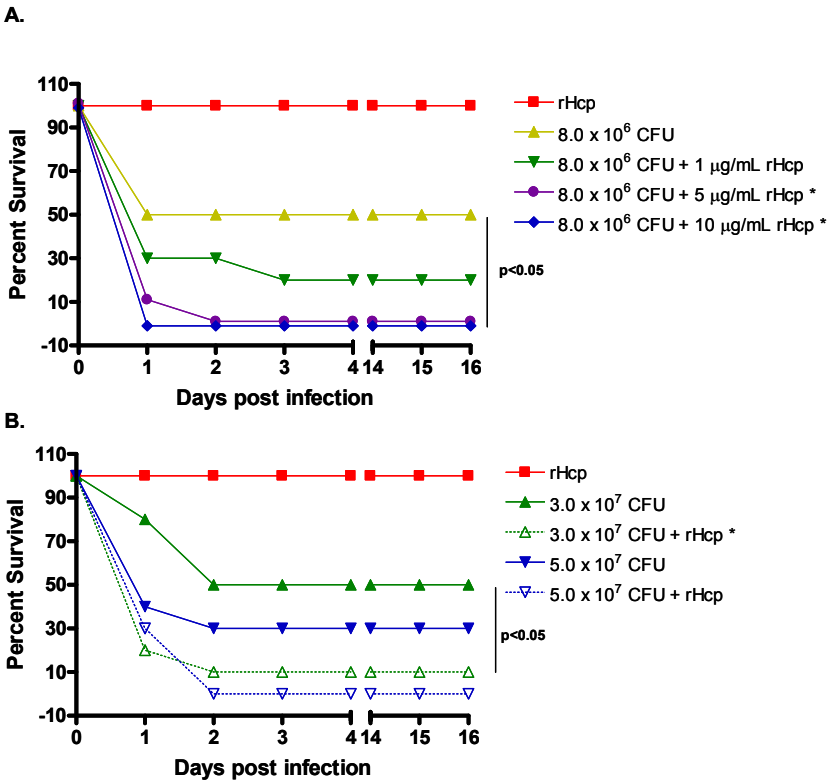

Figure 5.2: Hcp increases the virulence of the *A. hydrophila* SSU *Δact/ΔvasH* mutant. **A**. Survival curves of mice (n=10) infected via the i.p. route with 8 x 10^6 cfu of *A. hydrophila* Δ*act*/Δ*vasH* mutant in the presence of different concentrations of rHcp. (*) Asterisks denote statistically significant differences compared to the group without the rHcp. **B**. Survival curves of mice (n=10) infected via the i.p. route with 3 x 10^7 and 5 x 10^7 cfu of the Δ*act*/Δ*vasH* mutant in the presence of rHcp (10 μg/mL). As a control, groups of mice (n=10) were infected with the same doses of the mutant bacteria without rHcp. As another control for rHcp toxicity, mice were inoculated with the highest concentration of rHcp without the bacteria. The deaths were recorded for 16 days after infection. (*) Asterisks represent groups of mice with statistically significant differences in mortality against the same doses of bacteria without the rHcp. Statistical significant differences were determined by the Logrank test with GraphPad Prism V.4 Software.

Hcp enhances the spread of the Δ*act*/Δ*vasH* mutant of *A. hydrophila* SSU in a mouse model

Since our data showed that the presence of rHcp increased the death rate of mice infected with sub-lethal doses of the *Δact/ΔvasH* mutant, we evaluated the bacterial load in mouse organs after 48 h of infection in the presence of rHcp. Our results indicated that, when compared to findings in animals infected with the

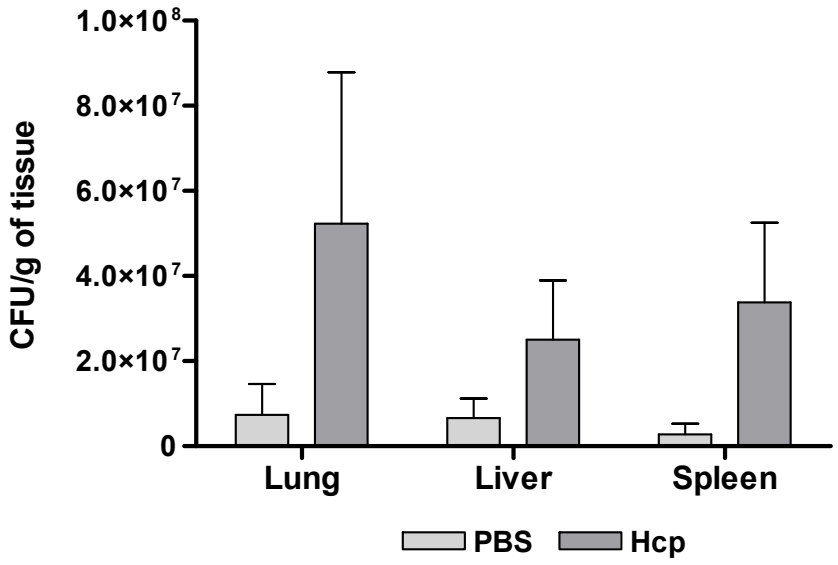

Figure 5.3: Hcp plays a role in the spread of *A. hydrophila* SSU *Δact/ΔvasH* mutant. Bacterial burden was measured in the lungs, livers and spleens after 48 h of i.p infection with 3 x 10^7 cfu of *A. hydrophila* Δ*act*/Δ*vasH* mutant in the presence of rHcp (10 μg/mL). The data were normalized with the organ weights. Mice infected with the same doses of bacteria without rHcp were used as a control. The graph represents data from 10 mice/group.

mutant bacteria alone, adding rHcp allowed the mutant bacteria to spread to different organs (lungs, livers and spleens) resulting in the animals' death due to a systemic effect (Fig. 5.3). These results support our contention that Hcp plays a role in innate immunity by avoiding bacterial clearance in the peritoneal cavity by phagocytosis, thus allowing organisms to multiply and spread to various organs.

Hcp binds to intraperitoneal immune cells of mice

Our earlier study showed that Hcp binds murine RAW 264.7 macrophages (193). To confirm that Hcp also binds to primary macrophages, we collected whole cell populations from the peritoneal cavity of mice and, after incubating them with rHcp, we performed a multicolored flow cytometry analysis by using antibodies against Hcp, F4/80, and Gr-1. Figure 5.4A shows the forward scatter versus the side scatter plot from the whole cell population isolated after the

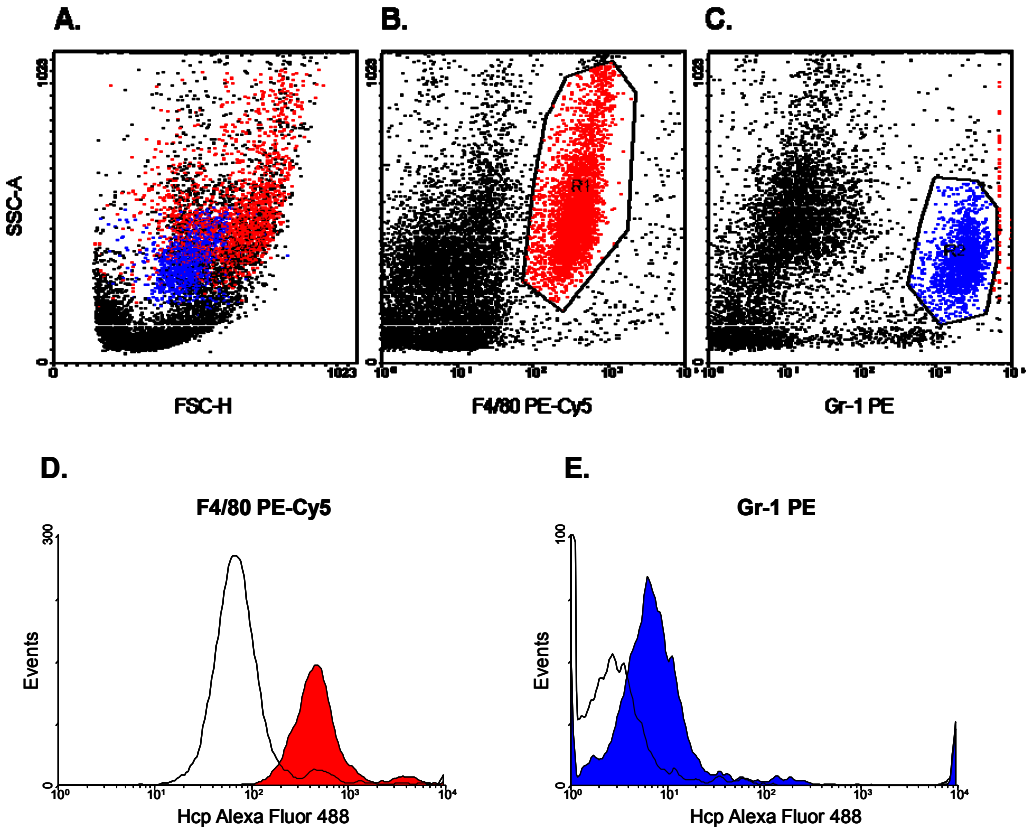

Figure 5.4: Hcp binds to intraperitoneal immune cells
Total i.p. cells were recovered after peritoneal lavages and incubated with rHcp (10 μg/mL). Subsequently, the cells were stained by using antibodies against Hcp (FITC), Gr-1 (PE), and F4/80 (PE-Cy5). **A**. Side scatter versus Forward scatter plot for the total i.p cells. **B**. Side scatter versus F4/80 PE-Cy5 plot from the total cell population gated for the F4/80 positive cells (red). **C**. Side scatters versus Gr-1 PE plot from the total cell population gated for the Gr-1-positive cells (blue). **D**. A histogram plot showing staining for Hcp (Alexa Fluor 488) in cells, which were F4/80, positive. The empty curve represents the isotype control, while the filled curve (red) represents cells incubated with anti-Hcp antibodies. **E**. A histogram plot showing staining for Hcp (Alexa Fluor 488) in cells, which were Gr-1, positive. The empty curve represents the isotype control and the filled curve (blue) cells incubated with anti-Hcp antibodies.

lavage. For analysis, we evaluated Hcp binding to cells gated both on F4/80 (macrophages) (Fig. 5.4B) and on Gr-1 (granulocytes) (Fig. 5.4C). As can be seen, Hcp was able to bind macrophages (Fig. 5.4D) and granulocytes (Fig. 5.4E), although the differential shift of the curve for granulocytes indicates that Hcp binding to this particular cell type was much less when compared to its isotype control and that for the macrophages. These results led us to speculate

that Hcp could bind to host cell membrane molecules (possibly receptors) which are common to both cell types; however, these surface components were more abundant on macrophages.

Hcp modulates the expression of activation markers on intraperitoneal immune cells

Since our data indicated that Hcp binds to cells involved in the innate immune response, we analyzed by flow cytometry the status of these cells localized in the peritoneal cavity after 4 h of infection with the *Δact/ΔvasH* mutant of *A. hydrophila* given along with two different concentrations of rHcp. Specifically, we analyzed changes in the percentage of macrophages (F4/80), granulocytes (Gr-1), and dendritic cells (CD11c). In addition, we examined the expression of CD69 (an early activation marker) and the major histocompatibility complex (MHC) class II on these cell types.

We noted a significant increase in the percentage of Gr-1-positive cells in the peritoneal cavity of animals infected with the *Δact/ΔvasH* mutant when compared to the uninfected mice (Fig. 5.5A). However, this difference was independent of the presence of Hcp. Additionally, our results showed no significant changes in the percentages of macrophages and DCs in the peritoneal cavity after 4 h of infection; although we noted marginal increases in CD11c-positive cells related to the increasing concentrations of Hcp (Fig. 5.5A).

We then analyzed the expression of CD69 and MHC-class II in the total cell population as well as in cells gated for F4/80, Gr-1, and CD11c after infection with the *Δact/ΔvasH* mutant and given rHcp. As shown in Fig. 5.5B, decreases in the percentages of CD69 in all cell types analyzed (F4/80 and Gr-1 positives) were noted, and these decreases were dependent on the concentration of the

rHcp used. Likewise, the percentages of MHC-class II-positive cells in the F4/80 cell type was decreased after mice were infected with the *Δact/ΔvasH* mutant and then given the higher dose of rHcp. However, the expression of MHC-class II showed an increase in CD11c cells, although the data were not statistically significant (Fig. 5.5C).

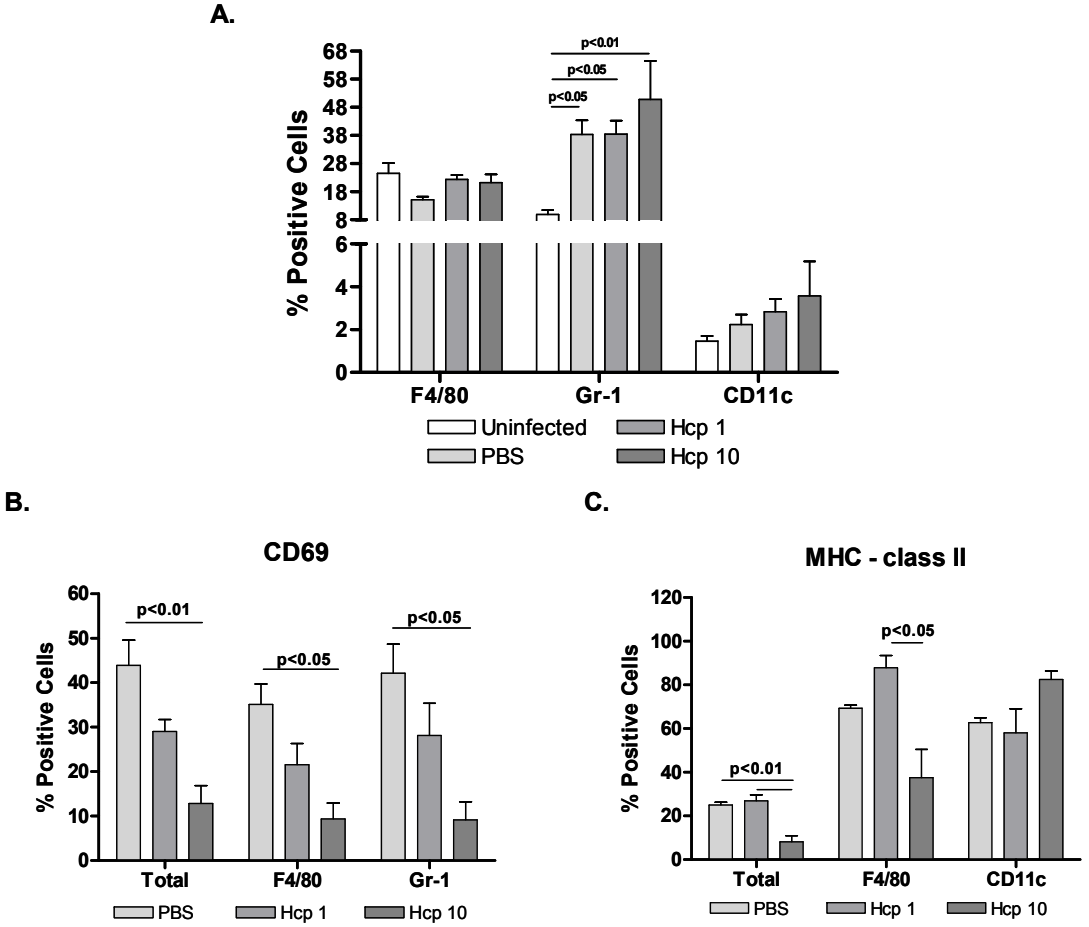

Figure 5.5: Hcp modulates the levels of activation markers on intraperitoneal immune cells.

Flow cytometric analysis of intraperitoneal cells recovered from lavages after 4 h of infection with 3 x 10^7 cfu of *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant in the presence of different doses of rHcp (1 and 10 μg/mL). The PBS group represents animals challenged with only bacteria without the rHcp but instead given PBS. **A**. Shows percentage of positive cells for F4/80 (macrophages), Gr-1 (granulocytes), and CD11c (DCs). **B**. Shows percentage of positive cells expressing CD69 and MHC-class II for the total population or for cells gated for F4/80, Gr-1, or CD11c. Statistically significant differences were measured by the one-way ANOVA test followed by the Tukey's test.

Hcp modifies cytokine/chemokine production profiles induced by the *A. hydrophila* SSU *Δact/ΔvasH* mutant

Since rHcp modulates the expression of activation markers on macrophages, granulocytes and DCs, we analyzed cytokine/chemokine patterns induced by the *Δact/ΔvasH* mutant in the peritoneal cavity after 4 h of infection (Table 5.1). We found that infection of mice with the mutant induced the production of a wide range of pro- and anti- inflammatory cytokines/chemokines and growth factors. However, the addition of rHcp, along with the *Δact/ΔvasH* mutant, during infection significantly modified the levels of some of these cytokines/chemokines. For example, the concentrations of IL-2, IL-10, IL-15, IL-12p70, MIP-1β, MIP-2, G-CSF, IL-6, and KC were increased by adding rHcp; however, the production of IFN-γ, IL-1α, and M-CSF was inhibited (Table 5.1).

Hcp does not have a significant impact on the activation status of intraperitoneal granulocytes and dendritic cells

Macrophages, immature DCs, and granulocytes are the main components of innate immunity and the first line of defense against invading organisms. Our data showed that rHcp binds to macrophages and granulocytes; however, the receptors or the binding targets for Hcp appeared to be more abundant on macrophages than in granulocytes (Fig 5.4). The flow cytometric analysis data on peritoneal cells showed a significant increase (4-fold) in the percentage of granulocytes after infection with the Δ*act*/Δ*vasH* mutant compared to that of the basal levels (uninfected) (Fig 5.5A, Gr-1 panel). These results, together with increases, dependent on rHcp concentrations, in cytokines/chemokines involved in neutrophil maturation and recruitment, such as G-CSF, KC, and MIP-2 (Fig. 5.6) may indicate that Hcp is not affecting the recruitment of granulocytes and

neutrophils in the peritoneal cavity of mice after infection with the *Δact/ΔvasH* mutant. However, reduction with rHcp of CD69 levels on the surface of granulocytes (Fig. 5.5B) indicated that this T6SS effector could have an effect in granulocyte activation.

The percentages of DCs (CD11c) were somewhat increased in mice infected with the *Δact/ΔvasH* mutant in the presence of Hcp (Fig. 5.5A). Importantly, these cells also showed increases in the percentage of the MHC-class II molecule in the presence of rHcp (10 µg/mL) (Fig. 5.5C). This was in

Table 5.1: Cytokine/Chemokine levels in the peritoneal lavages of mice

	A. hydrophila* SSU *Δact/ΔvasH	
	+ PBS	**+ rHcp**
Eotaxin	1018±58	1088±35
G-CSF	126849±18670	184895±11335 *
GM-CSF	128±12	142±24
IFN-γ	414±70	183±39 ***
IL-1α	276±11	240±14 *
IL-1β	811±68	872±90
IL-2	12±3	27±12 **
IL-3	ND	ND
IL-4	ND	ND
IL-5	129±22	100±18
IL-6	62608±11044	118731±22410 *
IL-7	ND	ND
IL-9	141±11	209±6 **
IL-10	109±32	192±32 *
IL-12p40	ND	ND
IL-12p70	123±3	133±3 *
IL-13	ND	ND
IL-15	14±1	20±0.5 ***
IL-17	1332±112	1774±259
IP-10	413±33	395±19
KC	19104±4320	36500±6353 *
LIF	ND	ND
LIX	560±55	568±11
MCP-1	16108±1562	17860±711
M-CSF	12±2	7±2 **
MIG	2180±173	1919±96
MIP-1α	401±29	481±18
MIP-1β	526±76	734±61 *
MIP-2	6288±935	10754±633 *
RANTES	207±15	238±17
TNF-α	16±2	22±3
VEGF	ND	ND

Concentrations are in pg/mL; mean ± SD.
ND: No detected; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

contrast to macrophages and granulocytes, which showed decreases in the expression levels of MHC-class II molecule. These results led us to suggest that Hcp might be inducing the maturation of DCs ($CD11c^+$/ MHC-Class II^+). However, low levels of IL-12p70, IFN-γ, and induction of IL-10 by rHcp in mice infected with the *Δact/ΔvasH* mutant would hamper their normal activation and induction of regulatory phenotypes (Fig. 5.6, Table 5.1).

Hcp induces an alternative pathway of macrophage activation

Activation of macrophages is an important mechanism in innate immunity against foreign invading organisms. There are two types of macrophage activation. M1 or classical activation is mainly a pro-inflammatory or T helper 1(Th1)-like response focused on removal and clearing of the infection or debris produced by an injury and M2, or alternative activation, is focused more on tissue re-modeling and wound healing. The latter is characterized by a low infiltration of cellular components and a Th2-like cytokine phenotype. Our data indicated that the presence of rHcp during infection with the *Δact/ΔvasH* mutant could modulate an alternative pathway of macrophage activation, which is supported by our data showing decreases in some pro-inflammatory cytokine levels, such as IFN-γ and IL-1α, and increases in anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-9 (Fig. 5.6, Table 5.1). These results were also supported by the low levels of M-CSF induced by infection with the *Δact/ΔvasH* mutant in mice, which were further reduced in the presence of Hcp (Fig. 5.6). Taken together, our data indicated minimal macrophage recruitment in the peritoneal cavity of mice after infection with the *Δact/ΔvasH* mutant of *A. hydrophila* (Fig 5.5A, F4/80 panel).

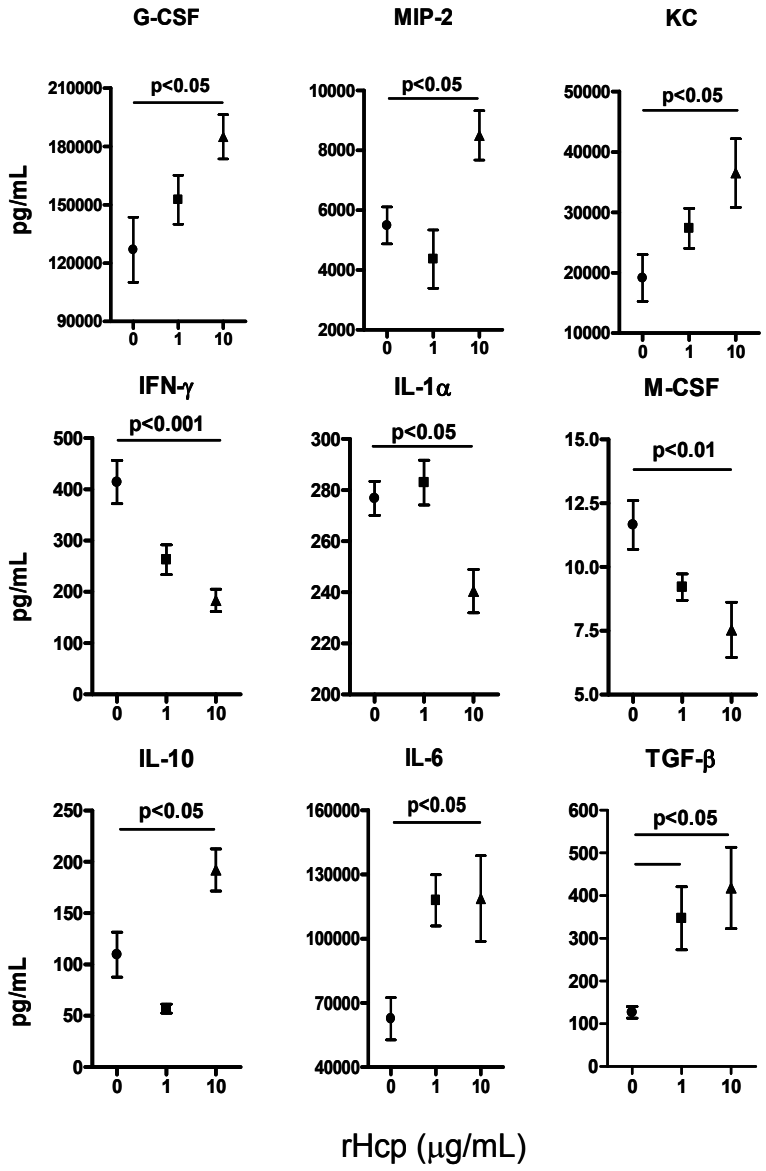

Figure 5.6: Hcp induce alternative activation in intraperitoneal Macrophages
rHcp induces an alternative pathway of macrophage activation in the peritoneal lavages of mice infected with 3 x 10^7 cfu of *A. hydrophila* SSU Δ*act*/Δ*vasH* mutant in the presence of different doses of rHcp (1 and 10 μg/mL), as determined by cytokine/chemokine profiling. Cytokines were measured by multiplex bead array on samples of intraperitoneal cavity lavages after 4 h of infection. Statistically significant differences were measured by the one-way ANOVA test, followed by the Tukey's test.

IL-6 has a dual role in immune regulation; this cytokine is considered as either pro-inflammatory or anti-inflammatory and produced by a wide panel of cell

types, which include fibroblasts, epithelial cells, macrophages and endothelial cells. Thus, IL-6, in the presence of low concentrations of IFN-γ and TNF-α, could induce the activation of a suppressor of cytokine signaling (SOCS) -3, resulting in a Th2- like response, mainly by the induction of IL-4 and IL-10. Peritoneal lavages of mice infected with the Δ*act*/Δ*vasH* mutant showed higher levels of IL-6, which further increased in the presence of rHcp (Fig. 5.6). These results, in combination with very low amounts of IFN-γ, IL-1α, M-CSF, TNF-α, and increases in IL-10 and TGF-β (Fig. 5.6, Table 5.1), point to a Th2 cytokine role for IL-6 in inducing the alternative pathway of macrophage activation by up-regulating SOCS proteins.

DISCUSSION

In this report, we have described the role of Hcp, a T6SS effector, in modulating the innate host immune response, specifically by inhibiting phagocytosis by macrophages. Our earlier study showed that the extent of phagocytosis of *ΔvasK* and *ΔvasH* mutants of *A. hydrophila* was significantly higher compared to that of the parental strain (193). Since the only common characteristic between these two mutant strains was that neither the Δ*vasK* nor the Δ*vasH* mutant secreted Hcp into the extracellular milieu, we considered that the secreted form of Hcp played a role in the inhibition of bacterial phagocytosis by macrophages.

Our results indeed confirmed that the presence of Hcp in the medium inhibited phagocytosis of the *Δact/ΔvasH* mutant by macrophages. Results using conditioned medium showed differences, albeit smaller, in the inhibition of phagocytosis of the mutant when Hcp was present. However, these differences

were more obvious when we used rHcp in the medium during infection of macrophages, and this inhibition in phagocytosis was inversely related to the concentration of Hcp used. We believe that, in addition to Hcp, conditioned medium has other secreted bacterial products that could affect the physiology of macrophages.

It has been reported that some bacterial proteins are able to interfere with phagocytosis by inducing host cell apoptosis, especially via caspase-1 activation (1, 48, 82, 232). We also showed that episomal expression of the *hcp* gene in epithelial cells induced apoptosis (193); however, there are no available data to show whether extracellular Hcp has any effect on the viability of eukaryotic cells. To confirm whether rHcp present in culture supernatants has any toxic effects on eukaryotic cells, we measured the incorporation of 7-AAD and the metabolism of MTT in RAW 264.7 cells. We did not detect any significant toxicity after 24 h incubation, except for a small increase in mitochondrial activity (measured by MTT) detected after 2 h of incubation. We believe this initial increase was due to the activation of macrophages by rHcp. Also, we found an increase in the percentage of 7-AAD-positive cells (~6% of the total population) as well as a decrease in the mitochondrial activity at 24 h that could be related to cell death after stimulation with rHcp. Since our goal was to show the effects of Hcp on innate immunity, and our experiments did not require incubation times longer than 4 h, we consider that the inhibition of phagocytosis *in vitro* due to Hcp present in the medium was independent of any toxic effects of Hcp on macrophages.

Introduction of mutation and/or deletion of genes in bacteria can cause changes in their normal physiology resulting in alterations in their growth rates.

However, the differences in phagocytosis of the *A. hydrophila ⊿act/⊿vasH* mutant compared to that of its parental strain could not be related to differences in growth rates caused by the deletion of the *vasH* gene.

There are several examples of bacterial proteins that target phagocytosis at different levels in order to establish an infection. Our results showed that the secretion of Hcp into the extracellular medium played an important role in inhibiting innate immunity mediated by macrophages *in vitro*. Similarly, the presence of Hcp during infection increased bacterial virulence and allowed bacterial spread to different mouse organs after infection with the *⊿act/⊿vasH* mutant of *A. hydrophila.* These data indicated that the increased virulence of this mutant in the presence of Hcp was associated with the inhibition of phagocytosis.

Previously, by using Western blot analysis, we reported that the secreted form of Hcp binds murine RAW 264.7 macrophages (193). In this report, we extended this observation and tested the binding of Hcp to primary intraperitoneal cells. We found that Hcp bound mainly to intraperitoneal macrophages, although some binding to granulocytes was also detected. We speculate that differences in Hcp binding between macrophages and granulocytes could be related to differences in the Hcp-binding receptor(s) of these two cell types. These results, together with the fact that after 4 h of infection with *A. hydrophila ⊿act/⊿vasH* mutant, there were increases in the percentage of Gr-1-positive cells, without any changes in the percentage of F4/80 positive cells in the peritoneal cavity, point to macrophages as the major cell target for Hcp. In addition, analysis of the activation status of intraperitoneal macrophages (F4/80) and granulocytes (Gr-1) showed that Hcp down-regulated the levels of the early activation markers, CD69 and MHC class II.

In accordance with these results, we found that the pattern of cytokines/chemokines in the peritoneal cavity, after 4 h of infection in the presence of rHcp, was indicative of an alternative pathway of activation or "deactivation" of macrophages led by the production of IL-10 and TGF-β (72, 124, 143). Macrophage activation is an important step in modulation of an immune response, and the classical activation of macrophages (M1) is characterized by the induction of Th1-like responses mediated principally by IFN-γ, while the alternative activation of macrophages (M2) induces a Th2- like immune response that is mainly mediated by the production of IL-4 and IL-13 (71, 124). However, a subdivision of alternative activation called "deactivation" has also been proposed (124, 144). This "deactivation" is mediated by the production of immunosuppressive cytokines IL-10 and TGF-β, down regulation of MHC–class II molecules and pro-inflammatory cytokines/chemokines, and low levels of IL-4 and IL-13 and low production of reactive oxygen and nitrogen intermediates (71, 85, 137). This kind of activation could predispose the host to infection by the induction of regulatory cells which are inhibitors of inflammation, even in the presence of inflammatory cytokines/chemokines (144).

IL-6 is an important modulator of the immune response due to its dual role as a Th1 cytokine, inducing the recruitment of cellular components or as a Th2 cytokine, inhibiting the production of IFN-γ and inducing the production of IL-10 (49, 50). In our study, we found that the *Δact/ΔvasH* mutant induced the secretion of high levels of IL-6 in the intraperitoneal cavity, which was even higher when rHcp was present. We believe IL-6, together with the production of IL-10 and low levels of IFN-γ, has a role in Th2-like differentiation by induction of SOCS-1 and -3. It has been reported that IL-6 could induce the expression of SOCSs (42, 49).

The SOCS family of regulators is involved in the suppression of NF-κβ signaling pathways, as well as in promoting IL-10 production (50). On the other hand, TGF-β, IL-9, and IL-10 also promote the production of SOCS proteins, which have been associated with impaired production of TNF-α, down regulation of nitric oxide synthase, and the expression of the IL-1ra antagonist gene (14, 117).

We previously reported that immunization of mice with rHcp conferred protection against future infections with lethal doses of WT *A. hydrophila* (193). Overall, the results reported in this study highlighted the importance of Hcp in early stages of *A. hydrophila* infection. Therefore, adaptive immunity mediated by antibodies could neutralize the effect that Hcp has on inhibition of phagocytosis and, thus, enhance bacterial clearing by opsonization mediated-phagocytosis.

We also have characterized other *A. hydrophila* SSU toxins with enzymatic activities that could impair phagocytosis. For example, VgrG1, a T6SS effector protein, has actin-ADP-ribosylation activity, which induces actin-depolymerization followed by host cell apoptosis (191). Another toxin is the T3SS effector protein AexU, which has both ADP-ribosylation (185) and Rho-GAP (GTP-ase activating protein) activities (unpublished data). AexU is able to induce a rounded phenotype, as well as it inhibits activation of NF-κβ when expressed in HeLa cells (185). Overall, *A. hydrophila* has developed multiple mechanisms to circumvent innate immunity to establish an infection.

In summary, Hcp binds to macrophages and induces the production of the immunosuppressive cytokines IL-10 and TGF-β that result in impaired recruitment and inhibition of phagocytosis. This is the first report highlighting how T6SS effector Hcp modulates the activation of macrophages to cause systemic infection in a mouse model.

Conclusions

- **A diarrheal isolate of *A. hydrophila* SSU carries a T6SS gene cluster.** By DNA sequence analysis, we noted the presence of a T6SS gene cluster in *A. hydrophila*. Analysis of this cluster revealed the presence of 20 open reading frames, some of which shared high similarity with the T6SS genes reported in other bacterial pathogens.
- **The T6SS of *A. hydrophila* SSU is functional.** Western blot, 2-D gel and proteomics analyses showed the presence of Hcp protein, a standard marker for the T6SS functionality in different bacteria, in the culture supernatant of *A. hydrophila* SSU. In addition, a subtractive analysis of the secretome of the wildtype and the *vasH* mutant strain of *A. hydrophila* SSU showed the presence of 32 proteins secreted via the T6SS.
- **The T6SS gene cluster of *A. hydrophila* SSU is regulated by the RpoN family of transcriptional activators.** Deletion of the *vasH* gene, a sigma 54-activator and member of the *rpoN* family of transcriptional activators, shutdown the expression of the *hcp, vgrG2,* and *vgrG3* genes present in the T6SS gene cluster.
- **The T6SS is an important virulent determinant of *A. hydrophila* SSU.** Mice infected via the i.p. route with the mutant strain for the *vasH* gene showed better survival rates than the animals infected with the wildtype bacteria. On the other hand, the *A. hydrophila* SSU mutant strain for the T6SS cluster, *ΔvasH*, showed higher rates of phagocytosis than the wildtype bacteria.

- ***A. hydrophila* SSU induces rounded morphology in the eukaryotic host.** Co-cultures of *A. hydrophila* and the epithelial cell line HeLa cells showed that wildtype bacteria induced rounded phenotype in a cell-cell contact dependent manner. In contrast, the mutant bacteria for the T6SS gene cluster were unable to induce the same phenotype.
- **The T6SS of *A. hydrophila* SSU is able to secrete and translocate proteins into the host eukaryotic cells.** By Western blot analysis and tagging fusion proteins with β-lactamase, we demonstrated the translocation of two effector proteins, Hcp and VgrG1, by the T6SS into eukaryotic host cell cytoplasm with deleterious effects.
- **The VgrG1 protein from *A. hydrophila* has a COOH terminal extension with enzymatic activity.** By sequence analysis of the 32 differential proteins secreted via the T6SS, we found a member of the VgrG family, which has at its COOH terminal end a VIP-2 motif with actin-ADP-ribosylating activity.
- **The episomal production of full-length VgrG1 protein or its COOH terminal domain from *A. hydrophila* has cytotoxic effects in HeLa cells.** We showed that the VIP-2 motif present in VgrG1 of *A. hydrophila* was functional and retained its actin-ADP-ribosyltransferase activity. In addition, episomal expression and production of VgrG1 in HeLa cells was able to induce rounded morphology by depolymerization of actin filaments, and consequently, loss of viability and apoptosis mediated by activation of caspase 9 and caspase 3.
- **The Hcp protein from *A. hydrophila* is able to bind macrophages.** By Western blot and flow cytometric analyses, we showed that Hcp was able to

bind to RAW 264.7 murine macrophages. Hcp also bound primary murine macrophages.

- **The Hcp protein plays a role in inhibition of phagocytosis of *A. hydrophila.*** We found that addition of rHcp into the medium was able to inhibit phagocytosis of *A. hydrophila* SSU deleted for the *vasH* gene by RAW 264.7 murine macrophages.
- **The Hcp protein from *A. hydrophila* inhibits recruitment of macrophages into the peritoneal cavity by modulation of secretion of cytokines/chemokines.** Flow cytometric analysis of intraperitoneal cells after infection with *A. hydrophila ΔvasH* mutant strain showed that addition of rHcp inhibited the recruitment of macrophages. In addition, analysis of the cytokine/chemokine profile in peritoneal lavage fluid showed significant decreases in important chemoattractive factors, such as IFN-γ, M-CSF, and IL-1.
- **The Hcp protein down-regulates activation of macrophages.** Flow cytometric phenotypic analysis of intraperitoneal macrophages after infection with *A. hydrophila ΔvasH* mutant strain in presence of rHcp revealed significant reduction in the expression of activation markers, such as CD69 and MHC-class II molecules.
- **The Hcp protein induces a pattern of cytokines/chemokines involved in alternative activation or "deactivation" of macrophages.** Analysis of the cytokine/chemokine profile of intraperitoneal lavage fluid after infection with *A. hydrophila ΔvasH* mutant strain showed that the addition of rHcp induced a cytokine pattern characteristic of the alternative pathway of macrophage

activation, called “deactivation”, which is characterized by high levels of IL-10 and TGF-β and low levels of IL-4, IL-13, IFN-γ, and TNF-α.

In summary, the components of the T6SS of *A. hydrophila* constitute important virulence determinants with a significant potential for the development of preventive and/or therapeutic vaccines as well as targets for antimicrobial drug development.

Future Directions

- **To corroborate virulence associated with the T6SS of *A. hydrophila* in a gastric infection model *in vivo*.** Unfortunately, there are no simple animal models of gastroenteritis induced by *Aeromonas* infection. However, recently, Ma *et al* 2010 (123) reported the activity of VgrG1 from *V. cholerae* in a gastric mouse model of infection *in vivo*. Since both *A. hydrophila* and *V. cholerae* produce similar pathologies, this study represents a good approach to explore *A. hydrophila* gastrointestinal pathology.
- **To identify signals that target T6SS effector protein secretion in *A. hydrophila*.** Little is known about the regulation of T6SS in *A. hydrophila*. Expansion of this knowledge will allow correlation between different virulence factor networks, and, consequently, in understanding pathologies associated with *A. hydrophila* infections.
- **To investigate the presence of new effector proteins associated with the T6SS in *A. hydrophila*.** By proteomic analysis of the secretome associated with the T6SS of *A. hydrophila*, I showed the presence of Hcp and VgrG1; however, several protein spots found in this study remained to be further analyzed.
- **To identify macrophage cell surface molecules that interact with Hcp.** Identification of this molecule will allow understanding of the signaling cascade that Hcp is triggering in macrophages in order to modulate innate immunity. In addition, identification of Hcp motif interacting with “macrophage

receptor(s)” will allow design of drugs focused on the control of *A. hydrophila* infections.

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VITA

Giovanni Suarez was born on October 31st, 1972, in Bogota, Colombia. He grew up in Anolaima, a small town close to Bogota, where he attended the elementary and high school. He then moved to Bogota to attend the National University of Colombia for his Bachelor and Master degrees in Biology and Biochemistry. He worked in the Colombian National Cancer Institute before joining the University of Texas Medical Branch as a visiting scientist in 2002. In 2005, he matriculated at the UTMB Graduate School of Biomedical Sciences, where he was awarded with the McLaughlin Scholarship. During Giovanni's graduate student time, he attended two national and four local meetings where he presented posters. He was awarded with the Corporate Activities Travel Grant from ASM in 2009.

Education

B.S., June 1996, The National University of Colombia, Bogota, Colombia
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Publications

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Abstracts and Posters

1. **Suarez G,** Sierra JC, Kirtley M, and Chopra AK. Role of a type VI secretion system effector protein, Hcp, of *Aeromonas hydrophila* in modulating activation of host immune cells. McLaughlin Colloquium, Galveston, TX. 2010.

2. Khajanchi BK, Sha J, Kozlova E, Erova TE, **Suarez G**, Sierra JC, Chopra AK. N-Acyl homoserine lactones involved in quorum sensing (QS) modulate type 6 secretion system and virulence in a clinical isolate of *Aeromonas hydrophila.* ASM General Meeting. Philadelphia. PA. 2009.

3. **Suarez G,** Sierra JC, Sha J, Erova TE, Horneman A, and Chopra AK. A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* induces host cell toxicity by ADP-ribosylation of actin. ASM General Meeting. Philadelphia, PA. 2009.

4. Sierra JC, **Suarez G**, and Chopra AK. A type 3 secretion system (T3SS) effector AexU from *Aeromonas hydrophila* SSU has ADP-ribosyltransferase and GTPase-activating protein activity. ASM General Meeting. Philadelphia, PA. 2009.

5. **Suarez G,** Sierra JC, Sha J, Erova TE, Horneman A, and Chopra AK. A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* induces host cell toxicity by ADP-ribosylation of actin. Graduate Student Festival, NIH, Bethesda, MD. 2009.

6. Sierra JC., **Suarez G**, Sha J, Erova TE, Foltz SM and Chopra AK. Identification and biological characterization of new type III secretion system effector proteins from an emerging human pathogen *Aeromonas hydrophila*. McLaughlin Colloquium on Infection and Immunity. Galveston, TX. 2008

7. Sierra JC, **Suarez G**, Sha J, Fadl AA, Wang S, Foltz SM, Erova TE, Schein CH, and Chopra AK. A new type III secretion system effector protein in an emerging human pathogen *Aeromonas hydrophila*. McLaughlin Annual Colloquium. Galveston, TX. 2007

8. **Suarez G**, Sierra JC, Sha J, Erova TE, Foltz SM, and Chopra AK. Initial characterization of a new and novel type six secretion system (T6SS) in an emergent human pathogen *Aeromonas hydrophila.* McLaughlin Annual Colloquium on Infection & Immunity. Galveston, TX. 2007.

9. **Suarez G**, Sierra JC, Sha J, Fadl AA, Wang S, Foltz SM, Erova TE, Schein CH, and Chopra AK. A new type III secretion system effector protein in an emerging human pathogen *Aeromonas hydrophila.* Texas Branch 2006 ASM Meeting. Galveston, Texas. 2006

10. Fadl AA, Erova TE, Foltz SM, **Suarez G**, Klimpel GR, Peterson JW, Chopra AK. Deletion of Murein Lipoprotein (lpp) attenuates *Yersinia pestis* KIM/D27 and protects mice against lethal challenge with highly virulent *Y. pestis* CO92. Bugs, Drugs and Vaccines: Securing Our Future. Galveston, Texas, USA. 2006.

11. **Suarez G**, Sierra JC, Pinckuk IV, Reyes VE. Plasmacytoid dendritic cells contribution to the immune response to *Helicobacter pylori*. Presented at the Minority Research Trainee Forum in NIH. Bethesda, MD. March 2005.

12. Sierra JC, **Suarez G**, Beswick EJ, Das S, Pinchuk IV, Reyes VE. Class II MHC-dependence of the cytokine response by monocytes exposed to

Helicobacter pylori urease. Presented at Digestive Disease week, Chicago, 2005.

13. **Suarez G**, Sierra JC, Beswick EJ, Das S, Pinchuk IV, Reyes VE. Potencial role of plasmacytoid dendritic cells in the inflammatory response induced by *Helicobacter pylori*. Presented at Digestive Disease week, Chicago, 2005.
14. **Suarez G**, Barrera CA, Sierra JC, Beswick EJ, Das S, and Reyes VE. Characterization of gastric epithelial cell exosomes and their potential role in local immunity. McLaughlin Annual Colloquium on Infection & Immunity. Galveston, TX. 2004.
15. Beswick EJ, Bland DA, Das S, **Suarez G**, Sierra JC, Reyes VE. *Helicobacter pylori* urease binds to CD74 and stimulates gastric epithelial cell response associated with patogénesis. Presented at the American Gastroenterological Association Annual Meeting. May 2004.
16. Das S, **Suarez G**, Beswick EJ, Bland DA, and Reyes VE. Presence of B7-H1 on gastric epithelial cells and its potential role in regulating T cells during *Helicobacter pylori* infection. Presented at the American Gastroenterological Association Annual Meeting. May 2004.
17. Pinchuk IV, Barrera CA, Saada JI, Bland DA, **Suarez G**, Beswick EJ, Adegboyega PA, Mifflin RC, Powell DW and Reyes VE. MHC class II+ intestinal myofibroblasts as accessory cells for Staphylococcal enterotoxin–mediated T-cell activation and cytokine secretion. Presented at the American Gastroenterological Association Annual Meeting. May 2004.
18. Das S, **Suarez G**, Beswick EJ, Sierra JC, Bland DA, Reyes VE. Presence of B7-H1 (PD-L1) on gastric epithelial cells and its potential role in regulating T cells during *Helicobacter pylori* infection. McLaughlin Annual Colloquium on Infection & Immunity. Galveston, TX. 2004.
19. Guerrero-Plata MA, **Suarez G** and Garofalo RP. Respiratory syncytial virus infection induces IFN-independent maturation of dendritic cells and suppresses $CD4^+$ T cell proliferation. Experimental Biology Annual Meeting. American Association of Immunologists. Washington, D.C. USA. 2004.

Oral Presentations

1. **Suarez G**, Sierra JC, Pinchuk IV, Reyes VE. Plasmacytoid dendritic cells contribution to the immune response to *Helicobacter pylori*. Presented at the Minority Research Trainee Forum in NIH. Bethesda, MD. March 2005.

2. **Suarez G**, Barrera CA, Bland DA, Das S, Sierra JC, Beswick EJ, and Reyes VE. Gastric epithelial cells secrete exosomes that express MHC class II,

CD74, CD86 and CD80: implication in CD4+ T cell activation. Presented at the American Gastroenterological Association Annual Meeting and The Digestive Disease Week. New Orleans, May 2004.