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ROLE OF NEW VIRULENCE MECHANISMS/FACTORS (TYPE 3 SECRETION SYSTEM AND TOX-R REGULATED LIPOPROTEIN [TAGA]) IN THE PATHOGENESIS OF THE EMERGING HUMAN PATHOGEN *AEROMONAS HYDROPHILA*

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by

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To my children, Devika and Arjun, for their sacrifice, laughter and inspiration and to Vasu, for his love and patience

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Aeromonas hydrophila, a gram-negative bacterium that causes gastroenteritis, wound infections, septicemia, and other diseases in humans, produces many different virulence factors. A clinical isolate SSU of *A. hydrophila* possesses a cytotoxic enterotoxin Act, a potent virulence factor that is secreted into the environment through the bacterium's type 2 secretion system (T2SS) and possesses several biological activities, including cytotoxicity, enterotoxicity, and lethality in a mouse model. The purpose of this study was to identify new virulence factors that contribute to the pathogenesis of this bacterium. We identified and characterized a type 3 secretion system (T3SS) in *A. hydrophila* SSU. By marker-exchange mutagenesis of the *aopB* gene, a crucial gene involved in the formation of the translocon apparatus, the functionality of the T3SS was elucidated, both in *in vitro* and *in vivo* models. Further, the characterization of the regulatory gene DNA adenine methyltransferase (Dam) from SSU and its role in modulating the function of both the T3SS and Act was investigated. The role of the T3SS in influencing the phenomenon of quorum sensing (QS) in *A. hydrophila* SSU was also conducted. This study highlights a unique link between the T3SS and Act of *A. hydrophila* and the production of QS molecules or lactones. While searching for potential effector proteins secreted through the T3SS of *A. hydrophila* SSU, the identification of a new virulence factor, ToxR regulated lipoprotein (TagA), was revealed. TagA is a zinc metalloprotease which has only been identified in the gram-negative pathogens, *Escherichia coli* O157:H7 and *Vibrio cholerae*. In *A. hydrophila*, TagA has been shown to play a role in the inhibition of complement by binding to and cleaving the serpin C1-INH. By recruiting C1-INH to the surface of the bacteria and cleaving it, TagA is able to significantly prevent the activation of complement at the cell surface, ultimately increasing the serum resistance of the pathogen. TagA can also target C1-INH to erythrocyte surfaces and decrease the lysis that occurs in the presence of serum. Confocal fluorescence microscopy revealed that the serpin C1-INH binds to TagA on the surface of the bacteria.

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INTRODUCTION

Among the different species of *Aeromonas* isolated to date, *A. hydrophila* is predominantly implicated in causing human infections such as gastroenteritis, hemolytic uremic syndrome, necrotizing fasciitis, wound infections, and septicemia (Galindo *et al.*, 2006). Although originally placed in the family Vibrionaceae, *Aeromonas* species have now been placed in their own family Aeromonadaceae (Colwell *et al.*, 1986). *A. hydrophila* has been isolated from a wide variety of food and water sources and the increased resistance of this organism to antibiotics and to chlorination in water presents a significant threat to public health (Galindo *et al.*, 2006). The pathogenesis of *A. hydrophila* infection is complex and multifactorial, with the involvement of a number of virulence factors. Gastroenteritis is one of the most common diseases associated with *A. hydrophila* infection and is accompanied by either mild or severe dysenteric-like diarrhea. After initial colonization of human intestinal epithelial cells, *A. hydrophila* causes diarrhea by producing enterotoxins. Asao *et al*. first purified a 49- to 52-kilodalton (kDa) ß-hemolysin to homogeneity from a species of *Aeromonas* that induced fluid secretion in an animal model (Asao *et al.*, 1984). Subsequently, a cytotoxic enterotoxin (Act, containing 493 amino acid [aa] residues), known to be secreted through the *sec*-dependent type 2 secretion system (T2SS), was molecularly characterized from the *A. hydrophila* diarrheal isolate SSU (Ferguson *et al.*, 1997). Recent studies further indicated the presence of a type 3 protein secretion system (T3SS) in fish isolates of *A. salmonicida* as well as in *A. hydrophila* (Burr *et al.*, 2002, Vilches *et al.*, 2004, Yu *et al.*, 2004). Concurrently, we sequenced the complete T3SS chromosomal operon from a diarrheal isolate SSU of *A. hydrophila*. Bacterial proteins "injected" through the T3SS "syringe" stimulate or interfere with a number of host cell processes, from causing cytotoxicity within the host to inhibition of bacterial phagocytosis by macrophages, induction of apoptosis, and even suppression of host immune response (Abe *et al.*, 2005). Data presented in this study indicated that the T3SS of a clinical isolate of *A. hydrophila* SSU was involved in inducing cytotoxicity in murine macrophages (e.g. RAW 264.7) and

human colonic epithelial cells (e.g. HT-29). The role of this system in causing lethality in a mouse model was also demonstrated (Sha *et al.*, 2005). Further, the regulation of virulence factor encoding genes is crucial in the overall pathogenesis of bacterial infections. Alterations in the level of a key enzyme involved in the methylation of bacterial DNA, known as DNA adenine methyltransferase (Dam), can attenuate the virulence potential of a number of pathogens, including *Salmonella* species, *Yersinia pseudotuberculosis*, and *Vibrio cholerae* (Low *et al.*, 2001). As reported for *Y. pseudotuberculosis* and *V. cholerae*, *dam* is an essential gene that is required for the viability of *A. hydrophila* SSU (Julio *et al.*, 2001, Erova *et al.*, 2006). An investigation of the role of Dam in the virulence of *A. hydrophila* revealed that overproduction of the Dam enzyme decreased the cytotoxic effects that resulted from the T3SS, while increasing cytotoxic and hemolytic activities associated with the T2SS-secreted Act (Erova *et al*., 2006). Another mechanism of gene regulation used by human bacterial pathogens is a cell-to-cell communication process named quorum sensing (QS), which refers to the ability of bacteria to respond to chemical hormone-like molecules called autoinducers (Henke and Bassler, 2004, Waters and Bassler, 2005). When these autoinducers reach a critical threshold, the bacteria detect and respond to this signal by altering their gene expression. QS was first described in the regulation of bioluminescence in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi*, where it was also shown to regulate the T3SS in the latter pathogen (Henke and Bassler, 2004). Our study demonstrated a link between the type 2 - and type 3 - protein secretion systems and the phenomenon of QS in *A. hydrophila* SSU. Through the use of specific type T3SS mutants and T2SS – secreted toxin mutants, our investigation revealed that the absence of a functional T3SS, and deletion of a T2SS-secreted Act from *A. hydrophila*, SSU decreased autoinducer (i.e. lactone) production. While searching for potential T3SS secreted effector proteins, our study revealed the presence of a new virulence factor in *A. hydrophila* SSU (Pillai *et al.*, 2006)**.** This virulence factor, identified as ToxR regulated lipoprotein, is coded by a gene designated as "*tagA*" (a zinc metalloprotease). The identification and characterization of this gene and its encoded lipoprotein was only

recently reported in the literature where its role in inhibiting complement activation was elucidated in the enteric pathogen *Escherichia coli* O157:H7 (in which the gene is now designated as *stcE*) (Lathem *et al.*, 2004). TagA or StcE provided serum resistance to the bacterium and prevented activation of the classical pathway of complement by recruiting the serpin C1-esterase inhibitor (C1-INH) to the cell surface and cleaving it, ultimately releasing the inhibitor and attached C1 complex from the site of potential lytic attack complex formation. Our investigation extended studies on TagA further and showed **1**) the presence of TagA in *A. hydrophila*, with 64% homology with *E. coli* StcE, **2**) co-localization of C1-INH with TagA on the bacterial surface, and **3**) significance of TagA during infection *in vivo*. Overall, this study advanced the role of different virulence factors in the pathogenesis of *Aeromonas* infections and represented a step forward in better understanding how *A. hydrophila* may cause human infections.

CHAPTER 1:
REVIEW OF THE LITERATURE

DESCRIPTION OF THE GENUS *AEROMONAS* AND TAXONOMY

The species within the genus *Aeromonas* are composed of gram-negative, non-spore forming, rod-shaped, facultatively anaerobic bacteria. Many studies have demonstrated that *Aeromonas* species are distributed universally in fresh-water environments and are widely isolated from clinical, environmental and food samples, where they can survive and multiply even at low temperatures (Sinha *et al.*, 2004). Historically, the *Aeromonas* genus was placed in the family Vibrionaceae (Popoff, 1984); however, subsequent research indicated that aeromonads were sufficiently divergent from the members of the Vibrionaceae family to warrant placement of these bacteria in their own family, the Aeromonadaceae (Colwell *et al.*, 1986, Martinez-Murcia *et al.*, 1992, Kita-Tsukamoto *et al.*, 1993). The aeromonads share many biochemical characteristics with members of the family Enterobacteriaceae, from which they are primarily differentiated by being oxidase positive (Popoff, 1984). Recent years have seen a great increase in the scientific interest in members of the genus *Aeromonas* as human and animal pathogens. Much of this interest is due to the association of this gram-negative bacillus with gastrointestinal disease in humans (particularly children). However, other areas of increasing importance are emerging, including the complicated taxonomy of the genus and virulence-associated factors potentially operative during animal and human infections. Because the biochemistry, genetics, and serology of the motile *Aeromonas* taxon are heterogenous, the taxonomic positions of this genus have been unstable. Popoff and Vernon demonstrated that the motile aeromonads could be classified into two distinct species: *A. hydrophila* (composed of the organisms previously described as *A. punctata* and *A. liquefaciens*) and a new species that they named *A. sobria* (Popoff and

Veron, 1976). Phenotypically, motile aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to < 150 mcg of the vibriostatic agent 0/129 (2, 4-diamino, 6, 7-di-isopropyl pteridine). In addition, these bacteria produce 2, 3-butanediol and reduce nitrate to nitrite. Currently, the number of species recognized within this genus is 17. Of these, seven are recognized as major and minor human pathogens (Carnahan *et al.*, 1991). Aerokey II is a reliable and accurate system for the identification of most of the currently recognized *Aeromonas* species isolated from clinical specimens (Carnahan *et al.*, 1991). Among the 17 species identified to date, *A. hydrophila*, *A. caviae*, and *A. veronii* biotype variant sobria (*A. sobria*) are most commonly associated with infections in humans and account for more than 85% of all clinical isolates (Janda, 1991, Abbott *et al.*, 2003, Martin-Carnahan and Joseph, 2005).

ENVIRONMENTAL OCCURRENCE OF *AEROMONAS*

The potential for pathogenicity mediated by mesophilic *Aeromonas* is of great public health concern because of the prevalence and distribution of *Aeromonas* in aquatic environments and its feature as a contaminant of drinking water supplies (Gavriel *et al.*, 1998, Dumontet *et al.*, 2000). A recent study conducted in Lebanon recovered *Aeromonas* species from both the chlorinated water network as well as from the untreated undergroup water source (Tokajian and Hashwa, 2004). Enteropathogenic *Aeromonas* species were also found to be commonly present in untreated drinking water obtained from wells in Libya (Ghenghesh *et al.*, 2001). The species of *Aeromonas* recovered from these water supplies in both Lebanon and Libya included *A. hydrophila* and *A. caviae* as well as *A. veronii* and *A. jandaei*. This was a first study reporting the isolation and identification of *Aeromonas* species from an intermittent water distribution network in Lebanon and emphasizes the need to survey the incidence of these bacterial species in chlorinated drinking water (Tokajian and Hashwa, 2004). Many studies have in fact demonstrated the ability of *Aeromonas* to survive and grow in drinking water supplies,

despite water treatment strategies such as rapid/slow sand filtration, hyperchlorination/direct filtration and the use of granular activated carbon (Janda and Abbott, 1998, Brandi *et al.*, 1999, Borchardt *et al.*, 2003). It is known that this pathogen has the potential to grow in biofilms in water distribution systems, where it is resistant to chlorination. In fact, studies have shown that water temperature and free chlorine are the main factors which affect the growth of *Aeromonas* in water distribution systems (Burke *et al.*, 1984, Sisti *et al.*, 1998). Sisti *et al*.'s study observed a rapid decline in viability of *A. hydrophila* at low temperature (5°C), whereas at 20°C (the temperature resembling water in distribution systems during the summer), *A. hydrophila* displayed a greater resistance to chlorine (from 0.2-0.25 mg/l concentration) (Sisti *et al.*, 1998). A study by Mackerness *et al*. demonstrated that *A. hydrophila* could establish a biofilm, along with other heterotrophic bacteria, which could survive up to 0.6 mg/l of monochlorine, a concentration that was sufficient to remove *E. coli* - associated biofilms (Mackerness *et al.*, 1991). Subsequent studies revealed that 10% of the water distribution pipe lengths had aeromonads even after disinfection with up to 1 mg/l of chlorine (Holmes *et al.*, 1996).

The presence of this pathogen in fresh vegetables has been noted (Neyts *et al.*, 2000, Szabo *et al.*, 2000), as well as the expression of virulence factors by *Aeromonas* strains isolated from foods at refrigeration temperatures (Kirov *et al.*, 1993). The incidence of enteropathogenic *Aeromonas* species in minimally processed, ready-to-eat vegetables, could therefore present a potential food hazard. When decontamination procedures were evaluated to control the *Aeromonas* contamination of minimally processed vegetables, it was noted that strains of *A. caviae* and *A. hydrophila* were resistant to chlorination in water, from 0.1-0.5 mg/l free chlorine (Uyttendaele *et al.*, 2004). This study confirmed previous findings indicating that chlorine use alone is ineffective in eliminating *Aeromonas* species A study conducted by Chamorey *et al* indicated a minimum concentration of 0.95 ppm of chlorine needed for efficient reduction of *A. hydrophila* (Chamorey *et al.*, 1999). Further, findings by Sisti *et al*. demonstrated that normal concentrations of chlorine used in tap water (0.2-0.25 ppm) did

not reduce *Aeromonas* species at 20°C; however, a slight decline in bacterial numbers was noted at a cooler temperature of 5°C (Sisti *et al.*, 1998). Uyttendaele *et al*. observed that decontamination with a lactic acid solution, and not chlorine, showed the most potential to reduce *Aeromonas* species and to guarantee prolonged shelf-lives of fresh-cut vegetables (Uyttendaele *et al.*, 2004).

Other studies have shown that reconstituted pesticides may present a suitable environment for the survival and growth of pathogenic *Salmonella*, *Shigella*, *E. coli* O157:H7, and *Listeria monocytogenes* (Coghlan, 2000, Guan *et al.*, 2001). A recent investigation in Australia indicated that dam water used to reconstitute the fungicide Kumulus saw a predominance of *Aeromonas* species after incubation (Ng *et al.*, 2005). The preharvest application of pesticide solutions onto vegetable produce may be an important additional source of contamination by enteropathogens such as *Aeromonas*.

As mentioned above, *Aeromonas* species are adept at forming biofilms on water distribution pipes, presenting a challenge to maintaining the safety of public water systems. Tap water samples analyzed from 8 different locations in Mersin city, Turkey, indicated the presence of *Aeromonas* species in 4% of the samples (Emekdas *et al.*, 2006). The investigators of this study indicated that although the frequency of the isolates was under standard limits, these results indicate the presence of hemolytic *A. hydrophila* in municipally treated tap water samples. Further, all of the strains isolated were resistant to first generation beta lactam antibiotics (Emekdas *et al.*, 2006). Similarly, Figueras *et al*. noted that 6.9% of the drinking water samples in Zaragoza, Spain were found to harbor *Aeromonas* species (Figueras *et al.*, 2005). Surprisingly, they have also recovered isolates of the rare *A. culicola* species, known only to be present in the midgut of mosquitoes in India (Pidiyar *et al.*, 2002). Further, these water strains were all positive for the *act* gene, which again points out a public health significance posed by *Aeromonas* species (Figueras *et al.*, 2005).

Consistent results have been obtained attesting to the detection of *Aeromonas* species in drinking water distribution pipe biofilms (Szewzyk *et al.*, 2000, Chauret *et al.*, 2001). Investigators from another recent study demonstrated that aeromonads could be

isolated from biofilms in potable and recycled water environments indicating their potential to colonize and further compromise both systems (Bomo *et al.*, 2004). Further, as mentioned above, many *Aeromonas* strains isolated from drinking water possess multiple virulence factors and thus have the potential to be pathogenic (Sen and Rodgers, 2004, Sharma *et al.*, 2005). The prevalence of genes encoding virulence factors such as for elastase, lipase, flagella A and B, *act*, heat-labile cytotonic enterotoxin (*alt*), and heat-stabile cytotonic enterotoxin (*ast*), as well as for protease and amylase was found to be present in two separate studies conducted on samples from US drinking water utilities and on samples from the Narmada river in India (Sen and Rodgers, 2004, Sharma et al., 2005). These two studies highlight the importance of future research geared toward examining different *Aeromonas* isolates for the prevalence and distribution of potential virulence factors in order to better understand the correlation between these virulence markers and the health risk these bacteria present.

Despite the association of virulence factors with aeromonads isolated from drinking water, there is increasing evidence that strains isolated from the environment generally belong to different groups than clinically associated strains. Havelaar *et al*. typed 187 *Aeromonas* strains from human diarrheal stools and 263 strains from drinking water in the Netherlands by three different methods (serotyping, FAME [gas-liquid chromatography of cell wall fatty acid methyl esters], and cluster analysis and principal component analysis of FAME profiles) and found little similarity between the two (Havelaar *et al.*, 1992). Other studies have indicated that *A. hydrophila* prevalence may be related to hybridization groups. Both Kirov *et al.* (1994) and Hänninen (1994) found that hybridization group 1 (HG1) was associated with clinical specimens, while HG3 and to a lesser extent HG2 predominated in water and environmental samples (Hanninen, 1994, Kirov *et al.*, 1994).

In order to better understand the environmental factors that contribute to the growth and viability of *Aeromonas* species in water, a recent study by Maalej *et al*. looked at the simultaneous effects of water turbidity, sensitivity to sunlight and pH, conductivity of the water and water temperature, on the dynamics of *Aeromonas* in an

urban effluent and in seawater off the coast of Tunisia (Maalej *et al.*, 2003). They discovered that during the cold months, *Aeromonas* counts increased in the treated effluent, while decreasing rapidly in seawater. Conversely, during the warm period, *Aeromonas* species decreased in the effluent, but showed an increasing trend in the seawater (Maalej *et al.*, 2003). The model designed by this study proposes that variations in *Aeromonas* numbers in the treated effluent water system is due to turbidity of the water and decreased sensitivity of the bacterial cells to sunlight. However, variance in *Aeromonas* numbers in the marine ecosystem could be explained by increased sensitivity to sunlight and high conductivity of the water. The main results of this study suggest that the combination of the negative effects of sunlight and conductivity in seawater affects the capacity of *Aeromonas* to grow or recover during the cold months (Maalej *et al.*, 2003).

DISEASES ASSOCIATED WITH *AEROMONAS*

A. hydrophila causes a variety of diseases in humans, the most common of which are gastroenteritis, hemolytic uremic syndrome, wound infections such as cellulitis, myonecrosis, ecthyma gangrenosum, and septicemia (Janda and Abbott, 1998). *A. hydrophila* has been isolated from a wide variety of food and water sources and, as mentioned above, has displayed increased resistance to antibiotics and chlorination in water. These phenotypes of *A. hydrophila* present a significant threat to public health (Chopra *et al.*, 2004) and, as a result, the Environmental and Protection Agency (EPA) placed *A. hydrophila* on the "Contaminant Candidate List", and the monitoring of US water supplies for this organism began in 2002 (Borchardt *et al.*, 2003). The incidence of *Aeromonas* species was reported quite recently in the assessment of environmental floodwater samples in New Orleans, Louisiana following Hurricane Katrina (Presley *et al.*, 2006).

Gastroenteritis

The most common disease associated with *A. hydrophila* infection is gastroenteritis, which ranges from a mild, self-limiting watery diarrhea to a more severe, invasive *Shigella*-like dysenteric form. *A. hydrophila* has also been reported to cause chronic diarrhea, which can exceed one year in duration (Janda and Abbott, 1998). *Aeromonas* species have also been connected to travelers' diarrhea in adults. From a ten-year survey conducted in Tokyo, *A. hydrophila* was isolated from 5.5% of traveler's returning from developing countries (Yamada *et al.*, 1997). *Aeromonas* species were also isolated from 8.7% of Finnish tourists who had traveled to Morrocco (Hanninen *et al.*, 1995). *Aeromonas*-associated gastroenteritis has been reported in non-traveling adults as well. *A. hydrophila* was isolated from 31 adult patients with diarrhea in three Australian hospitals during a nine month period (Quinn, 1991) and from 224 individuals with gastroenteritis in Iowa over six months (Khaitovich and Gal'tseva, 1983). In Bangkok, Thailand, *Aeromonas* was reported as the most common enteric pathogen in adults with diarrhea (Echeverria *et al.*, 1983).

The incidence of *Aeromonas*-associated gastroenteritis in the pediatric population is especially problematic. Approximately 2-20% of the diarrheal cases from children around the world were induced solely by species of *Aeromonas*. Further, only 0-2% of children that harbored *Aeromonas* did not present with diarrheal symptoms (Galindo *et al.*, 2006). During a 2-year study in Chicago, *Aeromonas* species were the only potential bacterial enteropathogens isolated from children between 1 and 27 months of age who were suffering from diarrhea (Challapalli *et al.*, 1988). A recent study was carried out to document the presence of pathogenic *A. hydrophila* in diarrheal stool samples in children (< 6 yrs) at Coimbatore, India (Subashkumar *et al.*, 2006). The results of this study revealed that 21 out of 216 (9.7%) stool samples tested positive for *A. hydrophila* (Subashkumar *et al.*, 2006). These results were higher than the findings of 4.7% in Chennai, India (Vila *et al.*, 2003) and 1.28% and 1.4% of *A. hydrophila* from Mumbai, India (Deodhar *et al.*, 1991). It was also noted that *A. hydrophila* isolated from children with acute diarrhea in Coimbatore exhibited resistance to multiple antibiotics

(Subashkumar *et al.*, 2006).

A clinical study representing 630 healthy adult patients revealed that individuals typically possessed antibodies against aeromonads, while nearly half of the patients with diarrhea caused by other enteropathogens had *Aeromonas*-specific antibodies (Khaitovich and Gal'tseva, 1983). In a recent diarrhea outbreak in Brazil, 2,170 cases were reported from January to July, 2004. Of 582 stools examined, *Aeromonas* species were the most frequently isolated enteric pathogen (19.5%), with the main isolates being *A. caviae*, *A. veronii* biovar sobria and *A. veronii* biovar veronii. Other pathogens were isolated with much less frequency than *Aeromonas*, e.g. *V. cholerae* (3.1%); *Salmonella* species (1.4%) and *Shigella* species (0.5%). This study indicates the prevalence and often predominance of *Aeromonas* species in humans inflicted with diarrhea (Hofer *et al.*, 2006).

Wound Infections

Wound infections are the second most common source from which *A. hydrophila* is isolated (Kelly *et al.*, 1993). Like *Aeromonas*-associated gastroenteritis, clinical symptoms associated with wound infections caused by *Aeromonas* vary greatly from person to person, depending upon host immune status. Unlike gastroenteritis, however, these infections can have fatal or serious debilitating outcomes, such as amputations. The majority of wound infections attributed to *Aeromonas* involves the introduction of bacteria into humans with abrasions or penetrating injuries, often through exposure to *Aeromonas*-inhabited water (Voss *et al.*, 1992, Gold and Salit, 1993, Kelly *et al.*, 1993). Interestingly, the majority of cases of water-borne *Aeromonas* wound infections occur in fresh water, despite the fact that aeromonads can be recovered from saltwater and seafood and that the number of bacteria in these environments is similar to that of freshwater habitats (Semel and Trenholme, 1990, Holmes *et al.*, 1996, Hanninen *et al.*, 1997). *Aeromonas* associated wound infections fall into three categories: cellulitis, myonecrosis, and ecthyma gangrenosum. Cellulitis, the most frequently encountered *Aeromonas* wound infection, is an acute inflammation of subcutaneous tissue characterized by redness and induration that may arise from injury or secondary to sepsis. Myonecrosis and ecthyma gangrenosum, the two less frequently seen types of *Aeromonas* infections,

are typically found in patients that are immunocompromised (Janda *et al.*, 1997). Myonecrosis, or bullous lesion, is characterized by the liquefaction of muscles with blackening of the tissue which may be gangrenous with gas formation. Ecthyma gangrenosum is a cutaneous necrotic or gangrenous pustule that occurs secondary to sepsis. This type of infection can lead to amputation and is often fatal (Gold and Salit, 1993). Among survivors of the 2004 Tsunami that struck off the coast of Thailand, wound infections were the most frequently encountered problem and the majority of these wounds became infected due to contamination with metal and wood debris (Wattanawaitunechai *et al.*, 2005). *Aeromonas* species were reported as the most common organism (145 [22.6%] of 641 isolates from 305 patients) isolated from those patients transferred to four hospitals in Bangkok who presented with skin and soft-tissue infections (Hiransuthikul *et al.*, 2005). It was speculated that *Aeromonas* species were the most commonly isolated bacteria because most Tsunami survivors were exposed to contaminated fresh water due to flooding by the Tsunami wave. Most of the Tsunami victims with skin and soft-tissue infections had polymicrobial infection, most often with *Aeromonas* species and other enteric gram-negative bacilli such as *E. coli* or *P. aeruginosa* (Hiransuthikul *et al.*, 2005).

Septicemia and Other Infections

Septicemia caused by *Aeromonas* species most commonly occurs in immunocompromised adults or children under 2 years of age. This condition is most common subsequent to malignancy (40-50%), liver disease (15-30%), or diabetes (3-5%) (Janda *et al.*, 1994). *Aeromonas*-associated septicemia has been reported to occur secondary to other underlying disorders, such as pancreatitis (Pelayo Melero *et al.*, 1993), trauma (Simodynes and Cochran, 1982, Outin *et al.*, 1984, Madsen and Pedersen, 1995, Barillo *et al.*, 1996, Ender *et al.*, 1996, Kienzle *et al.*, 2000), cardiac anomalies (Outin *et al.*, 1984, Nakasone *et al.*, 2001), gastrointestinal disorders (Chang *et al.*, 1997, Cordoba Lopez *et al.*, 1999), anemia (Tanphaichitr *et al.*, 1984, Cigni *et al.*, 2003), and respiratory problems (Ender *et al.*, 1996, Takano *et al.*, 1996). The mortality rate in these individuals is quite high, ranging from 25-50% (Janda *et al.*, 1994). The mortality rate is particularly

high for individuals that develop septicemia in conjunction with *Aeromonas*-associated myonecrosis (>90%) or subsequent to severe burns (~67%) (Purdue and Hunt, 1988, Janda *et al.*, 1994, Ko and Chuang, 1995, Barillo *et al.*, 1996, Lin *et al.*, 1996).

In addition to gastroenteritis, wound infections, and septicemia, *Aeromonas* species can cause a variety of other infections, including hemolytic uremic syndrome (Bogdanovic *et al.*, 1991), meningitis (Jacob *et al.*, 1988, Parras *et al.*, 1993), peritonitis (Munoz *et al.*, 1994, Ruiz de Gonzalez *et al.*, 1994), respiratory tract disease (Gaussorgues *et al.*, 1987, Baddour and Baselski, 1988, Wells *et al.*, 1991, Goncalves *et al.*, 1992, Preutthipan *et al.*, 1993, Steciw and Colodny, 1994, Hur *et al.*, 1995, Takano *et al.*, 1996), and ocular infections (Carta *et al.*, 1994). In addition, cases of necrotizing fasciitis due to *A. hydrophila* has been reported in both immunocompromised as well as immunocompetent individuals (Lau *et al.*, 2000, Minnaganti *et al.*, 2000, Angel *et al.*, 2002, Cheng *et al.*, 2004, Abuhammour *et al.*, 2006). The fact that *Aeromonas* can cause such a wide variety of infections points to the role of the large number of virulence factors that these species produce.

AEROMONAS HYDROPHILA VIRULENCE FACTORS

A significant number of virulence genes have been described among *Aeromonas* species, including those that code for aerolysin, hemolysin, enterotoxins (both cytotoxic and cytotonic), proteases, lipases, hemagglutinins, T2SS and T3SSs, lipopolysaccharide (LPS) and surface (S) layer, as well as flagella, type 4 pili and adhesins (Galindo *et al.*, 2006). Molecular typing has often been used as a tool to understand whether any particular clone of *Aeromonas* species is more often associated with diarrhea. In a recent study conducted on *Aeromonas* species isolated from patients with acute diarrhea over a period of 2 years in Kolkata, India, it was discovered that these strains were generally heterogenous and no one particular clone was predominant in causing diarrhea in these individuals (Sinha *et al.*, 2004). For example, the *alt* and *act* genes, which encode heat-labile cytotonic and cytotoxic enterotoxins, were respectively found in 71.9% and 20.1% of the strains examined. However, only a small percentage (2.4%) of strains carried the

heat-stable cytotonic enterotoxin (*ast*) gene. The *hlyA* gene encoding a hemolysin, was found in 28% of the strains (Sinha *et al.*, 2004). The dominant combination of enterotoxin genes, i.e. *alt* and *act*, in the Kolkata strains was in contrast to our earlier study conducted in Bangladesh, in which none of the *Aeromonas* isolates were positive for the *act* gene only (Albert *et al.*, 2000). However, these isolates did exhibit an increased presence of the *ast* gene, which might be related to the large numbers of *A. trota* isolated in this region (Albert *et al.*, 2000). Further, in our study conducted in Bangladesh, one *A. hydrophila* strain possessed all three enterotoxin genes (*alt, ast, act*) (Albert *et al.*, 2000), whereas in the study performed in Kolkata, all three genes were found only in one *A. veronii* biovar sobria strain (Sinha *et al.*, 2004).

A. hydrophila cytotoxic enterotoxin (Act)

A 52 kDa cytotoxic enterotoxin (Act) from a diarrheal isolate SSU of *A. hydrophila* was shown to induce inflammation and intestinal damage both *in vitro* and *in vivo*, indicating the involvement of both macrophages and intestinal epithelial cells in the diseases caused by this pathogen (Ferguson *et al.*, 1997, Xu *et al.*, 1998, Sha *et al.*, 2002). The toxin Act is known to possess several biological activities, including hemolysis, cytotoxicity, enterotoxicity, and lethality to mice when injected intravenously (Chopra and Houston, 1999). Act undergoes processing at both the amino (N)- and carboxy (C)-terminal ends, as do other aerolysins produced by *Aeromonas* species (Howard and Buckley, 1985, Chakraborty *et al.*, 1986, Howard and Buckley, 1986, Chopra *et al.*, 1993). Act has a leader sequence 23 aa long, which is removed when the toxin enters the periplasmic space (Chopra *et al.*, 1993, Ferguson *et al.*, 1995). After the secretion of Act into the medium through the *sec*-dependent T2SS, an approximately 4- to 5-kDa polypeptide is cleaved from its C terminus by a protease produced by *A. hydrophila* SSU, resulting in the mature form of the toxin (Chopra *et al.*, 1993, Ferguson *et al.*, 1995). In order to elucidate the function of this toxin, subsequent studies revealed that Act was able to up-regulate the expression of genes encoding proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and

inducible nitric oxide synthase (iNOS) in the murine macrophage cell line RAW 264.7 (Chopra *et al.*, 2000, Ribardo *et al.*, 2002). More recently, Act was shown to induce a rapid mobilization of calcium from intracellular stores as well as to stimulate an influx of calcium from the extracellular medium. The rise in intracellular calcium influenced the production of TNF-α and prostaglandin E_2 (PGE_2) *via* activation of nuclear factor κB (NF-κB) and cyclic AMP-responsive element binding protein (CREB) transcription factors (Chopra *et al.*, 2000, Ribardo *et al.*, 2002, Galindo *et al.*, 2005). In summary, our data demonstrated a direct role for Act in the induction of the host inflammatory response (Chopra *et al.*, 2000).

BACTERIAL PROTEIN SECRETION SYSTEMS

The interaction of bacterial pathogens with host cells is characterized by specific factors located on the bacterial surface or those that are secreted into the extracellular space. These secreted bacterial proteins are diverse and numerous and exhibit a wide variety of functions, which include proteolysis, hemolysis, cytotoxicity, and protein phosphorylation and dephosphorylation (Hueck, 1998). However, only a few pathways exist by which these proteins are transported from the bacterial cytoplasm to the extracellular space. Four main pathways of protein secretion have been described in gram-negative bacteria and have been designated as types 1 to 4 (Fath and Kolter, 1993, Salmond and Reeves, 1993, Van Gijsegem *et al.*, 1993, Finlay and Falkow, 1997). A fifth system, named type 5 secretion (the simplest secretion apparatus) includes the autotransporter family and represents the largest family of protein-translocating outer membrane porins in gram-negative bacteria (Desvaux *et al.*, 2004). This secretion process was first described for the IgA1 protease produced by *Neiserria gonorrhoeae* (Pohlner *et al.*, 1987). Serine protease autotransporters secreted by members of the Enterobacteriaceae family, termed SPATE's, have been described (Henderson and Nataro, 2001). Examples of SPATE autotransporter proeins include, EspP (secreted by Shiga toxin-producing *E. coli*), Pet (of enteraggregative *E. coli*) and Tsh (secreted by strains of avian pathogenic *E. coli*) (Henderson and Nataro, 2001). Examples of

autotransporters have been described in many pathogenic bacterial species, including *H. pylori* (e.g. VacA), *Salmonella* (e.g. ApcE), *Pseudomonas* (e.g. PspA), and *Shigella* (e.g. SepA) (Henderson and Nataro, 2001). The primary structure of autotransporters is modular and composed of three domains (Pohlner *et al.*, 1987): **1**) the signal sequence **2**) the passenger domain and **3**) the translocation unit or β-domain. At the N-terminus, the signal sequence allows targeting of the protein to the inner membrane for its export into the periplasmic space. The passenger domain confers the diverse effector functions to the autotransporters. The translocation unit is the last main domain located at the C-terminal end of the protein and consists of a short linker region having an α-helical secondary structure and a β-domain that will adopt a β-barrel secondary structure when embedded in the outer membrane (Maurer *et al.*, 1999, Oliver *et al.*, 2003). Since no energy coupling or accessory factor seemed to be required for the translocation process, proteins secreted in this way received the name of autotransporters.

Recently, a new a virulence mechanism in a non-O1-non-O139 *V. cholerae* strain (which are primarily associated with isolated cases of extra-intestinal infection or gastroenteritis) was identified (Pukatzki *et al.*, 2006). This mechanism involved extracellular translocation of proteins that lacked N-terminal hydrophobic leader sequences. These genes were termed ‘‘VAS’’ genes for virulence-associated secretion, and were proposed to encode a prototypic type 6 (T6SS) secretion system. Interestingly, these *vas* genes were shown to be required for cytotoxicity of *V. cholerae* cells toward *Dictyostelium* amoebae and mammalian J774 macrophages by a contact-dependent mechanism and may in fact, represent a new addition to bacterial protein secretion systems (Pukatzki *et al.*, 2006). As many pathogenic gram-negative bacterial species carry VAS gene clusters, it is predicted that the primary function of the T6SS is to mediate extracellular export of virulence factors and their translocation into target eukaryotic cells.

The type 2 and type 4 protein secretion pathways involve a separate step of transport of effector proteins across the inner membrane prior to transport across the cell envelope. A signature of *sec*-dependent protein export is the presence of a short (30 aa or

shorter) mainly hydrophobic N-terminal signal sequence in the exported protein (Johnson *et al.*, 2006). The signal sequence aids protein export and is cleaved by a periplasmic signal peptidase when the exported protein reaches the periplasm with the help of inner membrane proteins. In the T2SS, transport across the outer membrane requires additional outer membrane proteins (Johnson *et al.*, 2006) . The best studied example of this protein secretion system is pullulanase secretion by *Klebisella oxytoca* (Pugsley, 1993) and as mentioned above, the pathway of secretion for Act (Chopra *et al.*, 1993). The type 4 secretion system is related to the systems involved in conjugative transfer of DNA (Ward *et al.*, 1988). It allows the secretion of **1**) nucleoprotein DNA conjugation intermediates, **2**) multi-subunit toxins such as the pertussis toxin of *Bordetella pertussis* or **3**) monomeric protein such as RecA (Hueck, 1998). Recently, this pathway has been divided into two subclasses: **1**) type 4_a corresponds to machinery assembly containing VirB homologs of the plant pathogen *Agrobacterium tumefaciens* and **2**) type 4_b corresponds to functional secretion systems assembled from Tra homologs of the Incl ColIb-P9 plasmid of *S. flexneri* (Christie *et al.*, 2005). In contrast to the type 2 and type 4 secretion pathways, type 1 and type 3 secretion systems are independent of the *sec* sequence and thus do not involve N-terminal processing of the secreted proteins. Further, protein secretion in these pathways occurs in a continuous process without the distinct presence of periplasmic intermediates. Type 1 secretion requires three secretory proteins: an inner membrane transport ATPase, an outer membrane protein which is exported *via* the *sec* pathway, and a membrane fusion protein which is anchored in the inner membrane and spans the periplasmic space (Fath and Kolter, 1993). The proteins secreted *via* this pathway are not subject to proteolytic cleavage. Type 1 secretion is exemplified by *E. coli* alpha-hemolysin (Fath and Kolter, 1993).

Type 3 secretion system (T3SS)

Like the type 1 secretion pathway, type 3 secretion is independent of the sec system. However, recent speculation assumes that assembly of the type 3 secretion apparatus probably requires the sec pathway, as several components of this apparatus carry sec-characteristic N-terminal signal sequences (Journet et al., 2005). The type 3

secretion apparatus is comprised of approximately 20 proteins, most of which are located in the inner membrane. The secretion of proteins through the apparatus requires a cytoplasmic or membrane-associated ATPase (Hueck, 1998).

Fig. 1.1 A

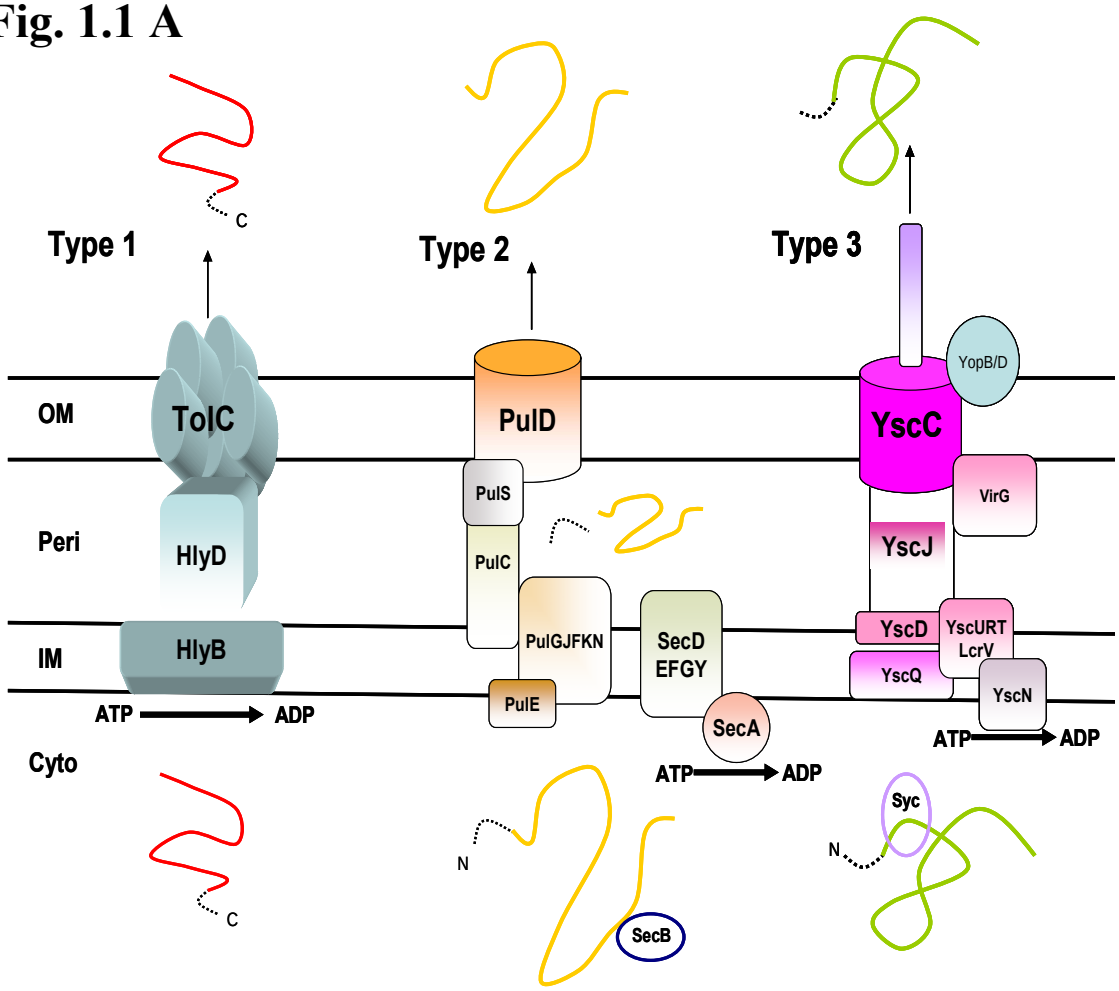

Figure 1.1. Bacterial protein secretion systems 1-6
(A) The type 1 secretion system is exemplified by alpha-hemolysin secreted by *E. coli*, type 2 by pullulanase secreted by *K. oxytoca* and type 3 by *Yersinia*. ATP hydrolysis by HlyB, SecA and YscN is indicated. The secretion signal in the secreted proteins is shown as a dashed line. N, amino terminus; C, carboxy terminus. It is believed that the secretion signal for the T3SS effector protein may reside in the 5'-region of its encoding mRNA. In the T2SS, the N-terminal signal sequence is cleaved off in the periplasmic space after export of the protein *via* the *sec* pathway. Type 2 and 3 secretion pathways

involve cytoplasmic chaperones (SecB and Syc, respectively) which bind to presecretory proteins. These two secretion systems also share homologous multimeric outer membrane proteins (PulD and YscC). However, the accessory proteins PulS and VirG differ in the two systems.

Fig. 1.1 B

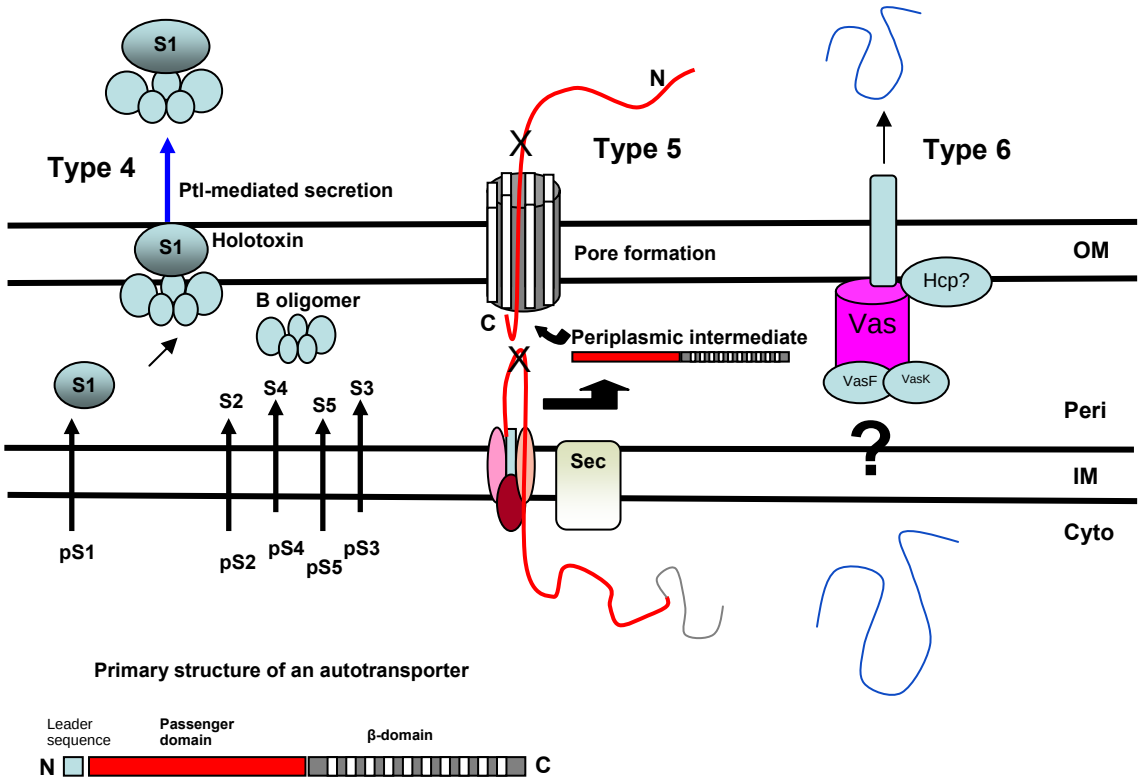

Figure 1.1. cont… Bacterial protein secretion systems 1-6
(B) Types 4-6 secretion systems. Type 4 secretion is illustrated by the secretion of pertussis toxin from *B. pertussis*. Individual polypeptide chains of the toxin (subunits S1-S5) are independently secreted across the IM by a *sec*-like system. The S1 subunit associates with the OM and acts a site for the assembly of the holotoxin, which once assembled, is transported by the *Bordetella* type 4 Ptl (pertussis toxin-linked) system across the OM of the bacteria. Types 5 and 6 secretion systems are shown as general pathways. Type 5 secretion systems represent the autotransporter system, whereby proteins are translocated across the OM via a transmembrane pore formed by a self-encoded β-barrel structure. The leader sequence directs secretion *via* the *sec* apparatus and is cleaved at the IM by a signal peptidase, releasing the remaining portion of the molecule into the periplasm. In the periplasm, the β-domain assumes a β-barrel shaped structure which inserts itself into the OM to form a pore. The passenger domain, after

insertion into the OM, is translocated to the bacterial surface where it may remain uncleaved and protrude from the bacterial surface as a large polyprotein (e.g. Hsr [for *Helicobacter* surface ring] protein of *Helicobacter mustelae*), or instead the protein may be cleaved from the β-domain but remain associated with it (e.g. AIDA-I protein [adhesin involved in diffuse adherence] of *E. coli*). The passenger domain can also be cleaved from the β-domain and released into the extracellular milieu (e.g. VacA [vacuolating cytotoxin] protein of *H. pylori* and IgA1 protease of *N. gonorrhoeae*). The T6SS is a newly described pathway which involves the translocation of proteins that lack N-terminal hydrophobic leader sequences. See the text for further descriptions of the secretion systems. OM, outer membrane; Peri, periplasm; IM, inner membrane and Cyto, cytoplasm. Figure modified from Hueck, 1998 and Henderson and Nataro, 2001.

Most of the inner membrane proteins are homologous to components of the flagellar biosynthesis apparatus of both gram-negative and gram-positive bacteria (Macnab, 2003). The injectisomes (or syringe-like organelles) of the T3SS allow bacteria to deliver proteins not only across the two bacterial membranes but also across the eukaryotic cell membrane. As seen in type 1 secretion, the proteins secreted *via* the type 3 pathway are not subjected to N-terminal processing during secretion. As the N-terminal sequences of proteins secreted through this pathway do not share any recognizable structural similarities that could function as a common secretion signal, it has been proposed that this signal resides in the 5' region of the mRNA which encodes the secreted proteins (Anderson and Schneewind, 1997). Interestingly, the secreted proteins require small cytoplasmic proteins with chaperone functions to protect the secreted factors from premature interaction with other components of the secretion system. In contrast to type 1 secretion, which is a true secretory system in that the secreted enzymes are active in the extracellular space, T3SSs seem to be dedicated machineries for the translocation of pathogenicity proteins into the cytosol of eukaryotic cells. Therefore, in some cases, protein secretion is regulated by contact with the surface of a target cell. For example, *Yersinia* species co-regulate expression of type 3 secreted proteins (Yops) with their secretion, i.e., expression is derepressed only when the secretion channels are opened. These channels are opened by contact of the bacteria with

a target cell; however, efficient secretion can also be induced *in vitro* by removal of Ca^{2+} from the growth medium (Pettersson *et al.*, 1996). In fact, Ca^{2+} appears to be required to keep the secretion channels shut in the absence of target cells and this *in vitro* phenomenon has been called the low-calcium response (Lcr) (Kupferberg and Higuchi, 1958, Higuchi *et al.*, 1959, Goguen *et al.*, 1984). The Lcr is characterized by the fact that the *Yersinia* T3SS is expressed only at 37°C in the absence of millimolar concentrations of Ca^{2+}. At 26°C (or at 37°C in the presence of Ca^{2+}), Yop secretion remains repressed. Temperature control directly acts on the transcriptional level of *yop* expression (Cornelis *et al.*, 1989, Lambert de Rouvroit *et al.*, 1992), while high Ca^{2+} inhibits the secretion of Yop proteins *via* the T3SS (Forsberg and Wolf-Watz, 1988, Forsberg *et al.*, 1991, Straley *et al.*, 1993). A recent study has also implicated the influence of Na^{+}, dicarboxylic amino acids such as L-glutamine and a slightly alkaline pH in modulating the Lcr of *Y. pestis* (Brubaker, 2005).

The translocation of bacterial virulence proteins into eukaryotic target cells is a common feature of most if not all T3SSs (Rosqvist *et al.*, 1994, Scofield *et al.*, 1996, Tang *et al.*, 1996, Wood *et al.*, 1996, Collazo and Galan, 1997, Yip and Strynadka, 2006). The term "translocation" is used to describe the transport of proteins from the bacterial cell through the eukaryotic plasma membrane into the cytosol of the target cell, while "secretion" refers to the transport of proteins from the bacterial cytoplasm to the extracellular space. In accordance with the homology of the type 3 secretion apparatus with bacterial flagellar structures, some T3SSs assemble supramolecular structures on the bacterial surface, which could be involved in translocation of effector proteins (Ginocchio *et al.*, 1994, Yip and Strynadka, 2006). While the mechanism of protein secretion is conserved among gram-negative bacteria, the secreted proteins themselves are highly divergent and the variety of diseases caused by these pathogens in different hosts is reflected by the multitude of type 3 secreted proteins. Many of the secreted proteins interact directly with host cell components to alter host cell signal transduction and most of the secreted proteins act inside the eukaryotic cytosol to, in effect, reprogram

the target cells to the benefit of the bacterium, or on rare occasions, to the benefit of both the organism and the host cell (Abe *et al.*, 2005).

The delivery of these effector proteins into the host cell is the only purpose of the injectisome. Translocation of the effectors across the eukaryotic cell membrane requires a set of proteins called the 'translocators' that are also secreted by the T3SS export machinery (Rosqvist *et al.*, 1994, Boland *et al.*, 1996). Generally, there are three translocators (e.g. YopB, YopD and LcrV in *Yersinia* species). When these translocators are secreted by bacteria in contact with erythrocytes, nucleated cells or even liposomes, they have been shown to form a pore on the surface of the host cell (Hakansson *et al.*, 1996, Blocker *et al.*, 1999). Homologs of YopB, purified SipB (*Salmonella*) or IpaB (*Shigella*), have been shown to penetrate into cultured mammalian cell membranes or liposomes without disturbing bilayer integrity (Boland *et al.*, 1996, McGhie *et al.*, 2002, Hume *et al.*, 2003, Goure *et al.*, 2004). However, the membranes of erythrocytes infected with *P. aeruginosa* contain only PopB and PopD and not PcrV, although PcrV is absolutely required for pore formation (Goure *et al.*, 2004). This suggests that the two hydrophobic translocators (YopB and YopD) form the pore together and that the hydrophilic LcrV acts as an extracellular chaperone.

It is generally assumed that the injectisome serves as a hollow conduit, allowing the exported proteins to travel across the two membranes and peptidoglycan layer in one step (Jin and He, 2001, Li *et al.*, 2002). These effector proteins, therefore, have to travel at least partially unfolded because the internal diameter of the needle would not allow passage of fully folded globular proteins (Feldman *et al.*, 2002). Upon close contact with a target cell, some injectisome channels become unplugged and secretion starts. The required contact is not achieved by the needle itself but by the interaction between bacterial outer membrane proteins called adhesions and integrins at the surface of a target cell (Cornelis, 2002). Because the injection process requires the very tight adhesion-mediated contact, the needle must retract, break down, or pierce the target cell membrane. It is plausible that YopB, YopD, and LcrV destabilize the host cell membrane, enabling the needle to pierce it, driven by the pressure that gradually builds

up as a result of the increased docking of the bacterium at the cell surface (Cornelis, 2002). The injectisome, therefore, is a nanomachine whose primary function is to inject effectors by the T3SS. It includes a T3SS apparatus which secretes not only these effectors but also the translocators and several proteins that are part of the injectisome itself (Stebbins and Galan, 2003).

DNA ADENINE METHYLTRANSFERASE (DAM)

The regulation of virulence factors/mechanisms is an important function carried out by bacteria as a means to control their pathogenic or virulent phenotype. One such method of regulation used by gram-negative bacterial species is DNA methylation. DNA methylation occurs in many different species throughout the living world, i.e. plants, and mammals, in addition to bacteria. Even the fruit fly, *Drosophila melanogaster* contains methylated DNA (Lyko *et al.*, 2000, Lyko, 2001). DNA methylation provides a mechanism by which additional information is imparted to DNA, which can alter the timing and targeting of cellular events (Marinus, 1996). DNA methylation can occur at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine. This process is catalyzed by enzymes known as DNA methyltransferases (MTases) (Palmer and Marinus, 1994). All MTases use S-adenosyl methionine as a methyl donor. The process of DNA methylation has historically been associated with DNA restriction-modification systems and was thought to be important in protecting cells from foreign DNAs such as tranposons and viral DNAs (Meselson *et al.*, 1972, Kobayashi *et al.*, 1999, Roberts and Macelis, 2000). Restriction-modification systems contain a DNA methylase that protects host DNA sequences from restriction by their cognate restriction enzymes, whose primary function is to digest unmodified foreign DNAs. Certain MTases, such as DNA adenine methyltransferase (Dam), which methylates N-6 of adenine in 5'-GATC-3' sequences, do not have cognate restriction enzymes associated with them (Reisenauer *et al.*, 1999). This methylase can participate in cellular regulatory events, including those that control bacterial virulence.

Dam is classified in the α group of MTases and homologs of the gene are widespread among enteric bacteria, such as *E. coli*, *Salmonella* species, *Serratia marcescens*, *Yersinia* species, and *V. cholerae* (Malone *et al.*, 1995). Dam methylation is not essential for the viability of *E. coli* (Bale *et al.*, 1979); however, *dam* does appear to be an essential gene in the pathogens, *V. cholerae* and *Y. pseudotuberculosis* (Julio *et al.*, 2001, Low *et al.*, 2001) and has recently shown to be essential for the viability of *A. hydrophila* (Erova *et al.*, 2006).

Adenine methylation can alter the interactions of regulatory proteins with DNA, either by a direct steric effect or by an indirect effect on DNA structure (Diekmann, 1987, Polaczek *et al.*, 1997, Polaczek *et al.*, 1998). Initial studies with *dam* mutants showed that Dam regulates the expression of certain genes in *E. coli*, including *trpR* (Peterson *et al.*, 1985), Tn*10* transposase (Roberts *et al.*, 1985), and *dnaA* (Braun and Wright, 1986). Methylation of a GATC site(s) within the consensus RNA polymerase binding site inhibits (*trpR* and Tn*10* transposase) or enhances (*dnaA*) transcription, by altering the interaction with the transcription apparatus.

Methylation can also alter the affinity of regulatory proteins for DNA. Conversely, DNA binding proteins have been shown to inhibit methylation of specific DNA sequences. For example, the SeqA protein involved in the timing of DNA replication binds specifically to hemimethylated DNA sequences near the origin of replication (*oriC*), thus sequestering oriC from Dam methylation for a portion of the cell cycle and maintaining it in a hemimethylated state (Lu *et al.*, 1994).

Regulation by hemimethylation

Dam can act as an efficient *de novo* methylase, methylating both nonmethylated and hemimethylated GATC sites with similar efficiency (Urieli-Shoval *et al.*, 1983). Dam plays a defining role in regulating a number of cellular functions such as DNA replication (Lu *et al.*, 1994, Bogan and Helmstetter, 1997, Kang *et al.*, 1999, Stancheva *et al.*, 1999), segregation of chromosomal DNA (Ogden *et al.*, 1988, Meury *et al.*, 1995), mismatch repair (Herman and Modrich, 1981, Marinus, 2000), and transposition (Dodson and Berg, 1989, Reznikoff, 1993). In these events, hemimethylated GATC sites, which

are present immediately following DNA replication, control the binding of proteins to nonspecific DNA target sites. Further, the segregation of newly replicated DNA may occur by binding of hemimethylated DNA to membrane-bound factors. For example, in methyl-directed mismatch repair, MutH binds to hemimethylated DNA and cleaves the non-methylated strand (Au *et al.*, 1992).

Regulation by DNA methylation patterns

Dam also plays pivotal roles in controlling gene expression by the formation of DNA methylation patterns (DMPs). DMPs are known to be present in eukaryotes where they also regulate gene expression (Wigler *et al.*, 1981, Bird and Wolffe, 1999). The first reported DMPs which regulate gene expression in prokaryotes are within the pyelonephritis-associated (*pap*) pilus operon of uropathogenic *E. coli* (Blyn *et al.*, 1990). By doing so, Dam regulates the expression of a group of pilus operons that plays an important role in urinary tract infections. It appears that most, if not all, DMPs are formed by the binding of regulatory proteins to upstream regulatory DNA sequences. Examples of regulatory proteins include GutR (van der Woude *et al.*, 1998), Lrp (Braaten *et al.*, 1994), histone-like nucleoid-structuring protein (H-NS) (White-Ziegler *et al.*, 1998), and OxyR (Haagmans and van der Woude, 2000). Studies with these regulatory elements have shown that purified proteins are able to block methylation of GATC sites that are contained in or near the DNA recognition sequence during an *in vitro* DNA methylation protection assay (van der Woude *et al.*, 1998). Inhibition of methylation could occur by direct steric occlusion of Dam binding or by alterations in DNA conformation which change the configuration of the Dam target (GATC) site (Polaczek *et al.*, 1998).

Role of Dam in virulence

The virulence of a number of pathogens, including *Salmonella* species (Garcia-Del Portillo *et al.*, 1999, Heithoff *et al.*, 1999, Heithoff *et al.*, 2001), *Y. pseudotuberculosis*, and *V. cholerae* (Julio *et al.*, 2001), has been attenuated due to alterations in the level of Dam. It is possible that pleiotropic effects not related to

alternations in gene expression may be responsible for the virulence defects of *dam* mutants, because Dam is known to play multiple roles in cell physiology. The growth rates, however, of *dam* mutant and Dam-overproducing *S. enterica* serovar Typhimurium were similar to that of wild-type *Salmonella* (Heithoff *et al.*, 2001). Further, levels of overproduction of Dam in *Y. pseudotuberculosis* and *V. cholerae* that inhibited virulence had no significant effect on *in vitro* growth rates (Julio *et al.*, 2001). Taken together, these data indicated that the virulence defects of *dam* mutants were directly the result of alterations in gene expression and not due to a nonspecific growth defect. This finding also holds true for the changes in virulence patterns seen in the Dam overproducing strains of *A. hydrophila* (Erova *et al.*, 2006). Dam has been reported to control the expression of a number of virulence genes such as the pyelonephritis-associated pilus phase variation of *E. coli* (Braaten *et al.*, 1994), type 3 protein secretion, cell invasion and M cell cytotoxicity associated with *S.* Typhimurium (Garcia-Del Portillo *et al.*, 1999) as well as expression and virulence of *Yersinia* T3SS virulence proteins and host immune responses to infection by these bacteria (Julio *et al.*, 2002). Deletion of *dam* erases DNA methylation patterns, which could alter the binding of regulatory proteins to a number of regions on the bacterial chromosome. In the absence of Dam, overexpression of genes could occur if GATC methylation blocked binding of an activator or enhanced the binding of a repressor. Conversely, underexpression of a gene would occur in the absence of Dam if GATC methylation blocked binding of a repressor or enhanced binding of an activator (Low *et al.*, 2001).

In the *pap* operon of *E. coli*, DNA methylation directly regulates the switch between pilus expression (phase ON) and nonexpression (phase OFF) by dictating the binding of Lrp (Braaten *et al.*, 1994). At low PapI levels, the regulatory protein Lrp binds with high affinity to promoter proximal sites, blocks transcription from the papBA promoter, and inhibits methylation of $GATC^{prox}$ (Weyand and Low, 2000). However, the promoter distal $GATC^{dist}$ site is not bound by Lrp and thus becomes methylated. Methylation of $GATC^{dist}$ inhibits movement of Lrp to the distal site and presumably locks cells in the phase-OFF state until DNA replication generates a hemimethylated $GATC^{dist}$

which binds Lrp with a higher affinity. Methylation of the promoter proximal $GATC^{prox}$ site is required for the expression of Pap pili (Braaten *et al.*, 1994). Mutations in the Lrp binding sites near $GATC^{prox}$ result in a phase-locked ON transcription phenotype that is Dam and PapI independent (Nou *et al.*, 1995). These results indicate that methylation of $GATC^{prox}$ may help displace Lrp from its promoter proximal DNA binding sites that overlap $GATC^{prox}$, with the aid of PapI (Kaltenbach *et al.*, 1995). Binding of Lrp-PapI at the promoter distal $GATC^{dist}$ site blocks its methylation, forming a DNA methylation pattern that is characteristic of cells expressing pili.

Recently, Dam was shown to be essential for the virulence of *S.* Typhimurium in a murine model of typhoid fever (Garcia-Del Portillo *et al.*, 1999, Heithoff *et al.*, 1999, Heithoff *et al.*, 2001). *Salmonella dam* mutants showed reduced M-cell cytotoxicity and invasion of enterocytes but appeared to grow normally within cells (Garcia-Del Portillo *et al.*, 1999). Dam-deficient *S.* Typhimurium up-regulated the expression of over 35 genes that were induced during infection (Heithoff *et al.*, 1999), including *spvB*, a cytotoxin which causes apoptosis of macrophages (Libby *et al.*, 1997). In contrast, Dam positively regulated the secretion of the SipA, SipB, and SipC proteins of the T3SS of *Salmonella* (Garcia-Del Portillo *et al.*, 1999). Thus, in the absence of Dam, virulence factors such as SpvB are predicted to be overexpressed, whereas other factors such as SipABC are underexpressed. It is hypothesized that this combination of overexpression and underexpression of virulence protein encoding genes inhibits virulence (Heithoff *et al.*, 2001).

TOXR REGULATED LIPOPROTEIN (TAGA)

ToxR regulon

Another regulatory system used by gram-negative bacterial species is represented by the ToxR regulon. The ToxR regulon has been identified as a major mechanism of gene regulation in *V. cholerae*. The genes encoding cholera toxin, i.e. *ctxAB*, are not well expressed when cloned in the heterologous background of *E. coli*. This observation

prompted a genetic screen for factors from *V. cholerae* which, when expressed in an *E. coli* strain having a *ctx-lacZ* operon fusion, would result in Lac^+ colonies due to activation of the fusion (Miller and Mekalanos, 1984). From this screen, clones were identified that were shown to encode *toxR*, which was later demonstrated to be an unusual regulatory protein in that it resided in the inner membrane and had an N-terminal cytoplasmic domain that shared extensive and important homology with the DNA-binding-transcription-activation domains of several response regulator proteins in the two-component family. The C-terminal domain of ToxR is in the periplasmic space and there it interacts with another protein called ToxS, also required for *ctxAB* expression; both of these proteins are encoded on the *toxRS* operon (Miller *et al.*, 1989, DiRita and Mekalanos, 1991, DiRita, 1992, DiRita *et al.*, 2000).

A search for gene fusions whose pattern of expression was similar to that of cholera toxin ultimately revealed several other genes that were also demonstrated to be regulated by ToxR. These included the toxin-coregulated pilus (TCP), the accessory colonization factor (ACF) and an outer membrane protein (OmpU) (Miller and Mekalanos, 1988, Peterson and Mekalanos, 1988, Skorupski and Taylor, 1997). Another outer membrane protein, OmpT, was regulated oppositely to those other factors and its gene expression was repressed directly by ToxR (Li *et al.*, 2000). In addition to cholera toxin, TCP and ACF, ToxR indirectly controls the expression of various genes such as those encoding for aldehyde dehydrogenase (AldA), ToxR-activated genes (TagA-D), and OmpU through its ability to control expression of another activator, ToxT (DiRita *et al.*, 1991) (Fig. 1.2). Although ToxR was originally identified for its ability to activate the cholera toxin operon in *E. coli* independently of other *V. cholerae* factors, mutants of *V. cholerae* lacking *toxT*, but expressing functional ToxR, did not express cholera toxin (Champion *et al.*, 1997).

The localization of several major regulatory factors, ToxR/ToxS and TcpP/TcpH, to the inner membrane poses the question of what role this placement may have in the regulation of gene expression. Some evidence suggests that the periplasmic domains of both ToxR and TCP are important for signaling across the inner membrane. Replacement

of the periplasmic domain of ToxR with the periplamsic protein alkaline phosphatase (PhoA) resulted in constitutive expression of cholera toxin, i.e., toxin expression occurred under normally non-permissive conditions (Miller *et al.*, 1987). This implies that the periplasmic domain of ToxR is a signal sensing domain that controls, for example, DNA binding or transcription activation by the cytoplasmic domain.

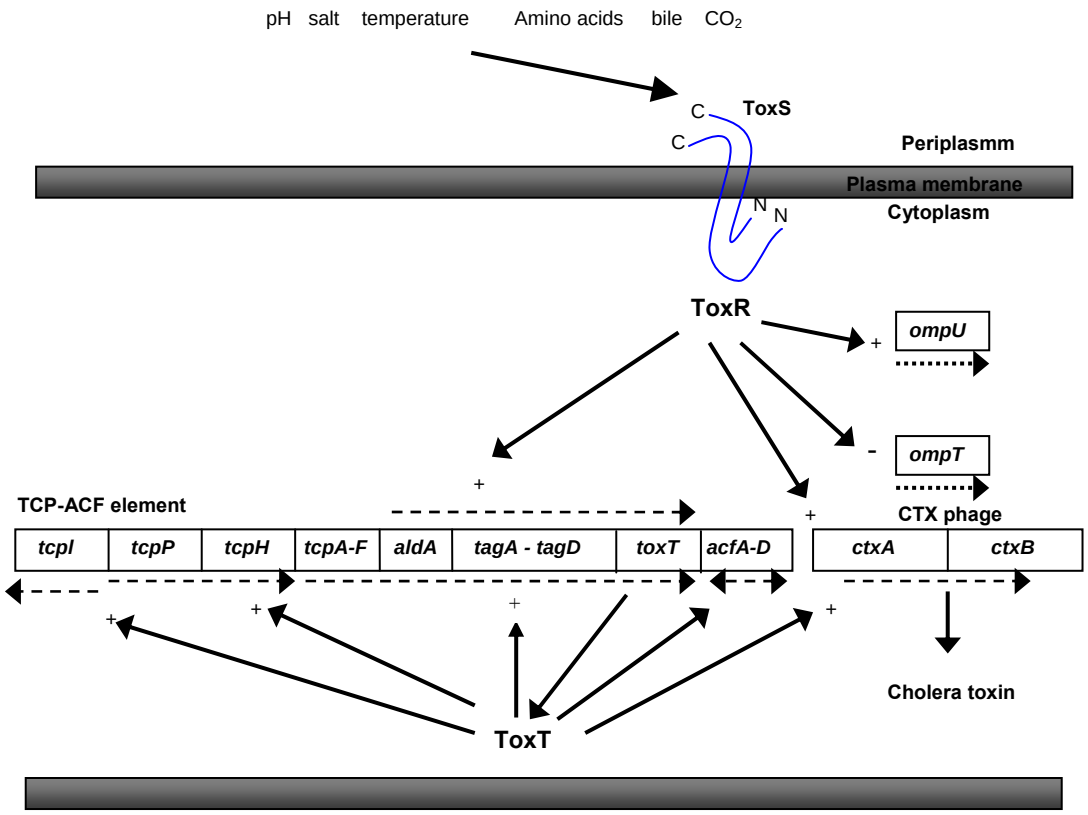

Fig. 1.2. ToxR regulon in *Vibrio* species
ToxR is a transmembrane DNA-binding protein situated in the bacterial cell inner membrane with a cytoplasmic N-terminal domain that functions in DNA binding and transcriptional activation and a periplasmic C-terminal domain which interacts with ToxS, another transmembrane protein. ToxR controls virulence gene expression by its ability to activate the expression of a positive regulatory gene, *toxT*. ToxT then directly activates a number of virulence genes, including TCP, ACFs, the *aldA* and *tagA-D* genes as well as the cholera toxin genes *ctxA* and *ctxB* in *V. cholerae*. The DNA between *tagA* and *tagD* contains three open reading frames (ORFs) with no obvious homology to any

sequence in the database. However, it is known that the *tagA-D* region is associated with epidemic and pandemic strains of *V. cholerae* and is absent from non-toxigenic environmental strains (Karaolis *et al.*, 1998). ToxR also directly controls the expression of genes in addition to *toxT* (i.e. *ompU* and *ompT*). The ToxR regulon can be thought of as two different branches depending on whether a gene is activated through the ToxT-dependent branch or regulated directly by ToxR (the ToxT-independent branch). In this figure, plus or minus signs indicate either a positive or negative effect of a particular regulatory protein on gene expression. Broken arrows show the gene transcripts. Figure modified from Skorupski and Taylor, 1997

TagA

As mentioned above, the Tox R regulon in *Vibrio* species (defined as the set of genes whose expression is under the direct or indirect control of ToxR) is comprised of the *ctx* and *tcp* genes, the genes encoding OmpU and OmpT, the *acf* genes, and the *tag* genes. TagA is included in this group of genes (DiRita *et al.*, 1991). Analysis of the primary aa sequence of the TagA signal sequence revealed the presence of a consensus *E. coli* signal peptidase II-processing site, suggesting that TagA represents a *Vibrio* lipoprotein (Harkey *et al.*, 1995). Metabolic labeling studies later showed that the TagA-PhoA fusion protein was fatty-acylated in *Vibrios* grown under culture conditions that promoted cholera toxin synthesis (Parsot *et al.*, 1991). However, the function of this gene was not elucidated in the *Vibrio* species. As no obvious role in virulence was noted by using a mouse model (Parsot and Mekalanos, 1991, Harkey *et al.*, 1995), these genes were thought to encode metabolic rather than virulence functions. It was subsequently reported that *tagA* is part of a 39.5-kb pathogenicity island (PAI) that includes the TCP-ACF cluster and is associated with epidemic and pandemic strains of *V. cholerae* (Karaolis *et al.*, 1998). It was not until a few years later that TagA was found to play an important role in another pathogenic organism, enterohemorraghic *E. coli* (EHEC) (Lathem *et al.*, 2002, 2003, 2004, Grys *et al.*, 2005, 2006).

Shiga toxigenic *E. coli* (STEC) organisms are an important cause of gastrointestinal disease in humans, particularly since these infections may result in life-threatening sequelae such as hemolytic-uremic syndrome (Karmali, 1989, Nataro and

Kaper, 1998, Paton and Paton, 1998). The STEC family is very diverse and strains belonging to a broad range of O:H serotypes have been associated with human disease. However, certain STEC subsets account for a disproportionately high number of serious infections. Members of one such subset have the capacity to produce attaching and effacing (A/E) lesions on intestinal mucosa, a property encoded on a pathogenicity island termed the locus for enterocyte effacement (LEE) (Frankel *et al.*, 1998, Nataro and Kaper, 1998, Jores *et al.*, 2004). LEE encodes a T3SS and *E. coli* secreted proteins (Esp) which deliver effector molecules to the host cell and disrupt the host cell cytoskeleton (Donnenberg *et al.*, 1997, Elliott *et al.*, 1998, Perna *et al.*, 1998). STEC strains belonging to serogroup O157 appear to be of particular concern for humans. This serogroup has been historically responsible for most major outbreaks of serious human STEC disease (Karmali, 1989, Nataro and Kaper, 1998, Paton and Paton, 1998). A useful approach to the identification of virulence-related gene products is to determine which STEC-specific proteins elicit a host immune response during infection. It was during such an attempt to identify additional virulence related gene products of O157:H7 STEC that a immunoreactive clone expressing TagA was isolated (Paton and Paton, 2002). This was accomplished by screening a cosmid DNA library of STEC strain EDL933 for clones reacting with convalescent HUS patient sera (Paton and Paton, 2002). This TagA exhibited 42% identity and 63% similarity with a 312-aa portion of the TagA lipoprotein of *V. cholerae* (Harkey *et al.*, 1995). Importantly, the *tagA* gene appeared to be present only in STEC strains belonging to serogroup O157 and was absent from all other STEC serogroups tested (Paton and Paton, 2002). In view of the strong immune response mounted by patients with O157 STEC disease, it was believed that antibodies to TagA may be a useful serological marker for O157 STEC infection, particularly in young children who may respond poorly to O157 O antigen (Paton and Paton, 2002).

StcE

The *tagA* gene identified in *E. coli* O157:H7 was later given the name StcE for secreted protease of C1 esterase inhibitor from EHEC (Lathem *et al.*, 2002). It was found to cleave the complement-associated serpin C1-INH (C1 esterase inhibitor) to produce

unique 60-65 kDa fragments. Serine protease inhibitors (serpins) form a superfamily of regulators that include α-1-antitrypsin, antithrombin, plasminogen activator type-1 and C1 esterase inhibitor (C1-INH) (Potempa *et al.*, 1994). Serpins act by changing the conformation of the proteases *via* insertion of the serpin reactive center loop (RCL) into the target molecule. This insertion irreversibly displaces the active serine of the protease from its catalytic partners (Huntington *et al.*, 2000). C1-INH, an extensively glycosylated attenuator of host injury, is the only inhibitor of activated C1r and C1s of the classical complement cascade, and the MASP 1 and 2 proteases of the mannose-binding ligand pathway. It is also a major inhibitor of Factor XIIa (Hageman Factor), Factor XIa, and kallikrein of the coagulation and the inflammation systems, respectively. Additionally, C1-INH competes with Factor B for binding to C3b to inhibit the activation of the alternative complement pathway (Jiang *et al.*, 2001). Circulating C1-INH has a molecular mass of 105 kDa, with post-translational glycosylations accounting for nearly half of its mass. Most glycosylations occur in the N-terminal 100 aa residues that are unique to this serpin. Desialylation does not affect the *in vitro* inhibitory activity of C1-INH, but reduces its circulating half-life in rabbits from >24 h to 3–5 min (Minta, 1981). C1-INH interacts with its target proteases to form large SDS-insoluble complexes that are subsequently removed from circulation (Caliezi *et al.*, 2000). Also, data suggest that surface associated C1-INH protects cells from proinflammatory events at their surface (Schmaier *et al.*, 1989, Schmaier *et al.*, 1993, Caliezi *et al.*, 2000). StcE was not observed to digest other serine protease inhibitors, extracellular matrix proteins or universal protease targets. It was also observed that StcE causes the aggregation of cultured human T cells but not macrophage-like cells or B cells, suggesting T cell-lineage specificity for this effect (Lathem *et al.*, 2002). Substitution of aspartic acid for glutamic acid at StcE position 435 within the consensus metalloprotease active site ablated its abilities to digest C1-INH and to aggregate T cells. StcE is secreted by the *etp* (EHEC type II secretion pathway) encoded on pO157, and extracellular StcE levels are positively regulated by the LEE-encoded regulator, Ler (Lathem *et al.*, 2002). StcE antigen and activity were also detected in the feces of a child with an *E. coli* O157:H7 infection,

demonstrating the expression of StcE during human disease. These results initially suggested that the cleavage of C1-INH by StcE could ablate the serpin's inhibitory activity on complement, which would ultimately lead to localized pro-inflammatory and coagulation responses resulting in tissue damage, intestinal edema and thrombotic abnormalities (Lathem *et al.*, 2002).

Subsequent work by Dr. Welch's group at the University of Wisconsin indicated that the cleavage of C1-INH by StcE, in fact, potentiated the activity of the inhibitor. StcE, once secreted out of the cell, could bind to the surfaces of bacteria or host erythrocytes, whereby it recruited C1-INH to these surfaces. By increasing the local concentration of C1-INH at these surfaces, Lathem *et al*. proposed that more activation of complement was prevented, ultimately leading to less lysis of erythrocytes or increased serum resistance of the bacteria within the host (Lathem *et al.*, 2004) (Fig. 1.3). Indeed it was demonstrated that removal of StcE abolished the ability of the inhibitor to bind to erythrocyte surfaces, as only StcE treated C1-INH, not native C1-INH, could bind to the cell surface (Lathem *et al.*, 2004).

QUORUM SENSING

Bacteria communicate with one another using chemical signal molecules. As in higher organisms, the information supplied by these molecules is critical for synchronizing the activities of large groups of cells. In bacteria, chemical communication involves producing, releasing, detecting, and responding to small hormone-like molecules termed autoinducers or lactones. This process, termed quorum sensing (QS), allows bacteria to monitor the environment for other bacteria and to alter behavior on a population-wide scale in response to changes in the number and/or species present in a community (Waters and Bassler, 2005, Reading and Sperandio, 2006). Most QS-controlled processes are unproductive when undertaken by an individual bacterium acting alone but become beneficial when carried out simultaneously by a large number of cells(Waters and Bassler, 2005, Reading and Sperandio, 2006). Thus, QS confuses the

distinction between prokaryotes and eukaryotes because it enables bacteria to act as multicellular organisms.

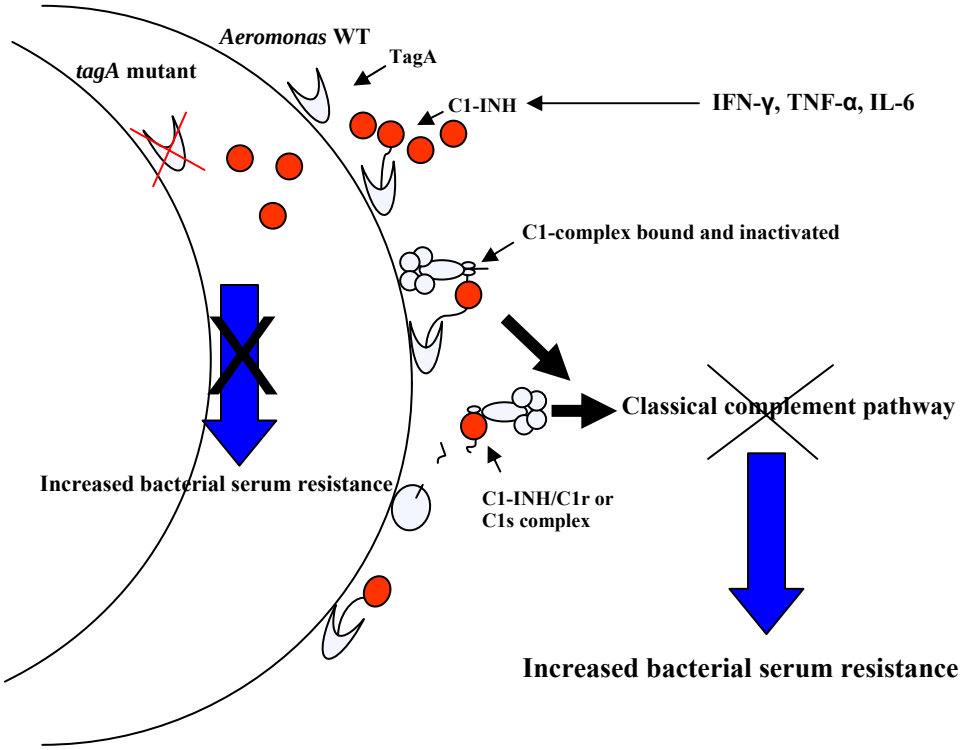

Fig. 1.3. Probable scenario of TagA's role in inhibiting complement *via* the classical complement pathway.
Levels of proinflammatory cytokines (e.g. TNFα, IL-6) are increased during *Aeromonas* infection, initially leading to the increased production of C1-INH. In the WT bacterium, surface TagA helps to recruit C1-INH. The metalloprotease (TagA) acts as a "bridge" by binding the NH_2-terminal domain of the serpin (C1-INH), sequestering it to the cell surface. It is also possible that TagA can act as a co-ligand to a molecule which is capable of binding C1-INH or TagA can direcly bind C1-INH. It is believed that the interaction between C1-INH and TagA is necessary for increasing the concentration of C1-INH at the cell surface (as demonstrated in erythrocytes by Lathem *et al*.). The cell-bound C1-INH can still bind to C1r and/or C1s of the C1 complex, thus inactivating the complex and leading to increased inhibition of complement and increased bacterial serum resistance. Alternatively, it is also possible that the potentiation of complement inhibition could occur by the following scenario: TagA, after having bound C1-INH to the cell

surface, cleaves within the NH_2 terminus of C1-INH, releasing the serpin/serine protease complex (C1r and C1s) and a smaller, NH_2-terminal cleavage fragment of C1-INH from the cell surface. By releasing the C1 complex from the surface of the bacterial cell, formation of the cytolytic membrane attack complex (MAC) is prevented and bacterial survival is enhanced. TagA is now free to bind another C1-INH molecule and repeat the cycle, ultimately increasing the serum resistance of the bacteria.

In the *tagA* mutant, the absence of TagA on the surface of the bacterium prevents the recruitment of the majority of C1-INH to the bacterial surface, whereby the potentiation of inhibition of complement cannot occur through either scenario mentioned above. Ultimately, the alternative pathway of complement will continue to operate and be activated by the presence of bacterial antigens, leading to eventual killing of the bacteria within the host. Figure modified from Lathem *et al*., 2004.

QS in *Vibrio* species

Research into acyl-homoserine lactone (AHL) based QS started in the late 1960s. The marine bioluminescent bacteria *V. fischeri* was being grown in liquid cultures and it was observed that the cultures produced light only when large numbers of bacteria were present (Stevens and Greenberg, 1997). The initial explanation for this was that the culture media contained an inhibitor of luminescence, which was removed by the bacteria when large numbers were present (Kempner and Hanson, 1968). This was suggested because when grown in media "conditioned" by exposure to the bacteria, luminescence could be induced even at low cell densities. It was later shown that the luminescence was initiated not by the removal of an inhibitor but by the accumulation of an activator molecule or "autoinducer" (Nealson *et al.*, 1970, Eberhard, 1972). This molecule is made by the bacteria and activates luminescence when it has accumulated to a high enough concentration. The bacteria are able to sense their cell density by monitoring the autoinducer concentration. This mechanism of cell density sensing was termed QS. Analysis of the genes involved in QS in *V. fischeri* was first carried out by Engebrecht *et al.* (1983). This led to the basic model for QS in *V. fischeri* which is now a paradigm for other similar QS systems (Engebrecht *et al.*, 1983).

For many years following this, it was thought that AHL-based QS was limited to marine bacteria such as *V. fischeri* and *V. harveyi*. In these bacteria, two proteins, LuxI and LuxR, control expression of the luciferase operon (*luxICDABE*) required for light

production. LuxI is the autoinducer synthase, which produces the AHLs. The AHL molecule produced by *V. fischeri* was first isolated and characterized in 1981 and identified as *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981, Engebrecht and Silverman, 1984). LuxR is the cytoplasmic autoinducer receptor/DNA-binding transcriptional activator (Engebrecht *et al.*, 1983). Following production, the AHL freely diffuses in and out of the cell and increases in concentration with increasing cell density (Kaplan and Greenberg, 1985). When the signal reaches a critical threshold concentration, it is bound by LuxR and this complex activates transcription of the operon encoding lucerifase (Stevens *et al.*, 1994). Importantly, the LuxR-AHL complex also induces expression of *luxI* because it is encoded in the luciferase operon. This regulatory configuration floods the environment with the signal. This creates a positive feedback loop that causes the entire population to switch into "QS-mode" and produce light. A large number of other gram-negative proteobacteria possess LuxIR-type proteins and communicate with AHL signals (Manefield and Turner, 2002). These systems are predominantly used for intraspecies communication as extreme specificity exists between the LuxR proteins and their cognate AHL signals. For example, LuxI-type proteins link and lactonize the methionine moiety from *S*-adenosylmethionine (SAM) to particular fatty acyl chains carried on acyl-acyl carrier proteins (More *et al.*, 1996, Parsek *et al.*, 1999).

V. harveyi is another bioluminescent marine bacterium and is known to possess two QS systems. Together they control functions such as bioluminescence (Bassler *et al.*, 1993, Bassler *et al.*, 1994), the T3SS (Henke and Bassler, 2004), as well as siderophore (Lilley and Bassler, 2000), polysaccharide (Lilley and Bassler, 2000), and metalloprotease production (Mok *et al.*, 2003). The System 1 autoinducer, *N*-(3-hydroxybutanoyl) homoserine lactone (HSL) (denoted HAI-1 for *harveyi* autoinducer 1), is produced by LuxM and is detected by LuxN (Cao and Meighen, 1989, Bassler *et al.*, 1993). The System 2 autoinducer, called AI-2 is a 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)dioxaborole-2,2,6,6A-tetraol, and the unborated precursor is made by LuxS (Surette *et al.*, 1999, Schauder *et al.*, 2001, Chen *et al.*, 2002). Two proteins, LuxP and

LuxQ, function together as the AI-2 sensor (Bassler *et al.*, 1994). LuxP is similar to periplasmic ribose binding proteins. Both LuxN and LuxQ are hybrid two-component proteins that contain sensor kinase and response regulator domains. The remainder of the signaling cascade is shared by System 1 and System 2. In *V. harveyi*, at low cell density (i.e., in the absence of the autoinducers), LuxN and LuxQ autophosphorylate and transfer a phosphate to the shared phosphotransferase protein LuxU, which in turn passes it to the response regulator, LuxO (Freeman and Bassler, 1999, Freeman and Bassler, 1999, Freeman *et al.*, 2000). Phosphorylated LuxO is active and, in conjunction with σ^{54}, activates the expression of genes encoding small regulatory RNAs (sRNAs). These sRNA, together with the RNA chaperone Hfq, destabilize the mRNA encoding the activator protein LuxR (Lilley and Bassler, 2000, Lenz *et al.*, 2004).

LuxR is required for expression of the target genes in the QS regulon (Martin *et al.*, 1989, Showalter *et al.*, 1990, Henke and Bassler, 2004, Lenz *et al.*, 2004). Because LuxR is required for transcription of *luxICDABE*, no light is produced under low cell density conditions. At high cell density, (i.e., in the presence of autoinducers), LuxN and LuxQ switch from kinases to phosphatases (Freeman *et al.*, 2000). Phosphate flow through the system is reversed, draining LuxO of phosphate (Freeman and Bassler, 1999). Unphosphorylated LuxO is inactive and thus cannot promote the expression of the sRNAs, leading to translation of *luxR* mRNA (Lenz *et al.*, 2004). LuxR binds the *luxICDABE* promoter and activates its expression, thus enabling *V. harveyi* to produce light. Many other genes are controlled by QS in *V. harveyi*. While all require LuxR for their regulation, some of them, like *lux* (the genes encoding bioluminescence), are induced at high cell densities, while others are repressed at high cell densities (Mok *et al.*, 2003, Henke and Bassler, 2004). Lux is not the exclusive target of QS regulation in *V. harveyi*, as an earlier genetic screen for AI-2-regulated genes revealed 10 additional targets (Mok *et al.*, 2003). Analysis of the regulation of these 10 targets showed that the autoinducers HAI-1 and AI-2 act synergistically and, furthermore, that both signals are required simultaneously for proper control of the target genes. This earlier work focused on information flow through the QS circuit and not on the functions of the target genes.

However, the analysis did reveal two genes with homology to components of T3SSs, suggesting that *V. harveyi* could possess a QS-regulated T3SS. It was later shown that the *V. harveyi* and *V. parahaemolyticus* T3SSs are functional and capable of secretion only under conditions that simulate low cell density and that transcription of both the *V. harveyi* and *V. parahaemolyticus* T3SS genes are repressed by autoinducers at high cell density (Henke and Bassler, 2004).

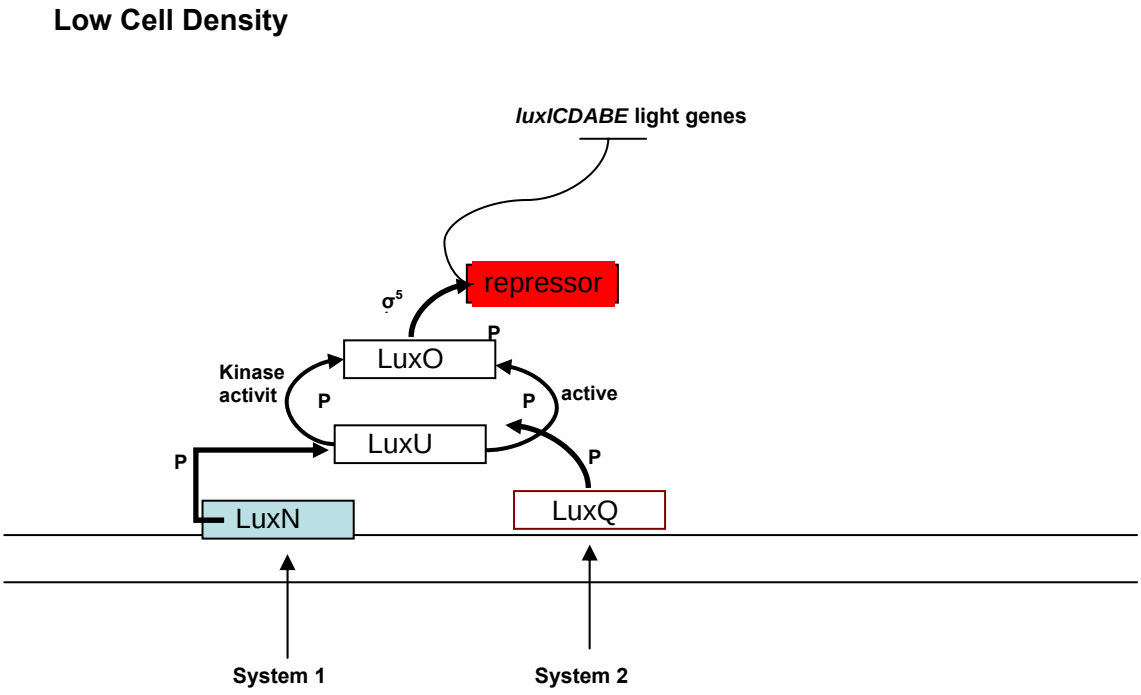

Fig. 1.4. QS system in *Vibrio* species
(A) QS in *V. harveyi* activates bioluminescence (*luxICDABE*) at high cell density. Two parallel signaling systems are employed to regulate light production. System 1 includes LuxM, an AHL signal synthase, and LuxN, a hybrid sensor/kinase that responds to the AHL signal. System 2 includes LuxSPQ. LuxS is the synthase for AI-2. AI-2 is then believed to bind to the periplasmic LuxP protein, and this complex interacts with the hybrid sensor/kinase, LuxQ. At low cell density, signal concentration is low, and LuxN and LuxQ act as kinases, resulting in autophosphorylation. The phosphate is then transferred to the cytoplasmic phosphotransferase LuxU, which in turn transfers the phosphate to the σ^{54}-dependent activator, LuxO. Upon phosphorylation, LuxO is

activated and, together with σ^{54}, activates the production of an unidentified repressor, which inhibits bioluminescence (*luxICDABE*).

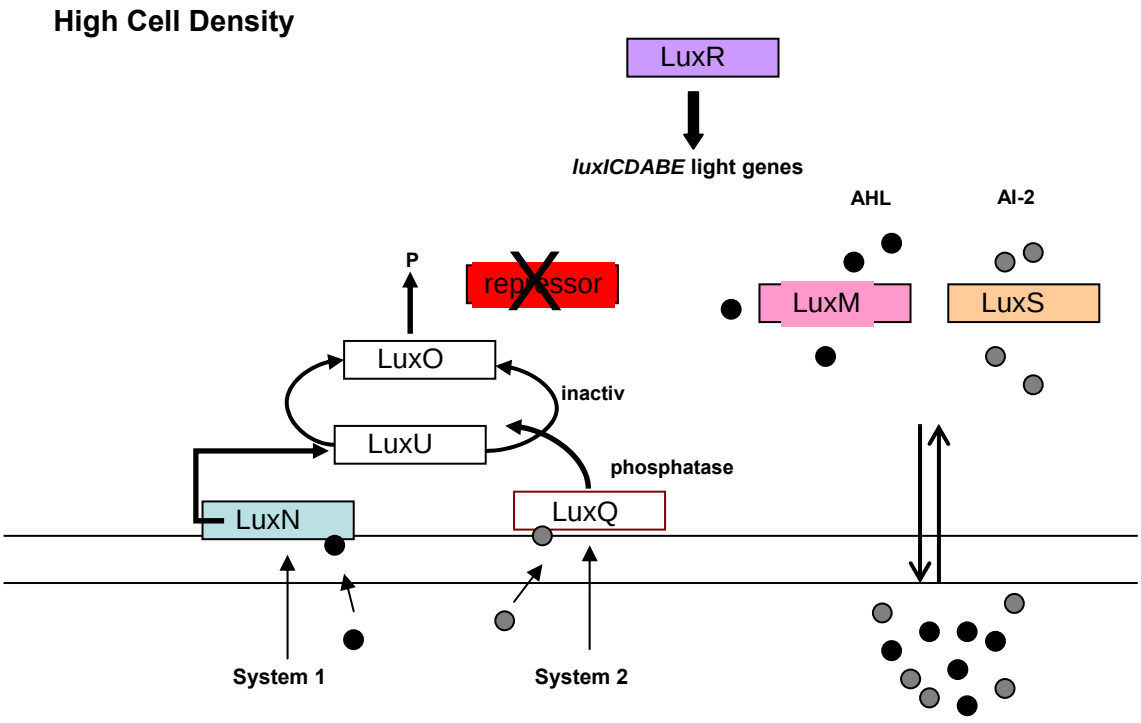

Fig. 1.4. cont… QS system in *Vibrio* species
(B) At high cell density, the AHL and AI-2 signal molecules are produced at a high level and can now interact with their cognate sensors. This interaction converts LuxN and LuxQ from kinases to phosphatases, resulting in the loss of phosphate from LuxU and LuxO. Consequently, LuxO is inactivated and the unidentified repressor is not expressed allowing the transcriptional activator to activate expression of the bioluminescence genes. Figure modified from Henke and Bassler, 2004.

QS in *Pseudomonas aeruginosa*

At around the same time period that a QS system was discovered in *Vibrio* species, other researchers discovered that the human pathogen *P. aeruginosa* also possesses a *V. fischeri*-like QS system (Gambello and Iglewski, 1991). In *P. aeruginosa*,

two QS systems have been identified: LasI/LasR and RhlI/RhlR, both of which represent LuxI/LuxR homologs (reviewed in (Juhas *et al.*, 2005)]. While LasI synthesizes the major AHL, *N*-(butanoyl)-L-homoserine lactone (BHL), RhlI produces *N*-(3-oxododecanoyl)-L-homoserine lactone (Juhas *et al.*, 2005). Mutations in either *rhlI* or *rhlR* resulted in the up-regulation of *exoS* (encoding the T3SS effector ExoS) expression during biofilm formation in *P. aeruginosa* (Hogardt *et al.*, 2004). This *exoS* up-regulation phenomenon in the *rhlI* mutant was repressed by adding BHL, indicating a negative regulatory effect on RhlR/BHL on *exoS* expression (Hogardt *et al.*, 2004). The QS system of *P. aeruginosa* was also shown to regulate the production of elastase, an important virulence factor in this bacterium (Pearson *et al.*, 1994). The AHL responsible for the induction of elastase was identified as 3-oxo-C12-homoserine lactone (HSL) (Pearson *et al.*, 1994, Bleves *et al.*, 2005).

A second QS system regulating rhamnolipid, hemolysin, and other important *Pseudomonas* virulence factors was discovered (Latifi *et al.*, 1995). Studies have shown that this system was responsible for the production of C4-HSL (Ochsner *et al.*, 1994, Winson *et al.*, 1995). QS in *P. aeruginosa* is involved in regulating the expression of a number of virulence factors, and as such, this regulation is believed to play an important role in the pathogenicity of this pathogen. Using a number of different animal models, this presumption has been confirmed. In the neonatal mouse model of pneumonia, a *lasR*-deficient strain of *P. aeruginosa* was found to have significantly decreased virulence compared to that in the parental strain (Tang *et al.*, 1996). Analysis of a *lasI* mutant, a *rhlI* mutant, and a *lasI/rhlI* double mutant in the same model revealed markedly decreased virulence, with the most notable reduction seen in the double I mutant (Pearson *et al.*, 2000). In a burned mouse model, strains deficient in *lasR*, *lasI*, *rhlI*, or both *lasI/rhlI* were found to be less virulent than the parental strain (Rumbaugh *et al.*, 1999, Rumbaugh *et al.*, 1999). In addition, the total number of bacteria recovered from the spleens, livers, and skin of mice infected with the different mutants was significantly lower than those for the parent strain (Rumbaugh *et al.*, 1999). These finding indicate that QS plays an important role in the dissemination of *P. aeruginosa* throughout the

body of burned mice. In the double I mutant, which was the least virulent strain, complementation with *lasI*, *rhlI*, or both *lasI/rhlI* on a multicopy plasmid significantly increased both *in vivo* virulence and the ability of the pathogen to spread within the burned skin of the infected animals (Rumbaugh *et al.*, 1999).

QS in *A. hydrophila*

The expression of several of the exoproducts produced by this bacterium is associated with high cell densities in the late exponential/stationary phase and therefore represents putative phenotypes for control by QS. It was shown that *A. hydrophila* and *A. salmonicida* possess homologs of the LuxI and LuxR QS proteins. These were designated AhyI, AhyR and AsaI, AsaR, respectively (Swift *et al.*, 1997). For both AhyI and AsaI, the major AHL produced is *N*-butanoylhomoserine lactone (C4-HSL) (Swift *et al.*, 1997). In *A. hydrophila*, an AhyR/C4-HSL-dependent QS system regulates both extracellular protease production and biofilm development (Swift *et al.*, 1999, Lynch *et al.*, 2002). Mutation of either *ahyI* or *ahyR* abolishes both serine and metalloprotease activities which can be restored in the *ahyI* mutant by the addition of exogenous C4-HSL (Swift *et al.*, 1999). During the biofilm mode of growth, an *ahyI* mutant fails to form large "mushroom-like" microcolonies characteristic of the differentiated biofilms made by the parent strain. In contrast, mutation of *ahyR* results in the formation of a biofilm which occupies a far greater proportion of the available surface area than the parent strain (Lynch *et al.*, 2002). The *ahyI* and *ahyR* genes comprise a divergon with a 62 base-pair (bp) intergenic region. This region contains a 12 bp symmetrical sequence analogous to the *V. fischeri lux* box and therefore, a potential binding site for AhyR. However, in contrast to the *V. fischeri* system where LuxR regulates *luxI* expression and hence AHL synthesis, in *A. hydrophila*, mutation of *ahyR* did not result in the loss of C4-HSL synthesis and this AHL was present in the stationary phase of both parent and *ahyR* mutant strains (Swift *et al.*, 1999). Recently, Kirke *et al*. (Kirke *et al.*, 2004) reported that AhyR regulates AhyI production in a growth phase-dependent manner in *A. hydrophila*, as AhyI accumulated in the exponential phase and degraded in the stationary phase (Kirke *et al.*, 2004). However, the AhyI levels were sustained in the stationary

phase of the *ahyR* mutant. Thus, the degradation of AhyI might be due to the production of a protease, or alternatively, a second QS circuit exists in which another LuxR homolog controls the production of AhyI in the *ahyR* mutant (Kirke *et al.*, 2004).

Lactone-based QS exists in many gram-negative bacteria, and microorganisms often produce more than one type of AHL (Swift *et al.*, 1997, Fuqua *et al.*, 2001, Michael *et al.*, 2001). Identification of AHLs in bacterial culture supernatants or in purified form requires appropriate bioassay strains. Two biosensor strains, namely, *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026, have been successfully used for detecting lactones in clinical isolates of *A. hydrophila* and *P. aeruginosa* (Zhu *et al.*, 2001). *C. violaceum* CV026 was used as a biosensor to detect AHLs with N-acyl side chains of four to eight carbons, especially BHL (McClean *et al.*, 1997). *A. tumefaciens* A136 was used as another biosensor (Fuqua and Winans, 1996), which is extremely sensitive to the 3-oxo derivatives with N-acyl chain length from six to 12 carbons, including *N*-(3-oxododecanoyl)-L-homoserine lactone (Shaw *et al.*, 1997). The observation that most strains of *A. hydrophila* and *P. aeruginosa* produced AHLs and BHLs that could be detected by both CV026 and A136 revealed that the majority of the isolates of these bacteria produced multiple AHLs (Zhu *et al.*, 2001). The reporter strain *A. tumefaciens* A136 (ATCC) (Zhu *et al.*, 2000) lacks the nopaline-type Ti plasmid and, therefore, does not produce its own lactones. However, the presence of a plasmid with *traR* and a *traG:lacZ* fusion allows the bacterium to respond to exogenously added lactones. The *traR* gene of *A. tumefaciens* resembles *luxR* of *V. fischeri*, in that it functions as a QS- transcriptional activator (Fuqua and Winans, 1994). TraR binds AHLs or BHLs, forming active homodimers, which, in turn, activate the *traG:lacZ* reporter gene (see Fig. 2.1).

A detailed understanding of the virulence factors of the human pathogen *A. hydrophila* SSU is paramount to understanding the mechanisms whereby this bacterium causes disease in the human host. The elucidation of new virulence mechanisms/factors used by the bacteria provide an avenue or target upon which research can be focused in order to better control infection by this bacterium. This study looked at both the *in vitro*

and *in vivo* effects of the above-mentioned virulence mechanisms/factors on host cells and animals. The *in vitro* studies were performed with macrophages and colonic epithelial cells, as in depth studies have previously been performed with these cells (Chopra *et al.*, 2000, Ribardo *et al.*, 2002) and because of the ease of growing these cells. Further, because *Aeromonas* causes gastroenteritis, human colonic epithelial cells were used in order to determine if these virulence mechanisms/factors might cause cytotoxicity and/or cell death in these cells. The overall objective of this proposal was to examine the effect of these virulence mechanisms/factors in contributing to the pathogenesis of a clinical isolate SSU of *A. hydrophila*.

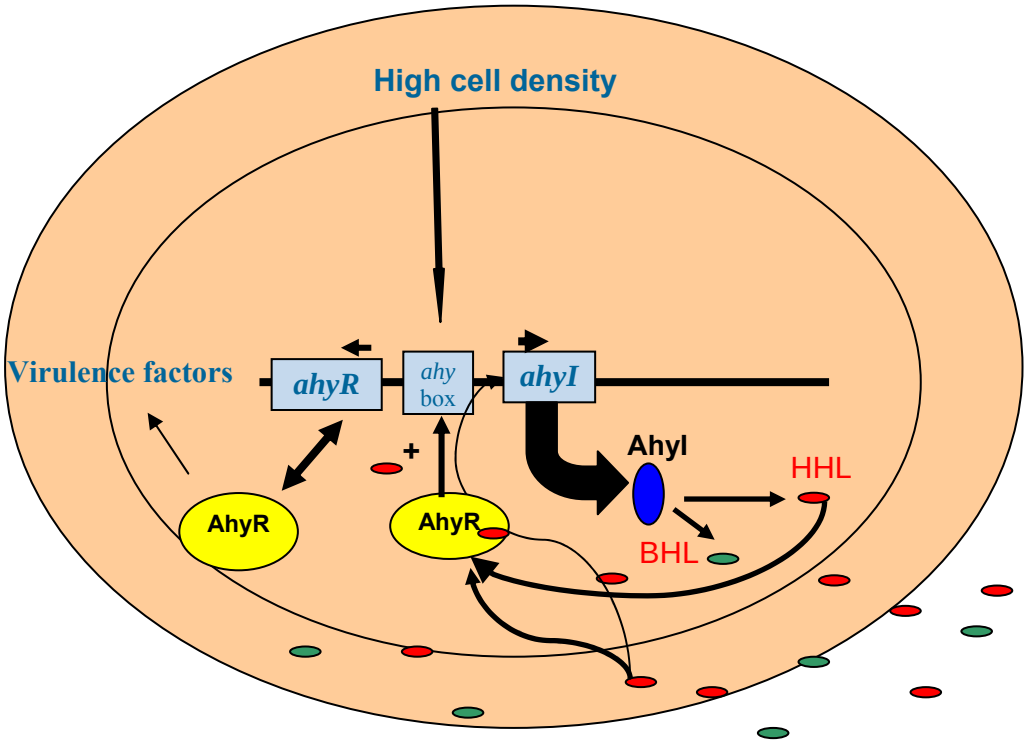

Fig. 1.5. Model of QS in *A. hydrophila* SSU
The cell-cell communication process of QS involves the production of extracellular signaling molecules, called autoinducers or lactones (AHL & BHL), which proportionally increase as the bacteria grow. When a critical threshold concentration of autoinducer is reached, the bacteria detect the signal, coordinately alter gene expression, and thus respond as a group. Lactone production also induces the expression of the transcription factor AhyR.

SPECIFIC AIMS

This project was initiated with the following specific aims:

Aim 1: To identify and characterize the type 3 secretion system (T3SS) from *A. hydrophila* SSU.

Aim 2: To prepare knock-out mutants of *A. hydrophila* for homologs of type 3 translocation proteins to elucidate the functionality of the T3SS in the virulence of *A. hydrophila* SSU.

Aim 3: To characterize the role of DNA adenine methyltransferase (Dam) and quorum sensing (QS) in the regulation of T3SS and T2SS genes and their expression.

Aim 4: To identify, sequence and characterize the *tagA* gene encoding ToxR-regulated lipoprotein from *A. hydrophila* SSU.

CHAPTER 2:
MATERIALS AND METHODS

Cell Culture

The murine macrophage (RAW 264.7) and human colonic epithelial (HT-29) cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). RAW 264.7 and HT-29 cells were cultured at 37°C and 5% CO_2 in Dulbecco's minimal essential medium (D-MEM) (Invitrogen, Gaithersburg, MD) containing 4.5 g glucose/Liter, 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics penicillin (100 U/mL) and streptomycin (Sp) (0.1 mg/mL).

Bacterial strains and plasmids

A. hydrophila SSU and its rifampin-resistant (Rif^r) derivative were previously described (Xu *et al.*, 1998, Sha *et al.*, 2002). Vectors pBluescript and pBR322 were used for cloning, and plasmids pBR*aopB* and pBR*tagA*, which contained the coding region of the *A. hydrophila aopB* and *tagA* genes, respectively, were used for complementation. A suicide vector pDMS197 with a conditional R6K origin of replication (*ori*), a levansucrase gene (*sacB*) from *Bacillus subtilis*, and a tetracycline resistance (Tc^r) gene was used for homologous recombination (Edwards *et al.*, 1998). The plasmid pHP45Ω, containing a spectinomycin and streptomycin resistance (Sm/Sp^r) gene cassette, was employed as a selective marker for generating the isogenic mutants. Ampicillin (Ap), tetracycline (Tc), kanamycin (Km), and spectinomycin and streptomycin (Sm/Sp) antibiotics were used at concentrations of 15, 25, 50, or 100 μg/ml, respectively, in Luria-Bertani (LB) medium or agar plates. The antibiotic rifampin (Rif) was utilized at a concentration of 40 μg/ml for bacterial growth and 300 μg/ml during conjugation experiments. Chromosomal DNA was isolated using a QIAamp® DNA Mini Kit, and digested plasmid DNA or DNA fragments from agarose gels were purified using a

QIAprep® Miniprep Kit (Qiagen, Inc., Valencia, CA). All radioactive materials, α^{32}P-dCTP and γ^{32}P-dATP were purchased from ICN, Irvine, CA.

Cloning and DNA sequence analysis of the *A. hydrophila* SSU T3SS

Among different genes that constitute a T3SS, sequences of some genes (e.g., *yscV* from *Yersinia* species and its homologs in other bacteria) are highly conserved (Foultier *et al.*, 2002). Therefore, primers (Table 2.1) were synthesized, based on the *yscV* gene of *Y. enterocolitica* T3SS (Snellings *et al.*, 2001). Next, PCR amplification of the corresponding gene (*ascV*) from the genome of *A. hydrophila* was performed under the following conditions: 96°C for 5 min (denaturation), followed by 35 cycles of 96°C for 1 min, 65°C for 1 min, and then 72°C for 1 min. The final extension was performed at 72°C for 7 min. The amplified *ascV* DNA fragment (2,166 bp) was sequenced, and additional primers were subsequently designed based on the sequence of the *ascV* gene, which allowed us to perform further chromosomal sequencing and to obtain a partial DNA sequence of the T3SS of *A. hydrophila* SSU. The fosmid library of *A. hydrophila* SSU (see below) was screened with an [α-^{32}P]dCTP-labeled *ascV* gene (2,166-bp) probe of *A. hydrophila* using colony blot hybridization in order to obtain the sequence of the entire T3SS and flanking regions (Sha *et al.*, 2001, Sha *et al.*, 2005). All of the DNA sequencing was performed with an automated DNA sequencer, 373XL (Applied Biosystems, Inc., Foster City, CA), in the Protein Chemistry Core Laboratory at the University of Texas Medical Branch, Galveston. The DNA sequence data were analyzed and compared with the databases using an online BCM Search Launcher (Baylor College of Medicine Human Genome Sequencing Center, Houston, TX) and the Clustal W program (Supercomputer Laboratory, Institute for Chemical Research, Kyoto University, Kyoto, Japan).

Fosmid library construction

A. hydrophila genomic DNA (gDNA) (5 μg) was sheared to generate fragments in the 25- to 40-kb size range. The sheared DNA was end repaired to generate blunt ends

and size selected using a 1% low-melting-point agarose gel. The size-selected DNA was then ligated to the dephosphorylated blunt-ended pEpiFOS-5 Fosmid vector (Epicenter, Madison, WI) and packaged using MaxPlax Lambda packaging extracts. *E. coli* EPI100 plating cells (Epicenter) were used as the host. Fosmid colonies were lifted onto nylon filters from LB agar plates containing 12.5 µg/ml chloramphenicol (Cm). The gene encoding Cm^r was carried by the fosmid vector. The filters were screened with the [α-^{32}P]dCTP-labeled *ascV*, *dam,* or *tagA* gene probe by colony blot hybridization. Positive colonies were identified and grown overnight in LB medium with Cm and 10 mM $MgSO_4$ for DNA isolation.

Colony and dot blot hybridization

Nylon membrane filters used for the colony or dot blots were removed from the agar plates and processed as described: (1) all the filters were soaked on 3 sheets of Whatman paper (Whatman International Ltd., Maidstone, England) soaked with 0.5 N NaOH for 7 min; (2) the filters were then soaked on 3 sheets of Whatman paper soaked with 1.0 M Tris, pH 7.4 for 2 min; (3) subsequently, the filters were soaked on 3 sheets of Whatman paper soaked with 1.5 M NaCl + 1 M Tris, pH 7.4 for 1 min. After these treatment steps, the filters were placed on a vacuum apparatus and treated with 70 ml of 1.5 M NaCl + 1 M Tris, pH 7.4, followed by treatment with 70 ml of chloroform (Sha *et al.*, 2002). The filters were then dried and baked at 80°C for 2 h. Filters were prewashed with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, and 0.1% sodium-dodecyl sulfate (SDS) at 42°C to remove cell debris. The filters were then prehybridized using Quickhyb solution at 68°C as described by the manufacturer (Stratagene, La Jolla, CA) and hybridized under high stringency conditions with the appropriate [α-^{32}P]dCTP labeled *ascV*, *dam* or *tagA* gene probe. After 2 to 3 h, the filters were washed with 2X SSC, 0.1% SDS for 40 min and 1X SSC, 0.1% SDS for 40 min at 68°C and exposed to autoradiograph film overnight at room temperature.

Primer sequence[a]	Position[b]	Purpose
Forward: 5′ CGTAAAGATATCATGCTGGC 3′ Reverse: 5′ TGGTTGGATATTAATCTGCTG 3′	+43 to +62 of *yscV* +2074 to +2094 of *yscV*	PCR amplification of the *ascV* gene from *A. hydrophila* SSU
Forward: 5′ GGGGTACCCCTCAACGACGTCAGCTCGC 3′ KpnI Reverse: 5′ CCGGAATTCCGGGTTCATCTTGCTGATCCCTTC 3′ EcoRI	−596 to −577 of *aopB* −15 to +9 of *aopB*	PCR amplification of the upstream flanking sequence to the *aopB* gene
Forward: 5′ CGGAATTCCGCTATCTAAGGAGTATTGC 3′ EcoRI Reverse: 5′ GCTCTAGAGCTTTGACCTCGTCTTCCTTGG 3′ XbaI	+1178 to +1198 of *aopB* +1928 to +1949 of *aopB*	PCR amplification of the downstream flanking sequence to the *apoB* gene
Forward: 5′ ATGAACCCGATCAGCAATGA 3′ Reverse: 5′ TTAGATAGCGGCCGGCCTGC 3′	+1 to +20 of *aopB* +1169 to +1188 of *aopB*	PCR amplification of the *aopB* coding region for complementation

Table 2.1. Sequences of the primers used in T3SS study.

Indicates primers used in amplifying *ascV* gene from *A. hydrophila* SSU, as well primers used to construct isogenic mutant of *aopB* and the complemented strain.

[a] Underlining indicates restriction enzyme sites in the primer.

[b] The nucleotide position of the primers was counted from the start codon of the genes. The upstream position relative to the start codon was designated with a – sign, while downstream nucleotide positions relative to the start codon were designated with a + sign.

Osmotic shock of cells for identification of T3SS needle structures under electron microscope (EM)

Osmotically shocked cells were prepared according to the method of Kubori *et al.* (Kubori *et al.*, 1992). Briefly, stock culture of *A. hydrophila* SSU was grown in 5 ml LB medium to OD_{600} of 0.8 and centrifuged to separate cell pellet and supernatant fractions. Bacterial cell pellets were resuspended in 10 ml of 1 M sucrose, 1 mM EDTA, and 150 mM Tris and incubated at room temperature for 5 min. The suspension was again

centrifuged and cells were rapidly dispersed in double distilled (dd) H_20 at 4ºC. The DNA was then digested by adding 30 µg of DNAse I/ml and 5 mM $MgCl_2$ and incubated at 37ºC for 15 min. The cells were washed with ddH_20 at 4ºC two times, collected and resuspended in 50 µl ddH_20. Samples were negatively stained with 2% phosphotungstic acid (pH 7.0) and observed under the Philips 201 electron microscope (EM410).

Isolation of needle structures

This procedure was carried out according to the protocol described by Kubori *et al.* (Kubori *et al.*, 1998) with the following modifications. Bacteria were grown overnight in 1 L of LB medium, pelleted, and resuspended in 1/10 volume of 0.5 M sucrose and 0.15 M Trisma base. Lysozyme and EDTA were added to a final concentration of 0.2 mg/ml and 1 mM, respectively. Samples were incubated for 1 h at 4ºC. A 1/10 volume of 10% lauryldimethylamine oxide (LDAO) was then added and samples centrifuged. The supernatant was adjusted to pH 10.5 with NaOH, incubated at 4ºC, and clarified further by centrifugation. The supernatant was then centrifuged at 200,000 g in a TL-100 rotor in a Beckmann (model TL-100) microultracentrifuge. The pellet was resuspended in 0.5 M sucrose, 0.15 M Tris base and 0.03% LDAO, adjusted to pH 10.5 and centrifuged at 250,000 g for 60 min at 4ºC, in the same microultracentrifuge. The pellet was resuspended in TET buffer (10 mM Tris, pH 8.0, 20 mM EDTA, 0.03% LDAO) and loaded onto a 30% CsCl solution for gradient formation and centrifuged for 15 h at 25,000 g and 20 ºC in a SW41 rotor in a Beckman ultracentrifuge (Optima L-90K). Fractions of 2 ml were centrifuged at 250,000 g for 60 min at 4ºC in the TL-100 microultracentrifuge and the pellet resuspended in 20 µl of phosphate-buffered saline (PBS) buffer. These samples were visualized directly under the Philips 201 electron microscope upon negative staining with 2% phosphotungstic acid (pH 7.0).

Generation and characterization of *aopB* and *act*/*aopB* mutants of *A. hydrophila* SSU

Two pairs of primers were synthesized that amplified the upstream and downstream flanking sequences to the *aopB* gene (Table 2.1) (Sha *et al.*, 2005). The resulting 604- and 783-bp DNA fragments were ligated together through a common *Eco*RI site and cloned into a pBluescript vector at *Kpn*I/*Xba*I restriction enzyme sites, resulting in a recombinant plasmid, pBlueUD. A Sm/Sp^r gene cassette flanked by an *Eco*RI site from plasmid pHP45 was inserted at the *Eco*RI site of pBlueUD plasmid to generate a recombinant plasmid, pBlueUDSm/Sp. After digestion with *Kpn*I/*Xba*I restriction enzymes, the DNA fragment from the above plasmid was ligated to a pDMS197 suicide vector at the compatible restriction enzyme sites, and the resulting plasmid (pDMS197UDSm/Sp) was transformed into an *E. coli* SM10λ*pir* strain (Sha *et al.*, 2002). The recombinant *E. coli* [pDMS197UDSm/Sp] was conjugated with either WT *A. hydrophila* SSU-R or its *act* mutant (Xu *et al.*, 1998, Sha *et al.*, 2002). The transconjugants were selected based on resistance to appropriate antibiotics and sucrose and subjected to further analysis (Sha *et al.*, 2002).

Southern blot analysis

Chromosomal DNA from isogenic mutants, as well as from WT *A. hydrophila*, was isolated, and an aliquot (10 µg) was digested with appropriate restriction enzymes and subjected to 0.8% agarose gel electrophoresis (Sha *et al.*, 2002). Next, the digested DNA was transferred to a nylon membrane and baked at 80°C for 2 h. Three DNA probes were used for Southern blot analysis. These probes represented the coding region of the target gene, a 2.0-kb Sm/Sp^r gene cassette from plasmid pHP45Ω obtained by *Bam*HI restriction enzyme digestion, and a suicide vector, pDMS197 (6.0 kb) (Sha *et al.*, 2002). The prehybridization and hybridization conditions were the same as those mentioned earlier for the colony blot hybridization.

Cell membrane integrity assays

The *aopB* and *act/aopB* mutants, or Dam-overproducing strain as well as the WT *A. hydrophila* or WT with pBAD vector alone (see below), were grown to an OD_{600} of 0.4 to 0.5 and diluted 50-fold. Next, various concentrations of Triton X-100 (TX-100; 0.5 to 5%), SDS (0.5 to 2%), and vancomycin (100 to 200 μg/ml) were added. The cultures were incubated at 37°C for 3 h with shaking (180 rpm), and the OD_{600} was measured. A 50% reduction in the OD of three independent experiments indicated sensitivity of the cultures to the above-mentioned detergents and/or antibiotic. These methods are described in the following reference (Sha *et al.*, 2004). Periplasmic RNase I leakage from the mutants or Dam-overproducing strain and WT *A. hydrophila* or WT with pBAD vector was also measured. Briefly, bacterial cells were streaked on LB agar plates containing 1.5% *Torula* yeast RNA (Sigma, St. Louis, MO) and incubated overnight at 37°C. After incubation, 10% trichloroacetic acid (TCA) was added onto the plates and the RNase leakage from bacterial cells was examined by measuring the diameter of the clear zones around the bacterial streaks (Cascales *et al.*, 2002).

***In vitro* binding assay**

HT-29 colonic epithelial cells were infected with above-mentioned strains, as well as with the WT *A. hydrophila* or WT with pBAD vector at a multiplicity of infection (MOI) of 10, and incubated at 4 or 37°C for 1 h. Unbound bacteria were aspirated, cells were washed four times with PBS and lysed with 0.1% TX-100, and various dilutions of the cell lysates were plated onto 1.5% (wt/vol) LB agar plates for determining colony forming units (CFUs) (Eichelberg and Galan, 2000, Sha *et al.*, 2004).

Complementation of the *A. hydrophila* SSU *aopB and tagA* mutants

The following primers were used to amplify the coding region of the *aopB* gene (1,188 bp) (Table 2.1) or the *tagA* gene (2,686 bp): *tagA*-N/*Eco*RI: 5'CCGGAATTCACAACCAGCTGGTATGGCAGG-3' and *tagA*-C/*Pvu*I: 5' ATCGATCGTCAGCGCGTCGCCAGCGGCATG-3'. The ends of the *aopB* DNA

fragment were made blunt by using a PCR polishing kit (Stratagene, La Jolla, CA). Subsequently, the blunt-ended DNA fragments were ligated to the pBR322 vector (GE, Piscataway, NJ) at the *Sca*I site, for *aopB*, or at the *Eco*RI/*Pvu*I sites for *tagA*. The recombinant plasmids (pBR*aopB* or pBR322*tagA*) were then electroporated into the *aopB* or *tagA* mutants of *A. hydrophila*. The pBR322 vector alone was also electroporated into WT *A. hydrophila*, as well as into the *aopB, act,* and *tagA* mutants (Sha *et al.*, 2002), and served as a proper negative control. The orientation of the *aopB* or *tagA* genes in the vector was examined by digestion of the recombinant plasmid with various restriction enzymes (e.g., *Eco*RI/*Pst*I, *Hin*dIII, *Eco*RI, *Pvu*I). The *aopB* and *tagA* genes were under the control of an ampicillin resistance gene promoter in the pBR322 vector.

Cloning of the *dam* gene under the *araBAD* promoter

To regulate *dam* gene expression, the pBAD-dam_{AhSSU} plasmid was generated using *dam*N-*Nco*I and *dam*C-*Pme*I primers (Table 2.2) by replacing the *Nco*I-*Pme*I fragment of the pBAD/Thio-E vector (Invitrogen) with the *dam* gene from *A. hydrophila* SSU under the vector's arabinose-inducible pBAD promoter. To induce expression of the gene from the plasmid, arabinose (0.2%) was added to the medium (Guzman *et al.*, 1995). This culture was referred to as the Dam-overproducing *A. hydrophila* strain. To construct the pBAD plasmid for use as a control, the pBAD/Thio-E vector was digested with *Nco*I and *Pme*I restriction endonucleases, treated with DNA polymerase I (Klenow fragment), and ligated. Both plasmids were then subjected to transformation/electroporation in *E. coli* JM109 and *A. hydrophila* SSU strains. The latter with pBAD vector alone was designated the *A. hydrophila* control strain.

Primer	Sequence (5′–3′)	Purpose
*dam*1	GGTGCATAAGGCGGATCGCA	PCR amplification of a portion (400 bp) of the *dam* gene from *A. hydrophila* SSU
*dam*2	TACATCCTTGCCGATATCAA	
*dam*3	GCGCCGAAGAGGATTGGGTCATCTATTGCGATCCG	DNA sequencing of *A. hydrophila* gDNA to obtain coding region of the *dam* gene
*dam*4	GGGTGGTTGTAAAGCCCGATCAGATCCGGATTGAT	
*dam*P1	CCATTTTAAAAAAGCGCGTGTTTTTTTCAT	Primer extension analysis
*dam*P2	CCCGGCCCATTTTAAAAAAGCGCGTGTTTT	
*dam*P3	TTTCCCCCGGCCCATTTTAAAAAAGCGCGT	
*dam*N-NdeI	GGAATTCCATATGAAAAAAACACGCGCTTTTTTAA	Cloning of the *dam* gene into pET-30a(+) vector
*dam*C-XhoI	CCGCTCGAGGCCGAGTGGCGCCAGTTCGGCGTCGCTCGGG	
*dam*N-NcoI	CATGCCATGGATGAAAAAAACACGCGCTTTTTTAAAATGG	Cloning of the *dam* gene into pBAD/Thio-E vector
*dam*C-PmeI	AGCTTTGTTTAAACGCCGAGTGGCGCCAGTTCGGCGTCGC	

Table 2.2 Primers used in sequencing, cloning, and mapping the promoter region of the dam gene as well as hyperexpression and purification of Dam
[a] Underlining indicates restriction endonuclease site.

Cytotoxicity and cell detachment assays

The WT *A. hydrophila* SSU, *act*, *aopB*, *act*/*aopB* mutants, complemented strains, or the Dam-overproducing strain and WT with pBAD vector were grown in 3 ml of LB medium in 50-ml disposable tubes and incubated at 37°C overnight with shaking (180 rpm). Supernatants and bacterial cells were separated and collected by centrifugation. The supernatants were filter sterilized (0.22 μm), and the bacterial cells were washed three times with PBS. RAW 264.7 murine macrophages or HT-29 colonic epithelial cell lines were seeded into 96-well plates (1 x10^5 cells/well) or chamber slides (1 x 10^6; Nalge Nunc International, Rochester, NY) and infected with the live bacterial cultures (as prepared above) at an MOI of 10. Host cells were also treated with 5 μl of bacterial culture supernatants. After incubation at 37°C for 2 to 4 h, the tissue culture medium was examined for the release of lactate dehydrogenase (LDH) enzyme using a CytoTox96 kit (Promega, Madison, WI).

Subsequent to bacterial infection, the morphology of the host cells was visualized in 20 to 25 fields by confocal microscopy using a Zeiss UV 510 Meta confocal microscope (Carl Zeiss, Inc., Thornwood, NY) (Sha *et al.*, 2004). The percentage of the

detached cells in the monolayer was calculated after staining with Giemsa stain. Briefly, the cells in the monolayer were fixed with 70% methanol for 5 min and then stained for 1 to 2 h. Subsequently, the monolayers were dissolved in a RIPA buffer (Ribardo *et al.*, 2002) and the plates were shaken gently for 1 h. The blue color that was released from the host cells was measured at 590 nm using a ELISA VERSAmax tunable microplate reader (Molecular Devices Corporation, Sunnyvale, CA) (Gentry and Dalrymple, 1980).

Motility assay

LB medium with 0.35% agar was used to characterize the motility phenotype of *A. hydrophila* control and Dam-overproducing strains. The overnight cultures grown in the presence of 0.2% arabinose were adjusted to the same optical density, and equal numbers of CFU (10^6) were stabbed into 0.35% LB agar plates containing 0.2% arabinose. Plates were incubated at 37°C overnight, and the motility was assayed by examining migration of bacteria through the agar from the center towards the periphery of the plate (Sha *et al.*, 2004).

Hemolytic activity

A. hydrophila SSU control and Dam-overproducing strains were grown overnight in LB medium with 0.2% arabinose and Ap (100 μg/ml). The culture supernatants were collected and treated with trypsin to convert the precursor form of T2SS-secreted Act to its mature form (Cascales *et al.*, 2002, Sha *et al.*, 2004, Fadl *et al.*, 2005). For the hemolytic activity assay, 100 μl of 1X (Dulbeco's PBS) DPBS was added to each of the wells of a 96-well microtiter plate. The above-treated culture supernatants were added to the first well in each row of the microtiter plate followed by serial twofold dilution and the addition of 100 μl of 3% rabbit erythrocytes (Colorado Serum Co., Denver, CO). The negative control included 1X DPBS and trypsin alone. The plate was incubated at 37°C for 1 h and observed for hemolytic activity associated with Act. The supernatants were taken from those wells that showed partial lysis of rabbit erythrocytes, and the hemoglobin release was recorded at 540 nm using a microplate ELISA reader. The

hemolytic titers were calculated as the value of the hemoglobin release multiplied by the dilution of the culture supernatant. The hemolytic units were reported per milliliter of culture filtrate per 10^8 CFU. For neutralization studies, culture filtrates were mixed with either preimmune or hyperimmune rabbit sera (laboratory stock; 1:10 dilution) containing antibodies to Act. After incubation at 37°C for 1 h, the hemolytic activity was measured.

Agrobacterium tumefaciens AHL bioassay (for QS)

The standard protocol using the *A. tumefaciens* reporter strain was followed. Briefly, overnight-grown *A. tumefaciens* (in LB medium at 30°C) was diluted to an OD_{600} of 0.2. An aliquot (2 ml) of diluted *A. tumefaciens* was mixed with 0.5-ml culture supernatants (filter sterilized) from *A. hydrophila* (WT, its mutants, and complemented strains) grown to various ODs (OD_{600}, 0.3 to 1.2) and incubated at 30°C for 5 h (Zhu *et al.*, 2000). This time allowed activation of *traR* by *A. hydrophila* lactones and initiation of transcription and amplification of the *traG:lacZ* reporter gene to produce detectable levels of β-galactosidase (Fig. 2.1). The pH of the supernatants was monitored, as the AHLs are rapidly inactivated under alkaline conditions (Yates *et al.*, 2002). The β-galactosidase activity was then measured according to the method of Miller and reported as Miller units (MU) (Miller, 1972). During the 5-h incubation, the *A. tumefaciens* grew from an OD_{600} of 0.2 to one of 0.6, which was still within the exponential growth phase. *A. tumefaciens* does not produce AHL-inactivating enzymes, such as lactonases, under these conditions (Zhang *et al.*, 2002).

ELISA

Aliquots (100 μl) of fivefold-diluted culture supernatants from the Dam-overproducing and the control strains of *A. hydrophila*, which were grown in the absence or presence of 0.2% arabinose overnight at 37°C (with shaking at 180 rpm), were used for coating the wells on the first row of 96-well microtiter plates in sodium bicarbonate buffer (pH 9.6). Each of the tested samples was done in triplicate and followed by twofold serial dilutions on the plates. After overnight incubation at 4°C, the plates

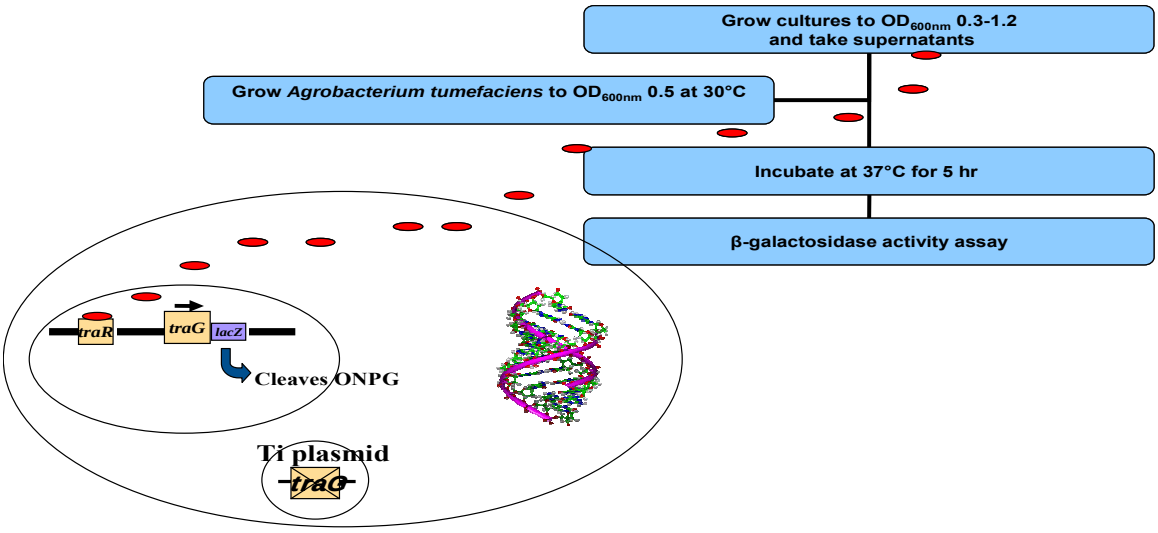

Fig. 2.1. *A. tumefaciens* A136 AHL bioassay.
A. tumefaciens A136 was used as a biosensor strain to detect AHLs. A136 is extremely sensitive to the 3-oxo- derivatives with *N*-acyl chain length from six to 12 carbons. This biosensor strain lacks the nonpaline Ti plasmid and thus does not produce its own lactones; however, it contains a plasmid with a *traR* and *traG*::*lacZ* transcriptional fusion and is therefore able to recognize exogenously supplied lactones from the test strain of *A. hydrophila* SSU (Zhu *et al.*, 2000). The assay was carried out as described above and the calculation shown here was used to quantify the production of lactones as Miller Units. T = Time of reaction in hours, from the time substrate was added to the development of yellow color and V = volume of reaction used in assay in mLs.

were washed three times with the wash buffer (PBS containing 0.05% Tween 20 and 0.1% bovine serum albumin [BSA]) and blocked with 2% BSA in PBS for 2 h at room temperature. After blocking, the plates were washed two times with wash buffer, and affinity-purified antibodies to Act (diluted 1:1,000) were added. After 1 h of incubation at room temperature, the plates were washed three times with wash buffer. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Southern Biotechnology Associates, Inc., Birmingham, AL) were added at a dilution of 1:2,000 for 1 h and the wells of the microtiter plates were washed four times with wash buffer,

followed by the addition of TMB substrate (3,3',5,5'-tetramethyl-benzidine; Sigma Chemical Co., St. Louis, MO) (Sha *et al.*, 2003, Erova *et al.*, 2006). The absorbance at OD_{370} was measured using the ELISA reader. The data were presented as ELISA titers/ml/10^8 CFU.

Primer Extension analysis

The primer extension system kit from Promega (Madison, WI) was used to determine the presumptive transcriptional start site of the *A. hydrophila dam* gene (dam_{AhSSU}) gene. Primers (10 pM each) complementary and downstream to the initiation codon of the dam_{AhSSU} gene (Table 2.2) were end labeled with 30 µCi of [γ-^{32}P]dATP. For each extension reaction mixture, 10 pM of labeled primer was mixed with 50 µg of RNA, heated to 58°C for 20 min, and cooled to room temperature. Subsequently, the mixture was incubated at 42°C with 1 U of avian myeloblastosis virus reverse transcriptase (Promega). After 30 min of incubation, the products were mixed with loading buffer, heated at 90°C for 10 min, and analyzed by running on a denaturing polyacrylamide gel containing 8% acrylamide (19:1 acrylamide-bis) and 7 M urea as described in the protocol (Promega). The length of the cDNA on the gel reflected the number of bases between the labeled nucleotide of the primer and the 5' end of the RNA and was compared with that of the labeled markers, which ranged in size from 24 to 311 bp.

Identification of the *tagA* gene on the chromosome of *A. hydrophila* SSU

The *tagA* gene in *A. hydrophila* was identified while searching for potential T3SS secreted effector proteins. One such effector protein (AexT) was recently identified in a fish isolate of *A. salmonicida* (Burr *et al.*, 2003). To evaluate any obvious differences in the protein profiling of *A. hydrophila* SSU and of *A. salmonicida* ATCC 49385 strain, the culture filtrates (1 liter) from these bacteria, grown at their optimal temperatures of 37°C and 26°C respectively, were concentrated by TCA (10% final concentration) precipitation. The precipitated proteins were resuspended in 300 µl of SDS-

polyacrylamide gel electrophoresis (PAGE) tracking buffer, and approximately 50-100 μg of total proteins were separated by SDS-12% PAGE (Rose *et al.*, 1989). The gels were then stained with either Coomassie Blue or SYPRO-Ruby (Bio-Rad, Hercules CA). A total of 15 bands that were unique in the supernatant of *A. hydrophila* were isolated from the stained gel, trypsin digested, and subjected to MS and tandem MS (MS-MS) analysis in the Proteomics Core Laboratory at UTMB, Galveston, TX.

One of the proteins of 85-90 kDa in size from *A. hydrophila* SSU exhibited homology (having a score of 0.025 based on MS analysis search using a Profound database [Genomic Solutions, Ann Arbor, MI]) to a T3SS effector protein homolog from the *hrp* (hypersensitivity and pathogenicity) T3SS gene cluster of *Erwinia amylovora* (AAF63400) (Wei *et al.*, 2000). To better characterize this 85-90 kDa polypeptide, the TCA precipitated proteins were separated by SDS-12% PAGE, transferred to PVDF membrane, and stained by Coomassie Blue (Chopra *et al.*, 1996, Sha *et al.*, 2003). Subsequently, the 85-90 kDa band was trypsin digested and the fragments were subjected to NH_2-terminal and internal sequencing at UTMB's Protein Chemistry Core Facility. Based on the sequence of three major tryptic digest peaks of this 85-90 kDa protein, a significant homology (64%) was noted within aa residues 109-130 of an unknown environmental protein obtained from the Sargasso Sea (EAI65408) (Venter *et al.*, 2004), based on a ClustalW alignment program. The homology with the *E. amylovora* T3SS effector protein homolog, however, was not as significant (22%) and was limited to a very small region of the 393 aa protein.

With a view that the 'unknown' protein could be of interest to us (as a potential T3SS effector protein from *A. hydrophila*), we designed specific 5' and 3' primers (AH5F 5'-ACCGCCTACTACCTGGAAGGAACGCCGGAGGAGGGG-3' and AH5R 5'-CCCCCTGGCTCAGTCGCAGGG-3'). These primers were designed based on the region in the unknown environmental sequence of highest homology beginning from aa residue 115 (a position that corresponded to nucleotide position 353 within the DNA sequence [based on reported 808 bp] of the unknown environmental gene) and the aa sequence obtained from two of the tryptic digest peaks of the 85 to 90-kDa polypeptide.

The 3' primer represented the terminal 21 bases of the 808-bp sequence of the unknown environmental gene. This strategy amplified, by polymerase chain reaction (PCR), a corresponding DNA fragment (455 bp) from the genomic DNA (gDNA) of *A. hydrophila* SSU, using conditions previously described (Sha *et al.*, 2005). DNA sequencing of the amplified fragment was performed using an automated DNA sequencer 373XL (Applied Biosystems, Inc.) in the Protein Chemistry Core Laboratory. Subsequently, our NCBI nucleic acid and Protein Blast searches demonstrated a significant identity and homology of this partial sequence to that of *stcE* of *E. coli* O157:H7 (AY714880).

Cloning and DNA sequence analysis of the *A. hydrophila* SSU *tagA* gene

By PCR amplification, using gDNA of *A. hydrophila* and primers designed to the 5' start and 3' stop coding region of the *E. coli* O157:H7 *stcE* gene, we first attempted to amplify the corresponding *tagA* gene from the gDNA of *A. hydrophila.* However, we were unsuccessful in amplifying a product, as it was later discovered that the 3' ends of *A. hydrophila tagA* and *E. coli* O157:H7 *stcE* genes were entirely different. Consequently, different strategies were used to obtain a full-length sequence of the *tagA* gene from *A. hydrophila*. Within the 455 bp of the sequenced *tagA* gene, a single *Bam*HI restriction enzyme site existed. Genomic DNA of *A. hydrophila* SSU was digested with the *Bam*HI enzyme and Southern blot hybridization using a [α-^{32}P]dCTP- labeled partial *tagA* gene (455 bp) as a probe under high stringency conditions, was performed (Sha *et al.*, 2002, Sha *et al.*, 2005). Briefly, the *Bam*HI-digested gDNA from WT *A. hydrophila* SSU (10 μg) was subjected to 0.8% agarose gel electrophoresis and Southern blot analysis (Sha *et al.*, 2002). Next, the digested DNA was transferred to a nylon membrane and baked at 80°C for 2 h. The conditions for prehybridization, hybridization, and washings of the filters were previously described (Sha *et al.*, 2002). Two bands, of sizes 2.3 kb and 4.4 kb, reacted with the *tagA* gene probe. Subsequently, DNA fragments within these size ranges were recovered from the agarose gel, purified, and cloned into a pBluescript vector at the *Bam*HI restriction enzyme site. The resulting plasmid libraries in *E. coli* DH5α were plated (150-200 colonies per LB agar plate with Ap [100 μg/ml]).

Colonies from the plates were transferred to nylon filters (Invitrogen, Gaithersburg, MD), which were then prehybridized and hybridized (Sha *et al.*, 2005) using the [α-^{32} P]dCTP-labeled *tagA* gene probe. After washing, the filters were exposed to X-ray films at -70°C for 2-12 h. The plasmid DNA isolated from the pure positive clones (after 2-3 rounds of purification) was digested with the *Bam*HI enzyme for correct identification of the clones. Both the 2.3- and 4.4-kb cloned DNA fragments were sequenced. An existing fosmid library that was prepared using gDNA of *A. hydrophila* SSU was also screened to obtain the entire sequence of the *tagA* gene (Sha *et al.*, 2005). The fosmid library was screened with the labeled *tagA* gene probe (455 bp) of *A. hydrophila* as described above. Finally, DNA isolated from the positive fosmid colonies was used as a template for DNA sequencing. The DNA sequence data were analyzed and compared with the databases using an online BCM Search Launcher, and the ClustalW program.

Purification of rDam and rTagA

PCR amplified from *A. hydrophila* SSU gDNA, the *dam* or *tagA* gene were cloned into a pET-30a(+) T7 promoter-based expression vector (Novagen, San Diego, CA) using primers with *Nde*I and *Xho*I and *Nde*I and *Bgl*II restriction enzyme sites, respectively. These primers had the following sequences: *dam*N-*Nde*I GGAATTCCATATGAAAAAAACACGCGCTTTTTTAA and *dam*C-*Xho*I CCGCTCGAGGCCGAGTGGCGCCAGTTCGGCGTCGCTCGGG or TagA5/*Nde*I: 5'-GGAATTCCATATGACCACATGCACCACACG-3' and TagA3/*Bgl*II: 5'-GAAGATCTTGCGCGTCGCCAGCGGCATGC-3'. To overexpress the *dam* or *tagA* gene in *E. coli* with a histidine (His) tag, the recombinant plasmid was transformed into the *E. coli* DE3 strain which harbored the T7 RNA polymerase gene on the chromosome. The *E. coli* (pET-30a-*dam*) culture was grown in 300 ml of the LB medium with Km (30 μg/ml) to an OD_{600} of 0.4-0.5 before induction with a final concentration of 1 mM IPTG for 3 h. The Dam-overproducing bacterial cells were harvested, resuspended in 10 ml of appropriate buffer (Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 5% glycerol), and disrupted by sonication. The cell lysate was passed through ProBond resin (ProBond,

Invitrogen, Carlsbad, CA) (3-ml bed volume in a 5-ml column) charged with nickel according to the manufacturer's recommendations. The resin was washed with a five-column volume of the wash buffer, followed by elution of the proteins using different concentrations (0.25 to 1 M) of imidazole. The purity of Dam was examined by SDS–12% PAGE gel electrophoresis followed by Coomassie blue staining of the gel.

As majority of the rTagA was present in the bacterial membrane fraction, a high concentration of urea was used to solubilize and purify the protein. Briefly, the bacterial cells were harvested, resuspended in 13 ml of appropriate buffer (8 M Urea, 20 mM NaH_2PO_4 and 500 mM NaCl, pH 7.8) and disrupted by sonication. The cell lysates were passed through the nickel-charged resin column (2-ml bed volume in a 10-ml column). The resin was washed with 3- column volumes of the wash buffer, and the TagA protein was eluted in 1-ml fractions (a total of 5 fractions) with a buffer containing 8 M urea, 20 mM NaH_2PO_4 and 500 mM NaCl, pH 4.0. The purity of the TagA protein from fractions 1-5 was examined by SDS-12% PAGE, followed by Coomassie Blue or SYPRO-Ruby staining of the gel. The eluted fractions 3 and 4, containing purified rTagA, were separately dialyzed against PBS and examined for enzymatic activity by testing for cleavage of C1-INH, which was obtained from Cortex Biochem, San Leandro, CA.

Proteolysis of C1-INH by TagA

Purified C1-INH (12 μg) was incubated with 1 μg of purified rTagA fusion protein in 120 μl of the AD buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.01% Tween-20) at room temperature. Subsequently, 20 μl of the reaction mixture was removed at various time points and subjected to Western blot analysis. To test cleavage of C1-INH by native TagA, WT *A. hydrophila* SSU, *tagA* mutant and complemented strains were grown to an OD_{600} of 0.5, washed once with PBS and resuspended in a final volume of 200 μl of AD buffer. Cells were either concentrated from 1 ml starting volume down to 200 μl of AD buffer (10^9 cells) or were diluted in a volume of 200 μl having 10^6 bacterial cells. This mixture was then incubated with 2 μg of

C1-INH for various time points 1 (0 or 8 h) upon which 20 µl of the reaction mixtures were removed and subjected to Western blot analysis.

Western blot analysis

Antibodies to C1-INH were purchased from Cederlane (Ontario, Canada) and antibodies to TagA were graciously gifted from Dr. Rodney Welch (University of Wisconsin, Madison WI). Western blot analysis was performed according to established procedures (Rose *et al.*, 1989) with slight modifications. Briefly, equal amounts of total protein were loaded and separated on SDS-10% PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and washed in 1X Tween (0.1%)-Tris-buffered saline (TTBS) three times for 5 min each. Primary antibodies diluted 1:1000 or 1:5000 in 5% milk or BSA (prepared in 1X TTBS) were allowed to incubate overnight at 4^{o}C. After washing, HRP-conjugated secondary antibody (Cell Signaling Technology) was diluted 1:20,000 in 5% milk and applied to membranes. Subsequently, membranes were washed and a chemiluminescence substrate (Pierce Biotechnology, Rockford IL) was applied and allowed to incubate at room temperature for 5 min, before exposing the membranes to X-ray films.

Lysis of sheep erythrocytes by TagA

Increasing concentrations of C1-INH (2, 8, and 16 µg) were mixed with 1 µg of rTagA, heated rTagA (80°C for 10 min to inactivate TagA), or elastase (as a negative control) from *P. aeruginosa* (Calbiochem, San Diego, CA) in a total volume of 149 µl AD buffer overnight at room temperature. The next day, sheep erythrocytes (Colorado Serum Co., Denver, CO) were opsonized with an anti-sheep red blood cell (RBC) antibody (Rockland Immunochemicals, Inc. Gilbertsville, PA) for 10 min. Human serum (0.5%, Cambrex, Baltimore MA) was mixed with the opsonized erythrocytes (10^7 cells) and added to the C1-INH/rTagA (or elastase) reaction mixture from overnight incubation in a total volume of 200 µl. The reaction was allowed to proceed for 1 h at 37°C. Next, 1 ml of AD buffer plus 10 mM EDTA was added to stop complement activity. Erythrocytes

were pelleted and the OD_{412} of the supernatant was measured using the VERSAmax microplate reader.

Protease activity

An aliquot (200 µl) of overnight culture filtrates (in the presence of 0.2% arabinose) from *A. hydrophila* control and Dam-overproducing strains was added to disposable 6-ml, snap-cap tubes which contained 800 µl of the DPBS and 5 mg of Hide azure powder substrate (Calbiochem, La Jolla, CA). The tubes were incubated in a shaker incubator at 37°C for 1 to 3 h. As the proteinase in the culture filtrates catalyzed the substrate, blue color was released from the substrate and was quantified at OD_{595}. The proteinase activity was calculated per ml of culture filtrate per 10^8 CFU. The substrate incubated with the LB medium alone served as a negative control.

To further test the protease activity of rTagA, a slight modification of the method described by Erova *et al*. was used (Erova *et al.*, 2006). Briefly, increasing concentrations of rTagA (25 ng, 50 ng, 100 ng, and 1µg), heated rTagA (1 µg-heated to 80°C for 10 min) or rTagA (1 µg) neutralized with α-StcE antibodies (1:20 dilution in PBS) were added to 500 µl of 1X DPBS and 5 mg of Hide azure powder substrate (Calbiochem, La Jolla, CA). The tubes were incubated in a shaker incubator at 37°C for 2.5 to 18 h. Blue color was quantified at OD_{595}. The substrate incubated with 1 µg of elastase from *P. aeruginosa* (Calbiochem, San Diego, CA) served as a positive control while substrate with PBS alone was used as negative control.

Serum Resistance

E. coli DH5α was grown to an OD_{600} of 0.5, washed once with PBS and resuspended in an equivalent volume of AD buffer. An aliquot (20 µl) of bacteria was added to the overnight room temperature incubation reaction of 8 µg C1-INH untreated or treated with 1 µg rTagA in a total volume of 176 µl of AD buffer. Human serum was then added to the bacteria and C1-INH/rTagA mixture to a final concentration of 2%. The reactions were incubated at 37°C for 1 h. The AD buffer was then added to 1 ml final

volume plus 10 mM EDTA to stop complement activity. A 10-fold serial dilution of this reaction mixture was plated on LB agar plates and the percentage of surviving bacteria was determined by dividing colony forming units (CFUs) by the total number of bacteria after 1 h in the absence of serum. As *A. hydrophila* SSU, when compared to *E. coli*, is naturally more serum resistant (Aguilar *et al.*, 1999), a modification of this method was used to determine increased serum resistance of *A. hydrophila* due to the presence of TagA. Both WT *A. hydrophila* and its *tagA* mutant were grown to an OD_{600} of 0.5. The bacterial cells were washed and resuspended in AD buffer and 20 µl of various bacterial strains was added to a reaction of AD buffer and 50% human serum. The reaction mixtures were incubated at 37°C for 3 h. The AD buffer was again added to a final volume of 1 ml along with 10 mM EDTA. A 10-fold serial dilution was plated out on LB-Rif agar plates and the percentage of surviving bacteria was determined as described above.

Sandwich Western blot analysis

Western blot analysis was performed as previously described (Ribardo *et al.*, 2002), with slight modification. Briefly, equal amounts (1 µg) of total protein (rTagA, C1-INH or cholera toxin) were loaded and separated on SDS 10%-PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and washed in 1X TTBS three times for 5 min each. After blocking, membranes were incubated with either rTagA (1 µg/ml in TTBS) or C1-INH (1 µg/ml in TTBS) at 4°C for 24 h with gentle shaking and then washed three times in TTBS. Primary antibodies were diluted 1:1000 (α-TagA) or 1:5000 (α-C1-INH) in 5% BSA (prepared in 1X TTBS) and allowed to incubate at room temperature for 2 h. After washing, HRP-conjugated secondary antibody (Santa Cruz) was diluted 1:20,000 in 5% milk and applied to membranes. Subsequently, membranes were washed and the chemiluminescence substrate (Pierce Biotechnology, Rockford IL) was applied and allowed to incubate at room temperature for 5 min, before exposing the membranes to X-ray films.

Binding of C1-INH to TagA on bacterial cell surface

Briefly, bacterial cells (WT *A. hydrophila*, its *tagA* mutant, and the *tagA* complemented strain) grown to an OD_{600} of 0.5 were washed with PBS and treated with 2 μg/ml of C1-INH for 2 h. Cells were then washed thoroughly and resuspended in PBS to a final concentration of 10^7 cells/20 μl. A 20-μl drop of bacterial cells was placed on a glass slide and allowed to air-dry. The cells were then fixed with 4% paraformaldehyde for 20 min, washed with PBS, and treated with the appropriate primary antibodies (α-StcE [diluted 1:500 in PBS], α-C1-INH [1:500 in PBS], or a combination of both) for 1 h. After washing 2X in PBS, the cells were incubated with fluorescein-conjugated secondary antibodies, purchased from Molecular Probes, Eugene, CA (Alexa-Fluor α-rabbit [1:100 in PBS], Texas Red α-goat [1:100 in PBS] or a combination of both) for 1 h. Cells were washed again and 5 μl of DAPI (Vector Laboratories Inc., Burlingame, CA) was added to stain the bacterial cell nuclei. Fluorescence labeling was visualized using the Zeiss 510 UV meta confocal microscope with an objective lens providing a total magnification of 2520X.

Animal Experiments

Groups of 10 Swiss Webster mice (Taconic Farms, CA) were infected by the intraperitoneal (i.p.) route with 5 x 10^7 to 1 x 10^8 bacteria (WT or *act*, *aopB*, or *act/aopB* mutants) or 4 x 10^7 bacteria (WT or *tagA* mutant) in accordance with approved animal care protocols. Deaths were recorded for 14-16 days postinfection. The bacterial doses used represented approximately two-three 50% lethal doses of WT *A. hydrophila* (Xu *et al.*, 1998). For animal studies, statistical analyses were performed using Fisher's exact test.

Statistics

All of the experiments were performed at least in triplicate and representative data are presented. Where applicable, the data were plotted as arithmetic mean ± standard de*via*tion, and Student's t test ($P \leq 0.05$) was used for data analysis.

CHAPTER 3:
IDENTIFICATION AND CHARACTERIZATION OF A TYPE 3 SECRETION SYSTEM (T3SS) IN *A. HYDROPHILA* AND ROLE OF QUORUM SENSING (QS) IN REGULATING BACTERIAL VIRULENCE[1,2]

INTRODUCTION

Recently, genes for a T3SS were identified in fish isolates of *Aeromonas* species (Burr *et al.*, 2002, Burr *et al.*, 2003, Burr *et al.*, 2003, Stuber *et al.*, 2003, Vilches *et al.*, 2004, Yu *et al.*, 2004). To date, more than 30 different effectors have been described as being translocated through the T3SS of several bacteria (Hueck, 1998, Foultier *et al.*, 2002). These effectors have been shown to possess multiple biological functions, such as cytoskeletal alterations and activation of intracellular signaling cascades within the host cells (Hueck, 1998, Foultier *et al.*, 2002, Viboud and Bliska, 2005). The T3SS was initially discovered in human and plant pathogens; however, the presence of a T3SS was recently reported in some endosymbionts and invertebrate pathogens, such as for the insect endosymbiont *Sodalis glossinidius* and *Photorhabdus luminescens*, an endosymbioant of nematodes pathogenic to insects (Freiberg *et al.*, 1997, Ffrench-

[1]Reproduced with permission from the following source: Infection and Immunity (http://www.iai.asm.org); Sha J.^, Pillai L^., (^ equal contribution) Fadl A.A., Galindo C.L., Erova T.E., and Chopra, A. K. 2005. The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. *Infect. Immun*. 73:6446-57.

[2] **Author contributions:** Lakshmi Pillai was involved in identifying and isolating T3SS needle structures by EM, performing QS assay using *A. tuemfaciens* reporter strain, helping Dr. Amin A. Fadl (McLaughlin postdoctoral fellow) in *in vivo* experiments and in writing portions of Introduction section and QS Results and Discussion section of the manuscript. Dr. Jian Sha (Assistant Professor) was involved in sequencing T3SS from SSU, as well as in preparing *aopB* and *act/aopB* isogenic mutants. Dr. Tatiana E. Erova (postdoctoral fellow) prepared the complemented strains of these mutants. Drs. J. Sha and A.A. Fadl were involved in mechanistic studies involving the mutants (i.e. LDH release assays, confocal microscopy to assess alterations in cell morphology, and membrane integrity and binding assays). Dr. J. Sha was involved in writing the final version of manuscript and submission to journal.

Constant *et al.*, 2000, Dale *et al.*, 2001, Foultier *et al.*, 2002). It appears that one of the functions of the T3SS might be to allow bacteria to establish transkingdom cell-cell communications, thus facilitating persistence and replication of the organism in the host (Foultier *et al.*, 2002).

In this study, we identified a T3SS gene cluster from the clinical isolate SSU of *A. hydrophila*. The *aopB* gene of the T3SS is involved in the formation of a needle complex and has been reported to be essential in inducing the T3SS-associated cytotoxicity in *in vitro* cell culture models. Further, the *aopB* mutants were noted to be less virulent in the animal models (Burr *et al.*, 2003, Vilches *et al.*, 2004, Yu *et al.*, 2004). Likewise, by preparing an *act* mutant, we demonstrated that the T2SS-secreted Act also played an important role in *Aeromonas* infections (Xu *et al.*, 1998). Therefore, we inactivated the *aopB* gene from the WT *A. hydrophila* SSU, as well as from a previously characterized *act*-minus mutant of *A. hydrophila* SSU (Xu *et al.*, 1998), to evaluate their relative contributions in the pathogenesis of *Aeromonas* infections. By using *aopB*, *act*, and *act/aopB* mutants of *A. hydrophila*, we demonstrated a role for the T3SS and Act in host cell cytotoxicity and animal lethality. Furthermore, we found a unique correlation of the T3SS and Act with AHL production in this bacterium. The effects of T3SS and Act deletion on cytotoxicity and lactone production could be complemented, indicating specific effects of the mutations on bacterial virulence.

RESULTS AND DISCUSSION

The T3SS of *A. hydrophila* SSU

An *Aeromonas* T3SS was first reported recently in the fish pathogen *A. salmonicida* (Burr *et al.*, 2002, Burr *et al.*, 2003, Burr *et al.*, 2003, Stuber *et al.*, 2003). We simultaneously and independently amplified, based on the DNA sequence of the *Y. enterocolitica yscV* gene, the corresponding gene (*ascV*) from a human diarrheal isolate SSU of *A. hydrophila*. Then, by using chromosomal DNA sequencing and screening of a fosmid library, we obtained the entire T3SS sequence of *A. hydrophila* SSU. A total of

750 fosmid clones were screened by colony blot hybridization, and we obtained eight positive clones that reacted with the *ascV* gene probe. The average length of the inserted DNA fragment in the fosmid clones was 25 kb. The entire T3SS operon contained 26,855 bp, encoding 35 genes, compared to only 20 genes identified for the *A. salmonicida* T3SS (Burr *et al.*, 2003) (Table 3.1). While we were sequencing and characterizing the T3SS from the human diarrheal isolate of *A. hydrophila*, a T3SS was reported for the *A. hydrophila* fish isolate AH1(Yu *et al.*, 2004). However, our SSU clinical isolate contained 10 additional T3SS genes, compared to the AH1 strain, indicating that these 10 additional genes might not exist in the AH1 strain or that the complete operon was not cloned and sequenced from this AH1 strain. Conservation was noted in the sequences of the T3SS genes of *A. hydrophila* SSU, the fish isolate AH1, *A. salmonicida*, and *Y. enterocolitica*; however, the sequences differed significantly for certain genes (Table 3.1). For example, the *aopB* and *aopD* genes, the products of which form the T3SS translocation apparatus (Burr *et al.*, 2003, Yu *et al.*, 2004), shared only 50 to 53% identity between the *A. hydrophila* strains AH1 and SSU. These differences might result in a more efficient injection of effector proteins into the respective fish and human host cells.

Interestingly, the *acrV* gene of *A. hydrophila* SSU was 147 to 165 bp shorter than that of *A. salmonicida* or *A. hydrophila* AH1. DNA sequence alignment revealed several deletions in the middle part of the gene, which resulted in a product that was 41 to 49 aa shorter. A similar deletion (41 aa) was found in LcrV, the *Y. enterocolitica* homolog of AcrV. The presence or absence of this stretch of aa residues in different *Aeromonas* strains might alter the function of AcrV in fish versus human hosts and requires further investigation. The T3SS of *A. hydrophila* SSU possessed two genes, *hscY/exsC* and *ascZ*/*exsD*, that had no homologs in the well-characterized *Y. enterocolitica* T3SS but were present in the T3SS of *P. aeruginosa* (Frank and Iglewski, 1991). The *exsD* gene product is an antitranscriptional activator of ExsA (Dasgupta *et al.*, 2004), while the *exsC* gene encodes exoenzyme S synthesis protein C precursor, which is involved in the synthesis of exoenzyme family proteins in *P. aeruginosa* (Frank and Iglewski, 1991). The

hscY/exsC and *ascZ*/*exsD* may function similarly in both *A. hydrophila* SSU and *P. aeruginosa*.

As our molecular characterization of the *A. hydrophila* SSU T3SS was near completion, a T3SS gene sequence from a fish isolate of *A. hydrophila* AH3 was submitted to GenBank (accession number AY528667) and subsequently published (Vilches *et al.*, 2004). The T3SS operon from both the SSU and AH3 strains contained 35 genes (Table 3.1). The general identities between various genes of the AH3 and SSU T3SS operons were 28 to 89% at the nucleotide level, and at the aa level, homologies of 38 to 97% were recorded (Table 3.1). When we compared the SSU to the AH3 strain, a 49-amino-acid deletion was found in AcrV of strain SSU, which was similar to the deletion in AcrV of *A. salmonicida* and *A. hydrophila* AH1. The sequence diversity in the gene encoding a 359-amino-acid-residue-long AscP was maximal between the SSU and AH3 strains (with only 38% homology at the aa level) (Table 3.1). Further comparison of the two strains showed that there were three deletions, of 11, 16, and 28 aa residues each, in the central part of AscP (between aa residues 187 and 228) in SSU, again highlighting a divergence in sequence between the T3SS of a fish and human isolate of *A. hydrophila*.

Recently, a dual function was proposed for YscP, the homolog of AscP, in *Y. enterocolitica* (Journet *et al.*, 2003, Agrain *et al.*, 2005). First, YscP may act as a molecular ruler that determines the length of the T3SS needle (Journet *et al.*, 2003), whereby the N and C termini of YscP anchor the central potion of the ruler (ruler domain), with the C terminus of YscP being attached to the basal body and the N terminus to the growing tip of the needle (Journet *et al.*, 2003). Deletion of either the N or C terminus of YscP has been shown to result in a loss of control in needle length. However, deletion of the ruler domain resulted in shorter needles at a rate of 1.9 Å per aa residue (Journet *et al.*, 2003), which could lead to less efficient injection of effectors into host cells (Mota *et al.*, 2005). We noticed that AscP in the clinical isolate SSU of *A. hydrophila* was 8 and 48 aa residues shorter in the ruler domain than AscP in the fish isolates AH1 and AH3, respectively. This size difference in the central ruler part could potentially lead to a shorter needle in isolate SSU that has less injection efficiency than

those in fish isolates AH1 and AH3. The effect on bacterial virulence of a shorter T3SS needle is worth pursuing and will be studied in our future research. Interestingly, AscP in *A. salmonicida* had the shortest needle of all *A. hydrophila* strains, with a 39- to 68-amino-acid-residue deletion in its ruler domain.

The second proposed function of YscP is that it may act as a type 3 secretion substrate specificity switch (T3S4) (Agrain *et al.*, 2005). In this model, the N terminus of YscP is believed to be attached to the growing needle, while the C terminus, containing the T3S4 domain, stays in the secretion apparatus and switches the substrate specificity from YscF to Yops by interacting with YscU in *Y. enterocolitica* (Agrain *et al.*, 2005). The T3S4 domain of YscP could be replaced by the T3S4 domain of AscP (*A. salmonicida*) or PscP (*P. aeruginosa*), indicating that the T3S4 domain is functional in *A. salmonicida* as well as in *P. aeruginosa* (Agrain *et al.*, 2005). Although in all of the above-mentioned *Aeromonas* strains the homology of AscP is only 38 to 57% (Table 3.1), the T3S4 domain is conserved with a homology of 67 to 85% at the aa level, indicating evolutionary relatedness.

Interestingly, DNA sequences flanking the T3SS operon were entirely different in the AH3 and SSU strains, indicating that the chromosomal location of the T3SS differed between *A. hydrophila* isolates of fish and human origin. More importantly, the T3SS of *A. salmonicida* was located on a plasmid, while it was found on the chromosome in *A. hydrophila* isolates (Burr *et al.*, 2003, Stuber *et al.*, 2003, Vilches *et al.*, 2004, Sha *et al.*, 2005).

Identification of the T3SS needle structure in membrane of *A. hydrophila* SSU

To investigate the membrane location and morphological features of the T3SS machinery, we examined the bacterial envelope of *A. hydrophila* by transmission electron microscopy (TEM). As shown in Fig. 3.1A, a needle complex was visible on the cell surface. The base of the structure was on the plane of the cytoplasmic membrane and extended to the outer membrane, where it was connected to a thinner structure (a needle).

Comparison of T3SS of *A. hydrophila* SSU and *Y. enterocolitica*

Homology % (Nucleotide /Amino acid) in:						Putative function[a]
A. hydrophila			*A. salmonicida* (fish isolate)	*Y. enterocolitica*		
SSU (Human isolate)	AH1 (fish isolates)	AH3				
ascU	84/91	88/91	87/91	*yscU*	66/70	Regulation of secretion
ascT	85/91	86/87	86/86	*yscT*	62/70	Intracellular trafficking and secretion
ascS	88/94	89/97	90/95	*yucS*	67/75	Intracellular trafficking and secretion
ascR	90/97	88/96	92/96	*yscR*	71/83	Intracellular trafficking and secretion
ascQ	82/84	76/74	76/75	*yscQ*	53/45	Intracellular trafficking and secretion
ascP	70/57	28/38	41/45	*yscP*	13/26	Regulation of secretion, molecular ruler
ascO	84/86	83/81	82/81	*yscO*	56/49	Regulation of secretion
ascN	86/96	88/96	88/96	*yscN*	71/86	ATP synthase
aopN	82/86	82/89	81/83	*yopN*	50/48	Regulation of translocation
acr1	87/90	82/88	84/89	*tyeA*	58/54	Translocation apparatus
acr2	80/86	83/86	83/87	*sycN*	60/56	Chaperone
ascX	79/84	77/80	79/80	*yscX*	57/47	Type III secretion apparatus
ascY	81/82	77/77	78/76	*yscY*	56/52	Type III secretion apparatus
ascV	86/95	87/95	88/94	*yscV*	71/83	Type III secretion apparatus
acrR	57/52	83/81	84/87	*lcrR*	45/52	Unknown
acrG	57/43	84/85	82/82	*lcrG*	56/41	Regulation of low-calcium response
acrV	17/38	79/76	81/77	*lcrV*	29/41	Protective antigen, anti-host factor
acrH	68/61	89/92	88/92	*sycD*	61/56	Chaperone
aopB	50/53	72/65	71/65	*yopB*	46/44	Pore-forming translocase, induction of apoptosis/programmed necrosi
aopD	53/57	75/70	71/61	*yopD*	43/40	Pore-forming translocase
hscY/exsC	79/83	76/80				Exoenzyme S synthesis protein C precursor
ascW/exsB	75/72	67/66		*virG*	7/33	Regulation of secretion
ascA/exsA	86/95	79/85		*virF*	59/59	Transcriptional activator
ascZ/exsD	78/78	68/65				Anti-transcriptional activator ExsA
ascB	77/82	75/78		*yscB*	47/44	Unknown
ascC		79/89		*yscC*	62/70	Outer membrane protein for transporting
ascD		73/74		*yscD*	56/44	Unknown
ascE		76/74		*yscE*	44/28	Unknown
ascF		70/75		*yscF*	43/56	Pore-forming translocase
ascG		73/78		*yscG*	43/53	Unknown
ascH		65/55		*yscH*	28/32	Unknown
ascI		82/82		*yscI*	58/50	Unknown
ascJ		81/91		*yscJ*	62/73	Intracellular trafficking and secretion
ascK		70/70		*yscK*	53/47	Unknown
ascL		74/83		*yscL*	62/69	Intracellular trafficking and secretion

Table 3.1. Homology of the T3SS genes in different *Aeromonas* strains and *Y. enterocolitica*

[a.] Based on Blast search in GenBank and cited literature (Sha *et al.*, 2005).

This type of needle structure is typical of those seen in *S.* Typhimurium, *S. flexneri* and EPEC (Kubori *et al.*, 1998, Sekiya *et al.*, 2001, Tamano *et al.*, 2002).

To further clarify the identity of the components of the *A. hydrophila* T3SS, we extracted the needle-like structures from the envelope and subjected them to CsCl density gradient centrifugation (Kubori *et al.*, 1998). The T3SS was present mainly in fractions 2 to 4 (Fig. 3.1B). In the TEM findings, we observed *A. hydrophila* needle structures to be similar to those of the EPEC T3SS (Sekiya *et al.*, 2001), in that the needle was long (≥ 100 nm-300 nm) and relatively thick. These needle structures were distinguishable from bacterial flagella in that the needles were thinner and shorter than the flagellar filaments.

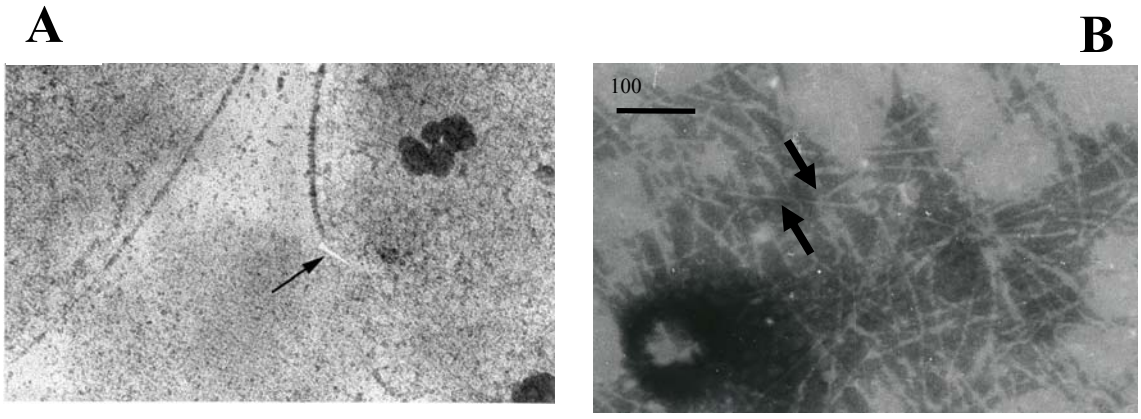

Fig. 3.1. T3SS needle structure of *A. hydrophila* SSU
Bacterial cells were osmotically shocked and needles isolated as previously described (Kubori *et al.*, 1992, 1998). (A) The electron micrograph (magnification 94,000X) indicates that the T3SS machinery is composed of a needle and a basal portion, with respective lengths of 62.5 ± 3 and 20 ± 1 nm. At least 20-25 microscopic fields were examined, and a representative field is shown. (B) An electron micrograph (magnification 141,000X) of negatively stained, isolated needle structures from *A. hydrophila*, purified in a 30% CsCl density gradient. Fraction 4 is shown (Bar = 100 nm). Arrows indicate the needle portion of a representative isolated needle structure.

Characterization of aopB and act/aopBmutants of *A. hydrophila*

Since homologs of AopB and AopD form the translocation apparatuses of other T3SS needle complexes (Burr *et al.*, 2003, Vilches *et al.*, 2004, Yu *et al.*, 2004), mutations in the *aopB* and *aopD* genes could block translocation of *A. hydrophila* effector

proteins. Therefore, we generated *aopB* deletion mutants in WT and *act*-minus strains of *A. hydrophila via* homologous recombination and confirmed their identity by Southern blot analysis. Briefly, digested chromosomal DNA from the *aopB* mutant reacted with the Sm/Sp^r gene cassette probe, but not with the probes to the *aopB* and suicide vector pDMS197, indicating the replacement of the *aopB* gene with the Sm/Sp^r gene cassette on the genome of this mutant. Further, an inability of the digested genomic DNA of this mutant to hybridize with the suicide vector probe indicated the loss of the suicide vector as the result of double-crossover homologous recombination (data not shown).

The growth rates of mutants (*aopB* and *act/aopB*) and their binding abilities for HT-29 cells were tested and compared to those of the WT bacterium. The mutants behaved very similar to the WT bacterium in terms of their growth rates and binding to the host cells. Nor was the membrane integrity of the mutants affected, as measured by their ability to grow in the presence of different concentrations of TX-100 and SDS as well as vancomycin. Moreover, the release of periplasmic RNase I was unaltered in the *aopB* and *act/aopB* mutants compared to that of the WT *A. hydrophila* SSU (data not shown). These findings showed that the membrane integrity of the mutants remained intact when the genes encoding *act* and *aopB* were deleted.

T3SS-associated cytotoxicity of *A. hydrophila* SSU

To test the function of the T3SS, RAW 264.7 murine macrophage cells were infected with WT *A. hydrophila*, its various mutants, or complemented strains, and cytotoxicity assays were performed. An LDH release of 18 to 20% was noted (following comparison to the positive control provided in the kit and adjusted to 100% cell lysis) during the initial phase (2 h) of infection with the WT bacterium or *act* mutant (Fig. 3.2A). In contrast, infection with *aopB* and *act/aopB* mutants resulted in 79% and 71% decreases in LDH release, respectively, compared to that in cells infected with the WT *A. hydrophila*, and these decreases were statistically significant at *P* values of 0.0004 and 0.0008 (Fig. 3.2A). The effects on cytotoxicity at 2 h, resulting from mutation in the *aopB* gene, could be complemented (Fig. 3.2A). The increase in cytotoxicity associated with the *aopB*-complemented strain (*aopB*$^+$) was significantly higher than that of the *aopB*

mutant ($P = 0.0002$) but was not significant when the LDH release was compared between the WT and *aopB*$^+$ strains ($P = 0.6$) and between WT and the *act* mutant at 2 h ($P = 0.3$).

More interestingly, after 4 h of infection, macrophage release of LDH was 8.1 times higher with the *aopB* mutant (with intact *act* gene) compared to findings after 2 h of infection with this mutant ($P = 0.0002$) (Fig. 3.2A). This higher LDH release after 4 h of infection with the *aopB* mutant may be due to the presence of Act, as no significant increase in LDH release occurred between 2 and 4 h of infection with the *act/aopB* mutant ($P = 0.7$), compared with the WT bacterium ($P < 0.0001$) (Fig. 3.2A). Act's effect on cytotoxicity at 4 h was almost fully restored when the *act* mutant was complemented with the *act* gene (*act*$^+$), reaching the level of that seen with WT *A. hydrophila* ($P = 0.7$). The increase in LDH release between the *act* mutant and *act*$^+$ strain at 4 h was statistically significant ($P = 0.003$).

Compared to that for the WT bacterium, the decrease in LDH release with the *act* mutant at 4 h was significant ($P = 0.004$); however, this decrease for the *aopB* mutant, compared to the rate in WT *A. hydrophila*, was not significant ($P = 0.1$), signifying the importance of Act in cytotoxicity. Between 2 and 4 h, the LDH release associated with the WT bacterium increased from 20% to 40% ($P = 0.0009$), while this increase for the *act* mutant was not significant ($P = 0.06$).

These LDH release data indicated that AopB played an important role in cytotoxicity during early (2-h) bacterial-host cell interactions (Burr *et al.*, 2003, Vilches *et al.*, 2004, Sha *et al.*, 2005), whereas Act's contribution towards cell toxicity became prominent only by 4 h postinfection. This was not surprising, as our previous studies demonstrated that Act could be detected in the culture medium only after 3 to 4 h of growth (Sha *et al.*, 2004). However, it is also plausible that the T3SS and Act might affect host cells in different ways with different time courses, which will need further investigation.

When HT-29 or RAW 264.7 cells were treated with overnight bacterial culture supernatants from the *act* mutant, LDH release was inhibited in both types of host cells, compared to when culture supernatants from WT *A. hydrophila* or *aopB* mutant were used, with *P* values of 0.008 for HT-29 and 0.001 for RAW cells, respectively (Fig. 3.2B and C). Similarly, bacterial culture supernatants from the *act*/*aopB* mutant were unable to induce any LDH release in macrophages or HT-29 cells over the same time period of 4 h ($P = 0.0005$ for RAW and 0.008 for HT-29 cells). However, we could fully restore Act-associated cytotoxicity by complementation (Fig. 3.2B and C). These data indicated that Act was the only significant cytotoxic factor present in overnight bacterial culture supernatants and, thus, was principally responsible for host cell damage and death, once secreted in sufficient quantities.

Microscopic alteration in the morphology of HT-29 and RAW 264.7 cells infected with the mutant strains of *A. hydrophila*, as well as the percentage of attached versus detached cells in the monolayer, revealed a trend similar to that observed for LDH release (Fig. 3.2 and 3.3). In the cell detachment assay, we recorded the host cells that remained attached to the wells of the plates after being stained with Giemsa stain. At 2 h postinfection, treatment with *aopB* or *act*/*aopB* mutant resulted in 95 to 98% fewer macrophages and intestinal epithelial cells that detached from the monolayers, compared to those infected with WT or *act* mutant bacteria (30 to 35%) ($P < 0.0001$) (Fig. 3.3C). Uninfected cells were designated as control. In concordance with the LDH release data at 4 h postinfection, a rapid host cell detachment (42%) was noted with the *aopB* mutant, reaching to the level seen with the WT *A. hydrophila* (51%). The difference in the cell detachment between WT and *aopB* mutant at 4 h was not statistically significant ($P = 0.07$) (Fig. 3.3C).

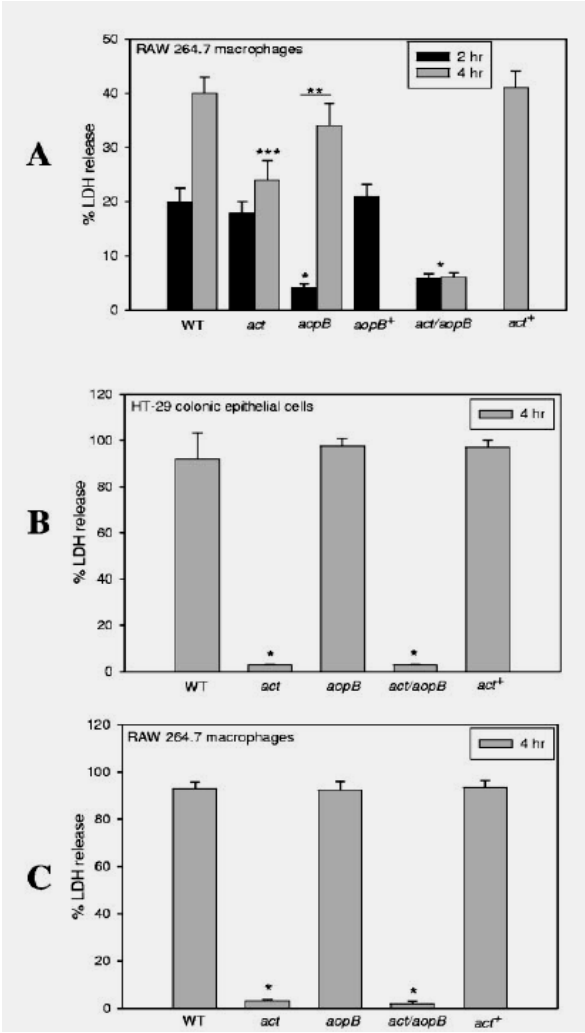

Fig. 3.2. Cytotoxicity induced by WT *A. hydrophila*, its various mutants, and complemented strains in RAW 264.7 and HT-29 cells.

(A) Macrophages in triplicate wells were infected with live *Aeromonas* strains at an MOI of 10 for 2 h and 4 h. After incubation, host cell culture supernatants were used to measure LDH release. (B & C) Cytotoxicity induced by overnight-grown bacterial culture supernatants from WT *A. hydrophila*, its various mutants, and complemented strains in HT-29 (B) and RAW 264.7 (C) cells. Arithmetic means ± standard de*via*tions from three independent experiments were used for plotting the data. Single asterisks denote statistically significant differences (by Student's t test) between various mutant strains and the WT bacterium. A double asterisk denotes a statistically significant difference between 2- and 4-h treatments of host cells with the *aopB* mutant. A triple

asterisk indicates a statistically significant difference between the *act* mutant and the WT bacterium at 4 h. The actual P values are presented in the text. The *aopB*$^{+}$ and *act*$^{+}$ represented complemented strains of *aopB* and *act* mutants

However, the host cells infected with the *act/aopB* mutant exhibited similar cell detachment patterns at 2 and at 4 h, indicating the significance of Act in cell toxicity when it is produced by the bacterium (Fig. 3.3C). We also noted that the decrease in cell detachment with the *act* mutant was statistically significant ($P = 0.001$) at 4 h compared to the WT-infected host cells. Likewise, the increase in cell detachment with the *aopB* mutant at 2 versus 4 h was also significant ($P = 0.0003$). The cell detachment assay results coincided with the morphology of the host cells as depicted in Fig. 3.3A and B. The HT-29 cells infected with either the WT or the *act* mutant were highly vacuolated and flattened after 2 h of infection. However, host cells infected with the *aopB* or *act*/*aopB* mutants showed normal morphology (Fig. 3.3A). Likewise, macrophages infected with the WT or the *act* mutant were rounded, while host cells infected with *aopB* and *act*/*aopB* mutants exhibited a normal morphology (Fig. 3.3B). These alterations in morphology are indicative of cytotoxicity, which we confirmed by the cell detachment and LDH release assays.

We do not know the precise mechanism by which AopB induces cell death of macrophages and intestinal epithelial cells. AopB homologs from other bacterial pathogens, such as *Y. pseudotuberculosis* (YopB), *S.* Typhimurium (SipB), *S. flexneri* (IpaB), *P. aeruginosa* (PopB), and *Bordetella bronchiseptica* (BopB), have been shown to function as the T3SS translocon (Hakansson *et al.*, 1996, Hueck, 1998, Kuwae *et al.*, 2003, Sundin *et al.*, 2004, Picking *et al.*, 2005). The translocon forms a pore in the host membrane, allowing translocation of the effectors into the cytosol of eukaryotic cells, which leads to cytotoxicity (Hakansson *et al.*, 1996, Hauser *et al.*, 1998, Hueck, 1998, Kuwae *et al.*, 2003, Sundin *et al.*, 2004, Picking *et al.*, 2005).

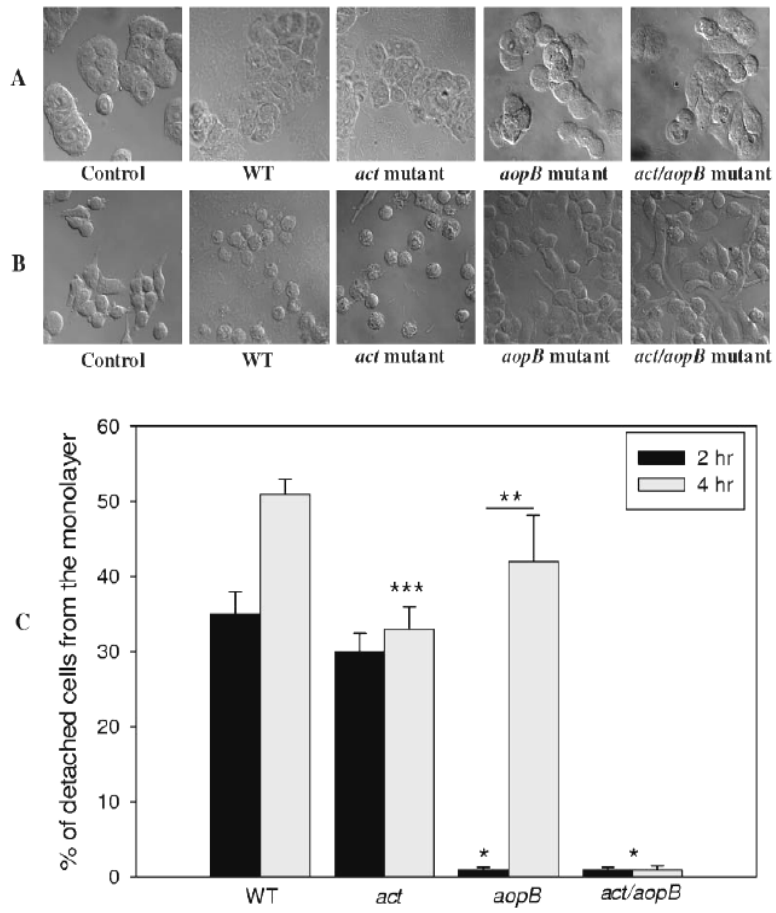

Fig. 3.3. Alteration of cell morphology induced by WT *A. hydrophila* and its various mutants in HT-29 (A) and RAW 264.7 (B) cells. (C) Cell detachment assay with HT-29 and RAW 264.7.

(A & B) Host cells were infected at an MOI of 10 and incubated for 2 h. Cells were then washed in PBS, fixed with 4% paraformaldehyde, and examined by confocal microscopy. Noninfected cells (control) and those infected with the *aopB* or *act/aopB* mutants exhibited normal cell morphology, while cell morphology was significantly altered (highly vacuolated and flattened [HT-29] or rounded [macrophages]) in cells infected with the WT or the *act* mutant. The experiment was performed in triplicate, and representative pictures are shown. For the cell detachment assay (C), HT-29 or RAW 264.7 cells were infected with the above-mentioned bacteria at an MOI of 10 for 2 and 4 h. The percentage of cells detached from the monolayers was calculated by Giemsa staining and solubilization of macrophages/HT-29 cells to release the blue color, the intensity of which was measured at 590 nm as described in Materials and Methods. Single asterisks denote statistically significant differences (by Student's *t* test) between various mutant strains and the WT bacterium. A double asterisk denotes a statistically significant difference between 2- and 4-h treatments of host cells with the *aopB* mutant. A triple asterisk indicates a statistically significant difference between the *act* mutant and the WT bacterium at 4 h.

Pore formation mediates the contact-dependent hemolytic activity in these bacteria and is an essential step for the translocation of effectors through the T3SS (High *et al.*, 1992, Hakansson *et al.*, 1996, Dacheux *et al.*, 2001, Kuwae *et al.*, 2003, Miki *et al.*, 2004). Further, IpaB, SipB, and BopB could act as effectors and translocate themselves into the host cell as well (Chen *et al.*, 1996, Collazo and Galan, 1997, Kuwae *et al.*, 2003). It has been reported that IpaB and SipB induce apoptosis through ICE (interleukin-1ß converting enzyme) or caspase 1 in professional phagocytes (Zychlinsky *et al.*, 1994, Chen *et al.*, 1996, van der Velden *et al.*, 2003); however, recent studies have implicated that the induced cytotoxicity (especially by SipB) has more features of necrosis and hence it was termed programmed necrosis (Brennan and Cookson, 2000, Guiney, 2005). BopB is essential in *Bordetella* T3SS-induced, caspase-1-independent necrosis (Kuwae *et al.*, 2003, Stockbauer *et al.*, 2003). However, it is not clear whether it is directly involved in the induction of necrosis (Kuwae *et al.*, 2003). In contrast, YopB does not translocate itself (Holmstrom *et al.*, 1997). However, it could activate proinflammatory signaling responses in *Yersinia*-infected epithelial cells (Ryndak *et al.*, 2005, Viboud and Bliska, 2005). The exact role of AopB in *Aeromonas* infections is not known and will be investigated in our future studies.

In *A. salmonicida*, the ADP ribosyltransferase toxin AexT (homolog of *P. aeruginosa* ExoT/S) was reported to function as a T3SS effector protein that caused cell death, and the role of AopB as the T3SS translocon was proposed (Braun *et al.*, 2002, Burr *et al.*, 2003). Ongoing work in our laboratory has identified an '*aexT-like*' gene to exist within the chromosome of *A. hydrophila* SSU; however it differs significantly in its C-terminal portion with the *aexT* gene of *A. salmonicida*.

In our *in vivo* studies, we noted that 100% of the animals infected with WT *A. hydrophila* SSU at doses of 5×10^7 to 1×10^8 organisms died within 48 h (Fig. 3.4). However, only 50 to 60% ($P = 0.02$ to 0.03 compared to WT bacteria) of the animals died when inoculated with the *act* or *aopB* mutant of *A. hydrophila* SSU. In contrast, 90% of the animals ($P = 0.001$ compared to WT bacteria) that were infected with the same doses of the *act/aopB* mutant survived over a test period of 16 days (Fig. 3.4), which indicated

that the presence of both the T3SS and Act was crucial for *Aeromonas*-mediated lethality in mice.

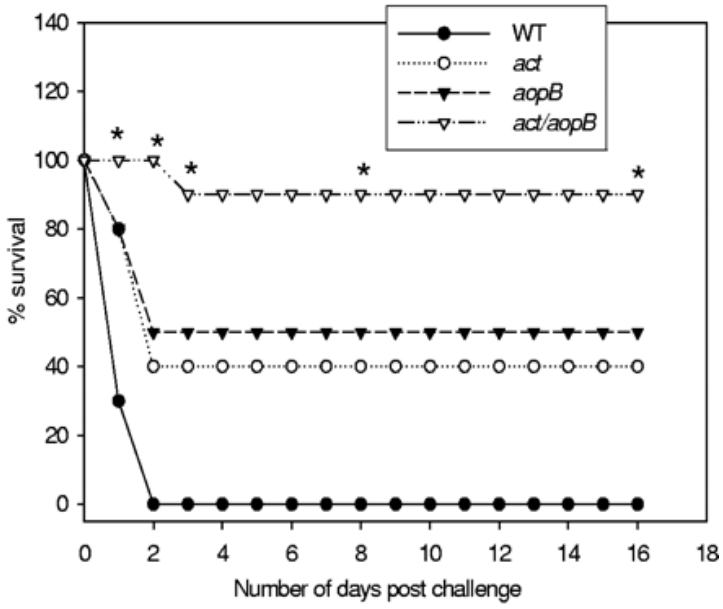

Fig. 3.4. Mutations in both the *act* and *aopB* genes rendered *A. hydrophila* SSU avirulent in a mouse model.
Swiss Webster mice (n = 10 per group) were injected intraperitoneally with three 50% lethal doses of indicated mutants and the WT *A. hydrophila* SSU and monitored for death over a period of 16 days. The data were statistically analyzed using Fisher's exact test. Three independent experiments were performed, and data from a typical experiment are shown. Asterisks denote statistically significant differences between *act/aopB* mutant and WT bacteria. The actual P values are presented in the text.

QS and the T3SS

Our studies indicated that both Act and T3SS contributed to the virulence of *A. hydrophila*. Recently, studies have shown that QS regulates T3SS in several bacteria. In enterohemorrhagic *E. coli*, the QS system LuxS/LuxPQ regulates the locus of the enterocyte effacement operon that, in turn, controls the T3SS (Sperandio *et al.*, 1999). On the other hand, in *V. harveyi* and *V. parahaemolyticus*, QS was shown to down-regulate the T3SS genes *vopD*, *vopN*, and *vopB* (*Vibrio* outer membrane proteins D, N, and B) at high cell density (Henke and Bassler, 2004).

As mentioned earlier, two QS systems have been identified in *P. aeruginosa*: LasI/LasR and RhlI/RhlR, both of which represent LuxI/LuxR homologs. While LasI synthesizes BHL, RhlI produces *N*-(3-oxododecanoyl)-L-homoserine lactone. Mutations

in *rhlI* or *rhlR* resulted in the up-regulation of *exoS* (encoding the T3SS effector ExoS) expression during biofilm formation in *P. aeruginosa*. This *exoS* up-regulation phenomenon in the RhlI mutant was repressed by adding BHL, indicating a negative regulatory effect of RhlR/BHL on *exoS* expression (Hogardt *et al.*, 2004). In addition to cell density, QS itself can also be regulated by a variety of factors, such as RpoS (σ^S) and RpoN (σ^{54}), which control the formation of flagella and pili and the production of exotoxin A in *P. aeruginosa* (Schuster *et al.*, 2004).

These studies led us to speculate whether deletion of a major virulence factor gene (*act*) or the T3SS *aopB* gene would alter QS autoinducer (lactone) production in *A. hydrophila* SSU. As shown in Fig. 3.5, WT *A. hydrophila* lactone production was significantly increased by high cell density, most notably between an OD_{600} of 0.9 and one of 1.2 (an increase in MU from 254 to 951 [$P = 0.0003$]), which is similar to the general trend observed for other gram-negative bacteria (Falcao *et al.*, 2004). Likewise, for the *act* mutant, there was a statistically significant ($P = 0.03$) increase in lactone production (from 331 to 642 MU) when the bacterium grew from an OD_{600} of 0.9 to one of 1.2. However, lactone production was reduced by 32% in the *act* mutant compared to that in WT *A. hydrophila* at an OD_{600} of 1.2 ($P = 0.02$) (Fig. 3.5).

More importantly, we did not observe any significant increase in lactone production by the *aopB* mutant grown to an OD_{600} of 0.9 or 1.2. Overall, there was a 64% reduction in lactone production between WT *A. hydrophila* and the *aopB* mutant at an OD_{600} of 1.2 ($P = 0.0001$). Lactone production was reduced even further in the *act/aopB* mutant, with a 92% decrease in lactone production compared to that of WT *A. hydrophila* when their growth reached an OD_{600} of 1.2 ($P < 0.0001$) (Fig. 3.5), possibly suggesting an additive effect of the *act* and *aopB* single gene deletions. We were able to restore QS in the *act* ($P = 0.04$) and *aopB* ($P = 0.006$) mutants after complementation (designated as act^+ and $aopB^+$), as measured by ß-galactosidase activity (Fig. 3.5). Statistically, no significant difference was noted in lactone production between WT *A. hydrophila* and the act^+ strain ($P = 0.2$) and between WT bacteria and the $aopB^+$ strain ($P = 0.5$).

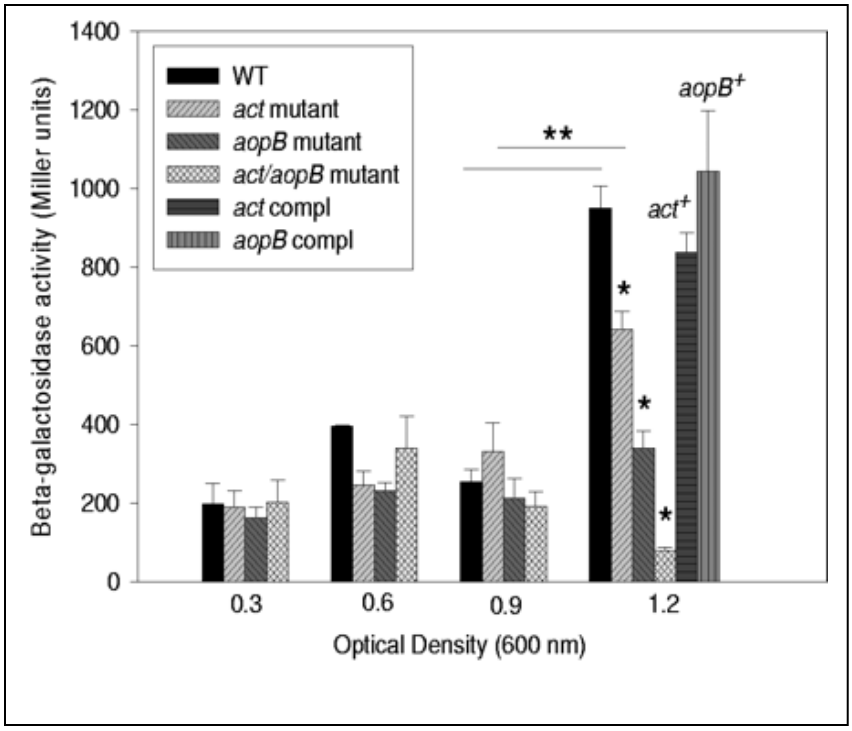

Fig. 3.5. Effect on lactone production by the T3SS and Act of *A. hydrophila* SSU.
WT *A. hydrophila*, its various mutants, and complemented strains were grown to various densities (OD_{600} of 0.3 to 1.2). The cultures were centrifuged, and the supernatants were measured for ß-galactosidase activity (presented in Miller units), indicative of AHL production, using an *A. tumefaciens* ß-galactosidase reporter strain. Three to five independent experiments were performed, and the arithmetic mean ± deviation was plotted. Single asterisks denote statistically significant differences (by Student's *t* test) in lactone production between WT and its various mutant derivatives at an OD_{600} of 1.2. A double asterisk denotes a statistically significant difference in lactone production in WT and its *act* mutant at ODs_{600} of 0.9 and 1.2. The increase in lactone production by the complemented strains was significantly higher than that in the corresponding mutants but was statistically insignificant compared to lactone production in the WT bacterium. Strains designated act^+ and $aopB^+$ indicate complementation of the corresponding mutants. The actual *P* values are presented in the text. Compl, complementation.

In conclusion, we showed, for the first time, a correlation between the presence of a T3SS, T2SS-secreted Act, and lactone production, in a diarrheal isolate of *A. hydrophila*. It is plausible that QS ensures that a sufficient number of bacteria are present to coordinate the expression of a virulence-associated gene(s) that could overwhelm host defenses. In addition, lactone-based quorum sensors are almost always integrated into other regulatory circuitry (Fuqua *et al.*, 2001). This effectively expands the range of environmental signals that influence target gene expression beyond population density

(Fuqua *et al.*, 2001). As we previously reported and mentioned here, Act is a type 2-secreted cytotoxic enterotoxin (Chopra *et al.*, 1993), and the expression of the *act* gene was affected by different environmental stimuli, including its regulation through the *fur* regulatory circuitry (Sha *et al.*, 2001). Act production increased as bacterial density increased (Sha *et al.*, 2004), and thus, it is plausible that *act* gene expression is also under the control of QS, which is a topic worth pursuing in the future. Complex regulatory networks exist in bacteria and are required for bacteria to assemble secretion machineries (e.g., type 2 and 3 secretion systems), as well as to produce and secrete proteins. It is therefore possible that the bacteria could "sense" and subsequently "react" to dysfunction of the components in these complex networks, thereby "shutting off" the production of signaling molecule lactones in an attempt to conserve "bacterial energy." This scenario emphasizes the complexity of coordinated virulence gene expression. QS has been shown to be regulated in the growth phase by the stationary phase sigma factor RpoS (also known as σ^s or σ^{38}) and by the alternative sigma factor RpoN (also known as σ^{54}) in the bacterium *P. aeruginosa* (Whiteley *et al.*, 2000, Heurlier *et al.*, 2003). It is plausible that the T3SS of *A. hydrophila* SSU regulate QS *via* a similar mechanism and requires further investigation. However, considerable further experimentation is needed to formulate more precisely the mechanisms of how the QS regulon is controlled by either T3SS or sigma factors and whether at the molecular level there is indeed cross-regulation between RpoS or RpoN, T3SS and AHL QS genes.

Although our data did not indicate a direct regulation of QS by either the T3SS or Act, we provided the first evidence for a positive correlation of these factors with QS, as their absence greatly reduced lactone production at high cell density, compared to the same conditions in the WT bacterium. Our future studies will focus on identifying the specific genes/regulators that are affected in these mutants.

CHAPTER 4:
CHARACTERIZATION AND ROLE OF DAM IN CONTRIBUTING TO VIRULENCE OF *A. HYDROPHILA* [3,4]

INTRODUCTION

The identification and characterization of a T3SS in the clinical isolate SSU of *A. hydrophila* as presented in Chapter 3 led us to search for additional genes that could alter bacterial virulence. Alteration of Dam activity has been shown to attenuate the virulence of several pathogens and confer protective immune responses in vaccinated animals (Garcia-Del Portillo *et al.*, 1999, Heithoff *et al.*, 1999, Heithoff *et al.*, 2001, Low *et al.*, 2001). The molecular basis of virulence attenuation and protection conferred in Dam mutant strains appears to involve ectopic gene expression and the resultant elaboration of an expanded repertoire of antigens. For example, in *Y. pseudotuberculosis*, Dam overproduction has been shown to attenuate virulence, confer protective immune responses, cause the secretion of several T3SS secreted Yops under conditions that are nonpermissive for secretion in wild-type strains, and alter host immune responses to *Yersinia* antigens (Julio *et al.*, 2001, Julio *et al.*, 2002). We therefore wanted to explore the possibility that Dam could play a regulatory role on the T3SS of *A. hydrophila*, as well as the T2SS, specifically with regard to the expression and production of Act.

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[3] Results of study published in following source and reproduced with permission here: Infection and Immunity (http://www.iai.asm.org); Erova, T.E., Pillai, L., Fadl, A.A., Sha, J., Wang, S., Galindo, C.L., and Chopra, A.K. DNA adenine methyltransferase influences the virulence of *Aeromonas hydrophila*. *Infect. Immun*. 74:410-424.

[4] **Author contributions:** Lakshmi Pillai was involved in construction of fosmid library and screening of fosmid clones for sequencing a portion of *dam* gene, primer extension and promoter mapping studies, and in writing portions of Introduction and Discussion sections of manuscript. Dr. Tatiana E. Erova (postdoctoral fellow) was involved in sequencing the full-length *dam* gene, mapping the promoter region and constructing the Dam-overproducing strain as well as purifying the recombinant Dam protein. Drs. Jian Sha (Assistant Professor), Amin A. Fadl (McLaughlin postdoctoral fellow), and T. E. Erova were involved in mechanistic studies of the Dam-overproducing strain. Dr. J. Sha performed final primer extension studies and both he and Dr. T.E. Erova contributed substantially in writing the manuscript.

As mentioned ealier, DNA methylation occurs in bacteria, plants, mammals, and, recently, and recently was also reported in *Drosophila melanogaster* (Lyko *et al.*, 2000, Lyko, 2001). MTases catalyze methylation of either the cytosine residues at the C-5 or N-4 position or at the N-6 position of adenine residue within the DNA (Walter *et al.*, 1992). Specifically, Dam exerts its function by DNA methylation at adenine residues in 5'-GATC-3' sequences (Palmer and Marinus, 1994). Methylation is a postreplicative process and the newly replicated DNA is methylated only on the parental strand. Therefore, this hemimethylated DNA is distinct from the rest of the chromosomal DNA. The hemimethylated status of newly synthesized DNA provides a time frame during which cellular processes, such as DNA replication (Russell and Zinder, 1987, Messer and Noyer-Weidner, 1988, Lobner-Olesen *et al.*, 2003), repair of mismatched bases, as well as alteration of gene expression occurs (Marinus, 1996).

Dam methylation has received significant interest recently because of its impact on the virulence of several pathogens such as *V. cholerae* (Mahan *et al.*, 2000, Julio *et al.*, 2001), *S. enterica* serovar Typhimurium (Garcia-Del Portillo *et al.*, 1999, Nicholson and Low, 2000, Dueger *et al.*, 2001, Dueger *et al.*, 2003), pathogenic *E. coli* strains (Calmann and Marinus, 2003, Lobner-Olesen *et al.*, 2003), *Y. pseudotuberculosis* (Julio *et al.*, 2002, Badie *et al.*, 2004), *Y. enterocolitica* (Falker *et al.*, 2005), *Haemophilus influenzae* (Bujnicki *et al.*, 2001, Zaleski and Piekarowicz, 2004), and others. However, in *S. flexneri*, the *dam* mutants showed no attenuation of virulence (Honma *et al.*, 2004). Overall, data seem to support the hypothesis that Dam could globally alter virulence gene expression in Gram-negative bacteria (Heithoff *et al.*, 1999, Low *et al.*, 2001). Therefore, Dam presents the exciting possibility that it may play a role in the virulence of a broad range of pathogens and, thus, further investigation is merited.

In this study, we showed that a diarrheal isolate SSU of *A. hydrophila* harbored the *dam* gene, and that its overexpression attenuated bacterial virulence, specifically that of T3SS-associated cytotoxicity, motility, and virulence in a mouse lethality model. This

is the first report of characterization of the *dam* gene from *Aeromonas* species. According to the recent nomenclature for methyltransferases and their genes, we denoted *A. hydrophila* SSU strain DNA adenine MTase as M.AhySSUDam and the *dam* gene as dam_{AhSSU} (Roberts *et al.*, 2003).

RESULTS

Cloning and sequencing of the *A. hydrophila* SSU *dam* gene

To determine whether *A. hydrophila* strain SSU contains the DNA adenine MTase gene, we initially used restriction endonucleases which are sensitive to methylation of adenine residues in 5'-GATC-3' sequences to digest gDNA. It is known that the *Dpn*I enzyme cuts the methylated GATC sequence, but not unmethylated GATC. Conversely, *Dpn*II does not cut methylated GATC, while it cuts the unmethylated GATC sequence (Roberts and Macelis, 2000). We noted that gDNA of *A. hydrophila* was sensitive to *DpnI* restriction endonuclease digestion and resistant to digestion with *Dpn*II restriction enzyme. These findings indicated that *A. hydrophila* might possess MTase activity with GATC specificity. As a result, we designed *dam*1 and *dam*2 primers (Table 2.2) that corresponded to the regions of highest conservation among the *dam* genes from different gram-negative bacteria (Fig. 4.1), such as the *Vibrio* species (e.g., *V. cholerae*, *V. fischeri*, and *V. parahaemolyticus*), *E. coli*, *S. enterica* serovar Typhimurium, and *Yersinia* species (e.g., *Y. pseudotuberculosis* and *Y. pestis*), to PCR amplify a portion of the *dam* gene (a 400-bp fragment) from the gDNA of *A. hydrophila* SSU. Upon Southern blot analysis under low-stringency conditions, this fragment reacted with an *E. coli dam* gene probe. Consequently, this 400-bp fragment was cloned into a pCR 2.1 vector and transformed in TOP10 chemically competent *E. coli* cells (TA cloning kit; Invitrogen). The DNA sequence analysis of this 400-bp fragment revealed 61% and 63% identities with the corresponding regions of the *dam* gene of *E. coli* and *V. cholerae*, respectively. The coding regions of the *dam* gene in *E. coli* and *V. cholerae* contained 834 and 831 bp, respectively (Brooks *et al.*, 1983, Julio *et al.*, 2001).

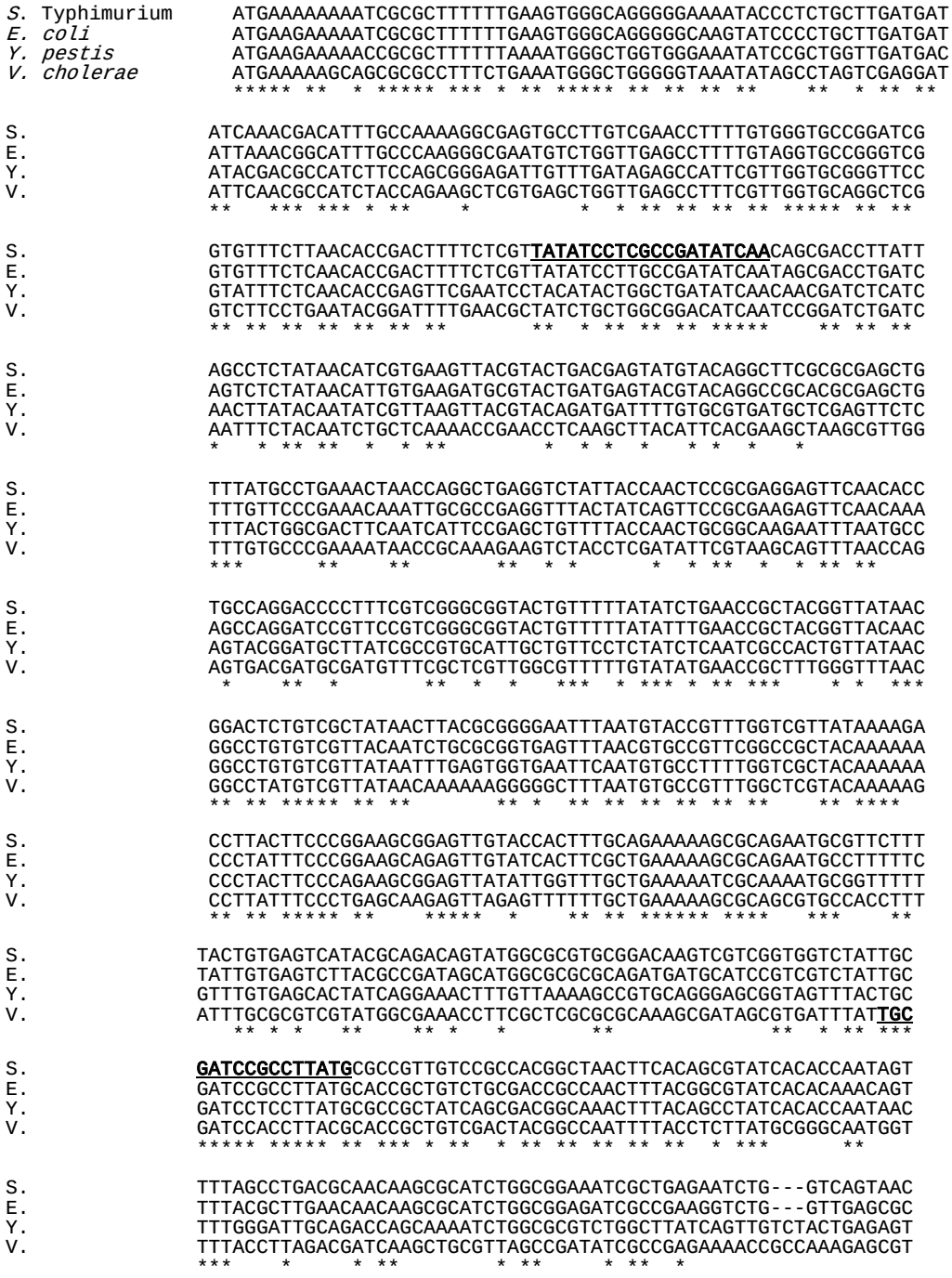


Fig. 4.1. Homology of *dam* gene among various gram-negative bacteria

Region of high homology among the *dam* gene from different gram-negative bacteria (i.e.

S. Typhimurium, *E. coli*, *Y. pestis*, and *V. cholerae*). The indicated region (in bold and underlined) was used to generate two primer to PCR amplify the *dam* gene from *A. hydrophila*. S = *S.* Tymphimurium; E = *E. coli*; Y = *Y. pestis*; V = *V. cholerae*

We used two strategies to obtain a complete sequence of the *A. hydrophila dam* gene coding region as well as of the flanking DNA sequences to the *dam* gene. The nucleic acid sequence of the entire dam_{AhSSU} gene (Fig. 4.1) was determined by gDNA sequencing using the additional primers *dam*3 and *dam*4 (Table 2.2). The construction of a fosmid library of *A. hydrophila* SSU gDNA enabled us to confirm the DNA sequence of the *dam* gene and to obtain its flanking sequences for preparing isogenic mutants. The 400-bp *dam* gene fragment generated by PCR amplification that reacted with the *E. coli dam* gene probe was used to screen the fosmid library of *A. hydrophila*. This probe did not react with the *E. coli dam* gene under high-stringency conditions and thus prevented identification of false-positive fosmid clones. Five positive fosmid clones that contained inserts of approximately 25 kb were obtained out of 800 clones that were screened. Further, this 400-bp *dam* gene fragment hybridized specifically with the gDNA digests (cut with various restriction enzymes) of *A. hydrophila* SSU under high-stringency conditions (data not shown). BLAST search of the DNA sequences obtained using the above-mentioned strategies at NCBI revealed an 873-bp ORF that had a high degree of identity with previously published bacterial *dam* sequences (Brooks *et al.*, 1983, Julio *et al.*, 2001). The gene encoded a protein of 291 aa residues with a molecular mass of 32.7 kDa. The complete DNA sequence of the dam_{AhSSU} gene with its corresponding aa sequence is depicted in Fig. 4.2. The overall identity of the *A. hydrophila* SSU *dam* gene with those of *V. cholerae*, *E. coli*, *S.* Typhimurium, and *Y. pseudotuberculosis* was 65%, 61%, 59%, and 58%, respectively. At the aa level, a maximum homology (68%) of M. AhySSUDam was noted with that of *V. fischeri*.

Analysis of the *dam*$_{AhSSU}$ gene presumptive transcriptional start site and promoter region.

Primer extension analysis was used to determine the presumptive transcriptional start site of the *dam* gene, and three primers were used. The *dam*P1 primer was designed to nucleotide positions 1 to 30 (nucleotide A of the *dam*$_{AhSSU}$ gene start codon [ATG] represented position 1). The *dam*P2 primer represented nucleotide positions 7 to 36, while the *dam*P3 primer spanned nucleotide positions 12 to 41 (Table 2.2 and Fig. 4.2). The primers were end labeled and hybridized to the RNA isolated from WT *A. hydrophila* SSU. The synthesized cDNA was analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflected the number of bases between the labeled primer and the 5' end of the *dam*$_{AhSSU}$ gene transcript. cDNA products with lengths of 54, 60, and 65 bp, were obtained with *dam*P1 (Fig. 4.3, lane 4), *dam*P2 (Fig. 4.3, lane 3), and *dam*P3 (Fig. 4.3, lane 2) primers, respectively. Based on the DNA sequence, primer extension analysis identified a presumptive transcriptional start site (G) at a position 24 nucleotides upstream of the ATG start codon of the *dam* gene (Fig. 4.3). Putative -10 (GGGTAGAAT) and -35 (TAGCCA) elements of the promoter were also identified in this region using a software program found at www.softberry.com.

Overproduction and purification of M.AhySSUDam

Initial attempts to delete the copy of the *dam* gene from the chromosome of *A. hydrophila* SSU were futile, as the gene turned out to be essential for the viability of this organism. Therefore, overexpression of the gene was used in order to study the effects of Dam on the virulence aspects of *A. hydrophila*. The *dam*$_{AhSSU}$ gene was overexpressed by cloning the gene into the pBAD/Thio-E vector system, which is under an arabinose-inducible pBAD promoter. Expression of the *dam* gene from the plasmid pBAD-ahySSUDam was induced when arabinose (0.2%) is added to the growth medium (Guzman *et al.*, 1995). Subsequently, M.AhySSUDam as a His tag fusion protein was purified using ProBond resin charged with nickel. Purified Dam was eluted from the column using 1 M imidazole. Based on SDS-polyacrylamide gel electrophoresis analysis

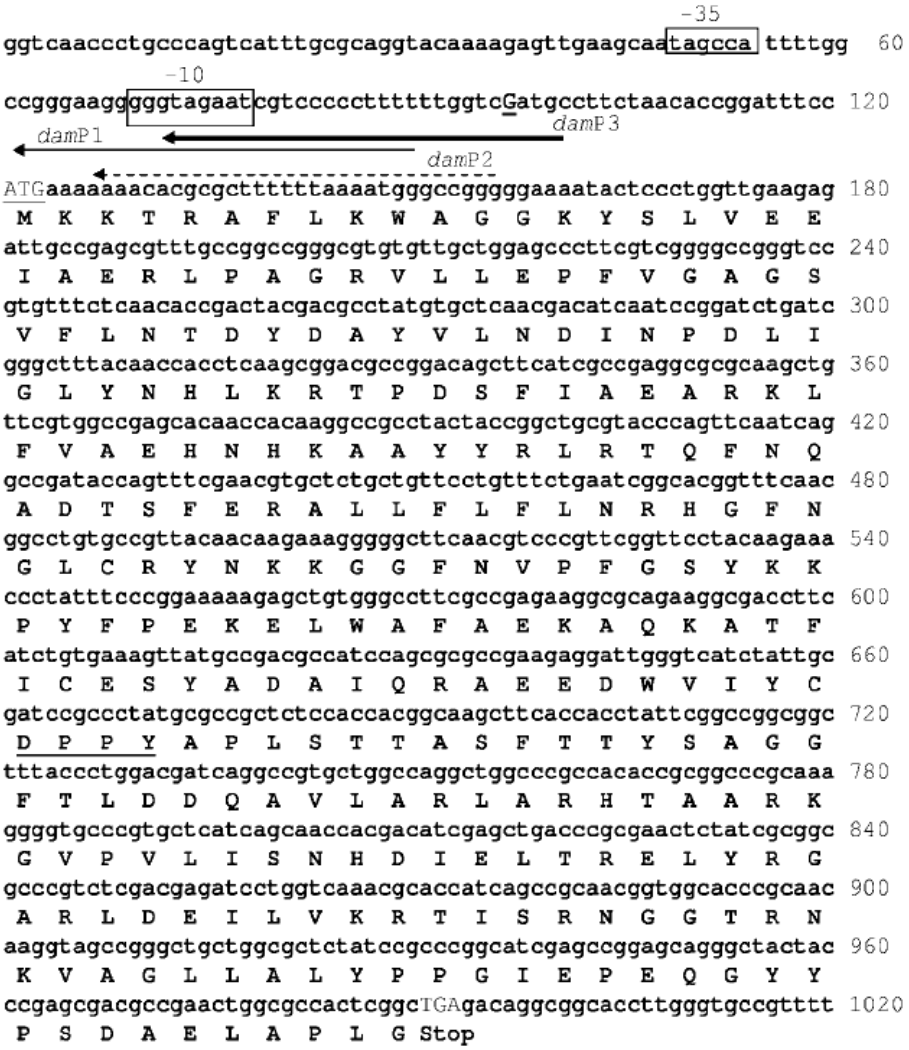


Fig. 4.2. Sequence of a 1,020-bp DNA fragment that encompasses the *dam*$_{AhSSU}$ gene of *A. hydrophila* SSU.

Presented is the nucleotide sequence of the coding region of the *dam*$_{AhSSU}$ gene with its deduced aa sequence. The start and stop codons of the *dam*$_{AhSSU}$ gene are in capital letters. Underlined DPPY aa residues represent the conserved catalytic motif for N6-methyladenine MTase. The sequences of the primers used for primer extension analysis are indicated with arrows. The potential transcriptional start site (G) is indicated by the capital letter and is underlined. The rectangular boxes represent putative -10 and -35 boxes.

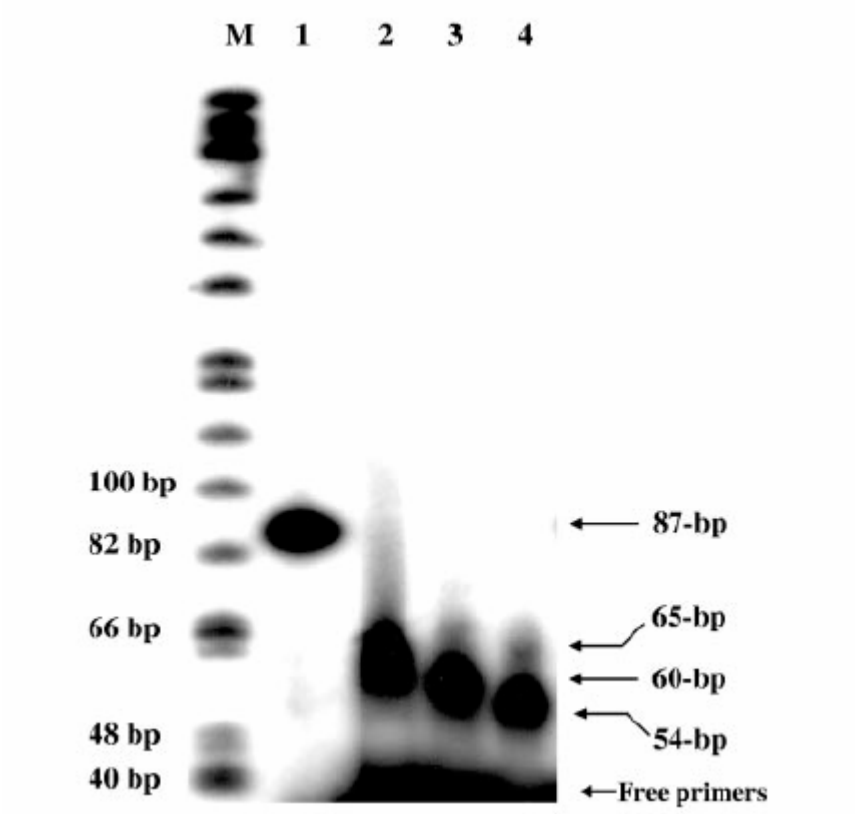


Fig. 4.3. Primer extension analysis to define the presumptive transcriptional start site of the *A. hydrophila* SSU *dam* gene.
The products from primer extension reactions were separated by electrophoresis on an 8% denaturing polyacrylamide gel. Lanes: (M) *Hinf*I-digested ΦX174 DNA markers (Promega); (1) cDNA product of the control RNA (87 bp); (2) cDNA product of *A. hydrophila* RNA with *dam*P3 primer (65 bp); (3) cDNA product of *A. hydrophila* RNA with *dam*P2 primer (60 bp); (4) cDNA product of *A. hydrophila* RNA with *dam*P1 primer (54 bp). The gel was exposed to X-ray film overnight at -70°C.

and Coomassie blue staining, a single protein band with a molecular mass of 34 kDa was detected. The molecular mass of purified Dam concurred with the predicted size of 291

aa residues (32,662 daltons [Da]) based on the DNA sequence plus the additional aa residues derived from the His tag region of the pET-30a(+) vector.

Effect of M.AhySSUDam overproduction on *A. hydrophila* SSU virulence.

To determine whether altered Dam production affected the virulence potential of *A. hydrophila* SSU, we evaluated various biological activities associated with *A. hydrophila* control and Dam-overproducing strains. Motility is an important pathogenic factor for bacteria to reach the host target tissue, to colonize, and then to cause disease. We noted that overproduction of Dam significantly reduced (58%) the motility of the bacterium (Fig. 4.4A). To determine if overproduction of Dam would have any effect on the cytotoxicity associated with the T3SS of *A. hydrophila*, RAW264.7 murine macrophage cells were infected with either the *A. hydrophila* control or the Dam-overproducing strain. As shown in Fig. 4.4B, T3SS-associated cytotoxicity (as measured by LDH release) was reduced significantly (55%; $P < 0.0001$) at 4.5 h after infection when the *dam* gene was overexpressed compared to that of the control *A. hydrophila* strain. The LDH release from macrophages by the control strain was similar to the positive control provided in the kit. No difference in the LDH release was noted between the control and Dam-overproducing strain at 3 h postinfection. However, at 3.5 and 4.0 h after infection, a decrease in the LDH release of 12% ($P = 0.0004$) and 30% ($P = 0.0001$) was noted between the control and *dam*-overproducing strains. Interestingly, when the cultures were grown in the absence of arabinose, no statistically significant difference was noted in LDH release from macrophages when the control strain was compared to that of the Dam-overproducing strain (Fig. 4.4B). This indicated that it was indeed the overproduction of Dam that caused the decrease in cytotoxicity associated with the T3SS.

In contrast, cytotoxicity associated with the T2SS-secreted Act on macrophages showed a 10-fold increase when the culture supernatant of the Dam-overproducing *A. hydrophila* strain was used compared with those of the *A. hydrophila* control strain (Fig. 4.5A). Another major biological activity associated with Act is hemolysis of red blood cells. Rabbit erythrocytes treated with culture supernatant from the Dam-overproducing

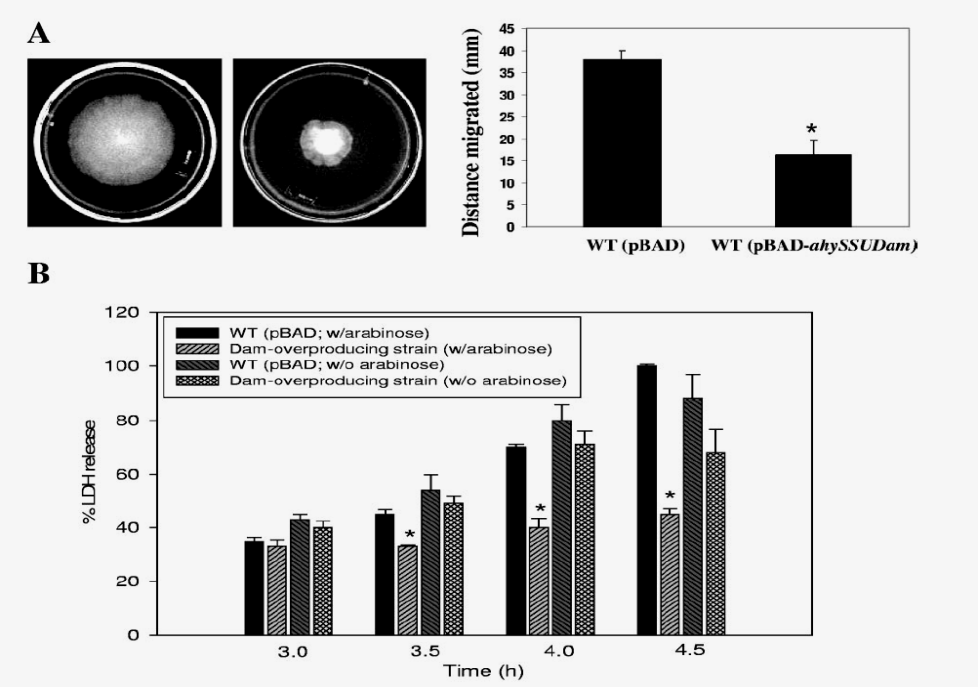


FIG. 4.4. Overproduction of Dam reduces (A) motility and (B) T3SS-associated cytotoxicity of *A. hydrophila* SSU.
(A) Motility assay results, with the motility phenotype of the *A. hydrophila* control strain (with the pBAD vector alone and designated as pBAD) and that of *A. hydrophila* (with pBAD-dam_{AhSSU}, *dam* overexpression induced with 0.2% arabinose). The bar graph shows the distances migrated by these two strains. (B) T3SS-associated cytotoxicity induced by *A. hydrophila* control and Dam-overproducing strains in RAW 264.7 macrophages. After infection of macrophages with the bacterial cells (for 3 to 4.5 h) at an MOI of 10, LDH release was measured. Both arabinose-induced and uninduced cultures were used for infection. Data from three wells were averaged, three independent experiments were performed, and data are plotted ±standard de*via*tion. The asterisks denote statistically significant differences ($P \leq 0.05$, as determined by Student's *t* test) between control and Dam-overproducing strains.

strain had a threefold higher hemoglobin release than the control strain (Fig. 4.5B), indicating that overproduction of Dam led to a concomitant increase in both the cytotoxicity and hemolysis associated with Act.

To demonstrate that these hemolytic and cytotoxic activities were indeed associated with Act, we neutralized the culture supernatants of the above-mentioned strains with Act-specific antibodies, which resulted in abrogation of these activities. We confirmed these observations by performing Northern blot analysis and noted an increased transcript of the *act* gene in the Dam-overproducing strain compared to the control strain (data not shown). Further, based on the ELISA data, increased production of Act was noted when Dam was overproduced in *A. hydrophila* (containing the pBAD-dam_{AhSSU} plasmid) compared to the control strain harboring pBAD vector alone in a medium containing arabinose (Fig. 4.5C). The ELISA values using antibodies to Act increased from 2.4 to 9.7/ml/10^8 CFU ($P = 0.0003$) when the Dam-overproducing strain was compared to that of the control strain. On the contrary, in the absence of arabinose, no difference in Act production was noted between the control and the Dam-overproducing strain. Expression of the genes (e.g., *aopB*, *aopD*, *ascV*, and *acrV*) that constitute the T3SS apparatus was not altered (data not shown).

The pathogenic and virulence characteristics of *A. hydrophila* are also associated with the production of T2SS-associated exoenzymes (e.g., proteases and lipases) (Janda and Abbott, 1998, Chopra and Houston, 1999). We noted increased production of proteinase (2.4-fold) with the *A. hydrophila* Dam-overproducing strain compared to its appropriate control in the culture supernatant (Fig. 4.5D). These results indicated that overproduction of Dam enzyme did, indeed, alter the virulence potential of *A. hydrophila* based on *in vitro* assays.

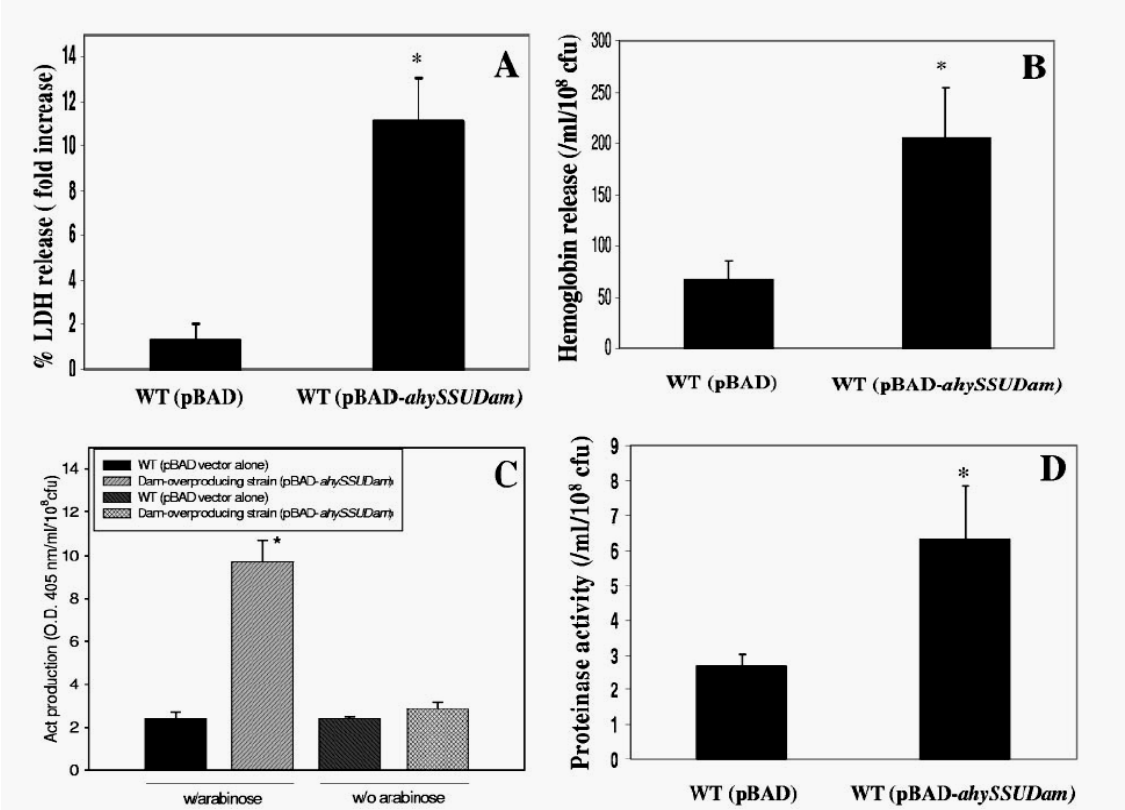


Fig. 4.5. Overproduction of Dam increases cytotoxic activity of T2SS-associated Act and proteinase activity of *A. hydrophila* SSU.
(A) Act-associated cytotoxicity induced by culture supernatants of *A. hydrophila* control and Dam-overproducing strains in RAW 264.7 macrophages. After treatment of macrophages with the culture filtrates (for 2 h), the LDH release was measured in the tissue culture supernatants (see Materials and Methods). The results were reported as fold increases in activity in the Dam-overproducing strain compared to the control strain. (B) Act-associated hemolytic activity in the culture supernatants of *A. hydrophila* control and Dam-overproducing strains, as measured by the release of hemoglobin (see Materials and Methods). (C) Increased production of Act based on ELISA in the culture supernatants of *A. hydrophila* Dam-overproducing and control strains when grown in the absence (w/o) and presence (w/) of arabinose. (D) Proteinase activity in the culture supernatants of *A. hydrophila* control and Dam-overproducing strains, as measured by hydrolysis of Hide azure powder. Data from three independent experiments were plotted with standard dev*ia*tions. The asterisks denote statistically significant differences ($P \leq 0.05$, as determined by Student's *t* test) between control and Dam-overproducing strains.

DISCUSSION

The role that Dam plays in altering bacterial virulence is only beginning to be understood (Low *et al.*, 2001), and studies have shown that adenine methylation can either directly or indirectly alter the interaction of regulatory proteins with DNA (Diekmann, 1987). In this study, we identified, cloned, and sequenced the *dam* gene from a diarrheal isolate, SSU of *A. hydrophila*. Further, the role of *A. hydrophila* Dam in altering bacterial virulence was evaluated using an *in vitro* system.

Previous studies indicated that Dam methylation played a role in *S. enterica* serovar Typhimurium cell envelope integrity (Pucciarelli *et al.*, 2002). These investigators showed that Dam mutants enhanced the release of extracellular proteins to the medium, with no obvious alteration in the T3SS-associated secretion of proteins. We therefore examined the effect of Dam on cell membrane integrity of *A. hydrophila* SSU. We noted that the growth phenotypes of the control and Dam-overproducing *A. hydrophila* strains as well their abilities to adhere to HT-29 colonic or mouse small intestinal epithelial cells were similar. Likewise, membrane permeability remained unaltered in the Dam-overproducing strain of *A. hydrophila* compared to the control strain. These data indicated that the virulence defect in the Dam-overproducing strain of *A. hydrophila* was directly the result of alterations in gene expression and not the pleiotropic effects of Dam on cell physiology (Heithoff *et al.*, 2001, Julio *et al.*, 2001, Low *et al.*, 2001).

Both deletion and overproduction of the *dam* gene have been shown to attenuate bacterial virulence (Heithoff *et al.*, 1999, Julio *et al.*, 2002, Badie *et al.*, 2004). We explored the role of Dam in the pathogenesis of *A. hydrophila* and showed decreased motility and cytotoxicity associated with the T3SS of the Dam-overproducing strain (Fig. 4.3A and B). Motility is an important virulence factor of many gram-negative pathogens and a significant invasion-related factor for bacteria such as *S. enterica* serovar Typhi (Liu *et al.*, 1988). Motility was shown to be decreased in a *dam* mutant of *E. coli* (Oshima *et al.*, 2002). Similarly, a decrease in motility might have contributed to the lack

of invasiveness of *S. enterica* serovar Typhi *dam* mutants (Liu *et al.*, 1988). Since invasiveness of *S. enterica* serovar Typhimurium to the host cells is also dependent upon the T3SS (Galan and Collmer, 1999), it was noted that the *dam* mutants were indeed defective in the secretion of *Salmonella* pathogenicity island 1-encoded effector proteins, including those proteins essential for the invasion of the bacterium (Garcia-Del Portillo *et al.*, 1999).

A possible mechanism by which overproduction of Dam leads to decreased T3SS-associated cytotoxicity in *A. hydrophila* could be explained by findings observed in *Y. pseudotuberculosis*. The overproduction of Dam in *Y. pseudotuberculosis* altered the expression and secretion of a T3SS-associated effector protein YopE (*Yersinia* outer membrane protein E), which is secreted by the WT bacterium under low calcium and high temperature (37°C) conditions and is also known to be antigenic (Julio *et al.*, 2002). Yops translocated into the host cell *via* the T3SS act to inhibit phagocytosis of the bacterium and to induce proinflammatory cytokine release (Badie *et al.*, 2004). The overproduction of Dam in *Y. pseudotuberculosis* disrupted both the thermal and calcium regulation of YopE synthesis and relaxed the thermal but not the calcium dependence of YopE secretion (Julio *et al.*, 2002). Currently, the effector proteins secreted by the *A. hydrophila* T3SS are not known. However, the phenomenon we observed of reduced T3SS cytotoxicity associated with Dam overproduction could be related to the altered secretion and/or synthesis of T3SS effectors. Our Northern blot analysis data indicated no alteration in the expression of T3SS apparatus genes *aopB*, *aopD*, *ascV*, and *acrV* in Dam-overproducing versus control *A. hydrophila* strains (data not shown). These data suggested that the T3SS machinery itself remained unaltered in the Dam-overproducing strain.

It is also possible that Dam overproduction in *A. hydrophila* may play a role in the increased expression of negative regulators of the T3SS or, conversely, the decreased expression of positive T3SS regulatory genes. An example of a T3SS regulator was recently elucidated in *P. aeruginosa*. A specific locus was defined (*sadARS*) which was comprised of genes for a putative sensor histidine kinase and two response regulators

(Kang *et al.*, 1999). Among the genes regulated by this three-component SadARS system are those required for the T3SS. This report showed that SadS and SadA were important for controlling expression of T3SS genes. SadA contains a helix-turn-helix motif and may regulate T3SS gene expression at the transcription level. In *B. pertussis*, the BvgA response regulator also contains a helix-turn-helix motif, and under activating conditions (in the Bvg^+ phase), this protein binds to virulence gene promoters and activates transcription (Merkel *et al.*, 1998). Recent data suggest that the SadARS regulatory system may function to promote biofilm formation, possibly, in part, by repressing expression of the T3SS (Kuchma *et al.*, 2005). Similarly, it was reported recently that ExsE is a negative regulator of the T3SS in *P. aeruginosa* (Rietsch *et al.*, 2005) and is secreted *via* the T3SS under conditions of low calcium. Therefore, it is intriguing to determine whether a homolog of ExsE exists in *A. hydrophila* and whether overproduction of Dam might prevent release of ExsE *via* the T3SS and hence reduced expression of the T3SS-secreted effectors.

In addition to T3SS-associated cytotoxicity, the biological activities associated with the T2SS-associated Act, a potent virulence factor of *A. hydrophila*, were also affected by M.AhySSUDam. Interestingly, overproduction of Dam augmented the virulence potential of Act. Both the cytotoxic and hemolytic activities associated with Act were markedly increased in the culture filtrate of the *A. hydrophila* Dam-overproducing strain compared to the control strain (Fig. 4.5A and B), indicating a positive effect on bacterial virulence by Dam. Indeed, *act* gene expression was increased in the Dam-overproducing strain of *A. hydrophila*, based on Northern blot analysis (data not shown) and ELISA (Fig. 4.5C), for the *act* transcript and Act protein, respectively. A similar pattern was noticed for proteinase production (Fig. 4.5D), which was upregulated in the Dam-overproducing strain compared to the control strain of *A. hydrophila*. Although the mechanism(s) by which Dam overproduction alters gene expression is far from clear, a recent study based on the microarray analysis of different mutants has implicated SeqA protein as playing an important role in the alteration of gene expression in the Dam-overproducing *E. coli* strain (Lobner-Olesen *et al.*, 2003). These investigators

noted that the absence of SeqA protein (*seqA* mutant) and high DNA methyltransferase levels (Dam-overproducing strain) affected global gene expression in an almost identical manner. However, a different pattern of gene expression was noted in the *dam* mutant of *E. coli* (Lobner-Olesen *et al.*, 2003). In addition to DNA initiation and replication, SeqA has been shown to exert its function in nucleoid organization through interaction with hemimethylated DNA (Lobner-Olesen *et al.*, 2003). Similar to other chromosome structure-maintaining proteins, such as H-NS, Fis, IHF, HU, etc. (Lobner-Olesen *et al.*, 2003), a global regulatory role has been proposed for SeqA (Lobner-Olesen *et al.*, 2003). It has been speculated that Dam and SeqA compete for binding to hemimethylated DNA behind the replication fork. Either deletion of the *seqA* gene or overproduction of Dam increases the negative superhelicity of the chromosome (Weitao *et al.*, 2000, Lobner-Olesen *et al.*, 2003), thus facilitating open complex formation by RNA polymerase on promoters in general, which leads to redistribution of the RNA polymerases in bacteria, resulting in up- and down-regulation of certain genes (Jensen and Pedersen, 1990, Lobner-Olesen *et al.*, 2003).

Previous studies also indicated that Dam overproduction in *Y. pseudotuberculosis* caused the ectopic secretion of LcrV (low calcium response protein V) under conditions that are nonpermissive for synthesis and secretion in the WT strain (i.e. under high-calcium and low-temperature conditions). LcrV is a *Yersinia* T3SS virulence protein involved in the expression and translocation of Yop proteins, as well as in the suppression of host inflammatory activities *via* interleukin-10 by activation of toll-like receptor 2 (Julio *et al.*, 2002, Brubaker, 2003, Badie *et al.*, 2004, Viboud and Bliska, 2005). It was demonstrated in *Y. pseudotuberculosis* that the protection conferred by the Dam-overproducing strain against the WT bacterium is highly dependent on the presence of LcrV (Badie *et al.*, 2004). Such dependence on LcrV may be due to its role as a principal immunogen and/or its role in the synthesis and localization of Yops, which may also contribute to the immunity observed in Dam-overproducing, *Yersinia*-vaccinated hosts (Badie *et al.*, 2004). A recent study indicated that oral immunization of mice with a *dam* mutant of *Y. pseudotuberculosis* protected them against infection with *Y. pestis*

(Taylor *et al.*, 2005). In fact, recent studies in our laboratory have indicated a similar phenomenon. Intraperitoneal injection of mice with a $2LD_{50}$ dose of Dam-overproducing *A. hydrophila* did not result in any lethality, while 100% of those animals injected with the WT bacterium died within 2-3 days. Furthermore, immunization of mice with the native Dam-overproducing strain at the same LD_{50} dose provided protection to animals after subsequent challenge with a lethal dose of the control WT strain (Erova *et al.*, 2006).

Taken together, the overproduction of Dam in *A. hydrophila* SSU altered the expression of two key virulence factors of this bacterium, namely T3SS- and T2SS-associated Act, in addition to motility and proteinase production. Overexpression of the *dam* gene might alter the expression of virulence genes in a positive or a negative way. Perhaps this dual nature of Dam in *A. hydrophila* may be responsible for causing diseases *via* aberrant virulence gene expression. Although expression of the *act* gene was increased in the Dam-overproducing strain compared to the WT *A. hydrophila*, overall virulence of the bacterium appears to depend upon the interplay between the T3SS- and/or T2SS-associated Act, and possibly other factors.

CHAPTER 5:
MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A TOX-R REGULATED LIPOPROTEIN FROM A CLINICAL ISOLATE OF *A. HYDROPHILA*[5,6]

INTRODUCTION

In our attempt to identify new virulence factors in isolate SSU of *A. hydrophila*, we performed mass-spectrometric analysis of several secreted proteins to assess the potential of T3SS effector proteins in this bacterium. One of the proteins we identified by this method exhibited homology to a ToxR-regulated lipoprotein or TagA, recently identified in the enteric pathogens *V. cholerae* (Harkey *et al.*, 1995) and *E. coli* O157:H7 (Lathem *et al.*, 2002, Lathem *et al.*, 2004, Grys *et al.*, 2005). Based on recent studies on the mechanism of action of *E. coli* O157:H7 TagA (now designated as StcE), it was noted that this protein potentiated the activity of the C1-INH (Lathem *et al.*, 2004). Henceforth, the gene from *E. coli* O157:H7 will be referred to as *stcE* and its gene product as StcE. We use the designation of *tagA* to refer to the gene from *A. hydrophila* SSU, because this nomenclature was originally adopted for the gene in both *V. cholerae* and *E. coli* O157:H7 (Harkey *et al.*, 1995, Paton and Paton, 2002). The C1- INH belongs to the superfamily of serine protease inhibitors (also referred to as serpins) and is the only inhibitor of activated C1r and C1s of the classical complement cascade, contact activation

[5]Reproduced with permission from the following source: Infection and Immunity (http://www.iai.asm.org); Pillai L., Sha J., Erova T.E., Fadl, A.A., Khajanchi B.K., and Chopra A.K. 2006. Molecular and functional characterization of a ToxR regulated lipoprotein from a clinical isolate of *Aeromonas hydrophila*. *Infect. Immun.* 74:3742-3755.

[6]**Author contributions:** Lakshmi Pillai was involved in initial identification of TagA based on SDS-PAGE and MS analysis and sequencing a portion of the gene with homology to the type 3 effector protein and *stcE*. L. Pillai was also involved in purifying recombinant TagA and in performing all mechanistic and confocal studies. L. Pillai and Dr. Amin A. Fadl (McLaughlin postdoctoral fellow) were involved in *in vivo* studies. L. Pillai was involved in writing the manuscript and preparing it for submission to journal. Dr. Jian Sha (Assistant Professor) was involved in sequencing full-length *tagA*, preparing its isogenic mutant and in hyperexpression of the gene in pET30 system. Dr. Tatiana Erova was involved in preparing the *tagA* complemented strain.

pathway, and intrinsic pathway of coagulation (Caliezi *et al.*, 2000). It is therefore endowed with anti inflammatory properties. Serpins, such as C1-INH, inhibit the action of their respective serine proteases by mimicking the three dimensional structure of the protease's normal substrate, thus blocking the enzyme's activity (Davis, 2004). Importantly, these proteases cleave within the serpins, leading to formation of a covalent bond linking the two molecules. A massive allosteric change in the serpin's tertiary structure causes the attached protease to be moved to a site where it can be destroyed (Davis, 2004). Almost 20% of the proteins found in blood plasma are serpins and their abundance reflects the fact that serpins inhibit excessive proteolysis, which affects host clotting and complement systems (Carugati *et al.*, 2001). Metalloproteases are widely spread in many pathogenic bacteria, where they play crucial functions related to colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection of the host (Vollmer *et al.*, 1996, Potempa *et al.*, 2000, Supuran *et al.*, 2002). StcE is a metalloprotease with a catalytical metal ion, Zn^{2+}, and hence is known as a zinc metalloprotease (Lathem *et al.*, 2002). The cleavage of C1-INH by StcE was initially believed to abrogate the activity of the esterase inhibitor, ultimately resulting in pro-inflammatory and coagulation responses culminating in localized tissue damage, intestinal edema and thrombotic abnormalities due to the increased activation of complement (Lathem *et al.*, 2002). However, recent data indicated StcE might increase and prolong survival of the bacterium within the host by preventing complement activation and the subsequent tissue damage (Lathem *et al.*, 2004). In addition to C1-INH, two other substrates were recently identified for StcE in *E. coli* O157:H7 (Grys *et al.*, 2005). The substrates, gp340 and mucin 7, are heavily glycosylated proteins found in human saliva, and, therefore, the cleavage of these substrates by StcE could potentially allow the bacterium to establish a successful infection in the host by evading its mucosal defenses (Grys *et al.*, 2005).

Here, we reported the first identification and characterization of TagA in an *Aeromonas* species. We provided evidence that the *tagA* gene was functional in the

clinical isolate SSU of *A. hydrophila* and that purified TagA interacted with and cleaved C1-INH. By cleaving C1-INH, TagA potentiated the activity of this serpin, resulting in more inhibition of complement. We also constructed a *tagA* isogenic mutant, which lost the ability to bind C1-INH and to cleave the serpin; these activities were restored after complementation of the *tagA* mutant. Finally, we demonstrated that TagA provided increased serum resistance to *A. hydrophila* SSU as well as to *E. coli* DH5α and contributed significantly to bacterial virulence in a mouse model.

RESULTS

Identification and cloning of the *tagA* gene from *A. hydrophila* SSU

The presence of a T3SS in the diarrheal isolate SSU of *A. hydrophila* (Sha *et al.*, 2005) led us to postulate the functionality of the system by the secretion of effector proteins. Consequently, we concentrated culture supernatants of *A. hydrophila* by TCA precipitation and separated the resulting proteins by SDS-12% PAGE. Unique protein bands present in the supernatant of *A. hydrophila*, but not in *A. salmonicida,* were isolated from the stained gel, trypsin digested, and analyzed by MS and tandem MS (MS-MS) analysis. One of the proteins (AH5; 85-90 kDa) yielded some homology to a T3SS-associated effector protein homolog from a plant pathogen *E. amylovora* (Wei *et al.*, 2000). Consequently, this protein was further subjected to NH_2-terminal and internal sequencing. Three major tryptic digest peaks were sequenced and ClustalW alignment of two tryptic digest sequences, a total of 22 aa residues identified by sequencing, also exhibited a 64% homology at the aa level within residues 109-130 of an unknown environmental protein from the Sargasso Sea (Venter *et al.*, 2004).

Believing this protein could be of interest to us, we designed primers against the 'unknown gene' sequence to determine the identity of our AH5 gene. Subsequent sequencing of a 455-bp fragment amplified from the gDNA of *A. hydrophila* SSU resulted in a match with the *stcE* gene of *E. coli* O157:H7 (Lathem *et al.*, 2002). Interestingly, updated NCBI BLAST homology searches with the 'unknown' sequence

also resulted in a significant match (52% at the aa level) with the *stcE* gene. Consequently, we cloned and sequenced the entire *tagA* gene, along with its flanking upstream and downstream sequences, by screening two recombinant plasmid libraries of *A. hydrophila* SSU (for details see Materials and Methods section). We screened approximately 3,000 colonies of each library to obtain 4-5 positive clones.

We also used an *A. hydrophila* SSU fosmid library previously prepared in the laboratory to obtain and confirm the sequence of the *tagA* gene and its flanking sequences. Five positive fosmid clones that contained inserts of approximately 25 kb reacted with the *tagA* gene probe out of 500 screened colonies. The full-length *tagA* gene encoded a protein of 793 aa with a molecular mass of 89 kDa. The overall homology at the aa level of *A. hydrophila* SSU TagA with that of *E. coli* O157:H7 StcE was 64% (Figure 5.1), and the identity at the nucleotide level was 60%. It is important to note that the *A. hydrophila tagA* gene was considerable shorter (by 285 bp) at the 3' end than its homolog in *E. coli* O157:H7. However, the TagA of *A. hydrophila* possessed the unique ligand binding site (which is underlined) (HEVGHNYGLGH) common to all zinc metalloproteases (Jung *et al.*, 1999), suggesting this protein should be functional (Figure 5.1). Furthermore, *A. hydrophila* SSU TagA also had a hydrophobic leader sequence (69 bp), an indication that it, like *E. coli* O157:H7 StcE, would be secreted out of the bacterial cell. Interestingly, TagA of *A. hydrophila* SSU shared limited identity (< 40%, and in the region that spanned the metalloprotease active site) with the only other ToxR-regulated lipoprotein sequence in the GenBank database of unknown function from *V. cholerae* (U12265). This poses an interesting evolutionary perspective on how *A. hydrophila* acquired this gene, i.e., whether it was horizontally acquired from the pathogenic *E. coli* O157:H7 strain, transferred from *V. cholerae*, or acquired from a third, unidentified source.

Fig. 5.1. AA sequence comparison of TagA from *A. hydrophila* SSU and *E. coli* O157:H7 StcE.

The aa sequence of *A. hydrophila* TagA was obtained after cloning the *tagA* gene in the pBluescript cloning vector and by sequencing of the fosmid library clones. The sequence of 793 aa residues of TagA was aligned by ClustalW Protein Sequence Alignment with the published sequence of *E. coli* O157:H7 StcE. The conserved zinc metalloprotease-active-site of the enzyme is in bold and highlighted. **-**, not found; *, conserved aa residues, **:**, identical aa residues. **.**, functionally similar aa residues. Numbers on the right indicate the positions of the aa residues.

Purification of *A. hydrophila* TagA

The *tagA* gene was overexpressed, and rTagA as a histidine-tagged fusion protein was purified using ProBond resin charged with nickel. Purified TagA was eluted from the column in 8 M urea buffer with 20 mM NaH_2PO_4 and 500 mM NaCl, pH 4.0. Based on SDS-12% PAGE and Coomassie blue or SYPRO-Ruby staining, a single protein band with a molecular mass of ~90 kDa was detected in eluted fractions 3 and 4 (Figure 5.2A). The molecular mass of purified TagA was in agreement with the predicted size of 793 aa residues (89,006 Da) based on the DNA sequence plus the additional aa residues derived from the histidine-tag region of the pET-30a(+) vector.

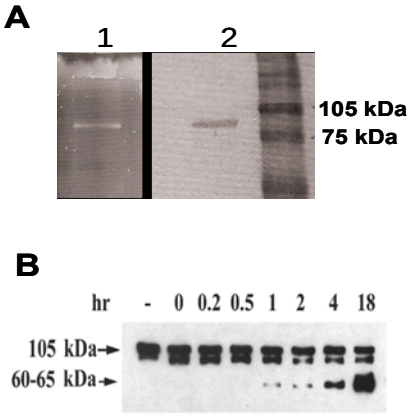


Fig. 5.2. Purification of rTagA and cleavage of C1-INH
(A) Purification of recombinant TagA. TagA was produced as a fusion protein with a His-tag in *E. coli* DE3 strain from pET30a vector as described in Materials and Methods. After dialysis, the purified protein from fractions 1-5 was examined by SDS-12% PAGE, followed by SYPRO-Ruby (lane 1) or Coomassie blue (lane 2) staining of the gel. Data from fraction 3 (representing homogeneous preparation) are shown. The molecular masses of the protein markers are indicated. (B) Time-dependent digestion of C1-INH by purified recombinant TagA. C1-INH (12 μg) was mixed with rTagA (1 μg) in 120 μl AD buffer. An aliquot (20 μl) of the reaction was removed at various time points (from 0 to 18 h) and subjected to Western blot analysis as described in Materials and Methods. The primary antibodies used were to C1-INH followed by HRP-conjugated rabbit anti-goat secondary antibodies. The membranes were then treated with the chemiluminescence substrate. The leftmost lane represents untreated C1-INH (2 μg). Lanes designated 0-18 indicate hours of incubation of rTagA with C1-INH in order to observe cleavage of the native C1-INH from 105 kDa to 60-65 kD polypeptide. The cleaved product of C1-INH (60-65 kDa) was observed after digestion with TagA. The presence of a doublet band for commercially available C1-INH was noted which could represent either the degradation product or different forms of C1-INH on a denaturing gel. This phenomenon was also evident in the Western blot figures of C1-INH cleavage by rStcE'-His (Lathem *et al.*, 2002).

Cleavage of C1-INH by TagA.

To determine functionality of *A. hydrophila* TagA, we examined cleavage of C1-INH with rTagA. We expected, based on previous studies (Lathem *et al.*, 2002), that TagA would cleave the native 105-kDa form of C1-INH to a 60-65 kDa product. It is known that proteases from several other bacteria, such as *Serratia marcescens* (Maeda and Molla, 1989), play a role in cleaving the C1-INH component of the complement system. Purified human C1-INH was mixed with rTagA and aliquots of the reaction were removed at specific times and subjected to immunoblot analysis using antibodies to C1-INH (Figure 5.2B). Over time, cleavage of the 105-kDa band, with the appearance of a ~65-kDa cleavage product was apparent. The intensity of the 65-kDa protein band increased significantly between 1-18 h. To determine if this cleavage of C1-INH would be seen with whole cells of *A. hydrophila*, we treated purified C1-INH with log-phase-grown WT *A. hydrophila, tagA* mutant or its complemented strain (*tagA*/pBR*tagA*). As shown in Figure 5.3, a specific cleavage pattern was evident only when C1-INH was treated with either the WT bacterium or its complemented strain (Figure 5.3A, lane 3 and Figure 5.3B, lanes 1 & 3) and not with the *tagA* mutant (Figure 5.3A, lane 1 and Figure 5.3B, lane 2). These data show functional protease activity of *A. hydrophila* TagA using C1-INH as a substrate.

C1-INH-mediated inhibition of complement is potentiated by *A. hydrophila* TagA

To evaluate *A. hydrophila* TagA's role in potentiating the inhibitory activity of C1-INH, we tested the effect of TagA-treated C1-INH on the lysis of erythrocytes in the presence of serum. Increasing concentrations of C1-INH (2, 8, and 16 µg) were either untreated or treated with 1 µg of rTagA overnight before we added opsonized sheep erythrocytes and human serum. As illustrated in Figure 5.4, increasing concentrations of C1-INH resulted in a dose-dependent decrease in hemoglobin release or erythrocyte lysis. However, when the inhibitor was pre-treated with 1 µg of rTagA, a significant reduction in the lysis of erythrocytes resulted due to increased complement inhibition (Figure 5.4). These results were specific to TagA, as C1-INH treated with heated (inactivated) rTagA

or with another metalloprotease, elastase from *P. aeruginosa*, did not reduce erythrocyte lysis at a concentration of 2 μg of C1-INH (the dose at which the greatest reduction in erythrocyte lysis was observed from untreated C1-INH to TagA-treated C1-INH). These data indicated the direct role of *A. hydrophila* SSU TagA in the potentiation of complement inhibition through its effect on C1-INH. Ultimately the bacterium can survive longer within the host by evading the detrimental effects of complement.

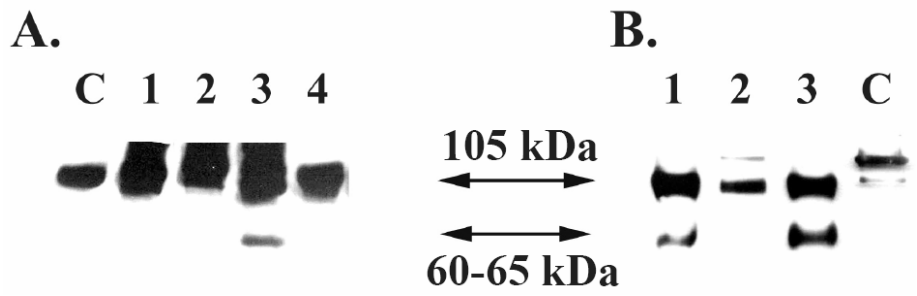


Fig. 5.3. Cleavage of C1-INH by WT and TagA mutant of *A. hydrophila* SSU.
(A). Whole WT *A. hydrophila*, *tagA* mutant, or *E. coli* DH5α bacterial cells (10^6) were mixed with purified human C1-INH (20 μg) and aliquots of the reaction were removed at various time points (0 and 8 h) for analysis by immunoblotting as described in the Materials and Methods section. The whole-cell of WT *A. hydrophila* SSU cleaved C1-INH from its native size of 105 kDa into a 60-65 kDa fragment (8 h) (lane 3). Lane 2 represents the absence of cleavage seen with the WT *A. hydrophila* SSU at 0 h. The whole-cell from the *tagA* mutant did not cleave C1-INH, even after 8 h of incubation (lane 1). *E. coli* DH5α cells not possessing the *tagA* gene were unable to cleave the inhibitor (lane 4), even after 8 h of incubation. A smearing pattern in this blot was due to the high load of bacterial proteins on the gel. (B) In this experiment, WT, *tagA* mutant or complemented strain (10^6 cells) were mixed with 20 μg of C1-INH and incubated for 8 h at room temperature. The absence of C1-INH cleavage noted with the *tagA* mutant was specific (lane 2), as this activity was restored in the complemented strain (lane 3) and was similar to the pattern seen with WT *A. hydrophila* SSU (lane 1). In both figures, lane C represents untreated C1-INH (2 μg). Once again, the presence of a doublet band for C1-INH was noted (Lathem *et al.*, 2002).

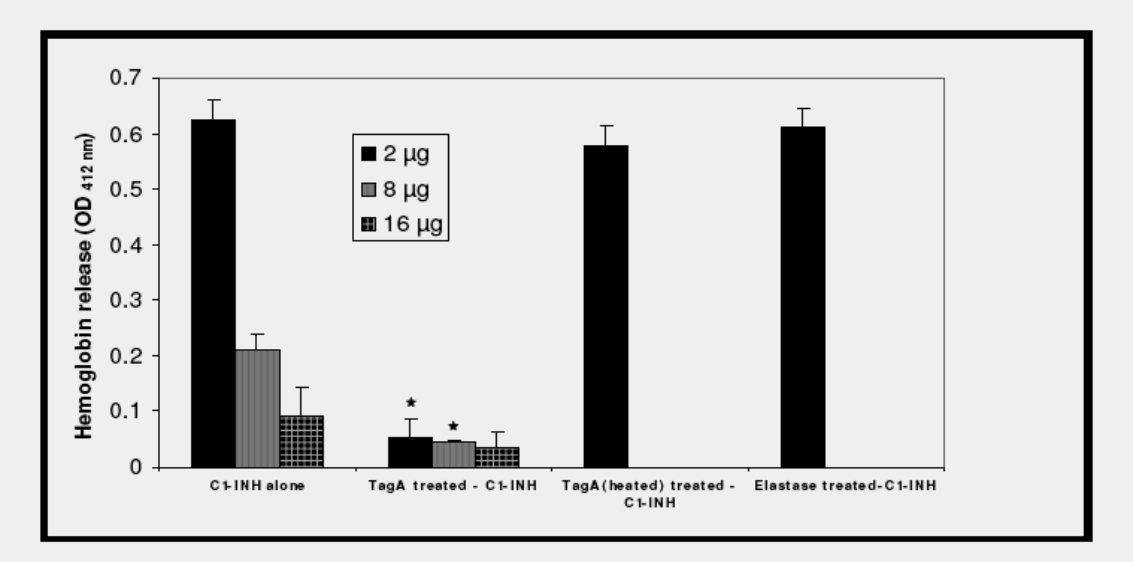


Fig. 5.4. The C1-INH-mediated inhibition of the classical complement cascade is potentiated by TagA from *A. hydrophila* SSU.
Increasing amounts of C1-INH (2, 8, and 16 µg) were either treated with 1 µg of rTagA (or heated rTagA), 1 µg of the metalloprotease elastase from *P. aeruginosa*, or were left untreated overnight at room temperature. The next day, opsonized sheep erythrocytes were mixed with the C1-INH overnight incubated reaction mixture as described in Materials and Methods. After incubation for 1 h at 37°C, the erythrocytes were pelleted and the OD_{412} of the supernatant was measured. * denotes statistically significant values ($P \leq 0.05$).

Having illustrated an important function for TagA in contributing to complement inhibition, we examined whether rTagA demonstrated protease activity only against the substrate C1-INH, or if other substrates were also able to be hydrolyzed by this enzyme. We noted that rTagA (25 ng-1 µg) was able to hydrolyze the chromogenic substrate Hide azure powder in a dose-dependent manner. As a positive control, *P. aeruginosa* elastase demonstrated high protease activity. The protease activity of TagA against this additional substrate was shown to be specific, as recombinant protein heated to 80°C for 10 min or rTagA neutralized by α-StcE antibodies was not able to hydrolyze the substrate. Further, the protease activity of rTagA was inhibited with 5 mM of EDTA, indicating requirement of a metal ion (Zn^{2+}) for the enzymatic activity (Fig. 5.5)

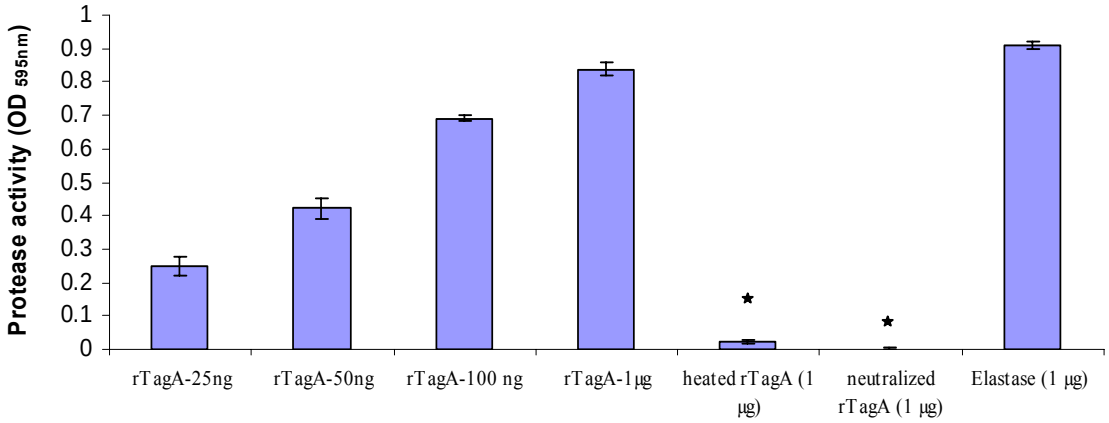


Fig. 5.5. TagA of *A. hydrophila* SSU demonstrates protease activity.
Increasing concentrations of rTagA, heated rTagA or rTagA neutralized with α-StcE antibodies were incubated with PBS and 5 mg of Hide azure powder substrate. The tubes were incubated in a shaker incubator at 37°C for 2-8 h. The blue color was quantified at OD_{595}. The substrate incubated with 1 µg of elastase from *P. aeruginosa* served as a positive control and represents OD after just 1 h of incubation. (* denotes statistically significant difference between protease activity of 1 µg of rTagA and heated or neutralized rTagA [1 µg], $P \leq 0.05$).

TagA increases the serum resistance of *E. coli* and that of *A. hydrophila* SSU.

Many pathogenic bacteria have developed mechanisms to evade the host immune system by preventing killing by complement activation (Figueroa and Densen, 1991). As *Aeromonas* strains are naturally serum resistant due to the presence of either a capsule or surface layer and/or certain outer membrane proteins (Merino *et al.*, 1991, Aguilar *et al.*, 1999), we first determined if rTagA of *A. hydrophila* SSU could impart serum resistance to *E. coli* DH5α, which is serum sensitive. *E. coli* strain DH5α grown to mid-log phase was incubated with human serum (2%) and 1µg of rTagA for 1 h at 37°C, serially diluted, and plated onto LB agar plates to determine CFUs and percentage of survival. *E. coli* grown in the presence of serum alone had a survival rate of only 5%. However, the addition of TagA-treated C1-INH significantly increased survival of the bacteria to 50%

in the presence of serum. The addition of TagA or C1-INH alone in the presence of serum did not significantly increase survival of the bacterium (Figure 5.6A).

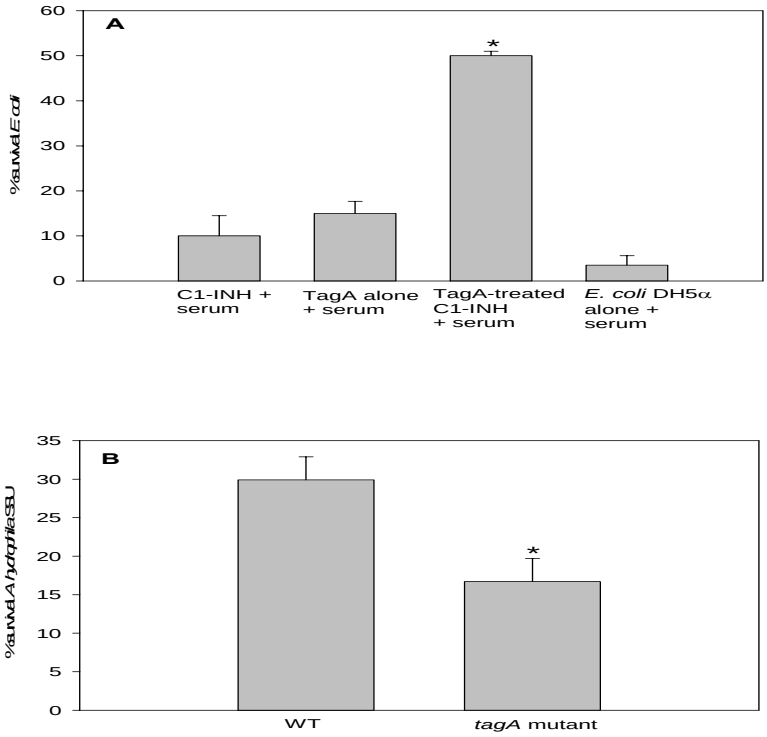


Fig. 5.6. TagA provides increased serum resistance to *E. coli* DH5α and *A. hydrophila* SSU by increasing the inhibitory activity of C1-INH.
(A). An aliquot (8 µg) of C1-INH was untreated or treated with 1 µg of rTagA and incubated overnight (o/n) at room temperature and the next day was added to an aliquot (20 µl) of mid-log-phase grown *E. coli* DH5α cells, as described in Materials and Methods. After incubation for 1 h at 37°C, the bacteria were serially diluted and plated onto LB agar plates. The percentage of surviving bacteria was calculated as described in Materials and Methods (*, P = 0.001, unpaired t-test). (B) TagA also contributes to the serum resistance of *A. hydrophila* SSU. WT bacterium or its *tagA* mutant was grown to mid-log phase, washed in PBS and resuspended in an equivalent amount of PBS. An aliquot (20 µl) of the bacterial cells was added to a mixture of PBS and 50% serum and incubated at 37°C for 3 h (Merino *et al.*, 1996). The percentage of surviving cells was calculated as described in Materials and Methods. (*, P = 0.04, unpaired t-test).

We then examined the role of TagA in the serum resistance of *A. hydrophila* SSU. For these experiments, we compared the survival of the WT bacterium with that of the *tagA* mutant in the presence of 50% human serum. A high concentration of human serum was required to kill *A. hydrophila*. Different human serum concentrations (5, 10, 15, 20, and 30%) were used initially to titrate the percentage of surviving WT cells (data not shown). However, only 50% human serum was shown to have any significant effect in killing WT *A. hydrophila* SSU as well as to demonstrate a significant reduction in the survival of the *tagA* mutant. As shown in Figure 5.6B, the *tagA* mutant was significantly less serum resistant compared to the WT *A. hydrophila* SSU.

Binding of *A. hydrophila* SSU TagA to human C1-INH

To further confirm the binding of *A. hydrophila* TagA to C1-INH, we performed sandwich Western blot analysis. In one set of experiments, the purified C1-INH was subjected to electrophoresis, followed by its transfer to a nitrocellulose membrane. This membrane was treated with rTagA before adding primary antibodies. When the membrane was probed with antibodies to StcE, a band of 105 kDa was detected corresponding to the size of purified human C1-INH (2 μg), which was run on the gel (Figure 5.7A, lane 1). Therefore, detection of a band similar to the size of that of C1-INH indicated binding of TagA to C1-INH on the membrane. The rTagA was also run on the gel as a positive control, indicating the native size of the protein (Figure 5.7A, lane 2). Similarly, when purified rTagA (1 μg) was first run on the gel and the membrane was treated with C1-INH, followed by antibodies to C1-INH, a band corresponding to the size of TagA (90 kDa) was detected (Figure 5.7B, lane 2). As a positive control, C1-INH (1 μg) was also run on the gel and a band corresponding to 105 kDa reacted to the C1-INH antibodies (Figure 5.7B, lane 1). No band was detected in either gel when cholera toxin (CT) was used (Figure 5.7A & B, lane 3), indicating a specific interaction between C1-INH and TagA. It is important to note that antibodies to C1-INH specifically reacted with purified human C1-INH and did not react non-specifically with rTagA and vice-a-versa (data not shown). Sandwich Western blot analysis data further substantiated interaction of TagA with its substrate C1-INH in both their native and denatured forms.

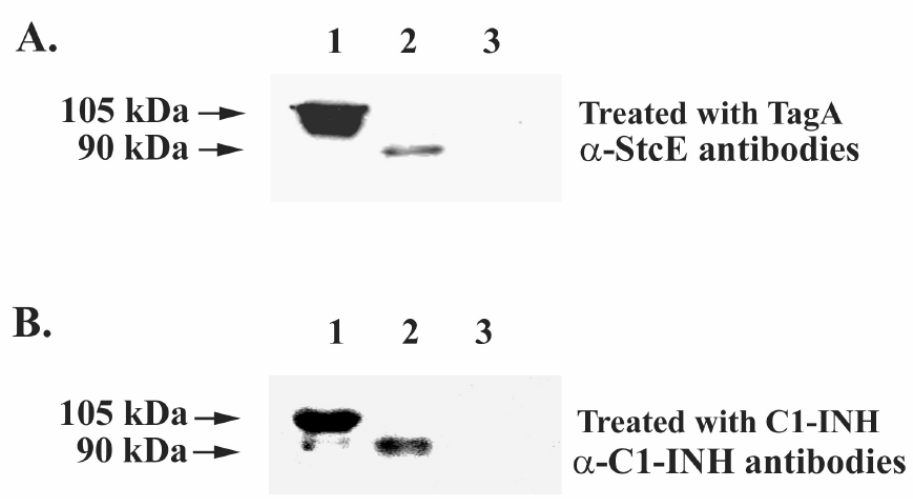


Fig. 5.7. Confirmation of the interaction of TagA with C1-INH by sandwich Western blot analysis.
C1-INH (2 μg), rTagA (1 μg), or cholera toxin (CT, 1 μg) was loaded on SDS-12% polyacrylamide gels, and electrophoresis was performed before transfer to nitrocellulose membranes. The membranes were subsequently incubated with either TagA or C1-INH and probed with anti-StcE or anti-C1-INH antibodies. (A) Sandwich Western blot analysis using C1- INH (2 μg). A 105-kDa-sized band (lane 1) was detected when membranes were first treated with rTagA (1 μg/ml) overnight at 4°C and then probed with anti-StcE antibodies. Lane 2 was used as a control to indicate the native size of TagA. No band was detected when CT was loaded on the gel instead of C1-INH (lane 3). (B) Sandwich Western blot analysis when TagA protein (1 μg) was loaded on the gel, incubated with C1-INH (1 μg/ml) overnight and probed with anti-C1-INH antibodies. Note binding of C1-INH specifically to TagA (lane 2, a 90-kDa band). Lane 1 was used to visualize the native size of the C1-INH protein. No band was detected when CT was loaded on the gel instead of TagA (lane 3).

Binding of C1-INH and TagA on bacterial surface of *A. hydrophila* SSU

The cleavage of C1-INH by StcE has been reported (Lathem *et al.*, 2002). Further, to elucidate the functional interaction of C1-INH and TagA and the specificity of binding of C1-INH to the bacterial surface of *A. hydrophila* SSU, we performed confocal fluorescence microscopy. Colocalization experiments were performed using polyclonal antibodies to both C1-INH and StcE and by using the WT bacterium, its *tagA* mutant, and

the complemented strain. Treating WT bacteria with C1-INH and then staining the cells with antibodies to both C1-INH and StcE, revealed a co-localization pattern consistent with the observed interaction between these two proteins (Figure 5.8A). The binding specificity of C1-INH to TagA was demonstrated using the *tagA* mutant and the complemented strain. As shown in the figure (Figure 5.8A & C), the WT and complemented strains bound individually to both of the fluorescently tagged antibodies. However, the *tagA* isogenic mutant did not express TagA on the surface, as seen from the lack of staining with anti-StcE antibodies and Alexa-Fluor labeled secondary antibodies, and hence did not show any co-localization of the metalloprotease and C1-INH (Figure 5.8B). The complemented strain, however, displayed co-localization of TagA and C1-INH on the bacterial surface similar to that seen in the WT bacterium (Figure 5.8C). It should be noted that uniform binding of the StcE and C1-INH antibodies to the bacterial surface was not evident probably because the bacterial cells were in different stages of growth (cells were not synchronized). Therefore, the level of expression of TagA on the bacterial surface might vary depending on the half-life of mRNA encoding TagA as well as the rate of degradation of this protein.

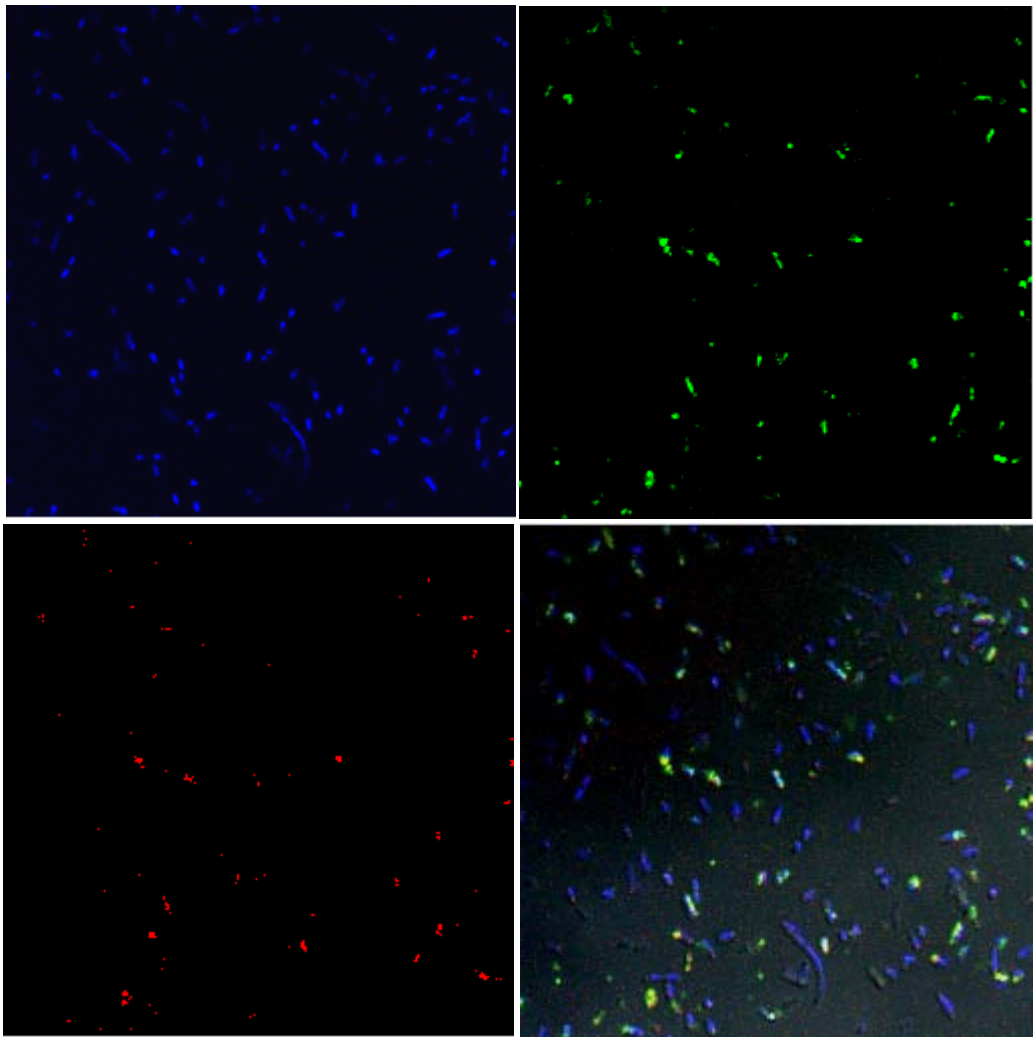
***In vivo* effects of TagA in *A. hydrophila* SSU**

In our *in vivo* studies, we noted that 100% of the animals infected with the 4 x 10^7 dose of WT *A. hydrophila* died within 48 h (Figure 5.9). However, only 40% ($p = 0.0038$ compared to WT bacteria) of the animals died when inoculated with the *tagA* mutant of *A. hydrophila* SSU at the same dose. These data indicated TagA played a significant role in contributing to the overall survival of the bacteria within the host. We also investigated the colonization of the *tagA* mutant bacteria in comparison to that of the WT *A. hydrophila*. The *in vivo* survival of WT *A. hydrophila* and its isogenic mutant was determined by counting the number of bacteria in the spleen of the mice on days 1 and 3 post-infection. We noted no significant difference in the number of WT and *tagA* mutant bacteria in the spleen on day 1 (data not shown). Further, the bacteria were quickly cleared from the host system, as day 3 showed almost no bacteria to be present in the tissue, with no significant difference between the WT and the mutant (data not shown).

These data indicated that the observed difference in survival of mice challenged with the *tagA* mutant bacteria was not due to an impaired ability of these bacteria to colonize host tissue.

Fig. 5.8A

WT **α-StcE**



α-C1-INH **Co-localization**

Fig. 5.8B

TagA mutant **α-StcE**

α-C1-INH **Co-localization**

Fig. 5.8C

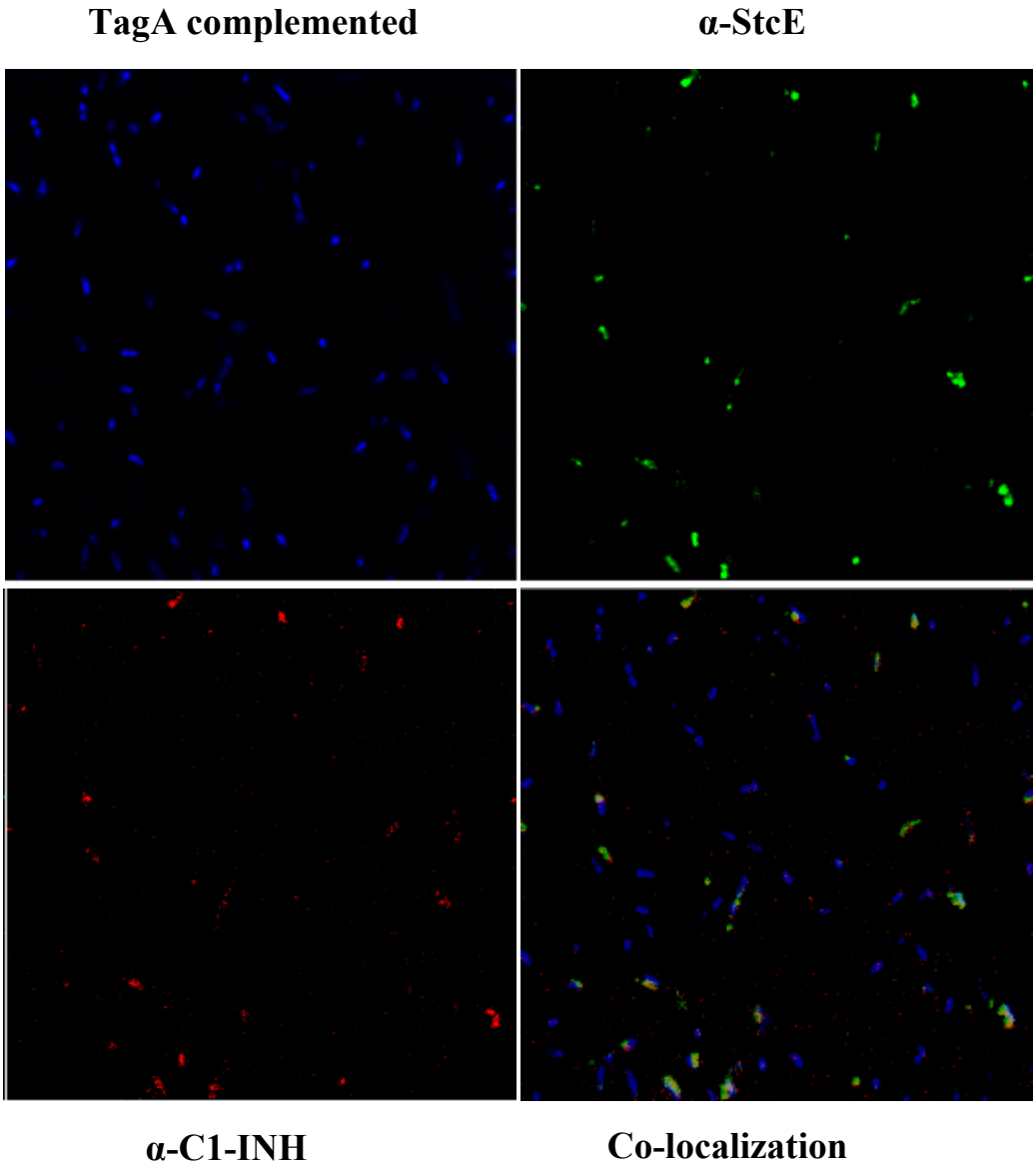


Fig. 5.8. Co-localization of C1-INH and TagA on the surface of *A. hydrophila* SSU and its *tagA* isogenic mutant.
WT *A. hydrophila* and *tagA* mutant were grown to an OD_{600} of 0.5, washed with PBS and treated with 2 µg of C1-INH for 2 h. Cells were then washed thoroughly with PBS several times and resuspended in PBS to a final concentration of 10^7 cells/20 µl. The cells were then fixed with 4% paraformaldehyde, washed with PBS, and then incubated with primary and fluorescein-conjugated secondary antibodies to both C1-INH and StcE

for 1 h, as described in Materials and Methods. After subsequent washes in PBS, followed by DAPI staining of the cells, binding was inspected by confocal fluorescence microscopy. The panels indicated as WT cells, TagA mutant, and TagA complemented strain show staining by DAPI. The panel indicated as α-StcE illustrates binding with anti-StcE antibodies and Alexa-Fluor labeled secondary antibodies, while the α-C1-INH panel indicates binding with anti-C1-INH antibodies and TX-Red-labeled secondary antibodies. The co-localization panel demonstrates binding of these two labeled proteins on the bacterial surface. (A) WT *A. hydrophila*. Approximately 70% of the total number of the cells in the field showed co-localization of TagA and C1-INH. (B) *tagA* mutant bacteria. No binding of TagA with C1-INH was seen. (C) pBR*tagA* complemented strain. Approximately 60% of the total number of cells in the field showed co-localization of TagA with C1-INH. At least 5 fields were visualized to calculate the percentage of positive cells. Total magnification, x2520.

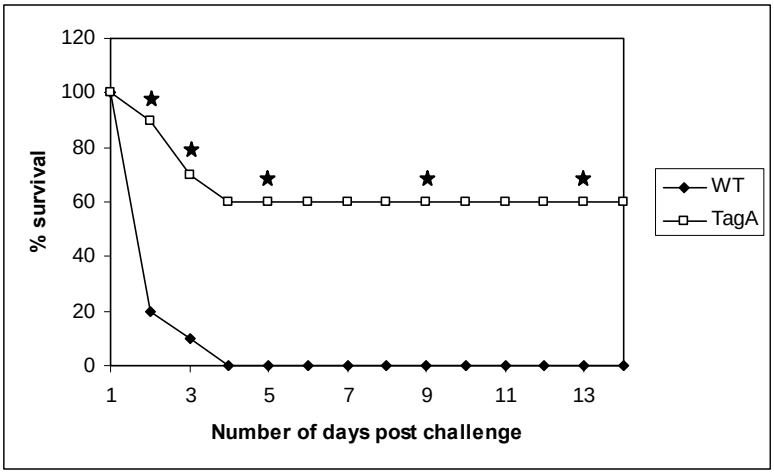


Fig. 5.9. TagA of *A. hydrophila* SSU contributes to the virulence of the bacterium. Swiss Webster mice (n = 10 per group) were injected intraperitoneally with two 50% lethal doses of WT *A. hydrophila* SSU. The same dose was used to infect mice with the *tagA* mutant and both groups were monitored for death over a 14-day period. The data were statistically analyzed using Fisher's exact test. Three independent experiments were performed, and data from a typical experiment are shown. * denotes statistically significant differences between *tagA* mutant and WT bacteria, $P \leq 0.05$.

DISCUSSION

The role of TagA in bacterial virulence is relatively new, with the presence of the *tagA* gene detected only in *V. cholerae* and *E. coli* O157:H7 (Harkey *et al.*, 1995, Lathem

et al., 2002, Lathem *et al.*, 2003, Lathem *et al.*, 2004). The functionality of this gene (designated as *stcE*) was recently elucidated only in the latter pathogen, where it is encoded on the pO157 virulence plasmid of *E. coli* O157:H7 (Lathem *et al.*, 2002, Lathem *et al.*, 2004, Grys *et al.*, 2005). In our study, we identified, cloned, and functionally characterized the chromosomally encoded *tagA* gene from a diarrheal isolate SSU of *A. hydrophila*. Further, the role of *A. hydrophila* TagA in altering bacterial virulence was evaluated using both *in vitro* and *in vivo* models. We showed for the first time the role of TagA in a mouse model of lethality as well as visualized colocalization of this metalloprotease to its target C1-INH on the bacterial surface.

TagA is regulated by the ToxR regulon, with the latter linked to the virulence potential of the *Vibrio* species. Although little is known regarding the host signals that impact the ToxR regulatory cascade, it is clear these intraintestinal signals play an important role in maximizing the ability of bacteria to survive and multiply within the host (Skorupski and Taylor, 1997). Whether TagA of *A. hydrophila* is regulated by a mechanism similar to that of *Vibrio* species is not known. TagA's role in bacterial pathogenesis is only now beginning to be understood.

To date, three proteases have been reported for *A. hydrophila*: 1) a thermostable 38-kDa metalloprotease (Rivero *et al.*, 1990, Loewy *et al.*, 1993); 2) a 19-kDa zinc protease (Loewy *et al.*, 1993); and 3) a 68-kDa temperature-labile serine protease (Rivero *et al.*, 1991). The 38-kDa metalloprotease (AhyB) has elastolytic activity and its isogenic mutant showed decreased virulence in rainbow trout (Cascon *et al.*, 2000). Colony blot hybridization analysis in our laboratory showed that TagA was widely distributed in *Aeromonas* species. Of 165 water isolates of *Aeromonas* screened for the *tagA* gene, 59% were positive. Further, 21% of 52 clinical isolates, obtained from patients with gastroenteritis, were also found to possess the *tagA* gene (data now shown). The presence of the *tagA* gene was noted in isolates obtained from patients with both gastroenteritis and wound infections.

When the metalloprotease StcE was initially identified in *E. coli* O157:H7, it was believed to contribute to the pathophysiological derangements of hemolytic uremic

syndrome (HUS), by degrading the serpin C1-INH (Lathem *et al.*, 2002). The degradation of C1-INH would result in the loss of control of multiple proteolytic cascades, including the classical and alternative complement pathways, intrinsic coagulation, and contact activation (Lathem *et al.*, 2002). *Aeromonas* species are also known to cause HUS (Bogdanovic *et al.*, 1991). Therefore, it is plausible that TagA could play a role in HUS during *Aeromonas* infections. However, we noted that the cleavage of C1-INH by TagA enhances, rather than inhibits, the serpin's ability to downregulate the classical complement cascade (as demonstrated in *E. coli* O157:H7), thereby protecting bacterial and host cells from the deadly lytic effects of complement activation (Lathem *et al.*, 2004). The mechanism by which TagA potentiates the ability of C1-INH to increase complement inhibition is not fully understood and requires further investigation.

The pathogenic and virulence characteristics of *A. hydrophila* have been shown to be associated with the production of T2SS-associated exoenzymes, such as proteases and lipases (Janda and Abbott, 1998, Chopra and Houston, 1999). Our results showed that TagA possessed protease activity, by acting on the substrates C1-INH and Hide azure powder. Earlier studies indicated that StcE from *E. coli* O157:H7 was not able to degrade casein, although elastase was able to do so, indicating the specificity of the metalloprotease StcE for the substrate C1-INH (Lathem *et al.*, 2002). However, later studies did increase the range of potential substrates for StcE to include mucin 7 and glycoprotein 340 (Grys *et al.*, 2005). We would like to explore further potential substrates that TagA could act upon on the gut mucosal surface, a major site of inflammation in gastroenteritis caused by *A. hydrophila*.

Previous studies showed that StcE did not play a role in the general adherence of *E. coli* O157:H7 to HEp-2 cells but contributed significantly to their intimate adherence (Grys *et al.*, 2005). Likewise, our results indicated that TagA did not contribute to the adherence of *A. hydrophila* cells to HT-29 intestinal epithelial cells (Merino *et al.*, 1997) (data not shown) as well as *in vivo*.

Lathem *et al*. (Lathem *et al.*, 2004) reported that native C1-INH could not bind to erythrocyte surfaces. However, when treated with StcE of *E. coli* O157:H7, the serpin was, in effect, tethered to the cell surface where it could prevent the activation of complement (Lathem *et al.*, 2004). In general, direct activation of complement *via* the classical pathway results in the formation and deposition of the key complement component C3b (Kraiczy and Wurzner, 2006). The deposition of C3b on activator cell surfaces leads to opsonization followed by formation of the MAC, resulting in complement-mediated killing. Therefore, it is often benefical for invading microorganisms to inhibit complement activation at the key site of the cascade (Kraiczy and Wurzner, 2006). Different serogroups of *A. hydrophila* are able to evade complement by preventing the formation of the MAC on their cell surface. For example, *A. hydrophila* strains devoid of the S-layer are resistant to complement-mediated killing because C3b is rapidly degraded, and, therefore, the lytic membrane attack complex is not formed (Merino *et al.*, 1996). Other factors can also contribute to the serum resistance of *A. hydrophila*, such as the long O-polysaccharide chain of lipopolysaccharide (LPS) and a capsule-like outer layer, which help protect the bacterium from the killing effects of complement (Merino *et al.*, 1991, Merino *et al.*, 1998, Aguilar *et al.*, 1999). Two capsular genes of *A. hydrophila* (serogroup O:34) were determined to confer serum resistance to the *E. coli* K12 serum-sensitive strains (Aguilar *et al.*, 1999). Our studies indicated that TagA from the clinical isolate SSU of *A. hydrophila* plays a similar role as it could confer serum resistance not only to the homologous strain but also to a serum-sensitive *E. coli* strain.

Lathem *et al*. reported that it is not the cleavage of C1-INH by StcE, but the interaction of StcE with C1-INH on the erythrocyte surface, which provided protection against complement-mediated lysis (Lathem *et al.*, 2004). This was based on studies indicating no significant difference in the amount of erythrocyte lysis between the C1-INH samples treated with either StcE or the proteolytically inactive StcE mutant. Furthermore, they showed that little to no C1-INH binding was measured on erythrocytes treated with only StcE, whereas increased deposition of C1-INH was evident on evident

on erythrocytes mixed with StcE-treated C1-INH (Lathem *et al.*, 2004). This was taken as further evidence that the specific conformation between the enzyme StcE and its substrate C1-INH (achieved during incubation of C1-INH with StcE) is crucial for the recruitment of C1-INH to the surface of erythrocytes. We have not tested the requirement of C1-INH cleavage by TagA for the potentiation of complement inhibition by the use of a TagA proteolytic mutant in *A. hydrophila*. However, we cannot rule out the possibility that the cleavage of C1-INH by TagA is necessary for the potentiation of its inhibitory activity, ultimately leading to bacterial serum resistance. The sequesteration of complement inhibitors to bacterial surfaces is known to reduce complement activity and promote serum resistance in bacteria. For example, the binding of the complement alternative pathway regulatory molecule, Factor H to the outer membrane protein OspE of *Borrelia burgdorferi* enables this pathogen to evade complement attack and phagocytosis (Hellwage *et al.*, 2001).

It was reported that StcE is expressed on the surface of *E. coli* and could act as a "bridge" between C1-INH and the cell surface (Lathem *et al.*, 2004). Using confocal fluorescence microscopy, we illustrated co-localization of these two proteins in WT *A. hydrophila* and in the *tagA* mutant complemented with the *tagA* gene on a plasmid. How does the binding of this metalloprotease to the inhibitor of the classical complement cascade relate to virulence of the bacterium within a host? This question was addressed by our animal studies.

We provided evidence for the first time of TagA's role *in vivo* and demonstrated reduced lethality in mice infected with the *tagA* mutant. If TagA is contributing to increased complement inhibition, then why would deletion of this gene result in less host killing? One would expect that the absence of this gene would lead to a more striking virulent phenotype of the bacterium, and hence more pathology such as inflammation and tissue damage would be expected in the host, eventually resulting in death. Perhaps TagA's role in serum resistance is quite significant. Without the coordinated effort of TagA and other factors, *A. hydrophila* could succumb to the bactericidal effects of human serum more readily. Further, TagA's role as a protease could play a significant role in

the *in vivo* situation. It is known that metalloproteases from other bacteria such as *P. aeruginosa* as well as from *A. hydrophila* are crucial to the virulence potential of these pathogens (Cascon *et al.*, 2000, Cowell *et al.*, 2003).

Other explanations can also be offered to describe the phenomenon observed *in vivo*. TagA may play a dual role in the virulence of *A. hydrophila*. In addition to potentiating the inhibition of complement, it could cause damage in the host by acting synergistically with other virulence factors. Vollmer *et al*. (Vollmer *et al.*, 1996) uncovered a novel mechanism *via* which microbial proteases possibly provoke long-range biological effects in the host cell. Specifically, this group discovered that certain membrane-anchored proteins, including several cytokines and cytokine receptors, were released into tissue culture supernatants of tissue fluid *in vivo* through the action of endogenous membrane-bound metalloproteinases. The shed molecules were then able to perform biological functions; for example, soluble Il-6 receptor (sIL-6R) could bind to bystander cells, rendering these cells sensitive to the action of IL-6 (Vollmer *et al.*, 1996). Our future studies will focus on elucidating TagA's effect on host immune status, specifically in regard to cytokine production/secretion. The other side of this possible dual nature of TagA is illustrated by another important metalloprotease, the lethal toxin of *Bacillus anthracis*, which cleaves upstream mitogen-activated protein kinases and promotes immune evasion of the bacterium by suppressing activation of macrophages and dendritic cells (Agrawal *et al.*, 2003, Paccani *et al.*, 2005).

This study highlighted a unique role for the *A. hydrophila* SSU metalloprotease TagA in potentiating the activity of the serpin C1-INH in inhibiting complement activation. Our data illustrated that this enzyme also contributed to the serum resistance of the bacterium and played a direct role in its virulence. Research conducted on metalloproteases, such as TagA, illuminated a fascinating role for these proteinases in bacterial virulence; in many cases, they are key players in subverting host immune defenses (Potempa *et al.*, 2000). It is obvious that bacterial proteases represent very attractive targets for the generation of a novel class of therapeutics, since these enzymes

are ubiquitously found in many bacterial species, and the inhibition of such critical enzymes would presumably lead to the death of the invading pathogen.

CHAPTER 6:
CONCLUSIONS

The aim of this study was to identify new virulence factors which contribute to the pathogenesis of *A. hydrophila* infections. The organism causes a wide variety of diseases, the most common of which is gastroenteritis. Further, the majority of non-intestinal infections caused by *A. hydrophila* and other species of *Aeromonas* are fatal. *A. hydrophila* has been isolated from fresh, brackish, and chlorinated waters as well as a variety of foods. These bacteria also grow well at refrigeration temperatures. *A. hydrophila* produces a number of virulence factors which contribute significantly to the pathologies of gastroenteritis and septicemia, cause excessive inflammation, and are destructive to host tissues. A detailed understanding of the mechanism of action of *A. hydrophila* virulence factors is crucial for the study of *A. hydrophila*-associated infections and the results of this study address this important issue.

Our data indicated the presence of a T3SS encoded within the chromosome of a diarrheal isolate SSU of *A. hydrophila*. The T3SS is present in many gram-negative bacteria, including other species of *Aeromonas*, i.e., *A. salmonicida* (the first *Aeromonas* species in which a T3SS was reported), in which it is encoded on a plasmid (Burr *et al.*, 2003, Stuber *et al.*, 2003). Our laboratory identified a T3SS in a human isolate of *A. hydrophila* simultaneously with two other groups which identified this protein secretion system in the fish isolates AH1 and AH3 of *A. hydrophila* (Vilches *et al.*, 2004, Yu *et al.*, 2004, Sha *et al.*, 2005). By generating isogenic mutants of the *aopB* gene, a crucial gene involved in formation of the translocation apparatus of the T3SS, and a double knockout mutant of the *act* cytotoxic enterotoxin and the *aopB* gene, we demonstrated that the T3SS of *A. hydrophila* SSU led to cytotoxicity in murine macrophages and human colonic epithelial cells (Sha *et al.*, 2005). We further demonstrated that the T3SS was responsible for inducing cytotoxicity in host cells during the early stages (2 h) of bacterial-host cell interactions. Conversely, Act contributed more significantly to *Aeromonas*-associated cytotoxicity during the later stages of infection. We also noted

minimal lethality (10%) in mice injected with the *act/aopB* double mutant at a 2 LD_{50} dose, which killed 100% of the animals injected with *WT A. hydrophila*. These results indicated that both Act and the T3SS played crucial roles in bacterial virulence. By transmission electron microscopy (TEM), we were the first group to demonstrate the presence of a T3SS needle structure in the *A. hydrophila* bacterial cell and we were further able to isolate specifically the needle portions of these structures, which could be viewed under TEM (Galindo *et al.*, 2006). Currently, our laboratory is investigating potential secreted proteins of the T3SS from *A. hydrophila*, as identification of bacterial virulence factors injected into host cells is paramount in understanding the pathogenicity associated with this system.

In order to understand the regulation of potential virulence factors and how this regulation contributes to the pathogenicity of *A. hydrophila*, our laboratory had previously characterized the role of the ferric-uptake regulator (*fur*) and glucose inhibited division gene (*gidA*) in modulating the biological effects of Act (Sha *et al.*, 2001, Sha *et al.*, 2004). In the search for additional genes that could alter bacterial virulence, this study reported characterization of the DNA adenine methyltransferase (Dam) from *A. hydrophila* SSU that modulates the function of both the T3SS and Act, which is secreted through a T2SS. DNA methylation of specific adenine residues in 5'GATC3' sequences ultimately impacts the virulence of several bacterial pathogens, through the coordinated effects of methylation on DNA replication, repair of mismatched bases and alterations in gene expression.

Through the use of restriction endonucleases, which are affected in their ability to digest gDNA due to methylation of adenine residues in 5'GATC3' sequences, we determined that *A. hydrophila* SSU possesses a *dam* gene. By initially designing primers to a region of highest conserved homology among the *dam* genes from different Gram-negative bacteria, such as the *Vibrio* species (e.g., *V. cholerae, V. fischeri,* and *V. parahaemolyticus*), *E. coli*, *S.* Typhimurium, and *Yersinia* species (e.g., *Y. pseudotuberculosis* and *Y. pestis*), we were able to PCR-amplify a portion of the *dam* gene (a 400-bp fragment) from the gDNA of *A. hydrophila* SSU**.** Next, we used a

combination of gDNA sequencing and construction of a fosmid library of *A. hydrophila* SSU gDNA to obtain the full-length sequence of *dam* (873-bp). By primer extension analysis, we mapped the promoter region of the *dam* gene, with putative -10 and -35 elements and a transcriptional start site (G) 24 bases upstream of the ATG start codon.

Next, we determined what role Dam played in regulating the virulence of *A. hydrophila* SSU. Attempts to obtain a knock-out mutant of the *dam* gene of *A. hydrophila* SSU were futile, indicating that the gene is essential for the viability of the bacterium. We therefore decided to overexpress the *dam* gene and test for the effects of Dam overproduction in *A. hydrophila*. The role of DNA methylation had also been reported in the viability of *V. cholerae*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (Julio *et al.*, 2001, Falker *et al.*, 2005). Further, it was shown that Dam overproduction led to attenuation in the virulence of these pathogens (Julio *et al.*, 2001, Falker *et al.*, 2005). The results of our study showed that the overproduction of Dam altered the expression of two key virulence factors of *A. hydrophila* SSU. Specifically, Dam overproduction decreased the cytotoxicity associated with the T3SS of *A. hydrophila*, while increasing the cytotoxicity and hemolytic activity associated with the T2SS-secreted Act. We showed that the increase in these biological functions was directly due to the increased expression of the *act* gene in the Dam-overproducing bacterium. Further, Dam overproduction decreased the motility of *A. hydrophila* SSU, a crucial factor involved in the ability of the bacterium to cause infection in the host. The increased production of another factor associated with the pathogenic characteristic of *A. hydrophila* was also observed, i.e. the protease activity.

This study demonstrated that the overexpression of Dam could alter the expression of virulence genes in a positive or negative way and perhaps, this combination of overexpression and underexpression of virulence proteins influences pathogenicity of the bacterium. Given the importance DNA methylation plays in the virulence of a growing list of pathogens, this study provided new insights into the regulatory role of Dam on the virulence factors of the human pathogen, *A. hydrophila*.

Past scientific research indicated that most bacteria function only as individuals

designed to compete with one another and to multiply rapidly under appropriate conditions. This concept has given way to the view that bacteria can communicate with each other and are capable of organizing into functional communities. *A. hydrophila* is an opportunistic pathogen that is known to readily attach to stainless steel to produce a thin biofilm and large microcolonies (Lynch *et al.*, 2002). Biofilms provide an advantageous environment for bacteria in otherwise harsh environments, offering them an increased flow of nutrients that raises their chances of survival and regrowth potential. *Aeromonas* species are of specific concern in forming biofilms because they can do so on the pipe surfaces of water distribution systems. Problems can then arise in these systems due to the detachment or sloughing off of the bacterial biofilms into the bulk water, which would represent a major health risk to consumers (Szewzyk *et al.*, 2000, Chauret *et al.*, 2001, Bomo *et al.*, 2004). Studies have indicated that the phenomenon of quorum sensing (QS) occurs within these biofilms, where it can regulate the formation of the biofilm itself (Lynch *et al.*, 2002). Not much is known in the literature about QS in the *Aeromonas* species It is known, however, that *A. hydrophila* possesses a *Vibrio* species LuxRI homolog system in which the AhyI molecules produce both AHL and BHL signaling molecules under conditions of high cell density and under the regulation of AhyR (Swift *et al.*, 1997). In *A. hydrophila*, QS is known to regulate expression of the genes encoding certain virulence factors such as protease production and biofilm formation (Swift *et al.*, 1999, Lynch *et al.*, 2002). A link has been established in other species of gram-negative bacteria between QS and expression of the T3SS. For example, QS has been shown to control T3SS gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *E. coli*, control expression of the *exoS* regulon (which comprises genes for a T3SS system and anti-host effector proteins) in *P. aeruginosa*, and regulate the T3SS in *V. harveyi* and *V. parahaemolyticus* (Sperandio *et al.*, 1999, Henke and Bassler, 2004, Hogardt *et al.*, 2004).

QS itself can be regulated by a variety of factors, in addition to cell density (Schuster *et al.*, 2004). This study demonstrated a positive correlation between the presence of the T3SS, Act, and production of lactone molecules (AHLs) in isolate SSU of

A. hydrophila (Sha *et al.*, 2005). Using *act*, *aopB*, and *act/aopB* knockout mutants, we demonstrated that deletion of these two virulence genes reduced production of lactones compared to WT bacteria, as detected using the biosensor strain *A. tumefaciens* A136. The most significant difference in AHL production between these mutants and the WT was seen during the phase of highest cell density, when the greatest amount of lactones was being produced by the WT *A. hydrophila*. This discovery underscores the complexities of bacterial cell-cell communication and regulation of virulence factor production in the context of the host environment.

Our laboratory also designed a bioassay to detect other lactone molecules produced by *A. hydrophila* that might not be detected using *A. tumefaciens*. Specifically, the biosensor strain *C. violaceum* CVO26 can detect BHL molecules and as *A. hydrophila* is known to produce both AHLs and BHLs, we designed a system to detect both types of lactone molecules.

While searching for potential effector protein secreted through the T3SS of *A. hydrophila* SSU, we identified a new virulence factor that contributes to the pathogenesis of this bacteria. The *tagA* or ToxR-regulated lipoprotein gene of *A. hydrophila* SSU exhibited 60% identity with that of a recently identified *stcE* gene from *E. coli* O157:H7, which encoded a protein (StcE) that provided serum resistance to the bacterium and prevented erythrocyte lysis. TagA accomplished this by controlling the classical pathway of complement activation *via* cleaving the complement C1-esterase inhibitor (C1-INH). We purified *A. hydrophila* TagA as a histidine-tagged fusion protein (rTagA) from *E. coli* DE3 strain using a T7 promoter-based pET30 expression vector and nickel affinity column chromatography. The rTagA cleaved C1-INH in a time-dependent manner. The *tagA* isogenic mutant of *A. hydrophila*, unlike its corresponding WT or the complemented strain, was unable to cleave C1-INH, which is required to potentiate the C1-INH-mediated lysis of host and bacterial cells. We also demonstrated co-localization of C1-INH and TagA on the bacterial surface by confocal fluorescence microscopy, which ultimately resulted in increased serum resistance of the WT bacterium. Likewise, we delineated the role of TagA in contributing to the enhanced ability of C1-INH to inhibit

the classical complement-mediated lysis of erythrocytes. Importantly, we provided evidence that the *tagA* mutant was significantly less virulent in a mouse model of infection (60%) when compared to animals challenged with the WT bacterium at a 2 LD_{50} dose, which resulted in 100% mortality within 48 h. Taken together, the results of this study provided new information on the role of TagA as a virulence factor in bacterial pathogenesis. This is the first report of TagA characterization from any species of *Aeromonas*. Fig. 6.1 depicts a schematic overview of the virulence factors outlined in this study and their regulation.

Our research in the future will continue to focus on the mechanism of action of these newly discovered virulence factors in *A. hydrophila* SSU. Specifically, we are focused on identifying and characterizing effector protein(s) secreted through the T3SS, delineating their mechanism(s) of action and their effect(s) on the host cell. We are also interested in pursuing studies to identify specific genes/regulators that are affected in the T3SS mutants, specifically in regard to how QS is affected. The *A. hydrophila* QS system is of particular interest, as the QS regulated phenotypes such as extracellular proteases are decreased in the presence of long chain AHLs like 3-oxo-C12-HSL. Thus the QS system could be a potential target for quorum sensing blockers based on the long chain AHLs which could be used as novel pharmaceutical agents to decrease the virulence of the bacteria.

Further, the role of TagA in bacterial virulence is only beginning to be understood and we are interested in pursuing studies to delineate the effect of TagA on other C1-INH regulated targets as well as mechanistic studies to understand exactly how TagA potentiates the inhibitory activity of the serpin C1-INH. It is important to possess thorough knowledge of the mechanism(s) of action of the different virulence factors of *A. hydrophila* as well as their effect on the host cell in order to develop a better understanding of *Aeromonas*-associated diseases which would provide the basis for better diagnostic and treatment strategies in the future.

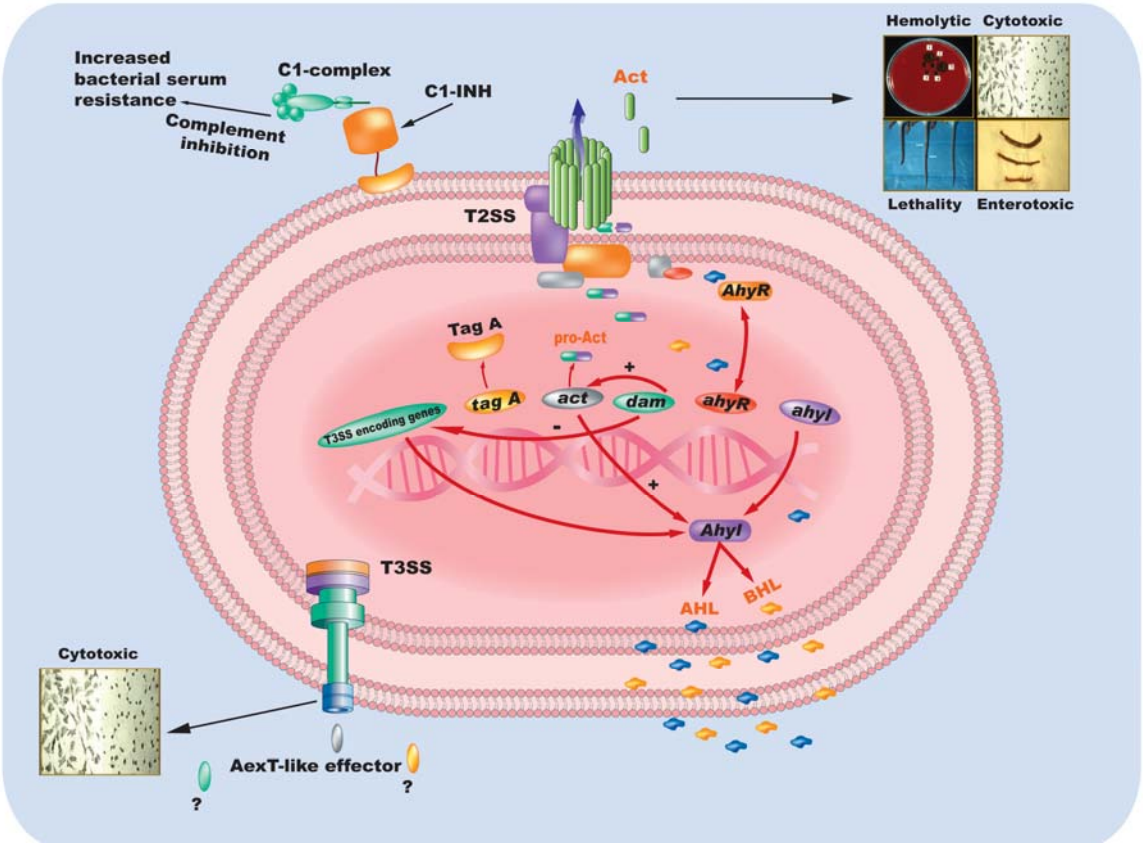


Fig. 6.1. Pathogenesis of *A. hydrophila* SSU is contributed by its various virulence factors.

This figure illustrates a schematic overview of the different virulence factors of *A. hydrophila*, which were elucidated in this study. As shown, the genes encoding the virulence factors, T3SS, TagA, Act, Dam, as well as the QS molecules AhyI and AhyR are located on the chromosome of this bacterium. Previous studies in the laboratory have elucidated the mechanism of action of the cytotoxic enterotoxin Act (secreted through a T2SS) and its effect on host cells. In this study, we specifically demonstrated that Act contributes to cytotoxicity of host cells during the later stages of infection by the pathogen, while the T3SS causes cytotoxicity early-on during an infection. Ongoing studies in the laboratory have indicated that an 'AexT-like' effector is secreted through the T3SS of *A. hydrophila*, which is similar to, yet distinct from the AexT effector of *A. salmonicida*. We are also actively pursuing studies to determine if any additional effector proteins are secreted through this T3SS. The results of this study further demonstrated that the cytotoxic effects mediated by the T3SS and Act were regulated by Dam. Specifically, the overproduction of Dam decreased the cytotoxic effects associated with the T3SS, while increasing the cytotoxicity and hemolytic activity of Act. We showed

that Dam overproduction explicitly targets the expression of *act*, at both the transcriptional and translational level. Further, our studies indicated a specific effect of both the T3SS and Act on the phenomenon of QS, as revealed by the decreased production of lactones in the *act* and *act*/*aopB* isogenic mutants. The mechanism of this effect is a subject of continued investigation in the laboratory. Other studies in our laboratory have also indicated a positive correlation between the overproduction of Dam and lactone production in *A. hydrophila* (data not shown). Finally, we have identified and characterized a new virulence factor in the chromosome of *A. hydrophila*, i.e. TagA. TagA's homolog in *E. coli* O157:H7 is known to be secreted through a T2SS. Our model indicates that TagA increases the serum resistance of *A. hydrophila* by recruiting the serpin C1-INH to the surface of the bacterium, cleaving it, and ultimately releasing it from the surface. We believe that TagA, in effect, prevents the activation of complement at the bacterial surface, which ultimately increases the survival of the bacterium within the harsh environment of the host. Figure drawn by S. Wang (Chopra lab).

REFERENCES

1. **Abbott, S. L., W. K. W. Cheung, and J. M. Janda.** 2003. The Genus *Aeromonas*: Biochemical characteristics, atypical reactions and phenotypic schemes. J. Clin Microbiol **41:**2348-2357.

2. **Abe, A., T. Matsuzawa, and A. Kuwae.** 2005. Type-III effectors: sophisticated bacterial virulence factors. C R Biol **328:**413-428.

3. **Abuhammour, W., R. A. Hasan, and D. Rogers.** 2006. Necrotizing fasciitis caused by *Aeromonas hydrophilia* in an immunocompetent child. Pediatr Emerg Care **22:**48-51.

4. **Agrain, C., I. Callebaut, L. Journet, I. Sorg, C. Paroz, L. J. Mota, and G. R. Cornelis.** 2005. Characterization of a Type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. Mol Microbiol **56:**54-67.

5. **Agrawal, A., J. Lingappa, S. H. Leppla, S. Agrawal, A. Jabbar, C. Quinn, and B. Pulendran.** 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. Nature **424:**329-334.

6. **Aguilar, A., S. Merino, M. M. Nogueras, M. Regue, and J. M. Tomas.** 1999. Two genes from the capsule of *Aeromonas hydrophila* (serogroup O:34) confer serum resistance to *Escherichia coli* K12 strains. Res Microbiol **150:**395-402.

7. **Albert, M. J., M. Ansaruzzaman, K. A. Talukder, A. K. Chopra, I. Kuhn, M. Rahman, A. S. Faruque, M. S. Islam, R. B. Sack, and R. Mollby.** 2000. Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. J Clin Microbiol **38:**3785-3790.

8. **Anderson, D. M., and O. Schneewind.** 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. Science **278:**1140-1143.

9. **Angel, M. F., F. Zhang, M. Jones, J. Henderson, and S. W. Chapman.** 2002. Necrotizing fasciitis of the upper extremity resulting from a water moccasin bite. South Med J **95:**1090-1094.

10. **Asao, T., Y. Kinoshita, S. Kozaki, T. Uemura, and G. Sakaguchi.** 1984. Purification and some properties of *Aeromonas hydrophila* hemolysin. Infect Immun **46:**122-127.

11. **Au, K. G., K. Welsh, and P. Modrich.** 1992. Initiation of methyl-directed mismatch repair. J Biol Chem **267:**12142-12148.

12. **Baddour, L. M., and V. S. Baselski.** 1988. Pneumonia due to *Aeromonas hydrophila*-complex: epidemiologic, clinical, and microbiologic features. South Med J **81:**461-463.

13. **Badie, G., D. M. Heithoff, and M. J. Mahan.** 2004. LcrV synthesis is altered by DNA adenine methylase overproduction in *Yersinia pseudotuberculosis* and is required to confer immunity in vaccinated hosts. Infect Immun **72:**6707-6710.

14. **Bale, A., M. d'Alarcao, and M. G. Marinus.** 1979. Characterization of DNA adenine methylation mutants of *Escherichia coli* K12. Mutat Res **59:**157-165.

15. **Barillo, D. J., A. T. McManus, W. G. Cioffi, W. F. McManus, S. H. Kim, and B. A. Pruitt, Jr.** 1996. *Aeromonas* bacteraemia in burn patients. Burns **22:**48-52.

16. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol Microbiol **9:**773-786.

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

18. **Bird, A. P., and A. P. Wolffe.** 1999. Methylation-induced repression--belts, braces, and chromatin. Cell **99:**451-454.

19. **Bleves, S., C. Soscia, P. Nogueira-Orlandi, A. Lazdunski, and A. Filloux.** 2005. Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. J Bacteriol **187:**3898-3902.

20. **Blocker, A., P. Gounon, E. Larquet, K. Niebuhr, V. Cabiaux, C. Parsot, and P. Sansonetti.** 1999. The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. J Cell Biol **147:**683-693.

21. **Blyn, L. B., B. A. Braaten, and D. A. Low.** 1990. Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. Embo J **9:**4045-4054.

22. **Bogan, J. A., and C. E. Helmstetter.** 1997. DNA sequestration and transcription in the oriC region of *Escherichia coli*. Mol Microbiol **26:**889-896.

23. **Bogdanovic, R., M. Cobeljic, M. Markovic, V. Nikolic, M. Ognjanovic, L. Sarjanovic, and D. Makic.** 1991. Haemolytic-uraemic syndrome associated with *Aeromonas hydrophila* enterocolitis. Pediatr Nephrol **5:**293-295.

24. **Boland, A., M. P. Sory, M. Iriarte, C. Kerbourch, P. Wattiau, and G. R. Cornelis.** 1996. Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y.enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. Embo J **15:**5191-5201.

25. **Bomo, A. M., M. V. Storey, and N. J. Ashbolt.** 2004. Detection, integration and persistence of aeromonads in water distribution pipe biofilms. J Water Health **2:**83-96.

26. **Borchardt, M. A., M. E. Stemper, and J. H. Standridge.** 2003. *Aeromonas* isolates from human diarrheic stool and groundwater compared by pulsed-field gel electrophoresis. Emerg Infect Dis **9:**224-228.

27. **Braaten, B. A., X. Nou, L. S. Kaltenbach, and D. A. Low.** 1994. Methylation patterns in pap regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. Cell **76:**577-588.

28. **Brandi, G., M. Sisti, F. Giardini, G. F. Schiavano, and A. Albano.** 1999. Survival ability of cytotoxic strains of motile *Aeromonas* spp. in different types of water. Lett Appl Microbiol **29:**211-215.

29. **Braun, M., K. Stuber, Y. Schlatter, T. Wahli, P. Kuhnert, and J. Frey.** 2002. Characterization of an ADP-ribosyltransferase toxin (AexT) from *Aeromonas salmonicida* subsp. salmonicida. J Bacteriol **184:**1851-1858.

30. **Braun, R. E., and A. Wright.** 1986. DNA methylation differentially enhances the expression of one of the two *E. coli* dnaA promoters *in vivo* and *in vitro*. Mol Gen Genet **202:**246-250.

31. **Brennan, M. A., and B. T. Cookson.** 2000. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. Mol Microbiol **38:**31-40.

32. **Brooks, J. E., R. M. Blumenthal, and T. R. Gingeras.** 1983. The isolation and characterization of the *Escherichia coli* DNA adenine methylase (*dam*) gene. Nucleic Acids Res **11:**837-851.

33. **Brubaker, R. R.** 2005. Influence of Na(+), dicarboxylic amino acids, and pH in modulating the low-calcium response of *Yersinia pestis*. Infect Immun **73:**4743-4752.

34. **Brubaker, R. R.** 2003. Interleukin-10 and inhibition of innate immunity to *Yersiniae*: roles of Yops and LcrV (V antigen). Infect Immun **71:**3673-3681.

35. **Bujnicki, J. M., M. Radlinska, P. Zaleski, and A. Piekarowicz.** 2001. Cloning of the *Haemophilus influenzae* Dam methyltransferase and analysis of its relationship to the Dam methyltransferase encoded by the HP1 phage. Acta Biochim Pol **48:**969-983.

36. **Burke, V., J. Robinson, M. Gracey, D. Peterson, and K. Partridge.** 1984. Isolation of *Aeromonas hydrophila* from a metropolitan water supply: seasonal correlation with clinical isolates. Appl Environ Microbiol **48:**361-366.

37. **Burr, S. E., K. Stuber, and J. Frey.** 2003. The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. salmonicida is translocated via a type III secretion pathway. J Bacteriol **185:**6583-6591.

38. **Burr, S. E., K. Stuber, T. Wahli, and J. Frey.** 2002. Evidence for a type III secretion system in *Aeromonas salmonicida* subsp. salmonicida. J Bacteriol **184:**5966-5970.

39. **Burr, S. E., T. Wahli, H. Segner, D. Pugovkin, and J. Frey.** 2003. Association of Type III secretion genes with virulence of *Aeromonas salmonicida* subsp. salmonicida. Dis Aquat Organ **57:**167-171.

40. **Caliezi, C., W. A. Wuillemin, S. Zeerleder, M. Redondo, B. Eisele, and C. E. Hack.** 2000. C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. Pharmacol Rev **52:**91-112.

41. **Calmann, M. A., and M. G. Marinus.** 2003. Regulated expression of the *Escherichia coli dam* gene. J Bacteriol **185:**5012-5014.

42. **Cao, J. G., and E. A. Meighen.** 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J Biol Chem **264:**21670-21676.

43. **Carnahan, A. M., S. Behram, and S. W. Joseph.** 1991. Aerokey II: a flexible key for identifying clinical *Aeromonas* species. J Clin Microbiol **29:**2843-2849.

44. **Carta, F., A. Pinna, S. Zanetti, A. Carta, M. Sotgiu, and G. Fadda.** 1994. Corneal ulcer caused by *Aeromonas* species. Am J Ophthalmol **118:**530-531.

45. **Carugati, A., E. Pappalardo, L. C. Zingale, and M. Cicardi.** 2001. C1-inhibitor deficiency and angioedema. Mol Immunol **38:**161-173.

46. **Cascales, E., A. Bernadac, M. Gavioli, J. C. Lazzaroni, and R. Lloubes.** 2002. Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. J Bacteriol **184:**754-759.

47. **Cascon, A., J. Yugueros, A. Temprano, M. Sanchez, C. Hernanz, J. M. Luengo, and G. Naharro.** 2000. A major secreted elastase is essential for pathogenicity of *Aeromonas hydrophila*. Infect Immun **68:**3233-3241.

48. **Chakraborty, T., B. Huhle, H. Bergbauer, and W. Goebel.** 1986. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. J Bacteriol **167:**368-374.

49. **Challapalli, M., B. R. Tess, D. G. Cunningham, A. K. Chopra, and C. W. Houston.** 1988. *Aeromonas*-associated diarrhea in children. Pediatr Infect Dis J **7:**693-698.

50. **Chamorey, E., M. Forel, and M. Drancourt.** 1999. An *in-vitro* evaluation of the activity of chlorine against environmental and nosocomial isolates of *Aeromonas hydrophila*. J Hosp Infect **41:**45-49.

51. **Champion, G. A., M. N. Neely, M. A. Brennan, and V. J. DiRita.** 1997. A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. Mol Microbiol **23:**323-331.

52. **Chang, C. Y., H. Thompson, N. Rodman, J. Bylander, and J. Thomas.** 1997. Pathogenic analysis of *Aeromonas hydrophila* septicemia. Ann Clin Lab Sci **27:**254-259.

53. **Chauret, C., C. Volk, R. Creason, J. Jarosh, J. Robinson, and C. Warnes.** 2001. Detection of *Aeromonas hydrophila* in a drinking-water distribution system: a field and pilot study. Can J Microbiol **47:**782-786.

54. **Chen, L. M., K. Kaniga, and J. E. Galan.** 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. Mol Microbiol **21:**1101-1115.

55. **Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545-549.

56. **Chen, Y., M. R. Smith, K. Thirumalai, and A. Zychlinsky.** 1996. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. Embo J **15:**3853-3860.

57. **Cheng, N. C., S. Y. Horng, S. C. Chang, and Y. B. Tang.** 2004. Nosocomial infection of *Aeromonas hydrophila* presenting as necrotizing fasciitis. J Formos Med Assoc **103:**53-57.

58. **Chopra, A. K., J. Graf, A. J. Horneman, and J. Johnson.** 2004. Virulence Factor Activity Relationships (VFARs) with Specific Emphasis on *Aeromonas* species based on October, 2004 Workshop in Baltimore, Maryland. U. S. Environmental Protection Agency, Office of research and Development.

59. **Chopra, A. K., and C. W. Houston.** 1999. Enterotoxins in *Aeromonas*-associated gastroenteritis. Microbes Infect **1:**1129-1137.

60. **Chopra, A. K., C. W. Houston, J. W. Peterson, and G. F. Jin.** 1993. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can J Microbiol **39:**513-523.

61. **Chopra, A. K., J. W. Peterson, X. J. Xu, D. H. Coppenhaver, and C. W. Houston.** 1996. Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila*. Microb Pathog **21:**357-377.

62. **Chopra, A. K., X. Xu, D. Ribardo, M. Gonzalez, K. Kuhl, J. W. Peterson, and C. W. Houston.** 2000. The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect Immun **68:**2808-2818.

63. **Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales.** 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol **59:**451-485.

64. **Cigni, A., P. A. Tomasi, A. Pais, S. Cossellu, R. Faedda, and A. E. Satta.** 2003. Fatal *Aeromonas hydrophila* septicemia in a 16-year-old patient with thalassemia. J Pediatr Hematol Oncol **25:**674-675.

65. **Coghlan, A. C.** 2000. Food poisoning bugs thrive in crop sprays., New Scientist, vol. 20.

66. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type III system of *Salmonella* Typhimurium directs the translocation of Sip proteins into the host cell. Mol Microbiol **24:**747-756.

67. **Colwell, R. R., M. R. MacDonell, and J. D. Ley.** 1986. Proposal to recognize the family Aeromonadaceae fam. nov. Int J Syst Bacteriol **36:**473-477.

68. **Cordoba Lopez, A., M. I. Bueno Alvarez-Arenas, R. J. Alzugaray Fraga, M. D. Veiga Gonzalez, and G. Corcho Sanchez.** 1999. Spontaneous bacterial

peritonitis and sepsis due to *Aeromonas hydrophila*. Gastroenterol Hepatol **22:**208.

69. **Cornelis, G. R.** 2002. *Yersinia* type III secretion: send in the effectors. J Cell Biol **158:**401-408.

70. **Cornelis, G. R., T. Biot, C. Lambert de Rouvroit, T. Michiels, B. Mulder, C. Sluiters, M. P. Sory, M. Van Bouchaute, and J. C. Vanooteghem.** 1989. The *Yersinia* yop regulon. Mol Microbiol **3:**1455-1459.

71. **Cowell, B. A., S. S. Twining, J. A. Hobden, M. S. Kwong, and S. M. Fleiszig.** 2003. Mutation of lasA and lasB reduces *Pseudomonas aeruginosa* invasion of epithelial cells. Microbiology **149:**2291-2299.

72. **Dacheux, D., J. Goure, J. Chabert, Y. Usson, and I. Attree.** 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. Mol Microbiol **40:**76-85.

73. **Dale, C., S. A. Young, D. T. Haydon, and S. C. Welburn.** 2001. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. Proc Natl Acad Sci U S A **98:**1883-1888.

74. **Dasgupta, N., G. L. Lykken, M. C. Wolfgang, and T. L. Yahr.** 2004. A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. Mol Microbiol **53:**297-308.

75. **Davis, A. E., 3rd.** 2004. Biological effects of C1 inhibitor. Drug News Perspect **17:**439-446.

76. **Deodhar, L. P., K. Saraswathi, and A. Varudkar.** 1991. *Aeromonas* spp. and their association with human diarrheal disease. J Clin Microbiol **29:**853-856.

77. **Desvaux, M., N. J. Parham, and I. R. Henderson.** 2004. The autotransporter secretion system. Res Microbiol **155:**53-60.

78. **Diekmann, S.** 1987. DNA methylation can enhance or induce DNA curvature. Embo J **6:**4213-4217.

79. **DiRita, V. J.** 1992. Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol Microbiol **6:**451-458.

80. **DiRita, V. J., N. C. Engleberg, A. Heath, A. Miller, J. A. Crawford, and R. Yu.** 2000. Virulence gene regulation inside and outside. Philos Trans R Soc Lond B Biol Sci **355:**657-665.

81. **DiRita, V. J., and J. J. Mekalanos.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell **64:**29-37.

82. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc Natl Acad Sci U S A **88:**5403-5407.

83. **Dodson, K. W., and D. E. Berg.** 1989. Factors affecting transposition activity of IS50 and Tn5 ends. Gene **76:**207-213.

84. **Donnenberg, M. S., J. B. Kaper, and B. B. Finlay.** 1997. Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. Trends Microbiol **5:**109-114.

85. **Dueger, E. L., J. K. House, D. M. Heithoff, and M. J. Mahan.** 2003. *Salmonella* DNA adenine methylase mutants elicit early and late onset protective immune responses in calves. Vaccine **21:**3249-3258.

86. **Dueger, E. L., J. K. House, D. M. Heithoff, and M. J. Mahan.** 2001. *Salmonella* DNA adenine methylase mutants elicit protective immune responses to homologous and heterologous serovars in chickens. Infect Immun **69:**7950-7954.

87. **Dumontet, S., K. Krovacek, S. B. Svenson, V. Pasquale, S. B. Baloda, and G. Figliuolo.** 2000. Prevalence and diversity of *Aeromonas* and *Vibrio* spp. in coastal waters of Southern Italy. Comp Immunol Microbiol Infect Dis **23:**53-72.

88. **Eberhard, A.** 1972. Inhibition and activation of bacterial luciferase synthesis. J Bacteriol **109:**1101-1105.

89. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444-2449.

90. **Echeverria, P., C. Pitarangsi, B. Eampokalap, S. Vibulbandhitkit, P. Boonthai, and B. Rowe.** 1983. A longitudinal study of the prevalence of bacterial enteric pathogens among adults with diarrhea in Bangkok, Thailand. Diagn Microbiol Infect Dis **1:**193-204.

91. **Edwards, R. A., L. H. Keller, and D. M. Schifferli.** 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene **207:**149-157.

92. **Eichelberg, K., and J. E. Galan.** 2000. The flagellar sigma factor FliA (sigma(28)) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. Infect Immun **68:**2735-2743.

93. **Elliott, S. J., L. A. Wainwright, T. K. McDaniel, K. G. Jarvis, Y. K. Deng, L. C. Lai, B. P. McNamara, M. S. Donnenberg, and J. B. Kaper.** 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. Mol Microbiol **28:**1-4.

94. **Emekdas, G., G. Aslan, S. Tezcan, M. S. Serin, C. Yildiz, H. Ozturhan, and R. Durmaz.** 2006. Detection of the frequency, antimicrobial susceptibility, and genotypic discrimination of *Aeromonas* strains isolated from municipally treated tap water samples by cultivation and AP-PCR. Int J Food Microbiol **107:**310-314.

95. **Ender, P. T., M. J. Dolan, D. Dolan, J. C. Farmer, and G. P. Melcher.** 1996. Near-drowning-associated *Aeromonas* pneumonia. J Emerg Med **14:**737-741.

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

97. **Engebrecht, J., and M. Silverman.** 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc Natl Acad Sci U S A **81:**4154-4158.

98. **Erova, T. E., A. A. Fadl, J. Sha, B. K. Khajanchi, L. Pillai, E. V. Kozlova, and A. K. Chopra.** 2006. Mutations within the Catalytic Motif of DNA Adenine Methyltransferase (Dam) of *Aeromonas hydrophila* Reverts the Virulence of the Dam-overproducing Strain to that of the Wild-type Phenotype. Infect Immun **(In Press)**.

99. **Erova, T. E., L. Pillai, A. A. Fadl, J. Sha, S. Wang, C. L. Galindo, and A. K. Chopra.** 2006. DNA adenine methyltransferase influences the virulence of *Aeromonas hydrophila*. Infect Immun **74:**410-424.

100. **Fadl, A. A., J. Sha, G. R. Klimpel, J. P. Olano, D. W. Niesel, and A. K. Chopra.** 2005. Murein lipoprotein is a critical outer membrane component involved in *Salmonella* enterica serovar Typhimurium systemic infection. Infect Immun **73:**1081-1096.

101. **Falcao, J. P., F. Sharp, and V. Sperandio.** 2004. Cell-to-cell signaling in intestinal pathogens. Curr Issues Intest Microbiol **5:**9-17.

102. **Falker, S., M. A. Schmidt, and G. Heusipp.** 2005. DNA methylation in *Yersinia enterocolitica*: role of the DNA adenine methyltransferase in mismatch repair and regulation of virulence factors. Microbiology **151:**2291-2299.

103. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol Rev **57:**995-1017.

104. **Feldman, M. F., S. Muller, E. Wuest, and G. R. Cornelis.** 2002. SycE allows secretion of YopE-DHFR hybrids by the *Yersinia enterocolitica* type III Ysc system. Mol Microbiol **46:**1183-1197.

105. **Ferguson, M. R., X. J. Xu, C. W. Houston, J. W. Peterson, and A. K. Chopra.** 1995. Amino-acid residues involved in biological functions of the cytolytic enterotoxin from *Aeromonas hydrophila*. Gene **156:**79-83.

106. **Ferguson, M. R., X. J. Xu, C. W. Houston, J. W. Peterson, D. H. Coppenhaver, V. L. Popov, and A. K. Chopra.** 1997. Hyperproduction, purification, and mechanism of action of the cytotoxic enterotoxin produced by *Aeromonas hydrophila*. Infect Immun **65:**4299-4308.

107. **Ffrench-Constant, R. H., N. Waterfield, V. Burland, N. T. Perna, P. J. Daborn, D. Bowen, and F. R. Blattner.** 2000. A genomic sample sequence of the entomopathogenic bacterium *Photorhabdus luminescens* W14: potential implications for virulence. Appl Environ Microbiol **66:**3310-3329.

108. **Figueras, M. J., A. Suarez-Franquet, M. R. Chacon, L. Soler, M. Navarro, C. Alejandre, B. Grasa, A. J. Martinez-Murcia, and J. Guarro.** 2005. First record of the rare species *Aeromonas culicicola* from a drinking water supply. Appl Environ Microbiol **71:**538-541.

109. **Figueroa, J. E., and P. Densen.** 1991. Infectious diseases associated with complement deficiencies. Clin Microbiol Rev **4:**359-395.

110. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev **61:**136-169.

111. **Forsberg, A., A. M. Viitanen, M. Skurnik, and H. Wolf-Watz.** 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol Microbiol **5:**977-986.

112. **Forsberg, A., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-plB1-encoded trans-acting elements controlled by temperature and calcium. Mol Microbiol **2:**121-133.

113. **Foultier, B., P. Troisfontaines, S. Muller, F. R. Opperdoes, and G. R. Cornelis.** 2002. Characterization of the ysa pathogenicity locus in the chromosome of *Yersinia enterocolitica* and phylogeny analysis of type III secretion systems. J Mol Evol **55:**37-51.

114. **Frank, D. W., and B. H. Iglewski.** 1991. Cloning and sequence analysis of a trans-regulatory locus required for exoenzyme S synthesis in *Pseudomonas aeruginosa*. J Bacteriol **173:**6460-6468.

115. **Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutton.** 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. Mol Microbiol **30:**911-921.

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

117. **Freeman, J. A., and B. L. Bassler.** 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. J Bacteriol **181:**899-906.

118. **Freeman, J. A., B. N. Lilley, and B. L. Bassler.** 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. Mol Microbiol **35:**139-149.

119. **Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394-401.

120. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet **35:**439-468.

121. **Fuqua, C., and S. C. Winans.** 1996. Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J Bacteriol **178:**435-440.

122. **Fuqua, C., and S. C. Winans.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J Bacteriol **176:**2796-2806.

123. **Galan, J. E., and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284:**1322-1328.

124. **Galindo, C. L., A. A. Fadl, J. Sha, L. Pillai, C. Gutierrez, Jr., and A. K. Chopra.** 2005. Microarray and proteomics analyses of human intestinal epithelial cells treated with the *Aeromonas hydrophila* cytotoxic enterotoxin. Infect Immun **73:**2628-2643.

125. **Galindo, C. L., J. Sha, A. A. Fadl, L. Pillai, and A. K. Chopra.** 2006. Host immune responses to *Aermonas* virulence factors. Curr Immun Rev **2:**13-26.

126. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. J Bacteriol **173:**3000-3009.

127. **Garcia-Del Portillo, F., M. G. Pucciarelli, and J. Casadesus.** 1999. DNA adenine methylase mutants of *Salmonella* Typhimurium show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc Natl Acad Sci U S A **96:**11578-11583.

128. **Gaussorgues, P., P. Bachmann, S. Tigaud, D. Piperno, J. M. Jaboulay, F. Salord, and D. Robert.** 1987. Pulmonary abscess: *Aeromonas hydrophila* caused by a complication of drowning in fresh water. Ann Med Interne (Paris) **138:**666-667.

129. **Gavriel, A. A., J. P. Landre, and A. J. Lamb.** 1998. Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland. J Appl Microbiol **84:**383-392.

130. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J Clin Microbiol **12:**361-366.

131. **Ghenghesh, K. S., A. El-Ghodban, R. Dkakni, S. Abeid, A. Altomi, T. Abdussalam, and K. Marialigeti.** 2001. Prevalence, species differentiation, haemolytic activity, and antibiotic susceptibility of aeromonads in untreated well water. Mem Inst Oswaldo Cruz **96:**169-173.

132. **Ginocchio, C. C., S. B. Olmsted, C. L. Wells, and J. E. Galan.** 1994. Contact with epithelial cells induces the formation of surface appendages on *Salmonella* Typhimurium. Cell **76:**717-724.

133. **Goguen, J. D., J. Yother, and S. C. Straley.** 1984. Genetic analysis of the low calcium response in *Yersinia pestis* mu d1(Ap lac) insertion mutants. J Bacteriol **160:**842-848.

134. **Gold, W. L., and I. E. Salit.** 1993. *Aeromonas hydrophila* infections of skin and soft tissue: report of 11 cases and review. Clin Infect Dis **16:**69-74.

135. **Goncalves, J. R., G. Brum, A. Fernandes, I. Biscaia, M. J. Correia, and J. Bastardo.** 1992. *Aeromonas hydrophila* fulminant pneumonia in a fit young man. Thorax **47:**482-483.

136. **Goure, J., A. Pastor, E. Faudry, J. Chabert, A. Dessen, and I. Attree.** 2004. The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. Infect Immun **72:**4741-4750.

137. **Grys, T. E., M. B. Siegel, W. W. Lathem, and R. A. Welch.** 2005. The StcE protease contributes to intimate adherence of enterohemorrhagic *Escherichia coli* O157:H7 to host cells. Infect Immun **73:**1295-1303.

138. **Grys, T. E., L. L. Walters, and R. A. Welch.** 2006. Characterization of the StcE protease activity of *Escherichia coli* O157:H7. J Bacteriol **188:**4646-4653.

139. **Guan, T. Y., G. Blank, A. Ismond, and R. V. Acker.** 2001. Fate of foodborne bacterial pathogens in pesticide products. Journal of the Science of Food and Agriculture **81:**503-512.

140. **Guiney, D. G.** 2005. The role of host cell death in *Salmonella* infections. Curr Top Microbiol Immunol **289:**131-150.

141. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177:**4121-4130.

142. **Haagmans, W., and M. van der Woude.** 2000. Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription. Mol Microbiol **35:**877-887.

143. **Hakansson, S., K. Schesser, C. Persson, E. E. Galyov, R. Rosqvist, F. Homble, and H. Wolf-Watz.** 1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. Embo J **15:**5812-5823.

144. **Hanninen, M. L.** 1994. Phenotypic characteristics of the three hybridization groups of *Aeromonas hydrophila* complex isolated from different sources. J Appl Bacteriol **76:**455-462.

145. **Hanninen, M. L., P. Oivanen, and V. Hirvela-Koski.** 1997. *Aeromonas* species in fish, fish-eggs, shrimp and freshwater. Int J Food Microbiol **34:**17-26.

146. **Hanninen, M. L., S. Salmi, L. Mattila, R. Taipalinen, and A. Siitonen.** 1995. Association of *Aeromonas* spp. with travellers' diarrhoea in Finland. J Med Microbiol **42:**26-31.

147. **Harkey, C. W., K. D. Everiss, and K. M. Peterson.** 1995. Isolation and characterization of a *Vibrio cholerae* gene (*tagA*) that encodes a ToxR-regulated lipoprotein. Gene **153:**81-84.

148. **Hauser, A. R., S. Fleiszig, P. J. Kang, K. Mostov, and J. N. Engel.** 1998. Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. Infect Immun **66:**1413-1420.

149. **Havelaar, A. H., F. M. Schets, A. van Silfhout, W. H. Jansen, G. Wieten, and D. van der Kooij.** 1992. Typing of *Aeromonas* strains from patients with diarrhoea and from drinking water. J Appl Bacteriol **72:**435-444.

150. **Heithoff, D. M., E. Y. Enioutina, R. A. Daynes, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. *Salmonella* DNA adenine methylase mutants confer cross-protective immunity. Infect Immun **69:**6725-6730.

151. **Heithoff, D. M., R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 1999. An essential role for DNA adenine methylation in bacterial virulence. Science **284:**967-970.

152. **Hellwage, J., T. Meri, T. Heikkila, A. Alitalo, J. Panelius, P. Lahdenne, I. J. Seppala, and S. Meri.** 2001. The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. J Biol Chem **276:**8427-8435.

153. **Henderson, I. R., and J. P. Nataro.** 2001. Virulence functions of autotransporter proteins. Infect Immun **69:**1231-1243.

154. **Henke, J. M., and B. L. Bassler.** 2004. Bacterial social engagements. Trends Cell Biol **14:**648-656.

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

156. **Herman, G. E., and P. Modrich.** 1981. *Escherichia coli* K-12 clones that overproduce dam methylase are hypermutable. J Bacteriol **145:**644-646.

157. **Heurlier, K., V. Denervaud, G. Pessi, C. Reimmann, and D. Haas.** 2003. Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. J Bacteriol **185:**2227-2235.

158. **High, N., J. Mounier, M. C. Prevost, and P. J. Sansonetti.** 1992. IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. Embo J **11:**1991-1999.

159. **Higuchi, K., L. L. Kupferberg, and J. L. Smith.** 1959. Studies on the nutrition and physiology of *Pasteurella pestis*. III. Effects of calcium ions on the growth of virulent and avirulent strains of *Pasteurella pestis*. J Bacteriol **77:**317-321.

160. **Hiransuthikul, N., W. Tantisiriwat, K. Lertutsahakul, A. Vibhagool, and P. Boonma.** 2005. Skin and soft-tissue infections among tsunami survivors in southern Thailand. Clin Infect Dis **41:**e93-96.

161. **Hofer, E., C. M. Reis, G. N. Theophilo, V. O. Cavalcanti, N. V. Lima, and F. Henriques Mde.** 2006. *Aeromonas* associated with an acute diarrhea outbreak in Sao Bento do Una, Pernambuco. Rev Soc Bras Med Trop **39:**217-220.

162. **Hogardt, M., M. Roeder, A. M. Schreff, L. Eberl, and J. Heesemann.** 2004. Expression of *Pseudomonas aeruginosa* exoS is controlled by quorum sensing and RpoS. Microbiology **150:**843-851.

163. **Holmes, P., L. M. Niccolls, and D. P. Sartory.** 1996. The ecology of mesophilic *Aeromonas* in the aquatic environment. John Wiley and Sons Ltd., West Sussex, UK.

164. **Holmstrom, A., J. Petterson, R. Rosqvist, S. Hakansson, F. Tafazoli, M. Fallman, K. E. Magnusson, H. Wolf-Watz, and A. Forsberg.** 1997. YopK of *Yersinia pseudotuberculosis* controls translocation of Yop effectors across the eukaryotic cell membrane. Mol Microbiol **24:**73-91.

165. **Honma, Y., R. E. Fernandez, and A. T. Maurelli.** 2004. A DNA adenine methylase mutant of *Shigella flexneri* shows no significant attenuation of virulence. Microbiology **150:**1073-1078.

166. **Howard, S. P., and J. T. Buckley.** 1985. Activation of the hole-forming toxin aerolysin by extracellular processing. J Bacteriol **163:**336-340.

167. **Howard, S. P., and J. T. Buckley.** 1986. Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. Mol Gen Genet **204:**289-295.

168. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev **62:**379-433.

169. **Hume, P. J., E. J. McGhie, R. D. Hayward, and V. Koronakis.** 2003. The purified *Shigella* IpaB and *Salmonella* SipB translocators share biochemical properties and membrane topology. Mol Microbiol **49:**425-439.

170. **Huntington, J. A., R. J. Read, and R. W. Carrell.** 2000. Structure of a serpin-protease complex shows inhibition by deformation. Nature **407:**923-926.

171. **Hur, T., K. C. Cheng, and J. M. Hsieh.** 1995. *Aeromonas hydrophila* lung abscess in a previously healthy man. Scand J Infect Dis **27:**295.

172. **Jacob, L., D. B. Carron, T. C. Haji, and D. W. Roberts.** 1988. An unusual case of pyogenic meningitis due to *Aeromonas sobria*. Br J Hosp Med **39:**449.

173. **Janda, J. M.** 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. Clin Microbiol Rev **4:**397-410.

174. **Janda, J. M., and S. L. Abbott.** 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding Panorama of species, disease presentations, and unanswered questions. Clin Infect Dis **27:**332-344.

175. **Janda, J. M., S. L. Abbott, and R. A. Brenden.** 1997. Overview of the etiology of wound infections with particular emphasis on community-acquired illnesses. Eur J Clin Microbiol Infect Dis **16:**189-201.

176. **Janda, J. M., L. S. Guthertz, R. P. Kokka, and T. Shimada.** 1994. *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. Clin Infect Dis **19:**77-83.

177. **Jensen, K. F., and S. Pedersen.** 1990. Metabolic growth rate control in *Escherichia coli* may be a consequence of subsaturation of the macromolecular biosynthetic apparatus with substrates and catalytic components. Microbiol Rev **54:**89-100.

178. **Jiang, H., E. Wagner, H. Zhang, and M. M. Frank.** 2001. Complement 1 inhibitor is a regulator of the alternative complement pathway. J Exp Med **194:**1609-1616.

179. **Jin, Q., and S. Y. He.** 2001. Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. Science **294:**2556-2558.

180. **Johnson, T. L., J. Abendroth, W. G. Hol, and M. Sandkvist.** 2006. Type II secretion: from structure to function. FEMS Microbiol Lett **255:**175-186.

181. **Jores, J., L. Rumer, and L. H. Wieler.** 2004. Impact of the locus of enterocyte effacement pathogenicity island on the evolution of pathogenic *Escherichia coli*. Int J Med Microbiol **294:**103-113.

182. **Journet, L., C. Agrain, P. Broz, and G. R. Cornelis.** 2003. The needle length of bacterial injectisomes is determined by a molecular ruler. Science **302:**1757-1760.

183. **Journet, L., K. T. Hughes, and G. R. Cornelis.** 2005. Type III secretion: a secretory pathway serving both motility and virulence (review). Mol Membr Biol **22:**41-50.

184. **Juhas, M., L. Eberl, and B. Tummler.** 2005. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. Environ Microbiol **7:**459-471.

185. **Julio, S. M., D. M. Heithoff, D. Provenzano, K. E. Klose, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*. Infect Immun **69:**7610-7615.

186. **Julio, S. M., D. M. Heithoff, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2002. DNA adenine methylase overproduction in *Yersinia pseudotuberculosis* alters YopE expression and secretion and host immune responses to infection. Infect Immun **70:**1006-1009.

187. **Jung, C. M., O. Matsushita, S. Katayama, J. Minami, J. Sakurai, and A. Okabe.** 1999. Identification of metal ligands in the *Clostridium histolyticum* ColH collagenase. J Bacteriol **181:**2816-2822.

188. **Kaltenbach, L. S., B. A. Braaten, and D. A. Low.** 1995. Specific binding of PapI to Lrp-pap DNA complexes. J Bacteriol **177:**6449-6455.

189. **Kang, S., H. Lee, J. S. Han, and D. S. Hwang.** 1999. Interaction of SeqA and Dam methylase on the hemimethylated origin of *Escherichia coli* chromosomal DNA replication. J Biol Chem **274:**11463-11468.

190. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J Bacteriol **163:**1210-1214.

191. **Karaolis, D. K., J. A. Johnson, C. C. Bailey, E. C. Boedeker, J. B. Kaper, and P. R. Reeves.** 1998. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci U S A **95:**3134-3139.

192. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin Microbiol Rev **2:**15-38.

193. **Kelly, K. A., J. M. Koehler, and L. R. Ashdown.** 1993. Spectrum of extraintestinal disease due to *Aeromonas* species in tropical Queensland, Australia. Clin Infect Dis **16:**574-579.

194. **Kempner, E. S., and F. E. Hanson.** 1968. Aspects of light production by *Photobacterium fischeri*. J Bacteriol **95:**975-979.

195. **Khaitovich, A. B., and G. V. Gal'tseva.** 1983. Serological diagnosis of diarrhoea caused by some representatives of the genera Vibrio and *Aeromonas*. J Hyg Epidemiol Microbiol Immunol **27:**211-218.

196. **Kienzle, N., M. Muller, and S. Pegg.** 2000. *Aeromonas* wound infection in burns. Burns **26:**478-482.

197. **Kirke, D. F., S. Swift, M. J. Lynch, and P. Williams.** 2004. The *Aeromonas hydrophila* LuxR homologue AhyR regulates the N-acyl homoserine lactone synthase, AhyI positively and negatively in a growth phase-dependent manner. FEMS Microbiol Lett **241:**109-117.

198. **Kirov, S. M., E. K. Ardestani, and L. J. Hayward.** 1993. The growth and expression of virulence factors at refrigeration temperature by *Aeromonas* strains isolated from foods. Int J Food Microbiol **20:**159-168.

199. **Kirov, S. M., J. A. Hudson, L. J. Hayward, and S. J. Mott.** 1994. Distribution of *Aeromonas hydrophila* hybridization groups and their virulence properties in Australian clinical and environmental strains. Lett Appl Microbiol **18:**71-73.

200. **Kita-Tsukamoto, K., H. Oyaizu, K. Nanba, and U. Simidu.** 1993. Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. Int J Syst Bacteriol **43:**8-19.

201. **Ko, W. C., and Y. C. Chuang.** 1995. *Aeromonas* bacteremia: review of 59 episodes. Clin Infect Dis **20:**1298-1304.

202. **Kobayashi, I., A. Nobusato, N. Kobayashi-Takahashi, and I. Uchiyama.** 1999. Shaping the genome--restriction-modification systems as mobile genetic elements. Curr Opin Genet Dev **9:**649-656.

203. **Kraiczy, P., and R. Wurzner.** 2006. Complement escape of human pathogenic bacteria by acquisition of complement regulators. Mol Immunol **43:**31-44.

204. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the *Salmonella* Typhimurium type III protein secretion system. Science **280:**602-605.

205. **Kubori, T., N. Shimamoto, S. Yamaguchi, K. Namba, and S. Aizawa.** 1992. Morphological pathway of flagellar assembly in *Salmonella* Typhimurium. J Mol Biol **226:**433-446.

206. **Kuchma, S. L., J. P. Connolly, and G. A. O'Toole.** 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. J Bacteriol **187:**1441-1454.

207. **Kupferberg, L. L., and K. Higuchi.** 1958. Role of calcium ions in the stimulation of growth of virulent strains of *Pasteurella pestis*. J Bacteriol **76:**120-121.

208. **Kuwae, A., M. Ohishi, M. Watanabe, M. Nagai, and A. Abe.** 2003. BopB is a type III secreted protein in *Bordetella bronchiseptica* and is required for cytotoxicity against cultured mammalian cells. Cell Microbiol **5:**973-983.

209. **Lambert de Rouvroit, C., C. Sluiters, and G. R. Cornelis.** 1992. Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol Microbiol **6:**395-409.

210. **Lathem, W. W., T. Bergsbaken, and R. A. Welch.** 2004. Potentiation of C1 esterase inhibitor by StcE, a metalloprotease secreted by *Escherichia coli* O157:H7. J Exp Med **199:**1077-1087.

211. **Lathem, W. W., T. Bergsbaken, S. E. Witowski, N. T. Perna, and R. A. Welch.** 2003. Acquisition of stcE, a C1 esterase inhibitor-specific metalloprotease, during the evolution of *Escherichia coli* O157:H7. J Infect Dis **187:**1907-1914.

212. **Lathem, W. W., T. E. Grys, S. E. Witowski, A. G. Torres, J. B. Kaper, P. I. Tarr, and R. A. Welch.** 2002. StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. Mol Microbiol **45:**277-288.

213. **Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol Microbiol **17:**333-343.

214. **Lau, S. M., M. Y. Peng, and F. Y. Chang.** 2000. Outcomes of *Aeromonas* bacteremia in patients with different types of underlying disease. J Microbiol Immunol Infect **33:**241-247.

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

216. **Li, C. C., J. A. Crawford, V. J. DiRita, and J. B. Kaper.** 2000. Molecular cloning and transcriptional regulation of ompT, a ToxR-repressed gene in *Vibrio cholerae*. Mol Microbiol **35:**189-203.

217. **Li, C. M., I. Brown, J. Mansfield, C. Stevens, T. Boureau, M. Romantschuk, and S. Taira.** 2002. The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. Embo J **21:**1909-1915.

218. **Libby, S. J., L. G. Adams, T. A. Ficht, C. Allen, H. A. Whitford, N. A. Buchmeier, S. Bossie, and D. G. Guiney.** 1997. The spv genes on the *Salmonella dublin* virulence plasmid are required for severe enteritis and systemic infection in the natural host. Infect Immun **65:**1786-1792.

219. **Lilley, B. N., and B. L. Bassler.** 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol Microbiol **36:**940-954.

220. **Lin, S. H., S. D. Shieh, Y. F. Lin, E. De Brauwer, H. W. Van Landuyt, B. Gordts, and J. R. Boelaert.** 1996. Fatal *Aeromonas hydrophila* bacteremia in a hemodialysis patient treated with deferoxamine. Am J Kidney Dis **27:**733-735.

221. **Liu, S. L., T. Ezaki, H. Miura, K. Matsui, and E. Yabuuchi.** 1988. Intact motility as a *Salmonella* Typhi invasion-related factor. Infect Immun **56:**1967-1973.

222. **Lobner-Olesen, A., M. G. Marinus, and F. G. Hansen.** 2003. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. Proc Natl Acad Sci U S A **100:**4672-4677.

223. **Loewy, A. G., U. V. Santer, M. Wieczorek, J. K. Blodgett, S. W. Jones, and J. C. Cheronis.** 1993. Purification and characterization of a novel zinc-proteinase from cultures of *Aeromonas hydrophila*. J Biol Chem **268:**9071-9078.

224. **Low, D. A., N. J. Weyand, and M. J. Mahan.** 2001. Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. Infect Immun **69:**7197-7204.

225. **Lu, M., J. L. Campbell, E. Boye, and N. Kleckner.** 1994. SeqA: a negative modulator of replication initiation in *E. coli*. Cell **77:**413-426.

226. **Lyko, F.** 2001. DNA methylation learns to fly. Trends Genet **17:**169-172.

227. **Lyko, F., B. H. Ramsahoye, and R. Jaenisch.** 2000. DNA methylation in *Drosophila melanogaster*. Nature **408:**538-540.

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

229. **Maalej, S., A. Mahjoubi, C. Elazri, and S. Dukan.** 2003. Simultaneous effects of environmental factors on motile *Aeromonas* dynamics in an urban effluent and in the natural seawater. Water Res **37:**2865-2874.

230. **Mackerness, C. W., J. S. Colbourne, and C. W. Keevil.** 1991. Growth of *Aeromonas hydrophila* and *Escherichia coli* in distribution system biofilm model. Proceedings of the U.K. Symposium of Health-related Water Microbiology.131-138.

231. **Macnab, R. M.** 2003. How bacteria assemble flagella. Annu Rev Microbiol **57:**77-100.

232. **Madsen, C. F., and N. R. Pedersen.** 1995. [Fulminant myonecrosis and sepsis caused by *Aeromonas hydrophila*]. Ugeskr Laeger **157:**3204-3205.

233. **Maeda, H., and A. Molla.** 1989. Pathogenic potentials of bacterial proteases. Clin Chim Acta **185:**357-367.

234. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu Rev Genet **34:**139-164.

235. **Malone, T., R. M. Blumenthal, and X. Cheng.** 1995. Structure-guided analysis reveals nine sequence motifs conserved among DNA amino-methyltransferases, and suggests a catalytic mechanism for these enzymes. J Mol Biol **253:**618-632.

236. **Manefield, M., and S. L. Turner.** 2002. Quorum sensing in context: out of molecular biology and into microbial ecology. Microbiology **148:**3762-3764.

237. **Marinus, M. G.** 1996. Methylation of DNA, p. 782-791. *In* F. C. N. e. *al*. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.

238. **Marinus, M. G.** 2000. Recombination is essential for viability of an *Escherichia coli dam* (DNA adenine methyltransferase) mutant. J Bacteriol **182:**463-468.

239. **Martin, M., R. Showalter, and M. Silverman.** 1989. Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi*. J Bacteriol **171:**2406-2414.

240. **Martin-Carnahan, A., and S. W. Joseph.** 2005. *Aeromonas*, p. 556-578. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), Bergey's Manual of Systemic Bacteriology, 2 ed, vol. 2. Springer-Verlag, New York, NY.

241. **Martinez-Murcia, A. J., S. Benlloch, and M. D. Collins.** 1992. Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. Int J Syst Bacteriol **42:**412-421.

242. **Maurer, J., J. Jose, and T. F. Meyer.** 1999. Characterization of the essential transport function of the AIDA-I autotransporter and evidence supporting structural predictions. J Bacteriol **181:**7014-7020.

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

244. **McGhie, E. J., P. J. Hume, R. D. Hayward, J. Torres, and V. Koronakis.** 2002. Topology of the *Salmonella* invasion protein SipB in a model bilayer. Mol Microbiol **44:**1309-1321.

245. **Merino, S., A. Aguilar, X. Rubires, N. Abitiu, M. Regue, and J. M. Tomas.** 1997. The role of the capsular polysaccharide of *Aeromonas hydrophila* serogroup O:34 in the adherence to and invasion of fish cell lines. Res Microbiol **148:**625-631.

246. **Merino, S., S. Camprubi, and J. M. Tomas.** 1991. The role of lipopolysaccharide in complement-killing of *Aeromonas hydrophila* strains of serotype O:34. J Gen Microbiol **137:**1583-1590.

247. **Merino, S., M. M. Nogueras, A. Aguilar, X. Rubires, S. Alberti, V. J. Benedi, and J. M. Tomas.** 1998. Activation of the complement classical pathway (C1q binding) by mesophilic *Aeromonas hydrophila* outer membrane protein. Infect Immun **66:**3825-3831.

248. **Merino, S., X. Rubires, A. Aguilar, S. Alberti, S. Hernandez-Alles, V. J. Benedi, and J. M. Tomas.** 1996. Mesophilic *Aeromonas* sp. serogroup O:11 resistance to complement-mediated killing. Infect Immun **64:**5302-5309.

249. **Merkel, T. J., C. Barros, and S. Stibitz.** 1998. Characterization of the bvgR locus of *Bordetella pertussis*. J Bacteriol **180:**1682-1690.

250. **Meselson, M., R. Yuan, and J. Heywood.** 1972. Restriction and modification of DNA. Annu Rev Biochem **41:**447-466.

251. **Messer, W., and M. Noyer-Weidner.** 1988. Timing and targeting: the biological functions of Dam methylation in *E. coli*. Cell **54:**735-737.

252. **Meury, J., A. Bahloul, and M. Kohiyama.** 1995. Importance of the replication origin sequestration in cell division of *Escherichia coli*. Biochimie **77:**875-879.

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

254. **Miki, T., N. Okada, Y. Shimada, and H. Danbara.** 2004. Characterization of *Salmonella* pathogenicity island 1 type III secretion-dependent hemolytic activity in *Salmonella enterica* serovar Typhimurium. Microb Pathog **37:**65-72.

255. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

256. **Miller, V. L., V. J. DiRita, and J. J. Mekalanos.** 1989. Identification of toxS, a regulatory gene whose product enhances toxR-mediated activation of the cholera toxin promoter. J Bacteriol **171:**1288-1293.

257. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J Bacteriol **170:**2575-2583.

258. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. Proc Natl Acad Sci U S A **81:**3471-3475.

259. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator toxR is a transmembrane DNA binding protein. Cell **48:**271-279.

260. **Minnaganti, V. R., P. J. Patel, D. Iancu, P. E. Schoch, and B. A. Cunha.** 2000. Necrotizing fasciitis caused by *Aeromonas hydrophila*. Heart Lung **29:**306-308.

261. **Minta, J. O.** 1981. The role of sialic acid in the functional activity and the hepatic clearance of C1-INH. J Immunol **126:**245-249.

262. **Mok, K. C., N. S. Wingreen, and B. L. Bassler.** 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. Embo J **22:**870-881.

263. **More, M. I., L. D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

264. **Mota, L. J., L. Journet, I. Sorg, C. Agrain, and G. R. Cornelis.** 2005. Bacterial injectisomes: needle length does matter. Science **307:**1278.

265. **Munoz, P., V. Fernandez-Baca, T. Pelaez, R. Sanchez, M. Rodriguez-Creixems, and E. Bouza.** 1994. *Aeromonas* peritonitis. Clin Infect Dis **18:**32-37.

266. **Nakasone, I., N. Yamane, T. Miyazaki, S. Onaga, and M. Higa.** 2001. [*Aeromonas* species infection with severe clinical manifestation in Okinawa, Japan -association with gas gangrene-]. Rinsho Biseibutshu Jinsoku Shindan Kenkyukai Shi **12:**15-21.

267. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev **11:**142-201.

268. **Nealson, K. H., T. Platt, and J. W. Hastings.** 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J Bacteriol **104:**313-322.

269. **Neyts, K., G. Huys, M. Uyttendaele, J. Swings, and J. Debevere.** 2000. Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. Lett Appl Microbiol **31:**359-363.

270. **Ng, P. J., G. H. Fleet, and G. M. Heard.** 2005. Pesticides as a source of microbial contamination of salad vegetables. Int J Food Microbiol **101:**237-250.

271. **Nicholson, B., and D. Low.** 2000. DNA methylation-dependent regulation of pef expression in *Salmonella* Typhimurium. Mol Microbiol **35:**728-742.

272. **Nou, X., B. Braaten, L. Kaltenbach, and D. A. Low.** 1995. Differential binding of Lrp to two sets of pap DNA binding sites mediated by Pap I regulates Pap phase variation in *Escherichia coli*. Embo J **14:**5785-5797.

273. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J Bacteriol **176:**2044-2054.

274. **Ogden, G. B., M. J. Pratt, and M. Schaechter.** 1988. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell **54:**127-135.

275. **Oliver, D. C., G. Huang, and R. C. Fernandez.** 2003. Identification of secretion determinants of the *Bordetella pertussis* BrkA autotransporter. J Bacteriol **185:**489-495.

276. **Oshima, T., C. Wada, Y. Kawagoe, T. Ara, M. Maeda, Y. Masuda, S. Hiraga, and H. Mori.** 2002. Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in *Escherichia coli*. Mol Microbiol **45:**673-695.

277. **Outin, H. D., A. Chatelin, E. Ronco, C. Nauciel, P. Gajdos, A. Barois, and M. Goulon.** 1984. [*Aeromonas hydrophila* septicemia: 3 cases, 1 with mediastinitis] Ann Med Interne (Paris) **135**:287-290.

278. **Paccani, S. R., F. Tonello, R. Ghittoni, M. Natale, L. Muraro, M. M. D'Elios, W. J. Tang, C. Montecucco, and C. T. Baldari.** 2005. Anthrax toxins suppress T lymphocyte activation by disrupting antigen receptor signaling. J Exp Med **201:**325-331.

279. **Palmer, B. R., and M. G. Marinus.** 1994. The dam and dcm strains of *Escherichia coli*--a review. Gene **143:**1-12.

280. **Parras, F., M. D. Diaz, J. Reina, S. Moreno, C. Guerrero, and E. Bouza.** 1993. Meningitis due to *Aeromonas* species: case report and review. Clin Infect Dis **17:**1058-1060.

281. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, Jr., and E. P. Greenberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci U S A **96:**4360-4365.

282. **Parsot, C., and J. J. Mekalanos.** 1991. Expression of the *Vibrio cholerae* gene encoding aldehyde dehydrogenase is under control of ToxR, the cholera toxin transcriptional activator. J Bacteriol **173:**2842-2851.

283. **Parsot, C., E. Taxman, and J. J. Mekalanos.** 1991. ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*. Proc Natl Acad Sci U S A **88:**1641-1645.

284. **Paton, A. W., and J. C. Paton.** 2002. Reactivity of convalescent-phase hemolytic-uremic syndrome patient sera with the megaplasmid-encoded TagA protein of Shiga toxigenic *Escherichia coli* O157. J Clin Microbiol **40:**1395-1399.

285. **Paton, J. C., and A. W. Paton.** 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev **11:**450-479.

286. **Pearson, J. P., M. Feldman, B. H. Iglewski, and A. Prince.** 2000. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect Immun **68:**4331-4334.

287. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc Natl Acad Sci U S A **91:**197-201.

288. **Pelayo Melero, M. J., A. Segura Garcia, E. Cortes Lopera, and L. Lu Cortez.** 1993. [Acute gastroenteritis from *Aeromonas* sobria and pancreatitis: apropos of a case]. Med Clin (Barc) **101:**238.

289. **Perna, N. T., G. F. Mayhew, G. Posfai, S. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner.** 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun **66:**3810-3817.

290. **Peterson, K. M., and J. J. Mekalanos.** 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect Immun **56:**2822-2829.

291. **Peterson, K. R., K. F. Wertman, D. W. Mount, and M. G. Marinus.** 1985. Viability of *Escherichia coli* K-12 DNA adenine methylase (dam) mutants requires increased expression of specific genes in the SOS regulon. Mol Gen Genet **201:**14-19.

292. **Pettersson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson, and H. Wolf-Watz.** 1996. Modulation of virulence factor expression by pathogen target cell contact. Science **273:**1231-1233.

293. **Picking, W. L., H. Nishioka, P. D. Hearn, M. A. Baxter, A. T. Harrington, A. Blocker, and W. D. Picking.** 2005. IpaD of *Shigella flexneri* is independently required for regulation of Ipa protein secretion and efficient insertion of IpaB and IpaC into host membranes. Infect Immun **73:**1432-1440.

294. **Pidiyar, V., A. Kaznowski, N. B. Narayan, M. Patole, and Y. S. Shouche.** 2002. *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. Int J Syst Evol Microbiol **52:**1723-1728.

295. **Pillai, L., J. Sha, T. E. Erova, A. A. Fadl, B. K. Khajanchi, and A. K. Chopra.** 2006. Molecular and Functional Characterization of a ToxR-Regulated

Lipoprotein from a Clinical Isolate of *Aeromonas hydrophila*. Infect Immun **74:**3742-3755.

296. **Pohlner, J., R. Halter, K. Beyreuther, and T. F. Meyer.** 1987. Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature **325:**458-462.

297. **Polaczek, P., K. Kwan, and J. L. Campbell.** 1998. GATC motifs may alter the conformation of DNA depending on sequence context and N6-adenine methylation status: possible implications for DNA-protein recognition. Mol Gen Genet **258:**488-493.

298. **Polaczek, P., K. Kwan, D. A. Liberies, and J. L. Campbell.** 1997. Role of architectural elements in combinatorial regulation of initiation of DNA replication in *Escherichia coli*. Mol Microbiol **26:**261-275.

299. **Popoff, M.** 1984. Genus II *Aeromonas*. Bergey's Manual of Systematic Bacteriology **I:**545.

300. **Popoff, M., and M. Veron.** 1976. A taxonomic study of the *Aeromonas hydrophila-Aeromonas punctata* group. J Gen Microbiol **94:**11-22.

301. **Potempa, J., A. Banbula, and J. Travis.** 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. Periodontol 2000 **24:**153-192.

302. **Potempa, J., E. Korzus, and J. Travis.** 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J Biol Chem **269:**15957-15960.

303. **Presley, S. M., T. R. Rainwater, G. P. Austin, S. G. Platt, J. C. Zak, G. P. Cobb, E. J. Marsland, K. Tian, B. Zhang, T. A. Anderson, S. B. Cox, M. T. Abel, B. D. Leftwich, J. R. Huddleston, R. M. Jeter, and R. J. Kendall.** 2006. Assessment of pathogens and toxicants in New Orleans, LA following Hurricane Katrina. Environ Sci Technol **40:**468-474.

304. **Preutthipan, A., T. Chantarojanasiri, S. Suwanjutha, and K. Tanpowpong.** 1993. *Aeromonas hydrophila* epiglottitis: a case report. J Med Assoc Thai **76 Suppl 2:**225-228.

305. **Pucciarelli, M. G., A. I. Prieto, J. Casadesus, and F. Garcia-del Portillo.** 2002. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. Microbiology **148:**1171-1182.

306. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol Rev **57:**50-108.

307. **Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg, and J. J. Mekalanos.** 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A **103:**1528-1533.

308. **Purdue, G. F., and J. L. Hunt.** 1988. *Aeromonas hydrophila* infection in burn patients. Burns Incl Therm Inj **14:**220-221.

309. **Quinn, j. p.** 1991. UHL enteric bacterial disease surveillance. Lab. Hotline **28:**2.

310. **Reading, N. C., and V. Sperandio.** 2006. Quorum sensing: the many languages of bacteria. FEMS Microbiol Lett **254:**1-11.

311. **Reisenauer, A., L. S. Kahng, S. McCollum, and L. Shapiro.** 1999. Bacterial DNA methylation: a cell cycle regulator? J Bacteriol **181:**5135-5139.

312. **Reznikoff, W. S.** 1993. The Tn5 transposon. Annu Rev Microbiol **47:**945-963.

313. **Ribardo, D. A., K. R. Kuhl, I. Boldogh, J. W. Peterson, C. W. Houston, and A. K. Chopra.** 2002. Early cell signaling by the cytotoxic enterotoxin of *Aeromonas hydrophila* in macrophages. Microb Pathog **32:**149-163.

314. **Rietsch, A., I. Vallet-Gely, S. L. Dove, and J. J. Mekalanos.** 2005. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **102:**8006-8011.

315. **Rivero, O., J. Anguita, D. Mateos, C. Paniagua, and G. Naharro.** 1991. Cloning and characterization of an extracellular temperature-labile serine protease gene from *Aeromonas hydrophila*. FEMS Microbiol Lett **65:**1-7.

316. **Rivero, O., J. Anguita, C. Paniagua, and G. Naharro.** 1990. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. J Bacteriol **172:**3905-3908.

317. **Roberts, D., B. C. Hoopes, W. R. McClure, and N. Kleckner.** 1985. IS10 transposition is regulated by DNA adenine methylation. Cell **43:**117-130.

318. **Roberts, R. J., M. Belfort, T. Bestor, A. S. Bhagwat, T. A. Bickle, J. Bitinaite, R. M. Blumenthal, S. Degtyarev, D. T. Dryden, K. Dybvig, K. Firman, E. S. Gromova, R. I. Gumport, S. E. Halford, S. Hattman, J. Heitman, D. P. Hornby, A. Janulaitis, A. Jeltsch, J. Josephsen, A. Kiss, T. R. Klaenhammer, I. Kobayashi, H. Kong, D. H. Kruger, S. Lacks, M. G. Marinus, M.**

Miyahara, R. D. Morgan, N. E. Murray, V. Nagaraja, A. Piekarowicz, A. Pingoud, E. Raleigh, D. N. Rao, N. Reich, V. E. Repin, E. U. Selker, P. C. Shaw, D. C. Stein, B. L. Stoddard, W. Szybalski, T. A. Trautner, J. L. Van Etten, J. M. Vitor, G. G. Wilson, and S. Y. Xu. 2003. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. Nucleic Acids Res **31:**1805-1812.

319. **Roberts, R. J., and D. Macelis.** 2000. REBASE - restriction enzymes and methylases. Nucleic Acids Res **28:**306-307.

320. **Rose, J. M., C. W. Houston, and A. Kurosky.** 1989. Bioactivity and immunological characterization of a cholera toxin-cross-reactive cytolytic enterotoxin from *Aeromonas hydrophila*. Infect Immun **57:**1170-1176.

321. **Rosqvist, R., K. E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. Embo J **13:**964-972.

322. **Ruiz de Gonzalez, P., C. Escolano, J. C. Rodriguez, C. Sillero, and G. Royo.** 1994. *Aeromonas sobria* spontaneous bacterial peritonitis and bacteremia. Am J Gastroenterol **89:**290-291.

323. **Rumbaugh, K. P., J. A. Griswold, and A. N. Hamood.** 1999. Contribution of the regulatory gene *lasR* to the pathogenesis of *Pseudomonas aeruginosa* infection of burned mice. J Burn Care Rehabil **20:**42-49.

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

325. **Russell, D. W., and N. D. Zinder.** 1987. Hemimethylation prevents DNA replication in *E. coli*. Cell **50:**1071-1079.

326. **Ryndak, M. B., H. Chung, E. London, and J. B. Bliska.** 2005. Role of predicted transmembrane domains for type III translocation, pore formation, and signaling by the *Yersinia pseudotuberculosis* YopB protein. Infect Immun **73:**2433-2443.

327. **Salmond, G. P., and P. J. Reeves.** 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. Trends Biochem Sci **18:**7-12.

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

329. **Schmaier, A. H., S. Amenta, T. Xiong, G. D. Heda, and A. M. Gewirtz.** 1993. Expression of platelet C1 inhibitor. Blood **82:**465-474.

330. **Schmaier, A. H., S. C. Murray, G. D. Heda, A. Farber, A. Kuo, K. McCrae, and D. B. Cines.** 1989. Synthesis and expression of C1 inhibitor by human umbilical vein endothelial cells. J Biol Chem **264:**18173-18179.

331. **Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg.** 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. Mol Microbiol **51:**973-985.

332. **Scofield, S. R., C. M. Tobias, J. P. Rathjen, J. H. Chang, D. T. Lavelle, R. W. Michelmore, and B. J. Staskawicz.** 1996. Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato. Science **274:**2063-2065.

333. **Sekiya, K., M. Ohishi, T. Ogino, K. Tamano, C. Sasakawa, and A. Abe.** 2001. Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A **98:**11638-11643.

334. **Semel, J. D., and G. Trenholme.** 1990. *Aeromonas hydrophila* water-associated traumatic wound infections: a review. J Trauma **30:**324-327.

335. **Sen, K., and M. Rodgers.** 2004. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol **97:**1077-1086.

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

337. **Sha, J., C. L. Galindo, V. Pancholi, V. L. Popov, Y. Zhao, C. W. Houston, and A. K. Chopra.** 2003. Differential expression of the enolase gene under in vivo versus in vitro growth conditions of *Aeromonas hydrophila*. Microb Pathog **34:**195-204.

338. **Sha, J., E. V. Kozlova, and A. K. Chopra.** 2002. Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun **70:**1924-1935.

339. **Sha, J., E. V. Kozlova, A. A. Fadl, J. P. Olano, C. W. Houston, J. W. Peterson, and A. K. Chopra.** 2004. Molecular characterization of a glucose-

inhibited division gene, gidA, that regulates cytotoxic enterotoxin of *Aeromonas hydrophila*. Infect Immun **72:**1084-1095.

340. **Sha, J., M. Lu, and A. K. Chopra.** 2001. Regulation of the cytotoxic enterotoxin gene in *Aeromonas hydrophila*: characterization of an iron uptake regulator. Infect Immun **69:**6370-6381.

341. **Sha, J., L. Pillai, A. A. Fadl, C. L. Galindo, T. E. Erova, and A. K. Chopra.** 2005. The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. Infect Immun **73:**6446-6457.

342. **Sharma, A., N. Dubey, and B. Sharan.** 2005. Characterization of aeromonads isolated from the river Narmada, India. Int J Hyg Environ Health **208:**425-433.

343. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc Natl Acad Sci U S A **94:**6036-6041.

344. **Showalter, R. E., M. O. Martin, and M. R. Silverman.** 1990. Cloning and nucleotide sequence of luxR, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. J Bacteriol **172:**2946-2954.

345. **Simodynes, E. E., and R. M. Cochran, 2nd.** 1982. *Aeromonas hydrophila* infection complicating an open tibial fracture. A case report. Clin Orthop Relat Res **171:**117-120.

346. **Sinha, S., T. Shimada, T. Ramamurthy, S. K. Bhattacharya, S. Yamasaki, Y. Takeda, and G. B. Nair.** 2004. Prevalence, serotype distribution, antibiotic susceptibility and genetic profiles of mesophilic *Aeromonas* species isolated from hospitalized diarrhoeal cases in Kolkata, India. J Med Microbiol **53:**527-534.

347. **Sisti, M., A. Albano, and G. Brandi.** 1998. Bactericidal effect of chlorine on motile *Aeromonas* spp. in drinking water supplies and influence of temperature on disinfection efficacy. Lett Appl Microbiol **26:**347-351.

348. **Skorupski, K., and R. K. Taylor.** 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol Microbiol **25:**1003-1009.

349. **Snellings, N. J., M. Popek, and L. E. Lindler.** 2001. Complete DNA sequence of *Yersinia enterocolitica* serotype 0:8 low-calcium-response plasmid reveals a new virulence plasmid-associated replicon. Infect Immun **69:**4627-4638.

350. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription

and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. Proc Natl Acad Sci U S A **96:**15196-15201.

351. **Stancheva, I., T. Koller, and J. M. Sogo.** 1999. Asymmetry of Dam remethylation on the leading and lagging arms of plasmid replicative intermediates. Embo J **18:**6542-6551.

352. **Stebbins, C. E., and J. E. Galan.** 2003. Priming virulence factors for delivery into the host. Nat Rev Mol Cell Biol **4:**738-743.

353. **Steciw, A., and S. M. Colodny.** 1994. Empyema due to *Aeromonas hydrophila*. Clin Infect Dis **18:**474-475.

354. **Stevens, A. M., K. M. Dolan, and E. P. Greenberg.** 1994. Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the lux promoter region. Proc Natl Acad Sci U S A **91:**12619-12623.

355. **Stevens, A. M., and E. P. Greenberg.** 1997. Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J Bacteriol **179:**557-562.

356. **Stockbauer, K. E., A. K. Foreman-Wykert, and J. F. Miller.** 2003. *Bordetella* type III secretion induces caspase 1-independent necrosis. Cell Microbiol **5:**123-132.

357. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca2+ in the *Yersinia* low-Ca2+ response. Mol Microbiol **8:**1005-1010.

358. **Stuber, K., S. E. Burr, M. Braun, T. Wahli, and J. Frey.** 2003. Type III secretion genes in *Aeromonas salmonicida* subsp salmonicida are located on a large thermolabile virulence plasmid. J Clin Microbiol **41:**3854-3856.

359. **Subashkumar, R., T. Thayumanavan, G. Vivekanandhan, and P. Lakshmanaperumalsamy.** 2006. Occurrence of *Aeromonas hydrophila* in acute gasteroenteritis among children. Indian J Med Res **123:**61-66.

360. **Sundin, C., J. Thelaus, J. E. Broms, and A. Forsberg.** 2004. Polarisation of type III translocation by *Pseudomonas aeruginosa* requires PcrG, PcrV and PopN. Microb Pathog **37:**313-322.

361. **Supuran, C. T., A. Scozzafava, and B. W. Clare.** 2002. Bacterial protease inhibitors. Med Res Rev **22:**329-372.

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

363. **Swift, S., A. V. Karlyshev, L. Fish, E. L. Durant, M. K. Winson, S. R. Chhabra, P. Williams, S. Macintyre, and G. S. Stewart.** 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J Bacteriol **179:**5271-5281.

364. **Swift, S., M. J. Lynch, L. Fish, D. F. Kirke, J. M. Tomas, G. S. Stewart, and P. Williams.** 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun **67:**5192-5199.

365. **Szabo, E. A., K. J. Scurrah, and J. M. Burrows.** 2000. Survey for psychrotrophic bacterial pathogens in minimally processed lettuce. Lett Appl Microbiol **30:**456-460.

366. **Szewzyk, U., R. Szewzyk, W. Manz, and K. H. Schleifer.** 2000. Microbiological safety of drinking water. Annu Rev Microbiol **54:**81-127.

367. **Takano, Y., Y. Asao, Y. Kohri, K. Oida, and T. Imanaka.** 1996. Fulminant pneumonia and sepsis due to *Aeromonas hydrophila* in an alcohol abuser. Intern Med **35:**410-412.

368. **Tamano, K., S. Aizawa, and C. Sasakawa.** 2002. Purification and detection of *Shigella* type III secretion needle complex. Methods Enzymol **358:**385-392.

369. **Tang, H. B., E. DiMango, R. Bryan, M. Gambello, B. H. Iglewski, J. B. Goldberg, and A. Prince.** 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect Immun **64:**37-43.

370. **Tang, X., R. D. Frederick, J. Zhou, D. A. Halterman, Y. Jia, and G. B. Martin.** 1996. Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase. Science **274:**2060-2063.

371. **Tanphaichitr, V. S., V. Suvatte, C. Mahasandana, and S. Tuchinda.** 1984. *Aeromonas* septicemia with shock in children with thalassemias. J Med Assoc Thai **67:**420-426.

372. **Taylor, V. L., R. W. Titball, and P. C. Oyston.** 2005. Oral immunization with a *dam* mutant of *Yersinia pseudotuberculosis* protects against plague. Microbiology **151:**1919-1926.

373. **Tokajian, S., and F. Hashwa.** 2004. Phenotypic and genotypic identification of *Aeromonas* spp. isolated from a chlorinated intermittent water distribution system in Lebanon. J Water Health **2:**115-122.

374. **Urieli-Shoval, S., Y. Gruenbaum, and A. Razin.** 1983. Sequence and substrate specificity of isolated DNA methylases from *Escherichia coli* C. J Bacteriol **153:**274-280.

375. **Uyttendaele, M., K. Neyts, H. Vanderswalmen, E. Notebaert, and J. Debevere.** 2004. Control of *Aeromonas* on minimally processed vegetables by decontamination with lactic acid, chlorinated water, or thyme essential oil solution. Int J Food Microbiol **90:**263-271.

376. **van der Velden, A. W., M. Velasquez, and M. N. Starnbach.** 2003. *Salmonella* rapidly kill dendritic cells via a caspase-1-dependent mechanism. J Immunol **171:**6742-6749.

377. **van der Woude, M., W. B. Hale, and D. A. Low.** 1998. Formation of DNA methylation patterns: nonmethylated GATC sequences in gut and pap operons. J Bacteriol **180:**5913-5920.

378. **Van Gijsegem, F., S. Genin, and C. Boucher.** 1993. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol **1:**175-180.

379. **Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y. H. Rogers, and H. O. Smith.** 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science **304:**66-74.

380. **Viboud, G. I., and J. B. Bliska.** 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol **59:**69-89.

381. **Vila, J., J. Ruiz, F. Gallardo, M. Vargas, L. Soler, M. J. Figueras, and J. Gascon.** 2003. *Aeromonas* spp. and traveler's diarrhea: clinical features and antimicrobial resistance. Emerg Infect Dis **9:**552-555.

382. **Vilches, S., C. Urgell, S. Merino, M. R. Chacon, L. Soler, G. Castro-Escarpulli, M. J. Figueras, and J. M. Tomas.** 2004. Complete type III secretion system of a mesophilic *Aeromonas hydrophila* strain. Appl Environ Microbiol **70:**6914-6919.

383. **Vollmer, P., I. Walev, S. Rose-John, and S. Bhakdi.** 1996. Novel pathogenic mechanism of microbial metalloproteinases: liberation of membrane-anchored molecules in biologically active form exemplified by studies with the human interleukin-6 receptor. Infect Immun **64:**3646-3651.

384. **Voss, L. M., K. H. Rhodes, and K. A. Johnson.** 1992. Musculoskeletal and soft tissue *Aeromonas* infection: an environmental disease. Mayo Clin Proc **67:**422-427.

385. **Walter, J., T. A. Trautner, and M. Noyer-Weidner.** 1992. High plasticity of multispecific DNA methyltransferases in the region carrying DNA target recognizing enzyme modules. Embo J **11:**4445-4450.

386. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the virB operon from an *Agrobacterium tumefaciens* Ti plasmid. J Biol Chem **263:**5804-5814.

387. **Waters, C. M., and B. L. Bassler.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol **21:**319-346.

388. **Wattanawaitunechai, C., S. J. Peacock, and P. Jitpratoom.** 2005. Tsunami in Thailand--disaster management in a district hospital. N Engl J Med **352:**962-964.

389. **Wei, Z., J. F. Kim, and S. V. Beer.** 2000. Regulation of hrp genes and type III protein secretion in *Erwinia amylovora* by HrpX/HrpY, a novel two-component system, and HrpS. Mol Plant Microbe Interact **13:**1251-1262.

390. **Weitao, T., K. Nordstrom, and S. Dasgupta.** 2000. *Escherichia coli* cell cycle control genes affect chromosome superhelicity. EMBO Rep **1:**494-499.

391. **Wells, R. G., G. M. Chertow, and E. R. Marcantonio.** 1991. Parapharyngeal soft-tissue infection with *Aeromonas hydrophila*. Head Neck **13:**528-530.

392. **Weyand, N. J., and D. A. Low.** 2000. Regulation of Pap phase variation. Lrp is sufficient for the establishment of the phase off pap DNA methylation pattern and repression of pap transcription in vitro. J Biol Chem **275:**3192-3200.

393. **Whiteley, M., M. R. Parsek, and E. P. Greenberg.** 2000. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. J Bacteriol **182:**4356-4360.

394. **White-Ziegler, C. A., M. L. Angus Hill, B. A. Braaten, M. W. van der Woude, and D. A. Low.** 1998. Thermoregulation of *Escherichia coli* pap transcription: H-NS is a temperature-dependent DNA methylation blocking factor. Mol Microbiol **28:**1121-1137.

395. **Wigler, M., D. Levy, and M. Perucho.** 1981. The somatic replication of DNA methylation. Cell **24:**33-40.

396. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. Salmond, B. W. Bycroft, and et al.** 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **92:**9427-9431.

397. **Wood, M. W., R. Rosqvist, P. B. Mullan, M. H. Edwards, and E. E. Galyov.** 1996. SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a sip-dependent mechanism and promotes bacterial entry. Mol Microbiol **22:**327-338.

398. **Xu, X. J., M. R. Ferguson, V. L. Popov, C. W. Houston, J. W. Peterson, and A. K. Chopra.** 1998. Role of a cytotoxic enterotoxin in *Aeromonas*-mediated infections: development of transposon and isogenic mutants. Infect Immun **66:**3501-3509.

399. **Yamada, S., S. Matsushita, S. Dejsirilert, and Y. Kudoh.** 1997. Incidence and clinical symptoms of *Aeromonas*-associated travellers' diarrhoea in Tokyo. Epidemiol Infect **119:**121-126.

400. **Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, R. E. Sockett, M. Goldner, Y. Dessaux, M. Camara, H. Smith, and P. Williams.** 2002. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun **70:**5635-5646.

401. **Yip, C. K., and N. C. Strynadka.** 2006. New structural insights into the bacterial type III secretion system. Trends Biochem Sci **31:**223-230.

402. **Yu, H. B., P. S. Rao, H. C. Lee, S. Vilches, S. Merino, J. M. Tomas, and K. Y. Leung.** 2004. A type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. Infect Immun **72:**1248-1256.

403. **Zaleski, P., and A. Piekarowicz.** 2004. Characterization of a *dam* mutant of *Haemophilus influenzae* Rd. Microbiology **150:**3773-3781.

404. **Zhang, H. B., L. H. Wang, and L. H. Zhang.** 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc Natl Acad Sci U S A **99:**4638-4643.

405. **Zhu, H., S. J. Thuruthyil, and M. D. Willcox.** 2000. Invasive strains of *Pseudomonas aeruginosa* are able to cause epithelial cell cytotoxicity that is dependent on bacterial cell density. Clin Experiment Ophthalmol **28:**201-204.

406. **Zhu, H., S. J. Thuruthyil, and M. D. Willcox.** 2001. Production of N-acyl homoserine lactones by gram-negative bacteria isolated from contact lens wearers. Clin Experiment Ophthalmol **29:**150-152.

407. **Zychlinsky, A., B. Kenny, R. Menard, M. C. Prevost, I. B. Holland, and P. J. Sansonetti.** 1994. IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. Mol Microbiol **11:**619-627.

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Publications

Pillai, L., J. Sha, T. Erova, A.A. Fadl, and A. K. Chopra. 2006. Molecular and Functional Characterization of a ToxR Regulated Lipoprotein (TagA) from a Clinical Isolate SSU of *Aeromonas hydrophila*. *Infection and Immunity*. 74: 3742-3755.

Erova, T.E., A.A. Fadl. J. Sha, B. Khajanchi, L. Pillai, and A.K. Chopra. 2006. Mutations within the Catalytic Motif of DNA Adenine Methyltransferase (Dam) of *Aeromonas hydrophila* Reverts the Virulence of the Dam-overproducing Strain to that of the Wild-type Phenotype. *Infection and Immunity*. (In Press)

Galindo, C. L., J. Sha, A.A. Fadl, L. Pillai, and A.K. Chopra. 2006. Host Immune Responses to *Aeromonas* Virulence Factors. *Current Immunology Reviews*. 2.13-26.

Erova, T.E., L. Pillai, A.A. Fadl, J. Sha, S. Wang, C. L. Galindo, and A. K. Chopra. 2006. DNA Adenine Methyltransferase Influences Virulence of *Aeromonas hydrophila*. *Infection and Immunity*. 74:410-24.

Sha, J., L. Pillai (equal contribution), A.A. Fadl, C.L. Galindo, T.E. Erova, and A. K. Chopra. 2005. The Type III Secretion System and Cytotoxic Enterotoxin Alter the Virulence of *Aeromonas hydrophila*. *Infection and Immunity* 73:6446-57.

Galindo, C.L., A.A. Fadl, J. Sha, L. Pillai, C. Gutierrez, and A. K. Chopra. 2005. Microarray and Proteomics Analyses of Human Intestinal Epithelial Cells Treated with the *Aeromonas hydrophila* Cytotoxic Enterotoxin. *Infection and Immunity*. 73:2628-43.

Galindo, C.L., J. Sha, D.A. Ribardo, A.A. Fadl, L. Pillai, and A. K. Chopra. 2003. Identification of *Aeromonas hydrophila* Cytotoxic Enterotoxin-Induced Genes in Macrophages using Microarrays. *Journal of Biological Chemistry*. 278:40198-212.

Abstracts

Pillai, L., J. Sha, T.E. Erova, A.A Fadl, and A.K. Chopra. Identification and characterization of a virulence factor, TagA, in a clinical isolate SSU of *Aeromonas hydrophila*. American Society for Microbiology General Meeting May 21-25, 2006 Orlando, Florida.

Pillai, L., J. Sha, T.E. Erova, A.A. Fadl, and A.K. Chopra. Identification and characterization of a novel virulence factor, TagA, in a clinical isolate of *Aeromonas hydrophila*. McLaughlin Colloquium April 26-27, 2006 Galveston, Texas.

L. Pillai, J. Sha, A.A. Fadl, C.L. Galindo, T.E. Erova, V.L. Popov, A.K. Chopra. The type III secretion system and cytotoxic enterotoxin modulate quorum sensing in *Aeromonas hydrophila*. American Society for Microbiology General Meeting June 5-9 Atlanta, Georgia.

Galindo, C.L., J. Sha, D.A. Ribardo, A.A. Fadl, L. Pillai and A.K. Chopra. Host cellular responses to the cytotoxic enterotoxin of *Aeromonas hydrophila*: A microarray approach. American Society for Microbiology General Meeting May 18-22 Washington, D.C.